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CARACTERIZACIÓN, PURIFICACIÓN Y EVALUACIÓN DE LA BIOACTIVIDAD DE FITOQUÍMICOS OBTENIDOS DE FUENTES NATURALES

Memoria presentada por

María de la Luz Cádiz Gurrea

Para optar al grado de

Doctor Internacional en Química por la Universidad de Granada

Tesis doctoral dirigida por

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Dr. Salvador Fernández Arroyo

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Memoria para optar al grado de Doctor Internacional en Química por la Universidad de Granada

Fdo. María de la Luz Cádiz Gurrea

El Prof. Dr. Antonio Segura Carretero, Catedrático del Departamento de Química Analítica de la Universidad de Granada y Director del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (CIDAF).

CERTIFICA:

Que el trabajo realizado en la presente Tesis Doctoral titulada: "**CARACTERIZACIÓN, PURIFICACIÓN Y EVALUACIÓN DE LA BIOACTIVIDAD DE FITOQUÍMICOS OBTENIDOS DE FUENTES NATURALES**", se ha realizado bajo su dirección y la del Dr. Salvador Fernández Arroyo, en los laboratorios del Departamento de Química Analítica de la Universidad de Granada, del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional, así como también de manera parcial durante sus estancias en las instalaciones del Instituto Imagine en el Hospital Necker de Paris (Francia) y en la Unitat de Recerca Biomèdica de la Universidad Rovira i Virgili (Reus, Tarragona), reuniendo todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dña. María de la Luz Cádiz Gurrea pueda optar al grado de Doctor Internacional en Química por la Universidad de Granada presentando la tesis como agrupación de publicaciones.

Y para que así conste, expido y firmo el presente certificado en Granada a 16 de Febrero de 2018.

Dr. Antonio Segura Carretero

La doctoranda **María de la Luz Cádiz Gurrea** y los directores de la tesis **Dr. Antonio Segura Carretero** y **Dr. Salvador Fernández Arroyo**, garantizamos, al firmar esta Tesis Doctoral, que el trabajo ha sido realizado por la doctoranda bajo nuestra dirección y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada, a 16 de Febrero de 2018

Directores de la tesis;

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Doctoranda;

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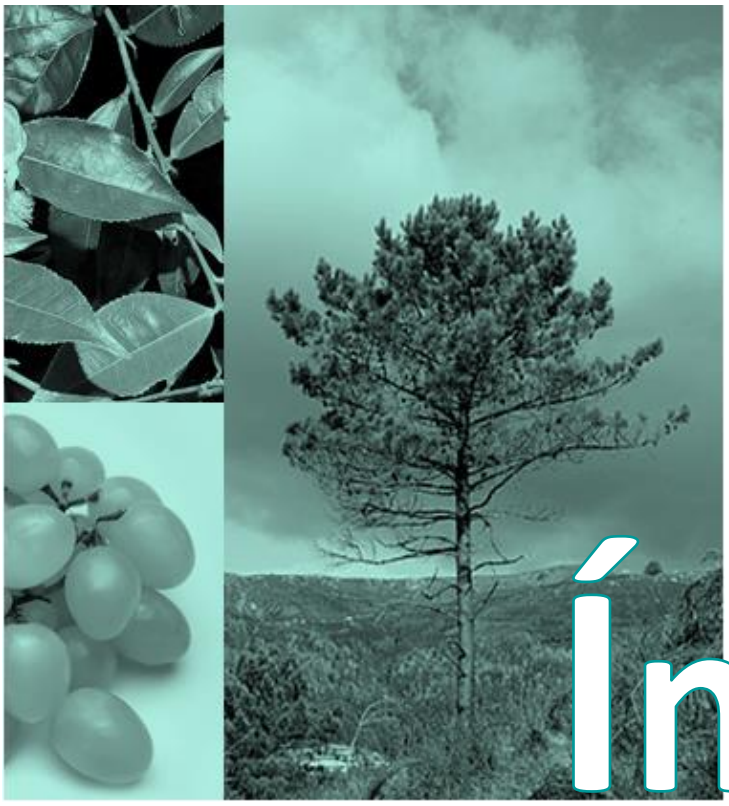

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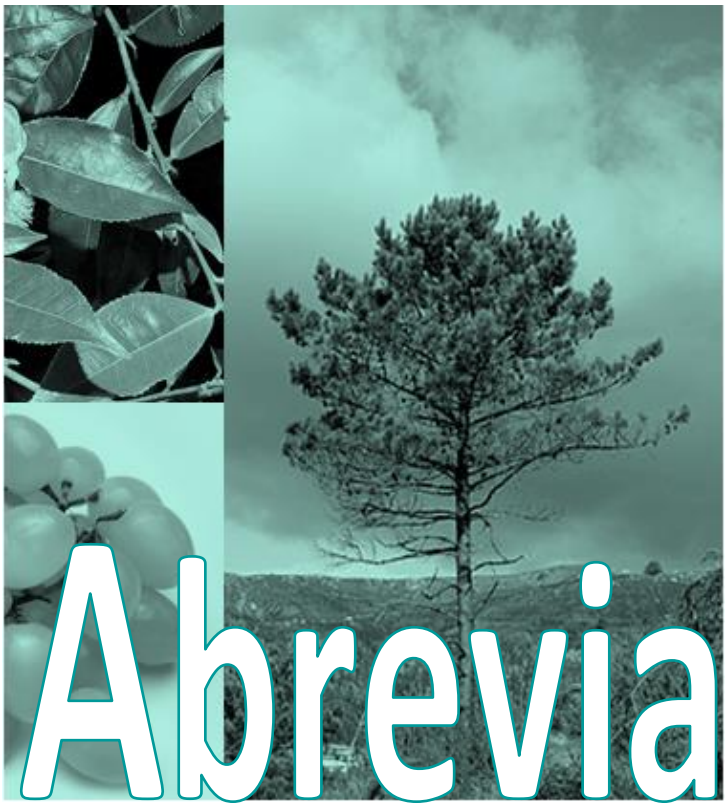

Abreviaturas

LISTA DE ABREVIATURAS

α-KG: α-cetoglutarato

AAPH: 2,2'-Azobis-(2-aminopropano)-dihidrocloruro

ABTS: ácido 2,2'-azinobis (3- etilbenzotiazolín)-6-sulfónico

ACH: acondroplasia

AICAR: 5-aminoimidazol-4-carboxamida ribonucleótido

AMPK: quinasa activada por AMP

ANOVA: análisis de varianza de una vía

AP-1: proteína activadora 1

ASE: extracción acelerada de disolventes

ATCC: colección americana de cultivos tipo

ATP: adenosina trifosfato

AUC: área bajo la curva

CAT: catalasa

COX-2: ciclooxigenasa 2

BPC: cromatograma de pico base

DAD: detector de batería de diodos

DEX: dexametasona

DMEM: medio de cultivo Eagle modificado de Dulbecco

DMSO: dimetilsulfóxido

EI: impacto electrónico

ELISA: ensayo por inmunoabsorción ligado a enzimas

ERK: quinasa reguladora de señales extracelulares

ESI: ionización por electrospray

EUA: extracción asistida por ultrasonidos

FBS: suero fetal bovino

FDA: administración de alimentos y medicamentos

FGFR: receptor de factores de crecimiento de fibroblastos

FOSHU: alimentos para uso específico de salud

FRAP: poder antioxidante reductor del hierro

FSC: fluido supercrítico

GAE: equivalentes de ácido gálico

GAPDH: glicelaldehído-3-fosfato deshidrogenasa

GC: cromatografía de gases

GPx: glutatión peroxidasa

GRAS: generalmente reconocido como seguro

GRed: glutatión reductasa

GSH: glutatión

H_2DCF-DA: 2′,7′-diclorofluoresceina diacetato

HAT: transferencia de átomos de hidrógeno

HEK: células embrionarias de riñón humano

HMBC: coherencia heteronuclear a múltiple enlace

HPC: calibración de alta precisión

HPLC: cromatografía líquida de alta resolución

HUVEC: células humanas endoteliales de la vena umbilical

IBMX: 3-isobutil-1-metilxantina

IFNγ: interferón-γ

IGF-1: factor de crecimiento insulínico 1

IK: inhibidor kB

IKK: IkB quinasa

IL-1β: interleuquina 1β

IL-2: interleuquina 2

IL-6: interleuquina 6

IL-8: interleuquina 8

ILSI: instituto internacional de ciencias de la vida

iNOS: óxido nítrico sintasa inducible

JNK: quinasa c-Jun amino terminal

LC: cromatografía líquida

LOX: lipooxigenasa

LPS: lipopolisacárido

MAE: extracción asistida por microondas

MCP-1: proteína quimiotáctica de monocitos 1

MEF: fibroblastos embrionarios de ratón

MDA: malondialdehido

MF: micro-filtración

MS: espectrometría de masas

MSPD: dispersión de matriz en fase sólida

M-SPE: extracción en fase sólida miniaturizada

MS/MS: espectrometría de masas en tándem

MTT: bromuro de 3-(4,5- di-metiltiazol-2-ilo)-2,5-difeniltetrazol

m/z*:* masa/carga

NAFLD: enfermedad del hígado graso no alcohólico

NF: nano-filtración

NF-κB: factor nuclear kappa B

NO: monóxido de nitrógeno

OI: ósmosis inversa

ORAC: capacidad de absorción de radicales de oxígeno

PBS: tampón fosfato salino

PCR: reacción en cadena de la polimerasa

PLA_2: fosfolipasa A_2

PI3K/AKT: fosfatidilinositol-3-quinasa/proteína quinasa B

PLCγ: fosfolipasa C gamma

PLE: extracción mediante líquidos presurizados

PON-1 KO: knock-out para paraoxonaxa-1

Q: cuadrupolo

RIPA: ensayo de radio inmunoprecipitación

RMN: resonancia magnética nuclear

ROS: especies reactivas del oxígeno

SAR: relación estructura-actividad

SET: transferencia de electrones

SFE: extracción con fluidos supercríticos

SLE: extracción sólido-líquido

SOD: superóxido dismutasa

SPE: extracción en fase sólida

sPLS-DA: análisis discriminante de mínimos cuadrados parciales sobre matrices dispersas

SPME: micro extracción en fase sólida

STAT1: activador de la transcripción 1

TCA: ciclo de los ácidos tricarboxílicos

TEAC: capacidad antioxidante equivalente al Trolox

tgMCP-1: ratón transgénico que sobreexpresa la MCP-1

TMS: trimetilclorosilano

TNF-α: factor de necrosis tumoral alfa

TOCSY: espectroscopía de correlación total

TOF: tiempo de vuelo

TPTZ: 2, 4, 6-tripiridil-s-triazina

Trolox: ácido-6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico

Ub: ubiquitina

UF: ultra-filtración

UV-VIS: ultravioleta-visible

VEGF: factor de crecimiento vascular endotelial

WT: tipo silvestre

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Resumen

Summary

RESUMEN

La presente Tesis Doctoral recoge herramientas de análisis, purificación y evaluación de la bioactividad de los compuestos fenólicos presentes en distintas especies vegetales, así como los resultados obtenidos tras la aplicación de todas ellas. Para describirlos, esta memoria se ha dividido en dos grandes secciones: **introducción** y **parte experimental**.

En la **INTRODUCCIÓN** se recoge información, en primer lugar, sobre la relación entre alimentación y salud a lo largo de los años y se detallan las principales características de las matrices vegetales que han sido objeto de estudio: *Sclerocarya birrea* (marula), *Eryngium bourgatii*, *Camellia sinensis, Pinus pinaster, Vitis vinifera, Theobroma cacao* y *Lippia citriodora* (hierbaluisa). A continuación, se describen los compuestos fenólicos, las rutas de donde surgen, su estructura y clasificación, para posteriormente centrarse en las etapas del proceso analítico como son la obtención, purificación y determinación de estos compuestos. Por último, se hace un recorrido por la bioactividad de los compuestos fenólicos, prestando especial atención a su papel como antioxidantes y antiinflamatorios y, también, a su relación con ciertas patologías como son la obesidad y las enfermedades raras de tipo esquelético. Además, se describen ensayos que permiten evaluar estos efectos beneficiosos.

En la **PARTE EXPERIMENTAL** se incluyen todos los trabajos que han sido llevados a cabo a lo largo del periodo de formación predoctoral y que está dividida a su vez en dos bloques. El ***Bloque 1*** está enfocado en la obtención, determinación y purificación de los compuestos fenólicos contenidos en las matrices vegetales contenidas en los distintos trabajos. Y el ***Bloque 2***, por su parte, está dirigido a la determinación de la bioactividad de estos compuestos.

Dentro del ***Bloque 1***, el ***Capítulo 1*** trata de la obtención de extractos ricos en compuestos fenólicos a partir de corteza de *S. birrea* mediante distintos sistemas de extracción tanto convencionales (SLE) como no convencionales (PLE y SFE) y, además, usando disolventes GRAS (agua y etanol). La identificación tentativa de los compuestos fenólicos presentes en cada uno de los extractos obtenidos

se llevó a cabo mediante la plataforma analítica HPLC-ESI-TOF-MS, encontrando compuestos que no habían sido descritos previamente en esta matriz. El ***Capítulo 2*** se realizó en colaboración con la Unitat de Recerca Biomèdica de la Universidad Rovira i Virgili en Reus (Tarragona, España), y se centra en el estudio de los compuestos fenólicos con capacidad antioxidante y antiinflamatoria contenidos en las hojas y flores del cardo *E. bourgatii*. La obtención de los extractos se llevó a cabo mediante SPE y la composición de su fracción fenólica fue determinada por primera vez mediante HPLC-ESI-QTOF-MS/MS. Siguiendo con la búsqueda de compuestos bioactivos, esta misma herramienta analítica fue utilizada en los ***Capítulos 3*** y ***4*** para la identificación de polifenoles, sobre todo proantocianidinas, de extractos comerciales de corteza de pino, té verde, semilla de uva y cacao. Además, se evaluó el contenido de polifenoles y flavan-3-oles totales por los métodos Folin-Ciocalteu y Vainillina, respectivamente, así como su capacidad antioxidante por los métodos FRAP, TEAC y ORAC. En el caso de la semilla de uva y el cacao (***Capítulo 4***), además se realizaron ensayos de capacidad antiinflamatoria en colaboración con la Unitat de Recerca Biomèdica de la Universidad Rovira i Virgili en Reus (Tarragona, España), con el objeto de evaluar la relación estructura-actividad de las procianidinas típicas del cacao y de las proantocianidinas con derivados de ácido gálico, más típicas de la uva y del té verde. Ya los ***Capítulos 5*** y ***6*** se centraron más en el estudio de distintas metodologías para la purificación de los compuestos fenólicos del cacao. Técnicas como la extracción en fase sólida, la HPLC semi-preparativa o el uso de membranas de micro-, ultra- y nano-filtración han servido para obtener extractos con un número más reducido de compuestos hasta llegar a fracciones purificadas. En el caso de la separación por membranas, esta metodología se llevó a cabo en colaboración con el Instituto de Biotecnología de la Universidad de Sfax (Túnez). Los compuestos contenidos en cada uno de los extractos obtenidos se determinaron mediante HPLC-ESI-TOF-MS y se evaluó su toxicidad para posteriores ensayos de bioactividad.

El ***Bloque 2*** se centra en el estudio de la bioactividad de los compuestos fenólicos. Para ello, los ***Capítulos 7*** y ***9*** ofrecen una amplia visión de los efectos beneficiosos para la salud que ofrecen los compuestos fenólicos contenidos en *L. citriodora* (***Capítulo 7***) y las proantocianidinas (***Capítulo 9***)

características de matrices como la corteza de marula, el té verde, la corteza de pino, la semilla de uva y el cacao. Siguiendo en la línea de la determinación de la bioactividad, el ***Capítulo 8*** recoge los resultados de un estudio realizado en colaboración con el Instituto de Biología Molecular y Celular (IBMC) de la Universidad Miguel Hernández de Elche (Alicante, España), donde se evalúa el efecto de los compuestos fenólicos purificados por HPLC semi-preparativa de *L. citriodora* en la obesidad a través de la modulación de la AMPK, siendo el verbascósido el que presentó mayor capacidad de activación. Algunos de los compuestos identificados, como la diooflavona o el iridoide shanzisido metil éster entre otros, fueron encontrados por primera vez en esta planta. El ***Capítulo 10***, que fue realizado en colaboración con la Unitat de Recerca Biomèdica de la Universidad Rovira i Virgili en Reus (Tarragona, España), se centra la atención en la evaluación del efecto de compuestos fenólicos que pertenecen a distintas familias en modelos de oxidación y de inflamación, para comprobar si se producen interacciones en el metabolismo energético. Para ello, se seleccionaron extractos de cacao, por ser rico en monómeros y polímeros de flavan-3-oles, y de *L. citriodora* por ser rica en fenilpropanoides, iridoides y otros flavonoides distintos a los presentes en el cacao, y se utilizó la herramienta analítica GC-EI-QTOF-MS para hacer el seguimiento de los intermediarios. Se vio que el cacao producía efecto parcial reparador en modelos de oxidación y la hierbaluisa en modelos de inflamación, aunque ambos extractos demostraron efectos beneficiosos en los dos modelos. En el ***Capítulo 11*** se recoge toda la información contenida en una patente realizada entre la Universidad de Granada y el Institut National de la Santé et de la Recherche Médicale (INSERM) de París (Francia) que describe el efecto de la (-)-epicatequina contenida en una fracción purificada de *T. cacao* para el tratamiento o la prevención de enfermedades relacionadas con el receptor tirosina quinasa como puede ser la acondroplasia.

Por último, parte de los trabajos experimentales realizados en los **Capítulos 10** y **11** se desarrollaron en diferentes estancias realizadas en la Unitat de Recerca Biomèdica de la Universidad Rovira i Virgili en Reus (Tarragona, España) y en el Institute Imagine/INSERM de París (Francia), respectivamente.

SUMMARY

The present doctoral dissertation encompasses different analytical approaches for identification, isolation and evaluation of bioactivity of phenolic compounds from natural sources. In order to describe them, this manuscript has been divided into two main parts: introduction and experimental section.

The ***INTRODUCTION*** presents noteworthy information concerning the relationship between food and health along the years and the main features of plants under study (*Sclerocarya birrea* (marula), *Eryngium bourgatii*, *Camellia sinensis, Pinus pinaster, Vitis vinifera, Theobroma cacao* and *Lippia citriodora* (lemon verbena)). Afterwards, chemical structure and classification of phenolic compounds are described as well as the different stages of an analytical procedure for obtaining, isolating and determining phenolic compounds used in the experimental section. Finally, their role as bioactive compounds is also included in this part, focusing on their antioxidant and antiinflamatory activities and their relationship with different diseases as obesity and skeletal disorders.

The **EXPERIMENTAL PART** includes all studies that has been carried out during the PhD and is sub-divided in two sections. ***Section 1*** focuses on the analytical procedure compiling obtainment, determination and isolation of phenolic compounds from selected vegetables. And, ***Section 2*** provides an extensive information about methods for bioactivity evaluation of these compounds.

In ***Section 1***, ***Chapter 1*** concerns the obtainment of phenolic-enriched extracts, suited for food applications, from *S. birrea* bark by conventional (SLE) and non-conventional (PLE and SFE) extraction systems and, in addition, using GRAS solvents (water and ethanol). The tentatively characterization of the phenolic compounds from each extract was carried out by HPLC-ESI-TOF-MS, where we found compounds which were identified for first time in marula bark. ***Chapter 2*** deals with the characterization of phenolic compounds with antioxidant and anti-inflammatory activities from *E. bourgatii* (leaves and inflorescences). The extraction process was carried out by SPE and this was the

first phenolic composition identification of *E. bourgatii* by HPLC-ESI-QTOF-MS/MS. This work was conducted in collaboration with the Biomedical Research Centre of Rovira i Virgili University from Reus (Spain). As a continuation of the search of bioactive phenolic compounds plant sources, the same analytical approach for identification of polyphenols, above all proanthocyanidins, from pine bark, green tea, grape seeds and cocoa commercial extracts, was applied in ***Chapters 3*** and ***4***. Moreover, the total phenolic compounds and flavan-3-ols were determined by Folin-Ciocalteu and Vanillin assays respectively, as well as the antioxidant capacity by FRAP, TEAC and ORAC assays. In case of grape seed and cocoa (***Chapter 4***), anti-inflammatory assays were also carried out, in collaboration with the Biomedical Research Centre of Rovira i Virgili University from Reus (Spain), in order to study the structure-activity relationship of procyanidins commons in cocoa and galloyl-derived proanthocyanidins from grape seeds or green tea. In ***Chapters 5*** and ***6***, different approaches for isolation such as solid phase extraction, semi-preparative HPLC or micro-, ultra- and nano-filtration membranes, were performed in order to obtain extracts with different phenolic composition and purity from *T. cacao*. In case of membrane separation, this methodology was performed in collaboration with Biotechnology Institute of Sfax University (Tunisia). This characterization was made by HPLC-ESI-TOF-MS and their toxicity was evaluated for subsequent bioactivity assays.

Section 2 deals with the study of bioactivity of phenolic compounds in selected plants. For this reason, ***Chapters 7*** and ***9*** provide a large perspective about beneficial effects on health of phenolic compounds from *L. citriodora* (***Chapter 7***) and common proanthocyanidins (***Chapter 9***) from marula, green tea, pine bark, grape seeds and cocoa. In this way, ***Chapter 8*** encompasses the results of a work, which was prepared in collaboration with the Institute of Molecular and Celular Biology (IBMC) of Miguel Hernández University from Elche (Spain), where the effect of purified phenolic compounds by semi-preparative HPLC from lemon verbena extract was evaluated in obesity model though the AMPK modulation. In this case, verbascoside presented the highest capacity of AMPK activation. Some of the identified phenolic compounds as diooflavone and an iridoid shanziside methyl ester, among others, were detected for first time in this extract. ***Chapter 10***, which was performance in collaboration with

the Biomedical Research Centre of Rovira i Virgili University from Reus (Spain), is focused on the evaluation of the effect of phenolic compounds from different families related with their chemical structures in oxidation and inflammatory models. The aim of this chapter was to study the interactions in the intermediates of energy metabolism. For this purpose, *T. cacao* and *L. citriodora* extracts were selected for being procyanidins monomers and polymers enriched in case of cocoa and phenylpropanoids, iridoids and other flavonoids enriched in case of lemon verbena. As an analytical tool, GC-EI-QTOF-MS was applied for monitoring the intermediates. Interestingly, it was noticed that *T. cacao* provided best results in oxidation model and *L. citriodora* in inflammation one, although both extracts showed positive effects in these two scenarios. ***Chapter 11*** compiles all data from a patent in agreement between University of Granada and National Institute of Biomedical Research and Human Health INSERM from Paris (France). This study describes the effect of (-)-epicatechin of purified fraction from T. cacao in the prevention or treatment of tyrosine-kinase receptor related disorders as achondroplasia.

Lastly, the studied in **Chapters 10** and **11** were partially conducted during different stays in the Biomedical Research Centre of Rovira i Virgili University from Reus (Spain) and Institute Imagine/INSERM from Paris (France), respectively.

Objetivos

OBJETIVOS

La búsqueda de extractos naturales de origen vegetal ricos en compuestos bioactivos como son los polifenoles y que pueden actuar como mejora o tratamiento de diversas enfermedades es un campo de la Ciencia muy activo en estos últimos años.

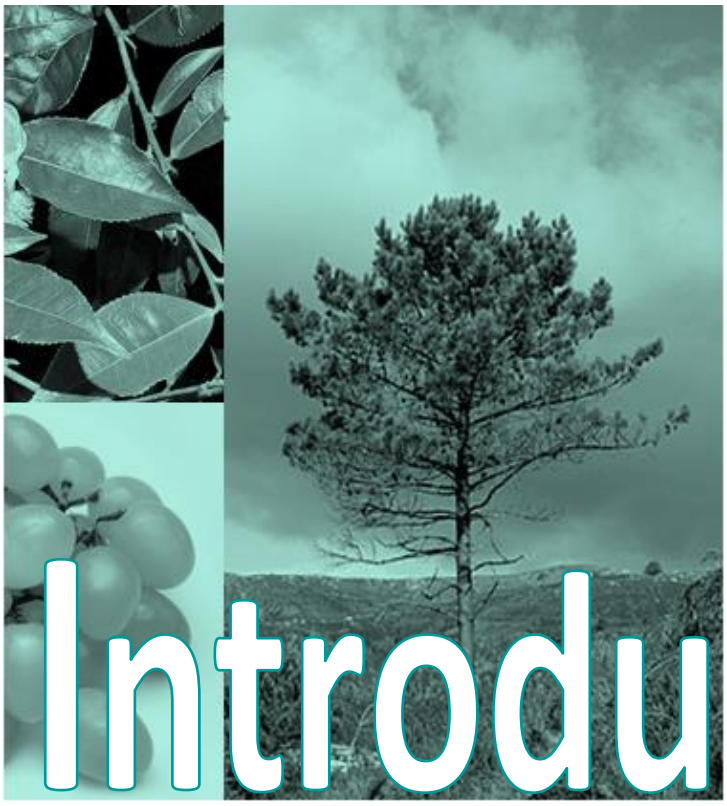
Por todo ello, el **objetivo global** de esta tesis doctoral ha sido obtener extractos enriquecidos en compuestos fenólicos a partir de diversas fuentes de origen vegetal como *Sclerocarya birrea*, *Eryngium bourgatii*, *Camellia sinensis, Pinus pinaster, Vitis vinifera, Theobroma cacao* y *Lippia citriodora*, mediante técnicas de extracción y purificación alternativas que permitan obtener estos compuestos a partir de nuevas tecnologías más respetuosas con el medio ambiente. Todo esto combinado con una caracterización detallada de los extractos y de las fracciones purificadas haciendo uso de técnicas separativas de alta resolución acopladas a espectrometría de masas como las plataformas analíticas HPLC-ESI-QTOF/TOF o GC-EI-QTOF. Para, finalmente, evaluar su bioactividad mediante ensayos tanto *in vitro* como *ex vivo* en modelos de oxidación e inflamación y de patologías relacionadas con la obesidad y displasias óseas.

Para la consecución de este ambicioso objetivo, se han establecido una serie de **objetivos específicos** que se describen a continuación:

- ✓ Obtener compuestos fenólicos de diferentes matrices de origen vegetal mediante técnicas de extracción convencionales (extracción sólido-líquido) y no convencionales (fluidos supercríticos y presurizados) para conseguir extractos ricos en estos compuestos que puedan presentar distintas actividades beneficiosas.
- ✓ Purificar los compuestos obtenidos mediante distintas tecnologías como la extracción en fase sólida, cromatografía líquida de alta resolución semi-preparativa y el uso de membranas de micro-, ultra- y nano-filtración, lo que permitirá el estudio más individualizado de la relación estructura-actividad.

- ✓ Caracterizar pormenorizadamente los compuestos contenidos en los extractos o en las fracciones purificadas mediante el uso de la cromatografía líquida de alta resolución acoplada a espectrometría de masas o mediante resonancia magnética nuclear.

- ✓ Estudiar la bioactividad de los extractos obtenidos o de las fracciones purificadas en modelos de oxidación e inflamación, así como evaluar su efecto en ciertas patologías como la obesidad o enfermedades raras de tipo esquelético.



Introducción

1. Alimentos y salud

La relación entre la **dieta** y la **salud** ya era bien reconocida desde la antigüedad. La utilización de las plantas como herramientas para la curación de diferentes trastornos de salud está ampliamente documentada desde la antigüedad en todas las culturas. En el libro más famoso y antiguo de la medicina tradicional China, el Huang Di Nei Jing, el canon interno de la medicina china del Emperador Amarillo que data de entre los siglos III y I a.C., se pueden encontrar descripciones de las propiedades curativas de algunas frutas y hortalizas. Su importancia es comparable a la que los tratados hipocráticos poseen para la medicina de Occidente. Por otro lado, los conocimientos sobre las plantas medicinales y el uso con finalidades terapéuticas de algunas plantas no alimenticias, tales como plantas aromáticas y arbustivas, se encuentra referido en algunas tablillas sumerias y mesopotámicas (**Figura 1**). La tablilla sumeria de Nippur (3000 a.C.) hace referencia a la preparación de extractos de peral, abeto, higuera o palmera entre otros, con finalidades médicas. La obra no concluida de farmacología china más antigua es el compendio titulado "Pen Tsao kang-mou" (2697 a.C.) que iba componiéndose con el transcurso de los siglos, basándose en la creencia de que los remedios para cualquier mal se encontraban ocultos en la naturaleza. Así, también integraban la lista sustancias desconocidas o no estudiadas a las cuales les asignaban un remedio todavía por descubrir, pero que en base a sus creencias debería existir. Ejemplo de esto, era la raíz mágica del Ginseng, cuyos efectos medicinales estaban todavía por demostrar, y que los europeos llegaron a valorar como una droga milagrosa, capaz de curarlo todo. Ya en la época clásica, Hipócrates ("*Que tu alimento sea tu medicina, y tu medicina sea tu alimento*" 400 a.C.) hizo referencia a la "*vix medicatrix naturae*" -la

Figura 1. Papiro de Ebers (1700 a.C.) donde los egipcios citan unas 700 plantas con fines medicinales.

naturaleza sana por sí misma- y posteriormente Discórides en el siglo I d.C. en su trabajo "De materia medica" describió unas 600 plantas usadas en tratamientos médicos. Posteriormente se han descrito numerosos textos que recogen la relación entre la alimentación y la salud y que llegan hasta nuestros días. No fue hasta los siglos XVIII y XIX cuando se dio lugar a un gran avance de estas ciencias. A comienzos del siglo XIX surgió la idea de usar un compuesto químicamente puro obtenido a partir de una planta como terapéutico. El hecho de que el farmacéutico alemán Friedrich W. A. Sertürner hiciera la primera separación de la morfina marcó una nueva era en el uso de las plantas medicinales y propició el nacimiento de una nueva línea de investigación encaminada en la extracción, purificación e identificación de los compuestos químicos que eran responsables de sus actividades beneficiosas (1). A finales de 1930, la alimentación y más concretamente los constituyentes de los alimentos habían comenzado a llamar la atención de los consumidores y de la industria alimentaria. Esto hizo que se abriera un nuevo campo de estudio para encontrar una explicación científica a la relación entre los alimentos y la salud que ha ido creciendo hasta nuestros días y que ha dado lugar a numerosos estudios en los últimos años (2–8).

Además, factores como el deseo de una mejor calidad de vida, reducir el coste sanitario como consecuencia del aumento de la esperanza de vida, sumado a un mayor y mejor concepto de la relación dieta-salud, han hecho que el concepto de una buena alimentación cambie sustancialmente.

1. Schmitz R. Friedrich Wilhelm Sertürner and the Discovery of Morphine. *Pharm Hist*. 1985;27(2):61–74.

2. Panagiotakos DB, Notara V, Kouvari M, Pitsavos C. The Mediterranean and other Dietary Patterns in Secondary Cardiovascular Disease Prevention: A Review. *Curr Vasc Pharmacol*. 2016;14(5):442–51.

3. Atkins JL, Wannamethee SG. Diet Quality and Cardiovascular Disease Prevention. In: *Preventive Nutrition*. Cham: Springer International Publishing; 2015. p. 245–54.

4. Langhans W. Food Components in Health Promotion and Disease Prevention. *J Agric Food Chem*. 2017.

5. Eilat-Adar S, Sinai T, Yosefy C, Henkin Y. Nutritional recommendations for cardiovascular disease prevention. Vol. 5, *Nutrients*. 2013. 3646-3683 p.

6. Barnard ND, Bush AI, Ceccarelli A, Cooper J, de Jager CA, Erickson KI, et al. Dietary and lifestyle guidelines for the prevention of Alzheimer's disease. *Neurobiol Aging*. 2014;35(SUPPL.2):S74–8.

7. Jones PJ, Jew S. Functional food development: concept to reality. *Trends Food Sci Technol*. 2007;18(7):387–90.

8. Abuajah CI, Ogbonna AC, Osuji CM. Functional components and medicinal properties of food: a review. *J Food Sci Technol*. 2015;52(5):2522–9.

De esta forma, la población puede mejorar su dieta mediante el consumo de alimentos formulados o fortificados con ingredientes funcionales que proporcionen efectos beneficiosos para la salud (9).

El término **alimento funcional** fue propuesto por primera vez en Japón en la década de los 80 con la publicación de la reglamentación para los "Alimentos para uso específico de salud" ("Foods for specified health use" o FOSHU) que surgió como una ayuda para reducir los costes sanitarios que derivaron de los efectos de la II Guerra Mundial en la salud de la población. La definición con mayor aceptación es la emitida por el International Life Sciences Institute en Europa (ILSI) en el año 1999 que establece que "un alimento funcional puede ser considerado como funcional si se ha demostrado de manera satisfactoria que posee un efecto beneficioso sobre una o varias funciones específicas en el organismo, más allá de los efectos nutricionales habituales, siendo esto relevante para la mejoría de la salud y el bienestar y/o la reducción del riesgo de enfermar" (10). La expansión de los alimentos funcionales fue inmediata y alcanzó los Estados Unidos. El papel de la Food and Drug Adminsitration (FDA) ha sido decisivo para que exista un marco legal que verifique la seguridad alimentaria de los productos ofertados. En lo que a Europa se refiere, la introducción de los alimentos funcionales no ha estado exenta de polémica debido a la falta de regulación específica. Después de un largo proceso de consulta se aprobó el Reglamento (CE) número 1924/2006 del Parlamento Europeo y del Consejo relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos (11).

Por otra parte, es importante no confundir alimento funcional con otros conceptos como nutracéutico, complemento alimenticio, aditivo, etc. El término **nutracéutico** (nutrición + farmacéutico) fue acuñado por el Dr. Stephen DeFelice en 1989 y se define como "un alimento, o parte

9. Perez-Gregorio R, Simal-Gandara J. A Critical Review of Bioactive Food Components, and of their Functional Mechanisms, Biological Effects and Health Outcomes. *Curr Pharm Des*. 2017;23(19). 2731-2741.

10. Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Roberfroid MB. Scientific Concepts of Functional Foods in Europe Consensus Document. *Br J Nutr*. 1999;81(4):S1–27.

11. UE. Reglamento (CE) No 1924/2006 del parlamento europeo y del consejo del 20 de diciembre de 2006 relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos. 2006;9–25.

de este, que proporciona beneficios en la salud ya sea en la prevención o en el tratamiento de enfermedades". Estos productos pueden ir desde nutrientes aislados hasta alimentos procesados (12). Ambas definiciones pueden resultar ambiguas, por lo que la tendencia actual es considerar un nutracéutico como un suplemento dietético que proporciona una forma concentrada de un agente bioactivo proveniente de un alimento, presentado en una matriz no alimenticia y utilizado para incrementar la salud en dosis que exceden a aquellas que pudieran ser aportadas por la dieta normal (13). A pesar de las diferencias que puedan existir entre ambos conceptos, el primer paso en su desarrollo es común y se fundamenta en la interacción entre uno y varios componentes de un alimento y una función beneficiosa para la salud.

2. Matrices vegetales como fuentes de compuestos bioactivos

Los metabolitos secundarios de las plantas representan una fuente importante de compuestos bioactivos utilizados en el ámbito de la investigación para el desarrollo de nuevos ingredientes funcionales en el campo de la industria agroalimentaria o de nuevos fármacos en la industria farmacéutica, de hecho numerosos principios activos usados actualmente son directa o indirectamente derivados de las plantas (14–17).

Es por ello que en la presente tesis doctoral se ha querido estudiar el potencial de las siguientes plantas (o partes de ellas), tanto desde un punto de vista analítico como de bioactividad.

12. DeFelice S. The nutraceutical revolution : its impact on food industry R & D. *Trends Food Sci Technol*. 1995;6(2):59–61.

13. Olaiya CO, Soetan KO, Esan AM. The role of nutraceuticals, functional foods and value added food products in the prevention and treatment of chronic diseases. *African J Food Sci*. 2016;10(10):185–93.

14. Teoh ES. Secondary Metabolites of Plants. In: *Medicinal Orchids of Asia*. Cham: Springer International Publishing; 2016. p. 59–73.

15. Balandrin MF, Klocke JA, Wurtele ES, Bollinger WH. Natural plant chemicals: sources of industrial and medicinal materials. *Science*. 1985;228(4704):1154-1160.

16. Crozier A, Yokota T, Jaganath IB, Marks S, Saltmarsh M, Clifford MN, et al. Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet. 2006. 208 p.

17. Hussain MS, Fareed S, Ansari S, Rahman MA, Ahmad IZ, Saeed M. Current approaches toward production of secondary plant metabolites. *J Pharm Bioallied Sci*. 2012;4(1):10–20.

2.1. *Sclerocarya birrea*

La marula, ***Sclerocarya birrea***, es un árbol de hoja caduca de tamaño medio a grande que puede alcanzar hasta 18 m y crece en África sobre terrenos arenosos. Perteneciente a la familia *Anacardiaceae* tiene el tronco recto de un solo tallo con la corteza manchada de gris y la copa redondeadas. Sus hojas, de unos 60 mm, son compuestas y crecen agrupadas en el extremo de las ramas (**Figura 2**). Las flores masculinas y femeninas florecen de septiembre a noviembre al sur del ecuador (18).

Figura 2. Árbol y detalle del tronco de *S. birrea*.

De entre los usos con los que cuenta el árbol, se pueden comer el fruto o sus productos derivados como bebidas que son ricos en vitamina C y minerales (19). Con respecto a las hojas, las larvas de la conocida mariposa verde africana se alimentan de estas. Además, dos tipos de orugas que sirven de alimento a poblaciones locales viven en la marula alimentándose de sus hojas. En algunas zonas de África, la corteza tiene usos antiinflamatorios y analgésicos y, además, frente a la disentería, diarrea, el reumatismo e incluso profilaxis frente a la malaria (20).

18. Ojewole JAO, Mawoza T, Chiwororo WDH, Owira PMO. *Sclerocarya birrea* (A. Rich) hochst. ['Marula'] (Anacardiaceae): a review of its phytochemistry, pharmacology and toxicology and its ethnomedicinal uses. *Phytother Res*. 2010;24(5):633-9.

19. Borochov-Neori H, Judeinstein S, Greenberg A, Fuhrman B, Attias J, Volkova N, et al. Phenolic Antioxidants and Antiatherogenic Effects of Marula (*Sclerocarrya birrea* Subsp. caffra) Fruit Juice in Healthy Humans. *J Agric Food Chem*. 2008;56(21):9884–91.

20. Mariod AA, Abdelwahab SI. *Sclerocarya birrea* (Marula), An African Tree of Nutritional and Medicinal Uses: A Review. *Food Rev Int*. 2012;28(4):375–88.

2.2. *Eryngium bourgatii*

El género ***Eryngium***, perteneciente a la familia *Apiaceae*, incluye unas 250 especies de plantas anuales y perennes, generalmente espinosas, que tienen una distribución generalizada por todo el mundo, principalmente en Eurasia, el norte de África, Sudamérica y Australia. Muchas especies tienen un largo historial de uso en la medicina tradicional para la preparación de remedios diuréticos, estimulantes del apetito, laxantes o antiinflamatorios (21).

Figura 3. Vista de la planta (izquierda) con detalle de la inflorescencia (derecha superior) y de hojas basales (derecha inferior)

Eryngium bourgatii, también conocido como cardo azul, es una planta herbácea espinosa perenne, de hasta 50 cm (**Figura 3**). Generalmente de color azul, al menos en los tallos y la inflorescencia. Su hábitat frecuente son pedregales de montañas. En España se puede encontrar en los Pirineos y en el Sistema Central entre otras zonas montañosas. Florece a final de primavera y en el verano.

Su fracción de aceite esencial ha sido estudiada y es rica en el diterpeno filocladan (22) destacado por su potencial antimicrobiano (23), aunque también contiene monoterpenos, cumarinas o ácidos fenólicos entre otros (21). Además, en sus raíces se han encontrado acetilenos como falcarinol, falcarinona o falcarinolona (24).

21. Wang P, Su Z, Yuan W, Deng G, Li S. Phytochemical constituents and pharmacological activities of Eryngium L.(Apiaceae). *Pharm Crop*. 2012;99–120.

22. Palá-Paúl J, Pérez-Alonso MJ, Velasco-Negueruela A, Vadaré J, Villa AM, Sanz J, et al. Essential oil composition of the different parts of *Eryngium bourgatii* Gouan from Spain. *J Chromatogr A*. 2005;1074(1–2):235–9.

23. Moiteiro C, Esteves T, Ramalho L, Rojas R, Alvarez S, Zacchino S, et al. Essential oil characterization of two Azorean *Cryptomeria japonica* populations and their biological evaluations. *Nat Prod Commun*. 2013;8(12):1785–90.

24. Lam J, Christensen L, Thomasen T. Acetylenes from roots of *Eryngium bourgatii*. *Phytochemistry*. 1992;31(8):2881-2882.

2.3. *Camellia sinensis*

La planta del té, ***Camellia sinensis***, procede del sur de China y sudeste de Asia, aunque hoy en día se cultiva en todo el mundo. Es un arbusto o árbol pequeño (1-9 m) perenne que posee una fuerte raíz principal. Las hojas son glabras y de bordes serrados, y las flores, axilares de color blanco-amarillento, pueden presentarse en solitario o en grupo de tres (**Figura 4**).

Figura 4. Detalle de las flores (izquierda) y hojas (derecha) del árbol de té.

El **té verde** es un tipo de té que no ha sufrido una oxidación durante su procesado puesto que las hojas se recogen frescas. Aproximadamente el 20-22 % del té producido y consumido es verde. Este tipo de té se consume principalmente en China, Japón y norte de África. El té ha sido utilizado como bebida medicinal para promover la salud en mente y cuerpo durante cinco milenios. La más antigua referencia de su consumo como ayuda para la salud data del año 2737 a.C. de parte de Shénnóng, segundo emperador de China (**Figura 5**).

Figura 5. El emperador Shénnóng junto al árbol de té.

En la actualidad existen numerosas evidencias de que el té verde es una fuente de compuestos que presentan numerosos efectos beneficiosos en la

salud humana como antioxidante, antiinflamatorio, anticancerígeno o protector frente al fotoenvejecimiento de la piel, entre otros (25–28). La composición química de sus hojas está ampliamente documentada siendo los polifenoles el grupo de compuestos mayoritario. Los efectos más beneficiosos del té verde están asociados a este grupo de compuestos, principalmente a las catequinas, que se corresponden con un 25-35% del peso seco de las hojas (29).

2.4. *Pinus pinaster*

Figura 6. *Pinus pinaster* (derecha) y detalle de la corteza (izquierda).

El pino marítimo (***Pinus pinaster***) es una especie invasora arbórea de la familia de las pináceas que se extiende por la zona mediterránea. Generalmente crece entre el nivel del mar y unos 800 m, aunque se ha llegado a encontrar a 2000 m. Es de tamaño mediano que puede alcanzar de 20 a 35 m de altura, con 1,2 m de tronco de corteza rojo anaranjada, gruesa y muy agrietada (**Figura 6**).

Usos muy diversos le han sido atribuidos a la **corteza de pino.** Indios nativos de Quebec introdujeron el té de corteza de pino al explorador francés Jacques Cartier y a su tripulación (año 1534), ya que resultaba ser un maravilloso remedio para prevenir el escorbuto. Fascinado por esta información, el profesor Jack Masqulier, que trabajaba con bioflavonoides contra esta enfermedad, determinó que el extracto de corteza de pino era rico en estos compuestos (30).

25. Roh E, Kim J-E, Kwon JY, Park JS, Bode AM, Dong Z, et al. Molecular mechanisms of green tea polyphenols with protective effects against skin photoaging. *Crit Rev Food Sci Nutr*. 2017;57(8):1631–7

26. Bravi F, La Vecchia C, Turati F. Green tea and liver cancer. *HepatoBiliary Surg Nutr*. 2017;6(2):127–9.

27. Cabrera C, Artacho R, Giménez R. Beneficial Effects of Green Tea - A Review. *J Am Coll Nutr*. 2006;25(2):79–99.

28. Nogueira L de P, Nogueira Neto JF, Klein MRST, Sanjuliani AF. Short-term Effects of Green Tea on Blood Pressure, Endothelial Function, and Metabolic Profile in Obese Prehypertensive Women: A Crossover Randomized Clinical Trial. *J Am Coll Nutr*. 2017;36(2):108–15.

29. Namal Senanayake SPJ. Green tea extract: Chemistry, antioxidant properties and food applications – A review. *J Funct Foods*. 2013;5(4):1529–41.

30. Maimoona A, Naeem I, Saddiqe Z, Jameel K. A review on biological, nutraceutical and clinical aspects of French maritime pine bark extract. *J Ethnopharmacol*. 2011;133(2):261–77.

Durante los últimos años, numerosas investigaciones se han centrado en el estudio de la composición química y de las propiedades farmacológicas que presentan esos compuestos, siendo los mayoritarios las procianidinas que gracias a su alto poder antioxidante, han demostrado efectos beneficiosos en enfermedades (31–34).

2.5. *Vitis vinifera*

La vid (***Vitis vinífera***) es una planta semileñosa y/o trepadora que en su forma silvestre puede alcanzar más de 30 m, pero que bajo el efecto de la poda humana queda reducida a un pequeño arbusto de 1 m. Se estima que los primeros sucesos de domesticación se produjeron hace más de 8000 años. Se cree originaria del suroeste de Asia y del centro y suroeste de Europa y, actualmente, se extiende por países de climas templados. Posee sarmientos flexibles y muy engrosados en los nudos sobre los que se disponen hojas grandes de estípulas caducas. Las flores son hermafroditas reunidas en panículas laterales opuestas a las hojas (**Figura 7**). Sus frutos, de tipo baya, crecen agrupados en racimos de entre 6 y 300 uvas (**Figura 8**) y pueden ser negras, moradas, amarillas, rosadas, anaranjadas o verdes.

Figura 7. Detalle de la planta *Vitis vinifera*.

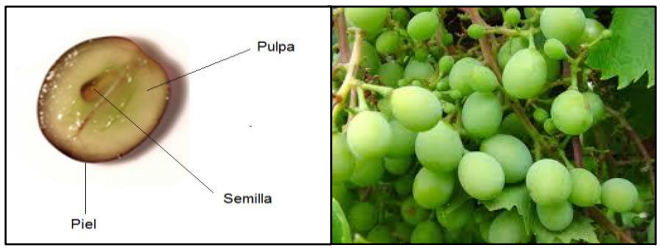


Figura 8. Detalle del fruto de la vid y esquema de las partes principales.

31. Nishioka K, Hidaka T, Nakamura S, Umemura T, Jitsuiki D, Soga J, et al. Pycnogenol, French Maritime Pine Bark Extract, Augments Endothelium-Dependent Vasodilation in Humans. *Hypertens Res*. 2007;30(9):775–80.

32. Karonen M, Loponen J, Ossipov V, Pihlaja K. Analysis of procyanidins in pine bark with reversed-phase and normal-phase high-performance liquid chromatography–electrospray ionization mass spectrometry. *Anal Chim Acta*. 2004;522(1):105–12.

33. Liu X, Wei J, Tan F, Zhou S, Würthwein G, Rohdewald P. Pycnogenol®, French maritime pine bark extract, improves endothelial function of hypertensive patients. *Life Sci*. 2004;74(7):855–62.

34. Devaraj S, Vega-López S, Kaul N, Schönlau F, Rohdewald P, Jialal I. Supplementation with a pine bark extract rich in polyphenols increases plasma antioxidant capacity and alters the plasma lipoprotein profile. *Lipids*. 2002;37(10):931–4.

En la estructura de la uva se pueden distinguir dos partes claramente diferenciadas, las semillas y el conjunto de tejidos que las envuelve (pericarpo), que comúnmente se divide en hollejo (piel) y la pulpa. Estos distintos tejidos contribuyen de forma diferencial a la composición química de la planta. La pulpa contiene agua y componentes mayoritarios del metabolismo primario como azúcares (glucosa y fructosa) y ácidos orgánicos como el málico y el tartárico, aunque las proporciones varían dependiendo del estado de maduración. La **semilla de uva** es uno de los subproductos alimentarios que proporciona mayor cantidad de beneficios en la salud. El hollejo y la semilla contienen gran número de compuestos del metabolismo secundario como son los compuestos fenólicos. Entre ellos se encuentran principalmente antocianos en la piel, responsables del color de la uva, y catequinas en sus formas libres o polimerizadas en la semilla, responsables del color y la astringencia, respectivamente (35).

Hoy en día hay un creciente interés en la recuperación de compuestos bioactivos a partir de los derivados de la uva en la industria del vino. Gracias al avance de las tecnologías se hace posible un enfoque integrado y sostenible para el aprovechamiento de estos subproductos, combinando la reducción del impacto medioambiental de los residuos y la obtención de nuevos productos de gran valor para las industrias farmacéutica, cosmética y agroalimentaria (36).

2.6. *Theobroma cacao*

El árbol del cacao (***Theobroma cacao***), de la familia de las malváceas, es un árbol perenne de talla pequeña que necesita de bastante humedad y calor para crecer. Puede alcanzar de 4 a 7 m de altura si es cultivado, aunque puede llegar a medir hasta 20 m en su forma silvestre. Sus hojas son grandes, alternas, colgantes y de punta larga y sus flores aparecen insertadas sobre el tronco o las viejas ramificaciones (**Figura 9**).

35. Yilmaz Y, Toledo RT. Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid. *J Agric Food Chem*. 2004;52(2):255–60.

36. Teixeira A, Baenas N, Dominguez-Perles R, Barros A, Rosa E, Moreno D, et al. Natural Bioactive Compounds from Winery By-Products as Health Promoters: A Review. *Int J Mol Sci*. 2014;15(9):15638–78.

Figura 9. Detalle del árbol del cacao.

Es originario de la cuenca del Amazonas de donde existe evidencia de su cultivo y consumo desde hace 5500 años. Conocido como "el alimento de los dioses", este árbol presenta frutos en forma de baya grande de color amarillo o purpúrea que pesa casi 500 g cuando madura.

Entre los mayas y aztecas, el chocolate era una bebida prestigiosa, de élite, reservada a la realeza, la nobleza, los mercaderes de larga distancia, y los guerreros de alto rango. Hacia el año 450 d.C., se empezaron a colocar vasos llenos de bebidas de chocolate en las tumbas de los reyes mayas. Estos vasos presentan textos jeroglíficos en los cuales se describe el sabor particular del chocolate servido.

Figura 10. Dibujo que muestra al dios del maíz brotando de una vaina de cacao en el libro *Popol Vuh*.

Según el *Popol Vuh* maya (**Figura 10**), el cacao era considerado uno de los cuatro árboles cósmicos situados en los rumbos del universo y, además, era un fruto relacionado metafóricamente con la sangre y el sacrificio. El chocolate entró en contacto con los europeos del renacimiento, pero fue en el barroco cuando se consumía en los palacios y mansiones de los ricos y poderosos. Los españoles despojaron a la bebida de su significado espiritual, dándole propiedades puramente medicinales, un remedio al sistema humoral galénico en la Europa del Barroco. Pronto se convirtió en bebida apreciada por su sabor, por su naturaleza que aplacaba el hambre y por el estímulo que proporcionaba. Nadie sabe con certeza cuándo exactamente llegó el cacao a España. Muchos autores acreditan a Hernán Cortés su introducción, pero no hay base histórica para ello. La primera oportunidad en que pudo hacerlo fue en 1519 antes de haber estado en Tenochtitlán. La primera evidencia documentada de la aparición del cacao en España es con los mayas

Kekchis que fueron llevados por los dominicos ante el príncipe Felipe, más tarde Rey Felipe II, en agradecimiento por su magnanimidad, en 1544 (37).

La principal utilidad del fruto del cacao es la producción de polvo y/o grasa o manteca de cacao, ambos utilizados fundamentalmente para la producción de chocolate. Este producto es consumido en muchos países y culturas como por ejemplo en Alemania, donde el chocolate proporciona más del 20 % de la ingesta de flavonoides totales en adultos e incluso el porcentaje es superior en niños. En América, es considerado como el tercer mayor contribuyente de antioxidantes a la dieta con casi 110 mg/día (38). Las propiedades antioxidantes del cacao han sido ampliamente estudiadas en los últimos años. Los productos derivados de esta planta son ricos en polifenoles, principalmente procianidinias, lo que les confiere beneficios saludables. Estos compuestos son frecuentemente degradados en el procesado industrial, por lo que nuevas técnicas para la obtención de productos ricos en polifenoles son objeto de estudio (38,39).

2.7. *Lippia citriodora*

El género *Lippia*, perteneciente a la familia *Verbenaceae*, cuenta con aproximadamente 200 especies de hierbas, arbustos y pequeños árboles distribuidos principalmente por países de Centro y Suramérica, y zonas tropicales de África. La mayoría de ellas son tradicionalmente usadas como remedios gastrointestinales y respiratorios (40). ***Lippia citriodora***, comúnmente conocida como hierbaluisa, es un arbusto perennifolio que puede alcanzar los 3 m de altura, en el que sus hojas de un color verde claro por el haz aparecen agrupadas en verticilos trímeros y despiden una fuerte fragancia a limón.

37. Dillinger TL, Barriga P, Escárcega S, Jimenez M, Lowe DS, Grivetti LE. Food of the gods: cure for humanity? A cultural history of the medicinal and ritual use of chocolate. *J Nutr*. 2000;130(8S Suppl):2057S–72S.

38. Rusconi M, Conti A. *Theobroma cacao* L., the Food of the Gods: A scientific approach beyond myths and claims. *Pharmacol Res*. 2010;61(1):5–13.

39. Schinella G, Mosca S, Cienfuegos-Jovellanos E, Pasamar MÁ, Muguerza B, Ramón D, et al. Antioxidant properties of polyphenol-rich cocoa products industrially processed. *Food Res Int*. 2010;43(6):1614–23.

40. Pascual ME, Slowing K, Carretero E, Sánchez Mata D, Villar A. Lippia: Traditional uses, chemistry and pharmacology: A review. *J Ethnopharmacol*. 2001;76(3):201–14.

Florece en verano, las flores son pequeñas, rosadas, blanquecinas o violáceas, agrupadas en panículas terminales (**Figura 11**). Nativa de Suramérica, crece de forma silvestre en los países andinos desde Ecuador hasta Argentina; también en Paraguay, Uruguay y Brasil. En el siglo XVII, tras las expediciones de conquista fue llevada a Europa, donde empezó a cultivarse en las zonas templadas (41).

Figura 11. Hierbaluisa con detalle de la inflorescencia (derecha) y de la hoja (izquierda)

La **hierbaluisa** tradicionalmente se ha empleado como especia y planta medicinal en el tratamiento de asma, fiebre, desórdenes gastrointestinales y enfermedades cutáneas (42). Su fracción de aceite esencial, rica en citral y limoneno, es generalmente incorporada a formulaciones cosméticas, y sus hojas y parte superior de las flores se usan para preparar infusiones y como aromatizantes para algunos postres.

Su composición química ha sido objeto de estudio en los últimos años revelando un elevado número de compuestos polares en sus hojas, tales como derivados de ácidos hidroxicinámicos, flavonoides e iridoides glicosados, siendo el verbascósido (fenilpropanoide) el más abundante (43). Es a estos compuestos a los que se les atribuye principalmente las propiedades beneficiosas de esta planta (44).

41. Wang T, Zhang Y, Chen Y, Wang S, Dong Y, Wang T, et al. Bioactive constituents from the aerial parts of *Lippia triphylla*. *Molecules*. 2015;20(12):21946–59.

42. Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, de Lourdes Bastos M. Studies on the antioxidant activity of *Lippia citriodora* infusion: scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid. *Biol Pharm Bull*. 2002;25(10):1324–7.

43. Bilia AR, Giomi M, Innocenti M, Gallori S, Vincieri FF. HPLC-DAD-ESI-MS analysis of the constituents of aqueous preparations of verbena and lemon verbena and evaluation of the antioxidant activity. *J Pharm Biomed Anal*. 2008;46(3):463–70.

44. Alipieva K, Korkina L, Orhan IE, Georgiev MI. Verbascoside - A review of its occurrence, (bio)synthesis and pharmacological significance. *Biotechnol Adv*. 2014;32(6):1065–76.

3. Compuestos fenólicos: biosíntesis y clasificación

Los **compuestos fenólicos**, metabolitos secundarios de las plantas puesto que no son esenciales para su supervivencia en su conjunto o de alguna de sus partes, constituyen uno de los principales grupos de estos metabolitos, con una gran diversidad de estructuras químicas y funciones. Comparten un esqueleto básico común conformado por un anillo aromático con uno o más sustituyentes hidroxilo. De hecho, conforman uno de los grupos de sustancias más numeroso y ampliamente distribuidos en el reino vegetal, con más de 8000 estructuras fenólicas conocidas actualmente (45).

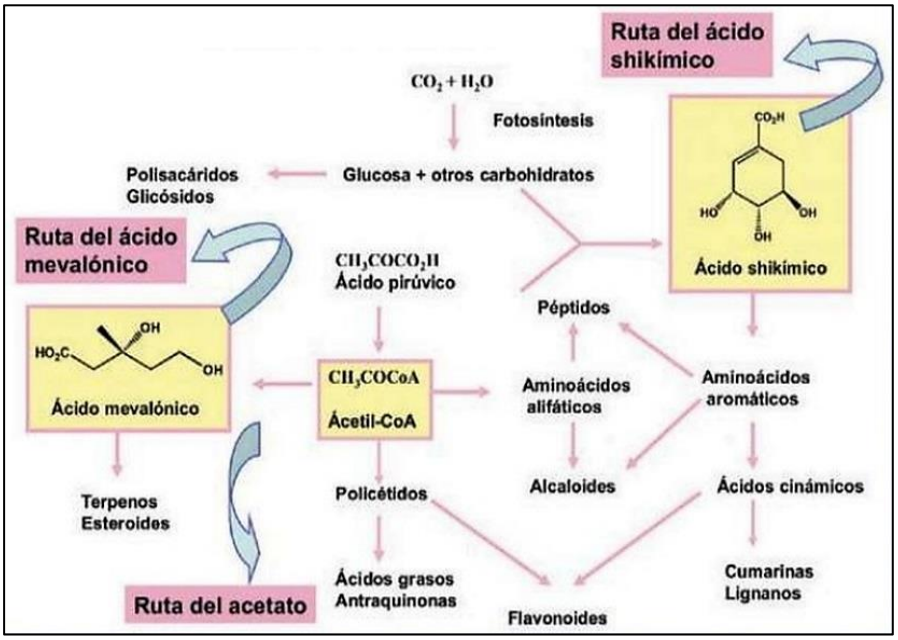


Figura 12. Esquema de las rutas biosintéticas de los compuestos fenólicos.

Surgen de dos principales rutas biosintéticas: la **ruta del ácido shikímico** y la **ruta del malonato-acetato** (**Figura 12**). Se pueden dividir en al menos 10 clases diferentes dependiendo de su estructura química básica (46).

45. Bravo L, Sources D, Significance N. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev.* 1998;56(11):317–33.

46. Turner R, Etienne N, Alonso MG, De Pascual-Teresa S, Minihane AM, Weinberg PD, et al. Antioxidant and anti-atherogenic activities of olive oil phenolics. *Int J Vitam Nutr Res*. 2005;75(1):61–70.

Estos compuestos son sintetizados por las plantas durante su desarrollo normal y cumplen una serie de funciones entre las que destacan su acción como atrayentes para la polinización y protección frente a agentes microbianos o a radiación UV. Además, también actúan en procesos de crecimiento y reproducción. Como consecuencia de alguna de estas funciones, el contenido de los compuestos puede incrementarse bajo ciertas condiciones de estrés, como por ejemplo altas temperaturas, presencia de patógenos, contaminación atmosférica o radiación UV. Otro tipo de factores externos como el tipo de cultivo, condiciones de crecimiento, proceso de maduración o condiciones de procesado y almacenamiento pueden afectar al nivel de estos compuestos en plantas o en sus productos derivados. Los compuestos fenólicos comúnmente se encuentran en verduras, frutas y otras fuentes de alimentos que forman una parte importante de nuestra dieta (47).

Entre las principales **familias de compuestos fenólicos** podemos encontrar los fenoles simples, benzoquinonas, ácidos fenólicos, ácidos fenilacéticos, acetofenonas, ácidos hidroxicinámicos, fenilpropenos, cumarinas, cromonas, naftoquinonas, xantonas, estilbenos, antraquinonas, flavonoides, lignanos, ligninos y taninos hidrosolubles. En la **Figura 13** se muestran las principales familias junto a su esqueleto básico. Dentro de los compuestos fenólicos, los **flavonoides** merecen una atención especial por ser la familia más importante y ampliamente distribuida en la naturaleza. Con más de 4000 especies identificadas, constan de 15 átomos de carbonos dispuestos en un esqueleto básico del tipo C_6-C_3-C_6 que puede sufrir modificaciones y adiciones de grupos funcionales (48), dando lugar a distintas clases (**Figura 14**). Debido a esta gran diversidad estructural, los polifenoles representan una fuente complementaria imprescindible frente a los compuestos que son sintetizados en laboratorio. Sin embargo, en la última década, los grandes cambios que han tenido lugar en el proceso de descubrimiento de nuevos medicamentos han introducido nuevos requerimientos en el campo de la investigación de los productos naturales.

47. Balasundram N, Sundram K, Samman S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem*. 2006;99(1):191–203.

48. Apak R, Güçlü K, Demirata B, Özyürek M, Çelik SE, Bektaşoğlu B, et al. Comparative Evaluation of Various Total Antioxidant Capacity Assays Applied to Phenolic Compounds with the CUPRAC Assay. *Molecules*. 2007;12(7):1496–547.

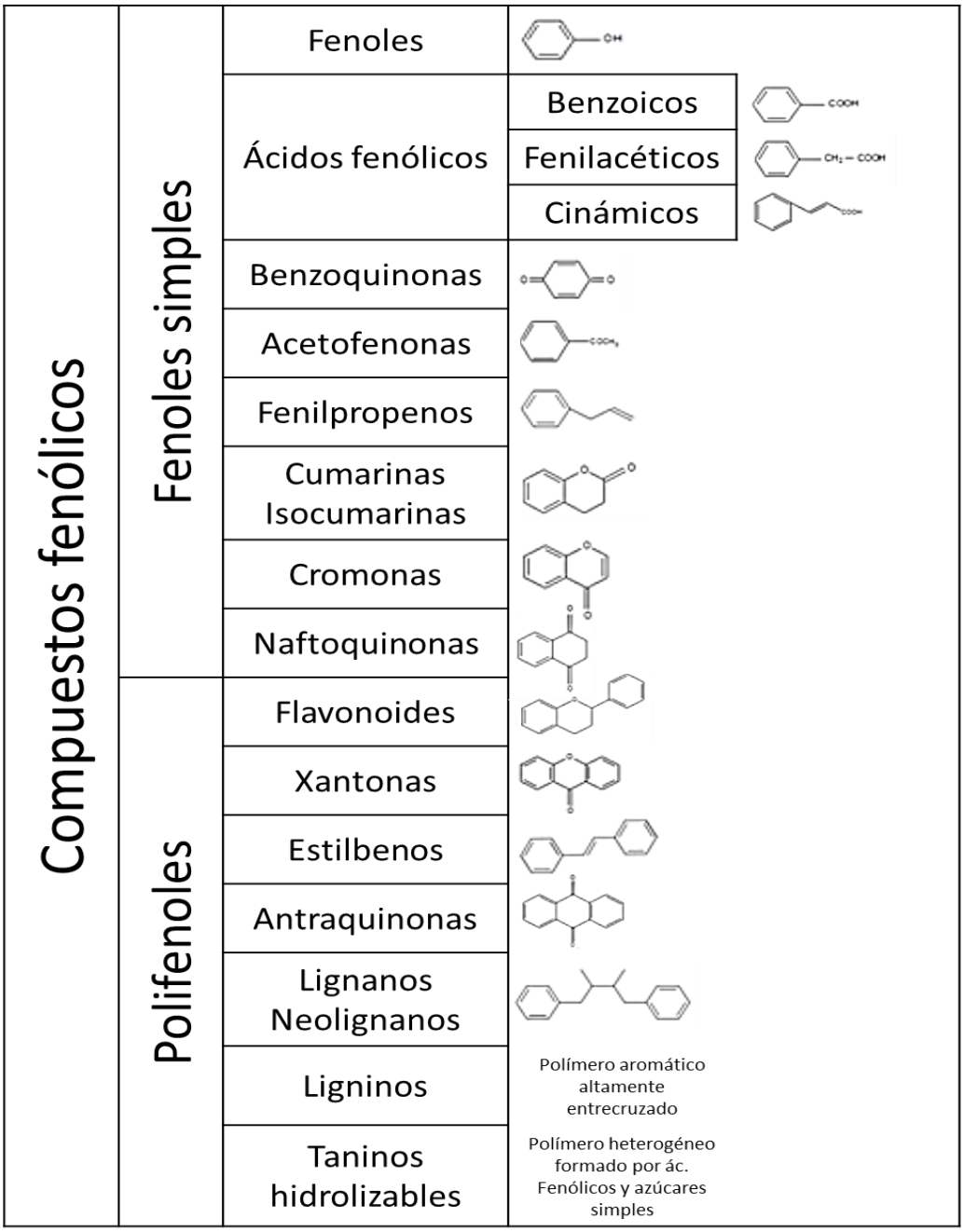


Figura 13. Clasificación de las principales familias de compuestos fenólicos

Estas nuevas tendencias van en la línea de identificar el candidato más prometedor que se encuentra contenido en una mezcla compleja y de acelerar el proceso de aislamiento o purificación de ese candidato, así como de evaluar su potencial (49,50).

49. Koehn FE, Carter GT. The evolving role of natural products in drug discovery. *Nat Rev Drug Discov*. 2005;4(3):206–20.

50. Cannell RJP, Dufresne C, Florence AJ, Lee I-S, Salituro GM, Shankland N, et al. Natural Products Isolation. In: *Methods in Biotechnology*. 1998. p. 343–408.

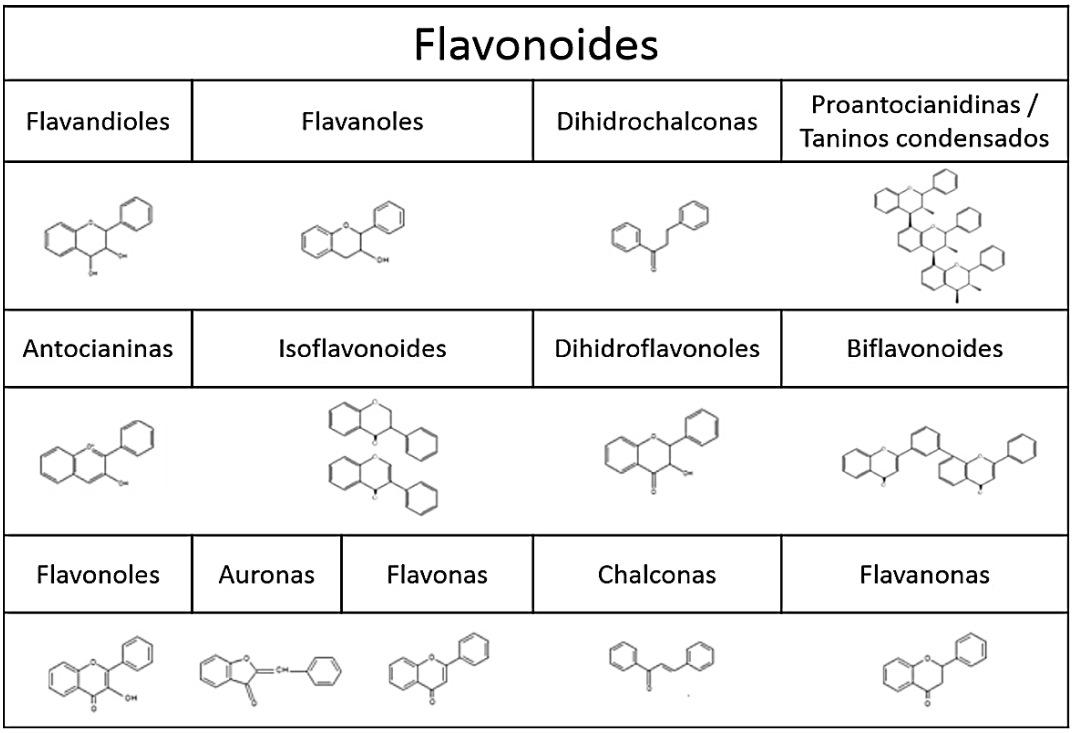


Figura 14. Clasificación de la familia de los flavonoides

4. Obtención de compuestos fenólicos: extracción y purificación

El estudio de los polifenoles ha acelerado sustancialmente su desarrollo gracias a la aparición de la fitoquímica como rama de la química y a la evolución de los estudios sistemáticos de clasificación quimiotaxonómicos, a los métodos de **extracción**, **purificación** y a los análisis de estos compuestos. Como ya se ha indicado anteriormente en esta memoria, los compuestos fenólicos constituyen un grupo muy diverso, que presenta una gran complejidad a nivel estructural. A esta complejidad se le suma el hecho de que en la naturaleza se presentan generalmente de forma conjugada, con uno o más residuos de azúcar unidos a los grupos hidroxilos, entre otros sustituyentes. En consecuencia, la identificación de los compuestos fenólicos presentes en una matriz de origen vegetal es una tarea sustancialmente compleja, que requiere el empleo de un conjunto de técnicas analíticas sofisticadas que nos ofrezcan información complementaria. En general, un procedimiento analítico con el fin de caracterizar compuestos fenólicos que estén contenidos en una matriz natural implica tres etapas

básicas: extracción y/o purificación de los compuestos de la muestra, separación analítica y detección e identificación.

4.1. Extracción de compuestos fenólicos

Esta etapa está orientada a extraer dichos compuestos de la matriz vegetal en la que se encuentren. Durante la **extracción** es necesario eliminar todos los interferentes presentes en la matriz que puedan afectar a la determinación, así como asegurarse de que la muestra sea un extracto compatible con la técnica analítica que se va a utilizar.

El proceso de extracción consiste en la separación de los compuestos de interés de la matriz sólida o líquida que los contiene, en base a las diferencias de solubilidades relativas de estos frente a los componentes de la matriz. Este proceso es especialmente crítico cuando se realizan análisis cuantitativos. En el caso de los polifenoles, la extracción es un paso de gran importancia ya que su distribución tisular en las plantas no es uniforme, se encuentran en altos niveles en partes externas y a nivel celular pueden localizarse en vacuolas si hablamos de polifenoles hidrofílicos, o en las paredes celulares en el caso de los insolubles (51). Debido a la amplia variedad de estructuras y propiedades de los compuestos fenólicos, junto con esta distribución desigual en la planta y, además, por el hecho de que puedan encontrarse unidos a otras macromoléculas en las matrices vegetales, no es posible hablar de procedimientos generales de extracción, sino que la elección de un procedimiento concreto dependerá de la matriz de la que se trate, de los compuestos que se quieran determinar y del tipo de información que se desee obtener (cualitativa o cuantitativa) (52). Generalmente, antes de llevar a cabo el proceso de extracción, las matrices vegetales se preparan mediante una serie de etapas previas que comprenden procesos de homogeneización, secado, liofilización o congelación.

51. Lattanzio V, Cardinali A, Linsalata V. Plant Phenolics: A Biochemical and Physiological Perspective. In: *Recent Advances in Polyphenol Research*. Oxford, UK: Wiley-Blackwell; 2012. p. 1–39.

52. Luthria DL. Significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods. *J Sci Food Agric*. 2006;86(14):2266–72.

Una vez preparadas para la extracción hay que tener en cuenta numerosos factores que influyen en esta etapa, como son el tipo de disolventes empleados, la temperatura, la presión o el número de ciclos de extracción, entre otros. Aunque la mayor influencia que puedan tener cada uno de estos, dependerá del tipo de técnica seleccionada. Actualmente han sido descritas numerosas metodologías para la extracción de compuestos fenólicos procedentes de matrices vegetales que pueden dividirse en dos grandes bloques: las técnicas convencionales y las no convencionales o "Green" (53,54).

4.1.1. Técnicas convencionales

Este tipo de extracción incluye técnicas basadas en la capacidad que diferentes disolventes presentan para extraer los compuestos de interés y, además, en la aplicación de calor y/o agitación. La **técnica clásica** más descrita en el campo de los compuestos contenidos en matrices vegetales ha sido la basada en la extracción **sólido-líquido** (SLE) (55,56). En este tipo de extracción, la solubilidad de los compuestos fenólicos en los disolventes usados juega un papel determinante. Esta solubilidad está condicionada por la naturaleza química del compuesto que, en el caso concreto de los polifenoles, puede variar dependiendo a la familia a la que pertenezcan. Para los compuestos fenólicos los más utilizados son metanol, etanol, propanol, acetona, agua, dimetilformamida, acetato de etilo, o las mezclas de alguno de ellos, obteniéndose así diferentes grupos de polifenoles, dependiendo del disolvente en cuestión (57). Sin embargo, cuando se trata de compuestos más polares, como pueden ser los ácidos fenólicos que no suelen extraerse con disolventes orgánicos, a menudo se utilizan mezclas de estos disolventes con agua a distintas proporciones.

53. Franco D, Sineiro J, Rubilar M, Sánchez M, Jerez M, Pinelo M, et al. Polyphenols from plant materials: Extraction and antioxidant power. *Electron J Environ Agric Food Chem*. 2008;7(8):3210–6.

54. Azmir J, Zaidul ISM, Rahman MM, Sharif KM, Mohamed A, Sahena F, et al. Techniques for extraction of bioactive compounds from plant materials: A review. *J Food Eng*. 2013;117(4):426–36.

55. Garcia-Salas P, Morales-Soto A, Segura-Carretero A, Fernández-Gutiérrez A. Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples. *Molecules*. 2010;15(12):8813–26.

56. Harnly JM, Bhagwat S, Lin L-Z. Profiling methods for the determination of phenolic compounds in foods and dietary supplements. *Anal Bioanal Chem*. 2007;389(1):47–61.

57. Antolovich M, Prenzler P, Robards K, Ryan D. Sample preparation in the determination of phenolic compounds in fruits. *Analyst*. 2000;125(5):989–1009.

Por ejemplo, los ácidos fenólicos libres y esterificados pueden ser extraídos utilizando una mezcla de metanol-acetona-agua. Los flavonoides son comúnmente extraídos del tejido vegetal con metanol, etanol, agua o sus combinaciones, pero en algunos casos estos solventes pueden encontrarse acidificados. Las antocianinas suelen obtenerse con metanol acidificado, que destruye las membranas celulares y simultáneamente disuelve y estabiliza las antocianinas. Otros factores como la temperatura, el tiempo de extracción, el número de ciclos sucesivos del proceso, la proporción entre volumen de disolvente y cantidad de muestra o las características de la misma, son determinantes en la obtención de los compuestos fenólicos (58,59). Además, hay que tener en cuenta las posibles interacciones que se puedan dar con otros componentes de la matriz, como pueden ser azúcares o proteínas, que pueden dar lugar a complejos con un grado de solubilidad muy bajo.

Aunque las técnicas de extracción convencionales son aplicadas frecuentemente a la extracción de polifenoles presentan una serie de inconvenientes como son una baja selectividad y porcentajes de recuperación, requieren tiempos largos de trabajo, y además utilizan grandes cantidades de disolventes que en muchos casos pueden tener potenciales efectos negativos sobre el medio ambiente y la salud humana. Además, los disolventes orgánicos son caros y su eliminación del producto final es muy costosa. Debido a estos inconvenientes, en los últimos años se han ido sustituyendo por métodos de extracción más novedosos, que por lo general utilizan alguna fuente de energía para aumentar la transferencia de los analitos al disolvente. Por otro lado, también hay un creciente interés por la búsqueda de técnicas que sean más respetuosas con el medio ambiente y no utilicen disolventes orgánicos.

Además de para la extracción, el uso de distintos disolventes mediante la SLE ha sido aplicado en el **capítulo 5** como una aproximación para el aislamiento de distintos grupos de compuestos fenólicos contenidos en una matriz muy compleja como es *T. cacao*.

58. Spigno G, Tramelli L, De Faveri DM. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *J Food Eng*. 2007;81(1):200–8.

59. Gironi F, Piemonte V. Temperature and solvent effects on polyphenol extraction process from chestnut tree wood. *Chem Eng Res Des.* 2011;89(7):857–62.

4.1.2. Técnicas no convencionales o "Green"

Desde la prehistoria, el hombre ha estado acompañado por procesos químicos. Muchos de los descubrimientos que han posibilitado su desarrollo tecnológico y la mejora de su calidad de vida, son todos procesos químicos que el hombre aprendió a utilizar para su provecho. Pero debido a este crecimiento, se va registrando un aumento en la generación de residuos perjudiciales, produciendo contaminación ambiental y dando origen al cambio climático global. A partir de esta situación de alerta, la química juega un doble papel, por un lado, debe atenuar y revertir este escenario y, por otro, debe continuar contribuyendo a incrementar la calidad de vida, generando procesos que eliminen o minimicen el riesgo y la contaminación en su diseño. Considerando este doble papel, surge la **Química Verde** o **Química Sustentable** (60). Es por ello que en la actualidad se imponen mayores restricciones sobre las técnica extractivas, incluyendo la disminución de los tiempos de extracción, el consumo reducido de solventes y energía, la prevención de la contaminación ambiental y el cuidado especial en el tratamiento de compuestos termolábiles (61). Por lo tanto, es de interés estudiar técnicas alternativas que permitan aumentar los rendimientos y pureza de los extractos y reducir los tiempos y riesgos de la extracción. Dentro de las técnicas alternativas propuestas en la literatura se incluyen la extracción asistida con microondas (MAE), ultrasonidos (EUA) y el uso de fluidos presurizados (PLE) y supercríticos (SFE). Dentro de este grupo de procesos, serán las tres últimas las que se tratarán a continuación ya que han sido utilizadas para la extracción de los compuestos fenólicos contenidos en las matrices objeto de estudio en la presente tesis.

La **EUA** es una de las técnicas que ha cobrado mucho interés recientemente, principalmente por su eficiencia, bajo costo, y por la posibilidad de realizar extracciones a bajas temperaturas, utilizando menores cantidades de disolvente que los procesos tradicionales (62).

60. Anastas PT, Warner. JC. Green chemistry: theory and practice. Green Chemistry. 2000. 132 p.

61. Chemat F, Vian MA, Cravotto G. Green extraction of natural products: Concept and principles. *Int J Mol Sci*. 2012;13(7):8615–27.

62. Vilkhu K, Mawson R, Simons L, Bates D. Applications and opportunities for ultrasound assisted extraction in the food industry — A review. *Innov Food Sci Emerg Technol*. 2008;9(2):161–9.

En esta técnica se combinan la alta potencia y la baja frecuencia para poder separar los compuestos de interés de la matriz que los contiene (63). Numerosas investigaciones de extractos vegetales se han servido de esta técnica para la extracción de los compuestos de interés (64). Es por ello que se ha utilizado para favorecer la obtención de proantocianidinas y otros compuestos fenólicos a partir de los extractos vegetales objeto de estudio en los **Capítulos 2**, **3**, **4**, **5**, **10** y **11**.

En el caso del **PLE** (**Figura 15**), la utilización de agua subcrítica constituye una alternativa interesante para la obtención y purificación de compuestos bioactivos a partir de productos naturales. Esta técnica se basa en el uso de disolvente a temperaturas y presiones lo suficientemente altas (por encima del punto de ebullición del disolvente) para que se mantenga en estado líquido. En estas

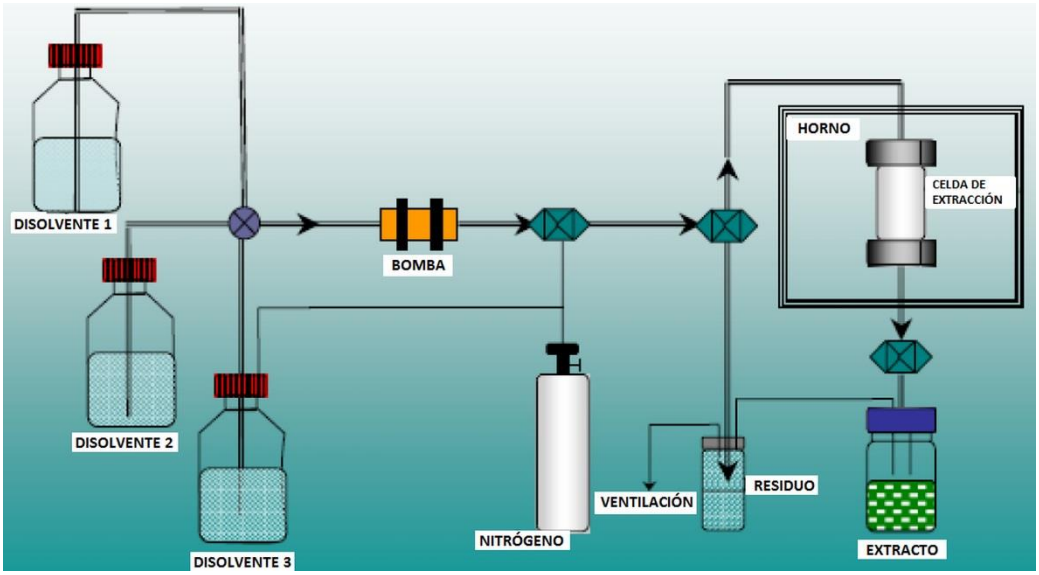


Figura 15. Esquema de un equipo de PLE.

condiciones se aumenta la solubilidad de los analitos y se disminuye la tensión superficial y la viscosidad del disolvente. Esto facilita la difusión del analito y la penetración de dicho disolvente en la matriz de extracción respectivamente, a la vez que facilita la transferencia de masa e incrementa la velocidad de difusión al disminuirse las fuerzas intermoleculares.

63. Albu S, Joyce E, Paniwnyk L, Lorimer JP, Mason TJ. Potential for the use of ultrasound in the extraction of antioxidants from *Rosmarinus officinalis* for the food and pharmaceutical industry. In: *Ultrasonics Sonochemistry*. 2004. p. 261–5.

64. Vinatoru M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrason Sonochem*. 2001;8(3):303–13.

La ventaja más importante con respecto a las técnicas de extracción convencionales es que los tiempos de extracción son más cortos, además de producirse un extracto de alta calidad, con un agente extractor de bajo costo (65).

Los principales parámetros a tener en cuenta en esta técnica son, al igual que en la SLE, la afinidad química del disolvente con el soluto, el tamaño de partícula, la temperatura y el tiempo de extracción. Las condiciones de extracción que pueden optimizarse son: el tamaño de partícula de la muestra, volumen, naturaleza y flujo del disolvente, temperatura, tiempo de extracción, número de ciclos y presión. De todas ellas, la naturaleza del disolvente y la temperatura son generalmente las que ejercen una mayor influencia. En el caso de la elección del disolvente de extracción, debe de ser capaz de solubilizar los analitos de interés, minimizando la co-extracción de otros componentes de la matriz ya que las altas temperaturas favorecen una disminución de la selectividad. En esta elección es muy importante tener en cuenta la volatilidad del disolvente y la compatibilidad con las siguientes etapas del tratamiento y del análisis de la muestra. Con respecto a la polaridad del disolvente, debería ser parecida a la de los compuestos de interés, y en el caso de que los analitos abarcaran un amplio rango de polaridad, se pueden encaminar los procesos mediante otras vías. Entre las más utilizadas se encuentran las mezclas de disolventes de alta y baja polaridad, que suelen proporcionar resultados eficaces. También se pueden llevar a cabo extracciones sucesivas, una con un disolvente no polar y otra con un disolvente polar. Y en otros casos, se ha logrado mejorar el proceso de extracción mediante adición de modificadores (tensioactivos, ácidos o bases) al disolvente utilizado, sobre todo si son disolventes orgánicos (66,67).

Entre las ventajas e inconvenientes que presenta esta técnica de extracción destacan los mencionados en la **Figura 16**.

65. Mustafa A, Turner C. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. *Anal Chim Acta*. 2011;703(1):8–18.

66. Ju ZY, Howard LR. Effects of Solvent and Temperature on Pressurized Liquid Extraction of Anthocyanins and Total Phenolics from Dried Red Grape Skin. *J Agric Food Chem*. 2003;51(18):5207–13.

67. Mukhopadhyay S, Luthria DL, Robbins RJ. Optimization of extraction process for phenolic acids from black cohosh (*Cimicifuga racemosa*) by pressurized liquid extraction. *J Sci Food Agric*. 2006;86(1):156–62.

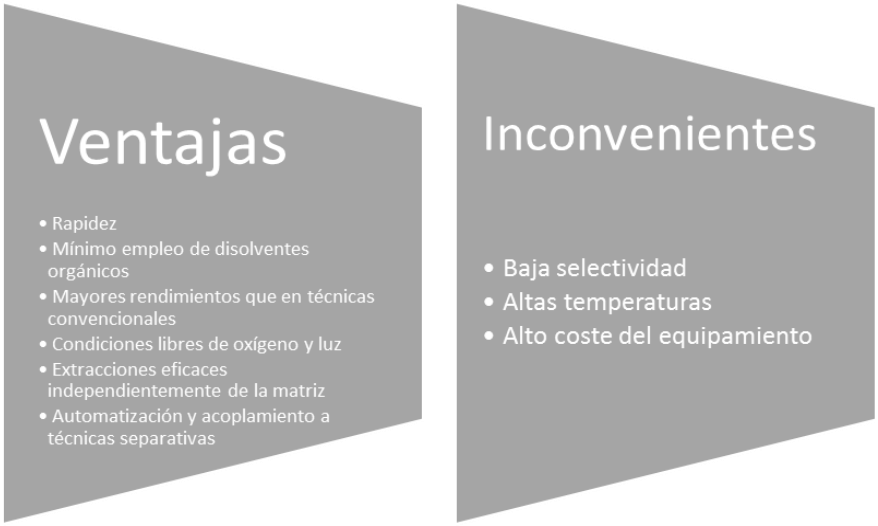


Figura 16. Ventajas e inconvenientes de la PLE.

Desde su introducción en 1996 por Richter y *col.* (68), sus aplicaciones se han focalizado en la extracción de contaminantes ambientales, pero gracias a las ventajas que presenta se ha ido introduciendo en la industria alimentaria. En el caso de los compuestos fenólicos, se ha utilizado la extracción con agua como disolvente para extraer antioxidantes de romero y salvia, antocianinas de piel de uva y de frutos rojos, catequinas de las hojas del té, flavonas de la piel de naranja o lignanos de semillas de lino, entre otros (69).

Con respecto a la **SFE**, fue en 1964 cuando se utilizó por primera vez en la industria alimentaria, dando lugar a una patente para descafeinar el café utilizando **dióxido de carbono supercrítico** como agente de extracción presentada por Zosel (70). Desde entonces, esta técnica ha promovido un gran interés y ha ampliado su campo de acción.

Se considera como un fluido supercrítico (FSC) a aquella sustancia que se encuentra en condiciones de temperatura y presión por encima de su punto crítico.

68. Richter BE, Jones BA, Ezzell JL, Porter NL, Avdalovic N, Pohl C. Accelerated Solvent Extraction: A Technique for Sample Preparation. *Anal Chem*. 1996;68(6):1033–9.

69. Wijngaard H, Hossain MB, Rai DK, Brunton N. Techniques to extract bioactive compounds from food by-products of plant origin. *Food Res Int*. 2012;46(2):505–13.

70. Zosel K. Method for separation of mixtures. 1964.

En estas condiciones, las propiedades de la sustancia son intermedias a las de los estados líquido y gas, como capacidad de difusión viscosidad y tensión superficial similares a las de un gas, y densidad y poder de solvatación similar a la de los líquidos. Estas características singulares de los FSCs hacen que sean particularmente adecuados para ser utilizados como agentes de extracción, en tiempos relativamente cortos y con altos rendimientos. Además al ser compresibles, pequeños cambios de presión pueden originar importantes cambios de densidad y poder disolvente (71). En la **Figura 17** se muestra el diagrama de fases de una sustancia pura donde la región supercrítica no queda definida por una línea continua ya que no tiene lugar el cambio de fase, sino que hay una transición continua que va desde el estado líquido a FSC por aumento de la temperatura a presión constante, o desde el estado gaseoso a FSC por incremento de la presión a temperatura constante. Cuando se emplean FSCs hay que tener en cuenta que la separación del soluto y el disolvente se consigue eficientemente mediante un simple proceso de expansión, lo que evita tener que aumentar la temperatura para eliminar el disolvente por evaporación, como sucede con los disolventes convencionales. Para que la solubilización de las sustancias sea eficaz, a veces es necesario recurrir al agregado de co-solventes o modificadores. Estos habitualmente son sustancias volátiles con afinidad por el soluto que se agregan en una concentración mucho mayor que la del soluto, pero mucho menor que la del disolvente, lo que amplía la posibilidad de interacciones entre las moléculas de disolvente, soluto y co-solventes.

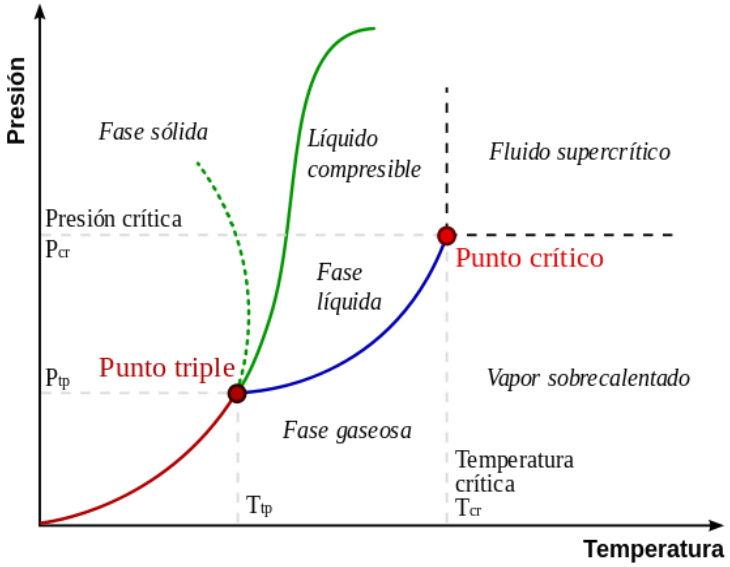


Figura 17. Diagrama de fases de una sustancia pura.

71. Herrero M, Cifuentes A, Ibañez E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review. *Food Chem*. 2006;98(1):136–48.

La instrumentación necesaria para realizar una extracción mediante FSC puede ser relativamente simple o altamente compleja, dependiendo de si es para sistemas a escala analítica o sistemas preparativos (escala piloto o industrial). La **Figura 18** muestra el esquema básico de un equipo de **SFE**. Las principales variables que intervienen en el proceso son: la densidad del fluido (presión y temperatura), el tipo de extracción (dinámica cuando se deja fluir continuamente a través de la celda de extracción, o estática cuando la celda de extracción es presurizada con el fluido, manteniendo cerrada la válvula de salida del extractor), el volumen y velocidad del fluido (tiempo de extracción), modificador (tipo y forma de adición), características de la muestra (tamaño de partícula, grado de humedad), tipo de restrictor (fijo o variable) y el sistema de colección (tipo y volumen de disolvente o material adsorbente, temperatura).

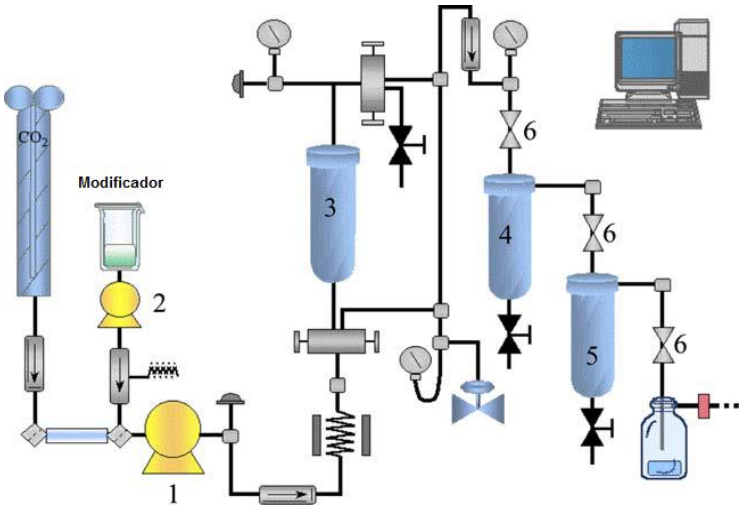


Figura 18. Esquema básico de un equipo de SFE a escala planta piloto equipado con: bomba de CO_2 (1), bomba del modificador (2), celda de extracción (3), colector 1 y 2 (4 y 5) y válvula (6).

Hay una amplia variedad de disolventes que pueden ser utilizados como FSC, entre los más habituales se encuentran: eteno, H_2O, metanol, CO_2, etano, óxido nitroso, SF_6, *n*-buteno o *n*-pentano (72). El CO_2 supercrítico es el disolvente ideal para la SFE ya que las condiciones de presión y temperatura que se necesitan para que alcance condiciones supercríticas son muy accesibles. La temperatura crítica del CO_2 (31,2 °C) es cercana a la temperatura ambiente, lo que lo convierte en el disolvente apropiado para manejar sustancias termolábiles. Además, la presión crítica es relativamente baja (74 bar).

72. McHugh M, Krukonis V. Supercritical Fluid Extraction: Principles and Practice. Vol. 53, Elsevier. 2013. 608 p.

Por otro, lado al ser un gas a presión atmosférica, se puede dejar escapar a la atmósfera tras la etapa de extracción, pudiéndose recoger los analitos en un pequeño volumen de disolvente sin utilizar prácticamente disolventes orgánicos, y así obtener extractos finales listos para ser analizados sin necesidad de efectuar posteriores etapas de concentración. Además, la utilización de CO_2 supercrítico como solvente tiene otras ventajas por no ser tóxico, inflamable, o corrosivo, no ser costoso, por presentar una baja viscosidad, alta difusividad, baja tensión superficial y elevada velocidad de transferencia de materia. Al no dejar residuos en la matriz a extraer, su utilización es adecuada en la industria agroalimentaria (incluido en la lista de la FDA de productos químicos reconocidos como inocuos para la salud). También permite obtener extractos de un elevado grado de pureza (el aroma, sabor, color y textura del producto extraído es similar al del producto original "fresco"). Además, está considerado por la industria alimentaria como GRAS (General Recognized As Safe). Realmente la única desventaja que presenta frente a otros fluidos supercríticos es su carácter apolar, limitación que puede ser fácilmente superada mediante la adición de pequeñas cantidades de co-solventes como se ha comentado previamente.

En cuanto a sus usos, el principal campo de aplicación de esta técnica se encuentra en la industria agroalimentaria, siendo muy empleada para la obtención de extractos herbales y aceites esenciales, aunque también se utiliza en el desgrasado de alimentos como el cacao o frutos secos y cereales o para la producción de productos dietéticos o bajos en colesterol. En el campo de las empresas de bebidas, la SFE se aplica en la eliminación del alcohol en la fabricación de bebidas desalcoholizadas y para la obtención de extracto de lúpulo para la elaboración de cerveza (73–76).

73. Reverchon E, De Marco I. Supercritical fluid extraction and fractionation of natural matter. *J Supercrit Fluids*. 2006;38(2):146–66.

74. Hamburger M, Baumann D, Adler S. Supercritical carbon dioxide extraction of selected medicinal plants--effects of high pressure and added ethanol on yield of extracted substances. *Phytochem Anal*. 2004;15:46–54.

75. de Melo MMR, Silvestre AJD, Silva CM. Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology. *J Supercrit Fluids*. 2014;92:115–76.

76. Ruiz-Rodríguez A, Fornari T, Jaime L, Vázquez E, Amador B, Nieto JA, et al. Supercritical CO_2 extraction applied toward the production of a functional beverage from wine. *J Supercrit Fluids*. 2012;61:92–100.

En cuanto a los compuestos fenólicos, esta técnica se ha utilizado con éxito para su extracción en semillas de uva, de naranjas, de hojas de romero y de olivo, extractos de propolis, *Ginkgo biloba*, etc. (77–79).

Tanto PLE como SFE han sido utilizadas en la presente tesis doctoral para la extracción de los compuestos fenólicos contenidos en *Sclerocarya birrea* (**Capítulo 6**).

4.2. Aislamiento y purificación de compuestos fenólicos

El proceso de investigación de nuevos compuestos con efectos beneficiosos en la salud requiere la generación de nuevas entidades moleculares y el estudio de su interacción con las dianas terapéuticas para las cuales han sido seleccionados. La fiabilidad de los datos obtenidos en los ensayos biológicos es fundamental para la interpretación de estos y, por tanto, para el progreso de los proyectos en estudio (80). Uno de los factores que pueden afectar a la certeza de los datos es la **pureza** de los compuestos estudiados. La realización de estudios de actividad biológica y de relación estructura-actividad (SAR) empleando extractos complejos, puede llevar a falsos positivos y a perder información sobre compuestos activos en bajas proporciones, y por supuesto a consumir recursos y tiempo en la confirmación de datos mediante la repetición de experimentos. Para obtener toda la información SAR de una lista de compuestos es necesario conocer la pureza de estos y verificar su estructura (81,82).

77. Passos CP, Silva RM, Da Silva FA, Coimbra MA, Silva CM. Supercritical fluid extraction of grape seed (*Vitis vinifera* L.) oil. Effect of the operating conditions upon oil composition and antioxidant capacity. *Chem Eng J*. 2010;160(2):634–40.

78. Le Floch F, Tena M., Ríos A, Valcárcel M. Supercritical fluid extraction of phenol compounds from olive leaves. *Talanta*. 1998;46(5):1123–30.

79. Junior MRM, Leite AV, Dragano NRV. Supercritical fluid extraction and stabilization of phenolic compounds from natural sources-Review (Supercritical extraction and stabilization of phenolic compounds). *Open Chem Eng J*. 2010;4:51–60.

80. Guintu C, Kwok M, Hanlon JJ, Spalding TA, Wolff K, Yin H, et al. Just-in-Time Purification: An Effective Solution for Cherry-Picking and Purifying Active Compounds from Large Legacy Libraries. *J Biomol Screen*. 2006 Dec;11(8):933–9.

81. Jiménez-Sánchez C, Olivares-Vicente M, Rodríguez-Pérez C, Herranz-López M, Lozano-Sánchez J, Segura-Carretero A, et al. AMPK modulatory activity of olive-tree leaves phenolic compounds: Bioassay-guided isolation on adipocyte model and in silico approach. *PLoS One*. 2017;12(3):1–22.

82. Xu Y, Jin Y, Wu Y, Tu Y. Isolation and purification of four individual theaflavins using semi-preparative High Performance Liquid Chromatography. *J Liq Chromatogr Relat Technol*. 2010;33(20):1791–1801.

Como se ha mencionado anteriormente, durante el proceso de extracción pueden obtenerse otros compuestos no deseados junto a los compuestos fenólicos como azúcares, ácidos orgánicos o proteínas, por lo que las etapas de extracción pueden ir seguidas de una etapa adicional de purificación. Pero otras veces, no se trata de este tipo de interferentes, sino de extractos fenólicos muy complejos que necesitan ser simplificados, sobre todo cuando se trata de asignar la responsabilidad de efectos beneficiosos en la salud a compuestos individuales. Para ello se hace uso de distintas técnicas de **aislamiento** o **purificación** de compuestos fenólicos. Las que se han llevado a cabo en la presente tesis doctoral son: el uso de distintos disolventes (explicado anteriormente en la sección de extracción), la extracción en fase sólida, la cromatografía líquida semi-preparativa, membranas de micro-, ultra- y nano-filtración, así como la combinación de alguna de ellas cuando ha sido necesario.

4.2.1. Extracción en fase sólida

La **extracción en fase sólida** (SPE) es una técnica utilizada para la preparación de muestras que comenzó a aplicarse a finales de 1970 por Burnham en estudios de contaminantes orgánicos en agua. Básicamente consiste en la extracción de un analito de interés mediante sistemas sólido-líquido, empleando principalmente cartuchos, discos o fibras. Se tiene como objetivo realizar la separación de ciertos componentes de una muestra mediante su distribución en dos fases: una estacionaria y otra móvil. La estacionaria es principalmente un sólido, que puede presentar características gomosas, retenido sobre un soporte, mientras que la fase móvil es líquida (83). La SPE permite desarrollar sistemas en miniatura, que son actualmente la tendencia dominante en campos como: química analítica, estudios ambientales, forenses, toxicológicos, clínicos, farmacéuticos, alimentarios, etc. Estos sistemas conducen a la automatización y al acoplamiento en línea para análisis, obteniendo entre otras ventajas: mayor sensibilidad, menor riesgo de pérdida del analito, ahorro de reactivos, disminución del tiempo de trabajo y menor cantidad y tratamiento de desechos de laboratorio.

83. Majors RE. New Designs and Formats in Solid-Phase Extraction Sample Preparation. LC-GC Eur. 2001;14(12):2–6.

Se han desarrollado varias modificaciones de la SPE destacándose: SPE en disco, extracción en fase sólida miniaturizada (M-SPE), micro dispositivos en línea, SPE en puntas de pipeta, dispersión de matriz en fase sólida (MSPD) y micro extracción en fase sólida (SPME).

En general, el procedimiento de purificación de los compuestos fenólicos mediante SPE se muestra en la **Figura 19** y cuenta con las etapas clave de:

1) Acondicionamiento: se activan el adsorbente y los grupos funcionales pasando un disolvente por la columna. Los adsorbentes pueden ser: hidrofóbicos, hidrofílicos, de intercambio o mixtos. Para activar adsorbentes hidrofóbicos se usa generalmente metanol o acetonitrilo, mientras que para los hidrofílicos se usa hexano o cloruro de metileno.
2) Carga de la muestra: los componentes de la matriz se retienen más o menos fuerte al pasar por el adsorbente.
3) Lavado: eliminación de cualquier resto de compuesto que pueda interferir manteniendo los analitos en el lecho del adsorbente.
4) Elución: paso de disolvente adecuado por la columna para eliminar la interacción analito-solvente y eluir el 100 % de los compuestos de interés.

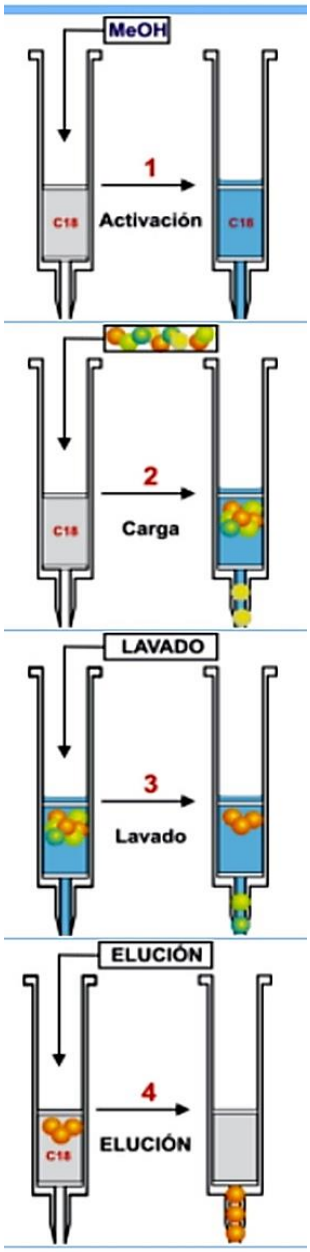


Figura 19. Etapas generales de la SPE.

Entre los usos de la SPE destacan principalmente la eliminación de interferencias que bloquean o inactivan la columna, y la concentración o enriquecimiento de trazas de un analito.

En el caso de la obtención y separación de distintos polifenoles mediante esta técnica se han publicado distintos procedimientos tanto para bebidas como cerveza sin alcohol (84), sidra y mostos de manzana (85), vino y zumos de uva (86,87), y cafés o tés (88), como también para otras matrices vegetales como bayas (89,90), recuperación de subproductos de la oliva (91,92), miel (93,94), etc.

4.2.2. Cromatografía líquida semi-preparativa

Tradicionalmente se han venido utilizando aproximaciones como las propuestas anteriormente ya que tienen la gran ventaja de su rapidez y facilidad para la automatización, pero la desventaja de que en la mayoría de los casos la pureza obtenida no es suficiente, haciéndose necesario un paso posterior de purificación. La **cromatografía de líquidos preparativa** es la herramienta más utilizada con el fin de obtener compuestos de alta pureza para su estudio biológico posterior.

84. Alonso García A, Cancho Grande B, Simal Gándara J. Development of a rapid method based on solid-phase extraction and liquid chromatography with ultraviolet absorbance detection for the determination of polyphenols in alcohol-free beers. *J Chromatogr A*. 2004;1054(1–2):175–80.

85. Suárez B, Picinelli A, Mangas JJ. Solid-phase extraction and high-performance liquid chromatographic determination of polyphenols in apple musts and ciders. *J Chromatogr A*. 1996;727(2):203–9.

86. Sun B, Leandro MC, de Freitas V, Spranger MI. Fractionation of red wine polyphenols by solid-phase extraction and liquid chromatography. *J Chromatogr A*. 2006;1128(1–2):27–38.

87. Aresta A, Cotugno P, Massari F, Zambonin C. Determination of Trans-resveratrol in Wines, Spirits, and Grape Juices Using Solid-Phase Micro Extraction Coupled to Liquid Chromatography with UV Diode-Array Detection. *Food Anal Methods*. 2018;11(2):426–31.

88. Abdul Mumin M, Farida Akhter K, Zainal Abedin M, Zakir Hossain M. Determination and Characterization of Caffeine in Tea , Coffee and Soft Drinks by Solid Phase Extraction and High Performance Liquid Chromatography (SPE–HPLC). *Malaysian J Chem*. 2006;8(1):045–51.

89. Kraemer-Schafhalter A, Fuchs H, Pfannhauser W. Solid-phase extraction (SPE)—a comparison of 16 materials for the purification of anthocyanins *Fromaronia melanocarpa* var Nero. *J Sci Food Agric*. 1998;78(3):435–40.

90. Denev P, Ciz M, Ambrozova G, Lojek A, Yanakieva I, Kratchanova M. Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties. *Food Chem*. 2010;123(4):1055–61.

91. Bertin L, Ferri F, Scoma A, Marchetti L, Fava F. Recovery of high added value natural polyphenols from actual olive mill wastewater through solid phase extraction. *Chem Eng J*. 2011;171(3):1287–93.

92. Delisi R, Pagliaro M, Saiano F, Ciriminna R. C18 alkyl-modified silica: A suitable tool for olive biophenol green extraction. *Chem Data Collect*. 2017;7–8:102–6.

93. Michalkiewicz A, Biesaga M, Pyrzynska K. Solid-phase extraction procedure for determination of phenolic acids and some flavonols in honey. *J Chromatogr A*. 2008;1187(1–2):18–24.

94. Pascual-Maté A, Osés SM, Fernández-Muiño MA, Sancho MT. Analysis of Polyphenols in Honey: Extraction, Separation and Quantification Procedures. *Sep Purif Rev*. 2017;1–17.

La cromatografía preparativa utilizada con este fin comprende desde la de baja o media presión a la de alta presión y desde la fase normal utilizando sílice sin derivatizar o funcionalizada (p. Ej.: NH_2, CN) a la fase reversa con fases estacionarias con un amplio intervalo de funcionalidades (C-18, C-8, CN, amida, fenilo, cianopropil, pentafluorofenil,…) que buscan cubrir el mayor intervalo de polaridades y necesidades de separación (95).

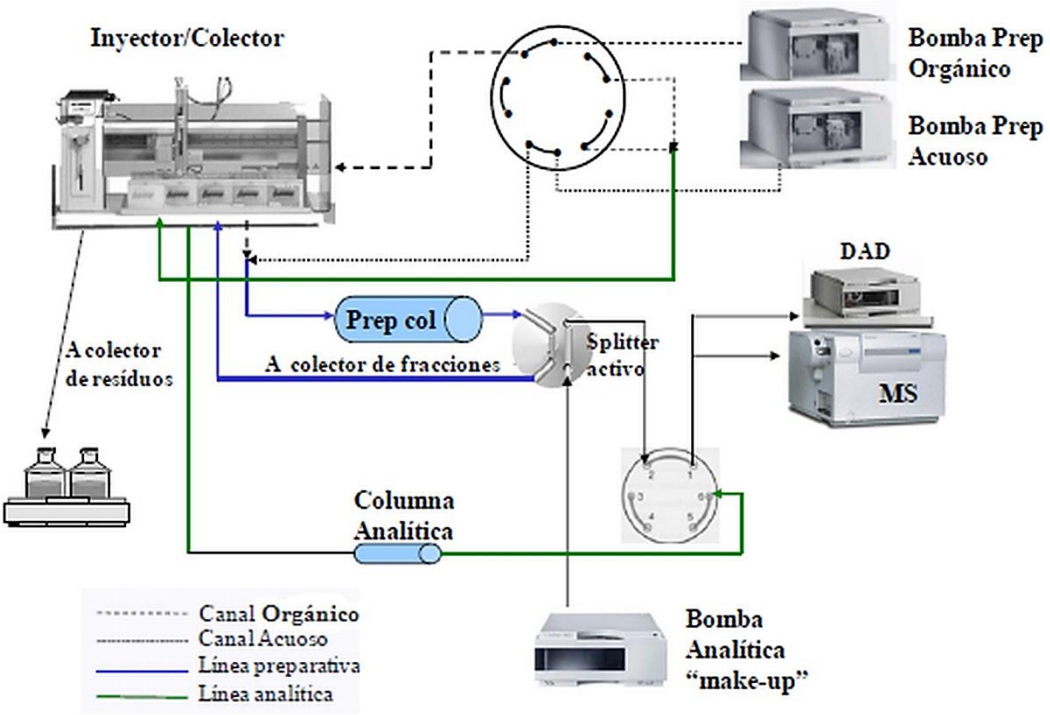


Figura 20. Esquema general de un equipo de HPLC semi-preparativa

Un equipo de HPLC semi-preparativo (**Figura 20**) puede estar compuesto de forma general por un inyector-colector (con un doble puerto analítico/preparativo), una bomba preparativa para el canal acuoso y otra para el orgánico, una bomba analítica (para adición de modificador), un horno de columna, un divisor de flujo activo y puede estar acoplado a un detector ultravioleta o a espectrometría de masas. En una configuración de este tipo, el sistema de bombeo binario genera el gradiente que lleva la muestra desde el inyector a la columna preparativa y de ahí al divisor de flujo, donde parte de la muestra es conducida a los detectores y el resto llega al colector de fracciones.

95. Marston A. Role of advances in chromatographic techniques in phytochemistry. *Phytochemistry*. 2007;68(22–24):2786–98.

Recientemente muchos autores han hecho uso de esta metodología para purificar compuestos fenólicos de distintas fuentes vegetales con la intención de facilitar la dilucidación de sus estructuras por RMN (96,97) y para el aislamiento de compuestos con propiedades beneficiosas para la salud (98–100).

4.2.3. Separación por membranas: micro-, ultra- y nano-filtración

En los últimos años, las técnicas convencionales o clásicas para resolver problemas de separación, tales como destilación, cristalización o extracción con disolventes se están viendo desplazadas en diferentes aplicaciones por procesos basados en el empleo de **membranas** como elemento separador. La separación por estos métodos abarca desde partículas sólidas, inmiscibles, que se hallan en fases líquidas o gaseosas, hasta la separación de solutos disueltos en fase líquida, pasando por la separación de mezclas de gases, tratándose en muchos casos de procesos de separación más rápidos, eficaces y económicos que los convencionales. Es importante resaltar que, en cualquiera de estos procesos, la membrana va a actuar como una barrera selectiva, permitiendo el paso de ciertos componentes y reteniendo otros en la mezcla. De esta forma, bien el permeado o bien la fase retenida se enriquece en uno o más componentes. Las membranas semipermeables o selectivas son aquellas membranas que permiten el paso de las moléculas de un disolvente (en general) a su través, pero retienen las moléculas del soluto disueltas en dicho disolvente impidiéndoles tal paso. En algunos casos las membranas permiten el paso de ciertos solutos impidiendo el paso de otros, por lo que también es posible realizar operaciones de fraccionamiento de diferentes compuestos.

96. Sudjaroen Y, Haubner R, Würtele G, Hull WE, Erben G, Spiegelhalder B, et al. Isolation and structure elucidation of phenolic antioxidants from Tamarind (*Tamarindus indica* L.) seeds and pericarp. *Food Chem Toxicol*. 2005;43(11):1673–82.

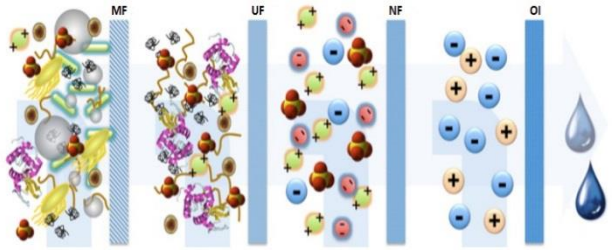
97. Bergman M, Varshavsky L, Gottlieb HE, Grossman S. The antioxidant activity of aqueous spinach extract: chemical identification of active fractions. *Phytochemistry*. 2001;58(1):143–52.

98. Yu R-J, Liu H-B, Yu Y, Liang L, Xu R, Liang C, et al. Anticancer activities of proanthocyanidins from the plant *Urceola huaitingii* and their synergistic effects in combination with chemotherapeutics. *Fitoterapia*. 2016;112:175–82.

99. Lai H-Y, Lim Y-Y, Kim K-H. Isolation and characterisation of a proanthocyanidin with antioxidative, antibacterial and anti-cancer properties from fern *Blechnum orientale*. *Pharmacogn Mag*. 2017;13(49):31–7.

100. Singh AP, Wilson T, Kalk AJ, Cheong J, Vorsa N. Isolation of specific cranberry flavonoids for biological activity assessment. *Food Chem*. 2009;116(4):963–8.

El transporte a través de cada uno de estos procesos de membrana se debe a la acción de una fuerza impulsora que actúa sobre el lado de la matriz a separar, cuya magnitud generalmente es proporcional a la velocidad de permeación. Los procesos de membrana más utilizados son aquellos en los que la fuerza impulsora es un gradiente de presión a través de la membrana. Estos procesos se clasifican en: micro-filtración (MF), ultra-filtración (UF), nano-filtración (NF) y ósmosis inversa (OI) (**Figura 21**). Cada proceso de membranas tiene su tamaño de poro específico, lo que afecta a las sustancias a separar. En la presente memoria se han utilizado los tres primeros.



	OI	NF	UF	MF
Membrana	Asimétrica	Asimétrica	Asimétrica	Simétrica Asimétrica
Espesor Capa Activa	150 µm 1-10 µm	150 µm 1-10 µm	150-250 µm 1-10 µm	10-150 µm
Tamaño de poro Estructura	< 0.002 µm Densa	< 0.002 µm Microporosa	0.2-0.02 µm Mesoporosa	4-0.02 µm Macroporosa
Retiene (ejemplos)	COBPM, COAPM Aminoácidos	COAPM, Azúcares Sales divalentes	Coloides Macromoléculas	Bacterias Partículas suspendidas
Naturaleza química	Orgánicas	Orgánicas	Inorgánicas Orgánicas	Inorgánicas Orgánicas
Presión	10 - 100 bar	5 - 35 bar	1 - 5 bar	< 2 bar

COBPM: Compuestos orgánicos de bajo peso molecular
COAPM: Compuestos orgánicos de alto peso molecular

Figura 21. Tipos de procesos de membranas por gradiente de presión.

Las membranas empleadas son de tipo poroso, y se confeccionan a partir de polímeros predominantemente hidrofílicos, tales como la polisulfona. Sus principales aplicaciones se encuentran en la clarificación de jugos de fruta, recuperación de proteína del suero lácteo y en el fraccionamiento y purificación de polisacáridos. Desde un punto de vista económico, las tecnologías OI, UF y MF cubren más del 60% del mercado mundial de la tecnología de membranas.

La **MF** retiene partículas en suspensión con un tamaño medio que oscila entre 0,05 a 10 µm; utiliza membranas porosas y el mecanismo de transporte está basado en la exclusión molecular. Las presiones de operación varían de 0,5 a 3 bar y se fabrican en un amplio rango de materiales, tanto orgánicos como inorgánicos. Este método que se remonta al año 1920, se emplea en la clarificación de zumos, vinos y bebidas, en la esterilización de fármacos, en la concentración de células, así como

en el pre-tratamiento de otros procesos posteriores, como la nanofiltración y ósmosis inversa, y en el tratamiento de aguas residuales (101).

La **UF** permite separar macromoléculas y coloides de una corriente líquida y utiliza el peso molecular como índice de rechazo. El intervalo de separación oscila entre 1000 a 150000 Dalton o entre 2 a 100 nm. Utiliza como principio fundamental de separación el mecanismo de exclusión por tamaño mediante membranas porosas, tanto orgánicas como inorgánicas. La primera membrana de UF fue desarrollada por Bechhold (nitrocelulosa). Las aplicaciones de la UF se centran en la concentración y purificación de corrientes asociadas a diversas industrias (lácteas, alimentos, textiles, metalúrgica, automóviles) y en el tratamiento de aguas residuales y efluentes industriales (102).

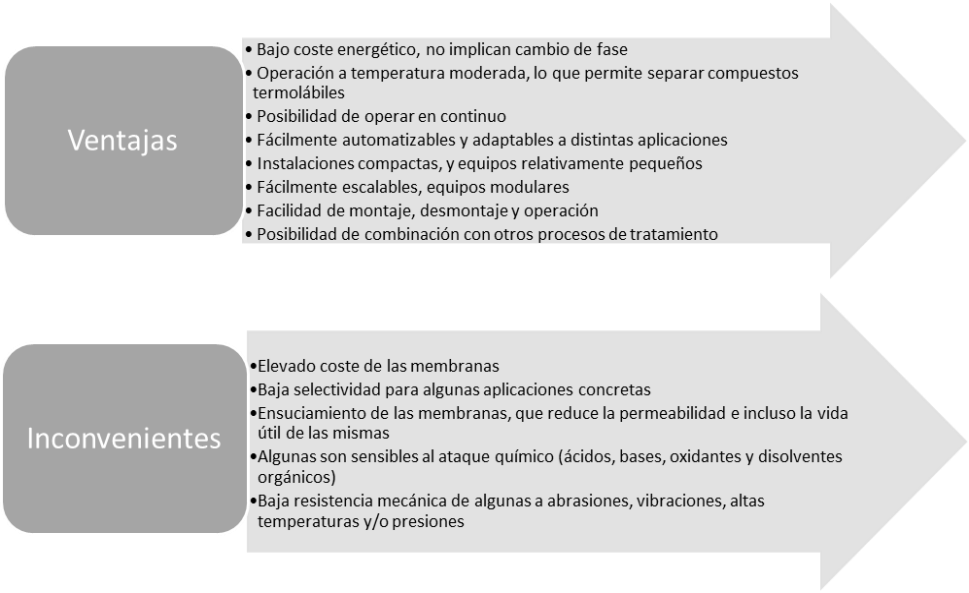


Figura 22. Ventajas e inconvenientes de los procesos con membranas.

La **NF** es un proceso de separación que usa membranas con un diámetro de poro inferior a 0,001 µm (1 nm). Retiene lactosa y otros componentes de gran tamaño, permitiendo permear solo iones minerales monovalentes y agua. El origen de la NF se remonta a 1970, como tecnología alterna a la OI, caracterizada por ser más permeables y utilizar presiones bajas.

101. Giorno L, Piacentini E, Bazzarelli F. The Principle of Microfiltration. In: *Encyclopedia of Membranes*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2015. p. 1–4.

102. Strathmann H, Giorno L, Drioli E. Introduction to membrane science and technology. Weinheim: Wiley-VCH; 2011. 473 p.

Los más grandes desarrollos de la NF se dieron con la mejora de las características de las membranas y el incremento de sus aplicaciones (103). En la **Figura 22** se indican las principales ventajas y desventajas que se encuentran en los procesos con membranas.

Las aplicaciones de los distintos tipos de membranas para la separación, purificación y concentración de compuestos fenólicos se centran principalmente en el tratamiento de aguas de deshecho en el procesado de alimentos (104). Para la obtención de polifenoles concentrados es necesario minimizar los contaminantes en estas aguas en las etapas preliminares. En el caso de la producción de aceite de oliva, el tratamiento del agua contaminada producida en almazaras reduce los gastos de gestión de dicho residuo y los impactos que producen sobre el medio ambiente. Pero además de la eliminación de los contaminantes, estas aguas son una gran fuente de polifenoles, principalmente de hidroxitirosol (105–107). Así mismo, en la industria vinícola también se hace necesario el tratamiento de los efluentes de las bodegas y es la MF una de las técnicas con las que se llega a recuperar hasta un 21 % de polifenoles y un 5.4 % de polisacáridos (108). Por otro lado, mediante combinación de las tres técnicas también se obtienen extractos ricos en oleuropeína a partir de hojas de olivo (109). En el caso de la recuperación de polifenoles de la semilla de uva se consigue hasta un 11,4 % del total del peso de la semilla mediante UF (110).

103. Van der Bruggen B, Geens J. Nanofiltration. In: *Advanced Membrane Technology and Applications*. Hoboken, NJ, USA: John Wiley & Sons, Inc.; p. 271–95.

104. Mohammadi T, Esmaeelifar A. Wastewater treatment of a vegetable oil factory by a hybrid ultrafiltration-activated carbon process. *J Memb Sci*. 2005;254(1–2):129–37.

105. Garcia-Castello E, Cassano A, Criscuoli A, Conidi C, Drioli E. Recovery and concentration of polyphenols from olive mill wastewaters by integrated membrane system. *Water Res*. 2010;44(13):3883–92.

106. Petrotos KB, Lellis T, Kokkora MI, Gkoutsidis PE. Purification of Olive Membrane Technology Mill Wastewater Using Microfiltration. *J Membr seperation Technol*. 2014;3:50–5.

107. Galanakis CM, Kotsiou K. Recovery of bioactive compounds from olive mill waste. In: *Olive Mill Waste*. Elsevier; 2017. p. 205–29.

108. Giacobbo A, do Prado JM, Meneguzzi A, Bernardes AM, de Pinho MN. Microfiltration for the recovery of polyphenols from winery effluents. *Sep Purif Technol*. 2015;143:12–8.

109. Khemakhem I, Gargouri OD, Dhouib A, Ayadi MA, Bouaziz M. Oleuropein rich extract from olive leaves by combining microfiltration, ultrafiltration and nanofiltration. *Sep Purif Technol*. 2017;172:310–7.

110. Nawaz H, Shi J, Mittal GS, Kakuda Y. Extraction of polyphenols from grape seeds and concentration by ultrafiltration. Sep Purif Technol. 2006;48(2):176–81.

La UF también ha demostrado su efectividad purificando catequinas del té verde (111). Otra aplicación para concentrar estos compuestos es a partir de zumos (112), como es el caso de la obtención de antocianinas con alto poder antioxidante a partir de zumo de fresa mediante NF (113) o de zumo de granada mediante UF y NF (114), entre otros.

5. Determinación analítica de compuestos fenólicos

La Química Analítica dispone de un amplio rango de técnicas que se fundamentan en las distintas propiedades físico-químicas que poseen los componentes de una muestra para lograr su **separación** y **determinación**. En el caso de las mezclas complejas, como pueden ser los extractos vegetales, el uso de técnicas donde la detección de los analitos se hace de manera continua a la separación es el más extendido.

Dentro de estas técnicas continuas, las más utilizadas para el análisis de compuestos fenólicos contenidos tanto en extractos como en fracciones purificadas son la cromatografía líquida (LC) y la cromatografía de gases (GC). Además, los sistemas de detección acoplados a estas técnicas separativas son de índole muy diversa, pero los más utilizados son la detección espectrofotométrica ultravioleta-visible (UV-VIS) y la espectrometría de masas (MS).

5.1. Técnicas separativas

Hasta mediados del siglo XX la separación de los compuestos de una mezcla compleja se llevaba a cabo mediante destilación, precipitación y extracción. Hoy en día, se utilizan especialmente la cromatografía y electroforesis (en menor medida) en la separación analítica.

111. Sousa L dos S, Cabral BV, Madrona GS, Cardoso VL, Reis MHM. Purification of polyphenols from green tea leaves by ultrasound assisted ultrafiltration process. *Sep Purif Technol*. 2016;168:188–98.

112. Cai M, Hou W, Lv Y, Sun P. Behavior and rejection mechanisms of fruit juice phenolic compounds in model solution during nanofiltration. *J Food Eng*. 2017;195:97–104.

113. Arend GD, Adorno WT, Rezzadori K, Di Luccio M, Chaves VC, Reginatto FH, et al. Concentration of phenolic compounds from strawberry (*Fragaria X ananassa* Duch) juice by nanofiltration membrane. *J Food Eng*. 2017;201:36–41.

114. Conidi C, Cassano A, Caiazzo F, Drioli E. Separation and purification of phenolic compounds from pomegranate juice by ultrafiltration and nanofiltration membranes. *J Food Eng*. 2017;195:1–13.

En el caso de la **cromatografía**, que ha sido la técnica utilizada en esta tesis doctoral, comprende un conjunto de técnicas que permiten separar componentes estrechamente relacionados estructuralmente presentes en mezclas complejas, lo que en muchas ocasiones resulta imposible por otros medios. La cromatografía se basa en la distinta distribución de las sustancias a separar entre dos fases, una móvil y otra estacionaria, en función de sus propiedades físico-químicas (carga, polaridad, potencial redox o masa molecular). Entre las más ampliamente utilizadas para el análisis de muestras ricas en compuestos fenólicos se pueden distinguir dos tipos de técnicas cromatográficas: cromatografía líquida y de gases.

5.1.1. Cromatografía líquida

La **cromatografía líquida** (LC) en columna fue inventada a principios del siglo XX por el botánico ruso Mikhail Tswett, quien empleó la técnica para separar varios pigmentos vegetales como clorofilas y xantofilas, haciendo pasar disoluciones de estos compuestos a través de una columna de vidrio rellena con carbonato de calcio. Las especies separadas aparecían como bandas coloreadas en la columna, lo que justifica el nombre que eligió para la técnica (del griego "*chroma"* que significa color, y *"graphein"* que significa escribir).

En los años 1950, Howard y Marlin introdujeron una nueva modalidad de cromatografía a la que llamaron de fase reversa. Hasta ese momento la cromatografía se había utilizado para separar sustancias polares usando una fase estacionaria polar y fases móviles apolares. Estos científicos revirtieron la polaridad de las fases móvil y estacionaria con el objetivo de separar ácidos grasos, de tal forma que usaron una fase estacionaria apolar y una fase móvil polar.

A finales de los años 1960 se vio que la eficiencia de la técnica podía aumentar disminuyendo el tamaño de partícula de la fase estacionaria. Así, empezaron a emplearse rellenos de columna con tamaño de partícula entre 3 y 10 µm. A la tecnología que se puso a punto para poder utilizar este tipo de columnas se le llamó **Cromatografía Líquida de Alta Resolución** (HPLC).

Los equipos de HPLC (**Figura 23**) disponen de varios módulos: bombas capaces de impulsar la fase móvil a través de las columnas cromatográficas (pueden ir precedidas de una pre-columna que impida que lleguen componentes de la muestra que puedan dañar la fase estacionaria) con alto grado de

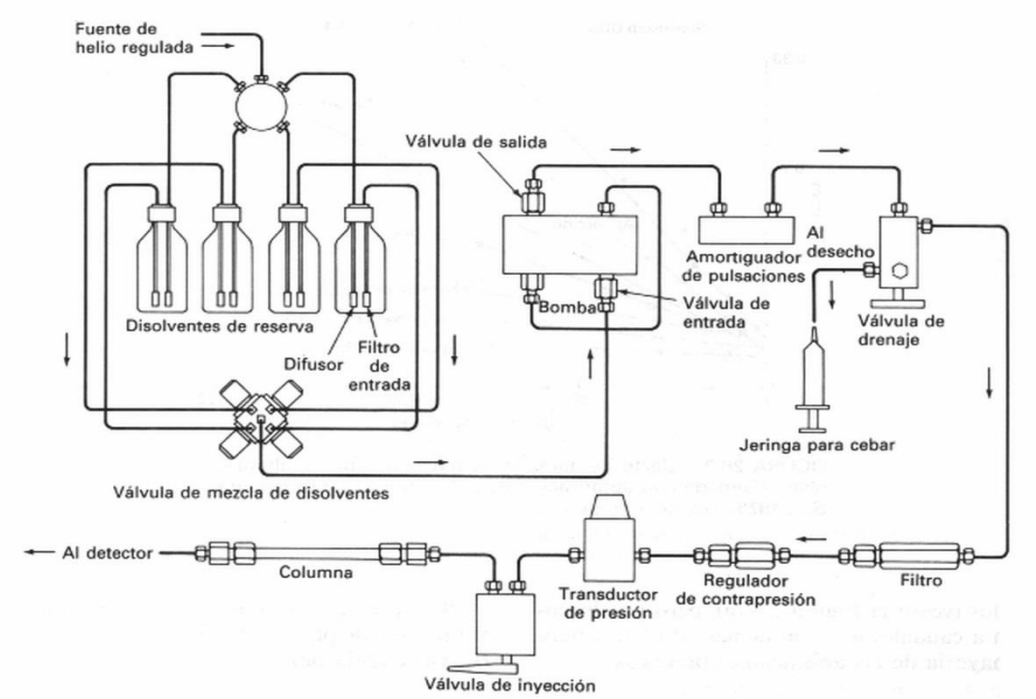


Figura 23. Esquema de un equipo de HPLC.

empaquetamiento proporcionando la presión necesaria para ello; un horno termostatizado; un sistema de inyección de muestra que permite su introducción en el flujo de la fase móvil de forma reproducible y sin despresurización del sistema; y en la mayoría de los casos, un detector acoplado con el que obtener parámetros cromatográficos como son el tiempo de retención y el área de pico de los compuestos que eluyen de la columna. El tiempo de retención del analito dependerá de lo fuerte que interaccione con la fase estacionaria, las dimensiones y el tamaño de partícula de la columna y de la composición, flujo y temperatura de la fase móvil utilizada. Los disolventes más utilizados son el agua, el metanol y el acetonitrilo. La fase acuosa puede contener tampones, sales, o compuestos como el ácido trifluoroacético, que ayudan a la separación de los compuestos.

Con respecto a la naturaleza de la fase estacionaria y del tipo de fenómeno físico que provoca la separación, se pueden diferenciar distintos modos de cromatografía líquida. Los más utilizados son:

- Cromatografía de adsorción, donde la separación se basa en repetidos pasos de adsorción-desorción.
- Cromatografía de partición, basada en la partición entre la fase móvil y la estacionaria.
- Cromatografía de intercambio iónico, en la que la fase estacionaria está compuesta por una superficie iónica de carga opuesta a la de los analitos.
- Cromatografía de exclusión molecular, donde la muestra se separa de acuerdo a su tamaño molecular a través de la columna rellena de un material con un tamaño de poro controlado.
- Cromatografía por afinidad, donde el sólido tiene propiedades de retención bioespecíficas.
- Cromatografía quiral, que puede considerarse dentro de la cromatografía de partición, donde el reactivo quiral va unido a la fase móvil o al soporte sólido.

La cromatografía de partición es la más ampliamente utilizada y en la práctica, pueden diferenciarse dos modalidades, dependiendo de la polaridad de las dos fases:

- Cromatografía en fase normal (NP-HPLC), donde la fase estacionaria es de naturaleza polar y la fase móvil es apolar. En este caso, las muestras polares son retenidas más fuertemente por la columna permitiendo, por tanto, la elución de componentes apolares en primer lugar.
- Cromatografía en fase inversa (RP-HPLC), donde la fase estacionaria es de naturaleza apolar y el disolvente de elución o fase móvil es polar. En este caso ocurre lo contrario que en fase normal, los compuestos apolares serán retenidos durante más tiempo en la columna.

Los compuestos fenólicos, por su carácter polar se separan, fundamentalmente, mediante cromatografía de partición en fase inversa, utilizando columnas rellenas de hidrocarburos como C8 (*n*-octil) o más comúnmente, C18 (*n*-octadecil). Respecto a las dimensiones de las columnas y tamaños de partícula, comercialmente hay disponibles una gran variedad de ellas, pero en el caso del análisis

de polifenoles, las columnas de HPLC convencional son las más usadas, con tamaños de partícula que oscilan entre 3 y 5 µm. La elución se lleva a cabo con una fase móvil de polaridad elevada como es el caso de disoluciones acuosas que pueden contener cierto porcentaje de disolventes orgánicos polares como acetonitrilo o metanol (115–117).

Esta técnica ha evolucionado notablemente, y a día de hoy, solo entre los años 2016 y 2017, se pueden encontrar más de 10.000 publicaciones acerca del uso del HPLC en el análisis de compuestos fenólicos (según ScienceDirect), lo cual da idea de la importancia y el potencial de esta técnica en la caracterización de los compuestos fenólicos en matrices vegetales. Es por ello, por lo que se ha utilizado para la separación de los compuestos fenólicos presentes en todas las matrices de estudio de la presente tesis doctoral.

5.1.2. Cromatografía de gases

La **cromatografía de gases** (GC) es una técnica cromatográfica donde la separación de los componentes de una muestra se produce gracias al equilibrio de distribución existente entre una fase móvil que es un gas y una fase estacionaria, que generalmente es un líquido. Entre las técnicas cromatográficas utilizadas con fines analíticos, la GC es probablemente la técnica de más amplia utilización ya que ninguna técnica analítica puede ofrecer su capacidad de separación o su sensibilidad a la hora de analizar compuestos volátiles. Por otra parte, el hecho de que con esta técnica las mezclas sean separadas en fase gaseosa, establece los límites de su uso, que estarán marcados fundamentalmente por la estabilidad térmica de los compuestos a separar. Por lo general, la aplicación de la cromatografía de gases está restringida a la separación de compuestos con un peso molecular menor de 1000 Da a una temperatura máxima de trabajo de aproximadamente 400 °C; dentro de estos límites la única limitación existente sería la estabilidad térmica de la muestra.

115. Ignat I, Volf I, Popa VI. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chem*. 2011;126(4):1821–35.

116. Tsao R, Yang R. Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography. *J Chromatogr A*. 2003;1018(1):29–40.

117. Kalili KM, de Villiers A. Recent developments in the HPLC separation of phenolic compounds. *J Sep Sci*. 2011;34(8):854–76.

Para realizar una separación mediante cromatografía de gases, se inyecta una pequeña cantidad de muestra en una corriente de un gas inerte a elevada temperatura. Esta corriente de gas atraviesa la columna cromatográfica, que separará los componentes de la mezcla por un mecanismo de partición o de adsorción o, en muchos casos, por medio de la mezcla de ambos. Los componentes separados emergerán de la columna a intervalos discretos y pasarán a través de algún sistema de detección adecuado, o bien serán dirigidos hacia un dispositivo de recogida de muestras.

La **Figura 24** muestra un esquema básico de un cromatógrafo de gases.

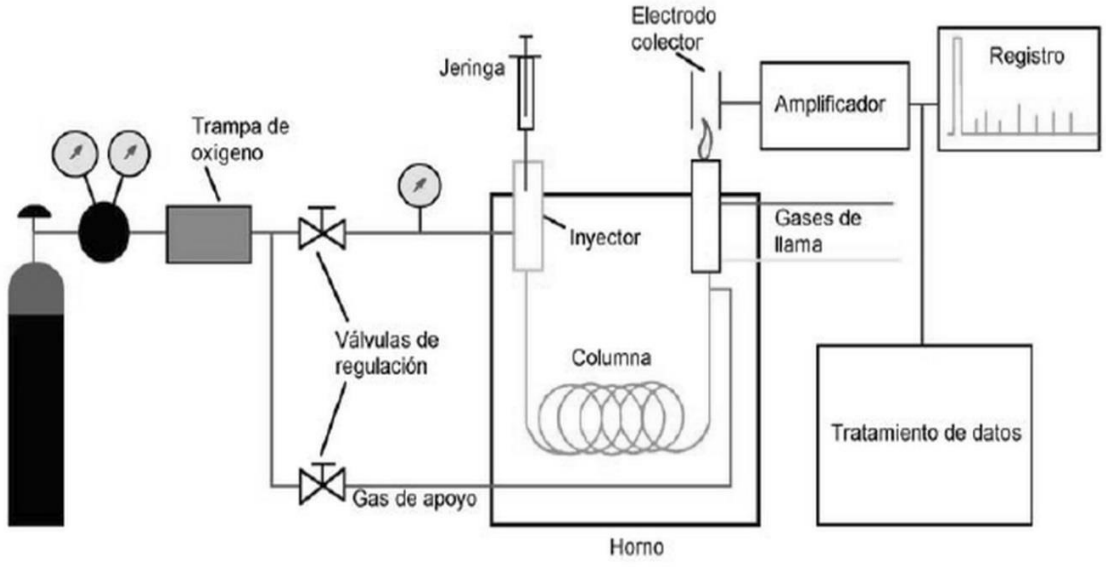


Figura 24. Esquema de un cromatógrafo de gases.

Esta es una técnica analítica que presenta ciertas desventajas en cuanto al análisis de compuestos fenólicos, ya que al ser compuestos caracterizados por la presencia de grupos hidroxilos con capacidad de formar enlaces de hidrógeno, esto se traduce en un aumento de su punto de ebullición y, como consecuencia, poseen una baja volatilidad. Además, el tratamiento de muestra para el análisis de estos compuestos mediante GC es complejo, ya que además de incluir las etapas anteriormente mencionadas, hay que eliminar los lípidos presentes en la muestra, realizar una hidrólisis ácida, básica o enzimática para liberar los enlaces glicosídicos, y finalmente realizar un proceso de derivatización

que transforme a los compuestos fenólicos en compuestos más volátiles (118). A pesar de estas desventajas, la GC se ha utilizado para el análisis de compuestos fenólicos en matrices como vino (119,120), aceite de oliva (121,122), plantas aromáticas (123), frutos rojos (124), hojas de papaya (124), etc., así como también en muestras biológicas (125,126), como es el caso de las muestras objeto de esta tesis doctoral (**Capítulo 10**).

5.2. Sistemas de detección

Una vez llevada a cabo la separación de los componentes de la muestra por alguna de las técnicas separativas, se requiere la presencia de un **detector** que produzca una señal medible cuando los analitos eluyan tras la separación.

Existen diversos tipos de detectores acoplables a cromatografía. La elección del detector idóneo para cada aplicación se hace en base a una serie de requisitos que aseguren que los analitos previamente separados serán detectados adecuadamente, estos pueden ser:

118. Zuo Y, Wang C, Zhan J. Separation, Characterization, and Quantitation of Benzoic and Phenolic Antioxidants in American Cranberry Fruit by GC−MS. *J Agric Food Chem*. 2002;50(13):3789–94.

119. Fariña L, Boido E, Carrau F, Dellacassa E. Determination of volatile phenols in red wines by dispersive liquid–liquid microextraction and gas chromatography–mass spectrometry detection. *J Chromatogr A*. 2007;1157(1–2):46–50.

120. Minuti L, Pellegrino RM, Tesei I. Simple extraction method and gas chromatography–mass spectrometry in the selective ion monitoring mode for the determination of phenols in wine. *J Chromatogr A*. 2006;1114(2):263–8.

121. Ríos JJ, Gil MJ, Gutiérrez-Rosales F. Solid-phase extraction gas chromatography-ion trap-mass spectrometry qualitative method for evaluation of phenolic compounds in virgin olive oil and structural confirmation of oleuropein and ligstroside aglycons and their oxidation products. *J Chromatogr A*. 2005;1093(1–2):167–76.

122. Angerosa F, D'Alessandro N, Corana F, Mellerio G. Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry. *J Chromatogr A*. 1996;736(1–2):195–203.

123. Proestos C, Sereli D, Komaitis M. Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. *Food Chem*. 2006;95(1):44–52.

124. Wang C, Zuo Y. Ultrasound-assisted hydrolysis and gas chromatography–mass spectrometric determination of phenolic compounds in cranberry products. *Food Chem*. 2011;128(2):562–8.

125. Riera-Borrull M, Rodríguez-Gallego E, Hernández-Aguilera A, Luciano F, Ras R, Cuyàs E, et al. Exploring the Process of Energy Generation in Pathophysiology by Targeted Metabolomics: Performance of a Simple and Quantitative Method. *J Am Soc Mass Spectrom*. 2016;27(1):168–77.

126. Peters S, Kaal E, Horsting I, Janssen H-G. An automated method for the analysis of phenolic acids in plasma based on ion-pairing micro-extraction coupled on-line to gas chromatography/mass spectrometry with in-liner derivatisation. *J Chromatogr A.* 2012;1226.

- Buena sensibilidad que asegure señales analíticas intensas en respuesta al analito, combinado con una baja fluctuación de la señal de fondo o ruido.
- Límites de detección bajos.
- Determinada selectividad a una serie de analitos o a uno determinado, evitando posibles interferencias en la señal por parte de otras sustancias presentes en la muestra.
- La respuesta debe ser rápida ante un pequeño cambio en la concentración del analito.
- La presencia del detector no debe perjudicar a la eficacia de la separación.
- Proporcionar señales fiables: reproducibles y estables en el tiempo.
- Idealmente, la relación señal/ruido debe ser nula en ausencia de analito.
- Proporcionar cambios en su señal en el margen más amplio posible de concentración o masas del analito, es decir, que presente un amplio intervalo lineal.

La elección de un detector se hace en base a la naturaleza y propiedades de los analitos a determinar, así como a la sensibilidad requerida y el tipo de información que se desea obtener (estructural, cuantitativa, etc.). Para la detección de compuestos fenólicos, los sistemas de detección más ampliamente utilizados son la espectroscopía UV/Vis y la MS (127–136), ambas utilizadas en esta memoria.

127. Constant H, Slowing K, Graham JG, Pezzuto JM, Cordell GA, Beecher CW. A general method for the dereplication of flavonoid glycosides utilizing high performance liquid chromatography/mass spectrometric analysis. *Phytochem Anal*. 1997;8:176–80.

128. Díaz-De-Cerio E, Verardo V, Gómez-Caravaca AM, Fernández-Gutiérrez A, Segura-Carretero A. Determination of polar compounds in guava leaves infusions and ultrasound aqueous extract by HPLC-ESI-MS. *J Chem*. 2015;2015.

129. Sarnoski PJ, Johnson J V., Reed K a., Tanko JM, O'Keefe SF. Separation and characterisation of proanthocyanidins in Virginia type peanut skins by LC–MSn. *Food Chem*. 2012;131(3):927–39.

130. Engström MT, Pälijärvi M, Fryganas C, Grabber JH, Mueller-Harvey I, Salminen JP. Rapid qualitative and quantitative analyses of proanthocyanidin oligomers and polymers by UPLC-MS/MS. *J Agric Food Chem*. 2014;62(15):3390–9.

131. Taamalli A, Arráez-Román D, Abaza L, Iswaldi I, Fernández-Gutiérrez A, Zarrouk M, et al. LC-MS-based metabolite profiling of methanolic extracts from the medicinal and aromatic species *Mentha pulegium* and *Origanum majorana*. *Phytochem Anal*. 2015;26(5):320–30.

132. Pérez-Magariño S, Revilla I, González-SanJosé ML, Beltrán S. Various applications of liquid chromatography–mass spectrometry to the analysis of phenolic compounds. *J Chromatogr A*. 1999;847(1–2):75–81.

133. Abu-Reidah IM, del Mar Contreras M, Arráez-Román D, Fernández-Gutiérrez A, Segura-Carretero A. UHPLC-ESI-QTOF-MS-based metabolic profiling of *Vicia faba* L. (Fabaceae) seeds as a key strategy for characterization in foodomics. *Electrophoresis*. 2014;35(11):1571–81.

134. Tan SN, Yong JWH, Teo CC, Ge L, Chan YW, Hew CS. Determination of metabolites in *Uncaria sinensis* by HPLC and GC-MS after green solvent microwave-assisted extraction. *Talanta*. 2011;83(3):891–8.

135. Rockenbach II, Jungfer E, Ritter C, Santiago-Schübel B, Thiele B, Fett R, et al. Characterization of flavan-3-ols in seeds of grape pomace by CE, HPLC-DAD-MSn and LC-ESI-FTICR-MS. Food Res Int. 2012;48(2):848–55.

136. Wang D, Lu J, Miao A, Xie Z, Yang D. HPLC-DAD-ESI-MS/MS analysis of polyphenols and purine alkaloids in leaves of 22 tea cultivars in China. *J Food Compos Anal*. 2008;21(5):361–9.

Aunque para la identificación inequívoca de compuestos de los que no se disponga de patrones es necesaria la resonancia magnética nuclear (RMN) (137,138), esta técnica presenta entre otros inconvenientes, ser poco sensible y su alto costes, pero se ha requerido en una parte de esta tesis (**Capítulo 11**).

5.2.1. Espectroscopía de absorción UV/VIS

Los inicios de la **espectroscopía** se remontan al año 1704 cuando Newton descubrió el fenómeno de dispersión de la luz. Pero no fue hasta mucho más adelante, cuando Bunsen y Kirchhoff en 1859 dieron a conocer que cada elemento químico posee un espectro de emisión de líneas características y construyeron un equipo capaz de medir esa emisión, llamado espectroscopio. Mientras que la espectroscopía de **emisión atómica** ya tenía varias aplicaciones prácticas a finales del siglo XIX, no fue hasta los años 30 del siglo XX, que la espectroscopía de **absorción molecular** empezó a desarrollarse al descubrir que las vitaminas (especialmente la vitamina A) absorbían radiación ultravioleta. En el año 1940, la casa comercial National Technologies Laboratories (la que más adelante pasó a ser Beckman Instruments) desarrolló el *DU UV-Vis Model A*, el primer espectrofotómetro comercial. En 1954 se desarrolló el primer equipo de doble haz, lo que permitía medidas más rápidas y precisas al permitir la medida de la muestra y la de una disolución de referencia al mismo tiempo. Hasta este momento se usaban lámparas de tungsteno para el espectro visible y lámparas de deuterio para el ultravioleta con la única opción de seleccionar una longitud de onda para la medida. En 1969 se fabricó el primer detector acoplable a HPLC, con longitud de onda variable sin necesidad de cambiar los filtros o las lámparas. Diez años después, en 1979, se inventó el detector de batería de diodos (DAD), cuya aparición permitió la detección simultánea de un rango de longitudes de onda en segundos con el fin de obtener espectros de absorción, especialmente en muestras que pasan rápidamente por una celda.

137. Owen R., Mier W, Giacosa A, Hull W., Spiegelhalder B, Bartsch H. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignansand squalene. *Food Chem Toxicol*. 2000;38(8):647–59.

138. Christophoridou S, Dais P, Tseng L-H, Spraul M. Separation and Identification of Phenolic Compounds in Olive Oil by Coupling High-Performance Liquid Chromatography with Postcolumn Solid-Phase Extraction to Nuclear Magnetic Resonance Spectroscopy (LC-SPE-NMR). *J Agric Food Chem*. 2005;53(12):4667–79.

La espectroscopía de absorción molecular **UV-Vis** se basa en la capacidad de las moléculas de absorber radiación en el rango de los 190-800 nm aproximadamente del espectro electromagnético, causando la promoción de un electrón de su estado fundamental a uno excitado. La espectroscopía UV-Vis mide, generalmente, la transmitancia del analito que transforma en absorbancia. Esta es definida como la cantidad de intensidad de luz que es capaz de absorber una molécula. Esta absorción está relacionada de forma lineal (dentro de un rango) con la concentración de la sustancia, siguiendo la ley de Lambert-Beer.

COMPUESTOS	BANDAS UV (nm)	
Ácidos benzoicos	270 - 280	
Ácidos hidroxicinámicos	305 - 325	
Cumarinas	220 - 230	310 - 350
Chalconas	220 - 270	340 - 390
Dihidrochalconas	~220	~280
Auronas	240 - 270	340 - 370
Flavonas	250 - 270	330 - 350
Flavonoles	250 - 270	350 - 380
Flavanonas	270 - 295	
Flavanoles	270 - 280	
Antocianidinas	240 - 280	450 - 560
Isoflavonas	245 - 270	300 - 340
Proantocianidinas	~280	

Figura 25. Bandas de absorción características de diversas familias de compuestos fenólicos.

Los múltiples enlaces conjugados presentes en los compuestos fenólicos los convierten en cromóforos que presentan bandas de absorción en la región UV (generalmente en torno a los 220 y 280 nm) e incluso en el visible como es el caso de las antocianidinas y algunos flavonoles (**Figura 25**). La espectroscopía de absorción UV-Vis tiene una gran aplicación en la identificación y cuantificación de multitud de moléculas y probablemente sea la técnica de análisis cuantitativo más utilizada en todo el mundo debido a su posibilidad para determinar un gran número de compuestos y grupos

funcionales y su facilidad de manejo, a pesar de que su sensibilidad pueda ser menor que la de otros sistemas de detección.

5.2.2. Espectrometría de masas

En los últimos años han aumentado exponencialmente las aplicaciones de la **espectrometría de masas** como sistema de detección acoplado a diversas técnicas separativas y en especial a la cromatografía líquida. Este auge de la técnica se debe principalmente a su selectividad y al hecho de que es uno de los pocos sistemas de detección que proporciona información estructural, evitando la inherente falta de sensibilidad de la resonancia magnética nuclear. Este acoplamiento también presenta la ventaja de que proporciona una segunda dimensión de separación ya que tras separar los compuestos según su tiempo de retención, se produce en el espectrómetro de masas una separación en función de la relación **masa/carga** (*m/z*).

Podría considerarse a Thomson como el padre de la espectrometría de masas. Se basó en los trabajos previos de Goldstein a finales del siglo XIX en los que descubrió que los rayos luminosos en un tubo de descarga que contenía gases a baja presión viajaban en línea recta desde los agujeros de un disco metálico usado como cátodo hasta la parte opuesta usada como ánodo. La continuación de estos estudios, junto con los descubrimientos de que esos rayos luminosos podían deflectarse en un campo magnético, llevó a Thomson a crear el primer equipo que era capaz de medir las diferencias en la relación *m/z* de los átomos. Varios años después, Aston refinó los equipos fabricados por Thomson y construyó el llamado espectrógrafo de masas.

Debido a que este tipo de detectores necesitan que la muestra se encuentre en estado gaseoso, los primeros acoplamientos con equipos de cromatografía se llevaron a cabo con la cromatografía de gases en los años 1960. No fue hasta la década de los 70, cuando se empezaron a desarrollar interfases capaces de desolvatar las muestras líquidas dejando las moléculas ionizadas en fase gaseosa, que se pudo realizar el acoplamiento con HPLC, aunque hasta los años 80 este acoplamiento no fue del todo práctico.

La espectrometría de masas se basa en la separación a vacío de iones en fase gaseosa de acuerdo con su relación *m/z*. Existen diversos tipos de espectrómetros de masas, pero todos incluyen los siguientes elementos básicos: un sistema de introducción de la muestra, un sistema de ionización, un acelerador de iones a través de un campo eléctrico, un analizador que dispersa los iones en función a su relación *m/z*, un detector de iones y, finalmente, un sistema de adquisición de datos que procesa la señal generada por el detector.

Existen distintos sistemas de ionización y analizadores de masas, los más utilizados son los que se muestran en la **Figura 26**. Para el trabajo experimental que se recoge en la presente memoria se han utilizado como sistema de ionización: la ionización por electrospray (ESI) y por impacto electrónico (EI), y como analizadores de masas: tiempo de vuelo (TOF) y cuadrupolo tiempo de vuelo (QTOF).

SISTEMAS DE IONIZACIÓN	ANALIZADORES DE MASAS
Impacto electrónico (EI)	Cuadrupolo (Q)
Ionización química a presión atmosférica (APCI)	Triple Cuadrupolo (QqQ)
Ionización por electrospray (ESI)	Trampa de iones (IT)
Desorción/ionización por una matriz (MALDI)	**Tiempo de vuelo (TOF)**
Flujo continuo y bombardeo con átomos rápidos (CF-FAB)	**Cuadrupolo Tiempo de vuelo (QTOF)**
Plasma acoplado inductivamente (ICP)	Transformada de Fourier-Resonancia ciclotrónica (FT-ICR)
Ionización por termonebulización (FS)	Orbitrap

Figura 26. Sistemas de ionización y analizadores de masas más utilizados.

En la ESI, el proceso de formación del **electrospray** (**Figura 27**) se lleva a cabo a presión atmosférica e intervienen diversos mecanismos al mismo tiempo. La muestra, procedente del capilar de

separación, y con la ayuda de un gas nebulizador, se carga y dispersa simultáneamente. El disolvente se va evaporando de las microgotas formadas (desolvatación) y estas van aumentando su densidad de carga eléctrica.

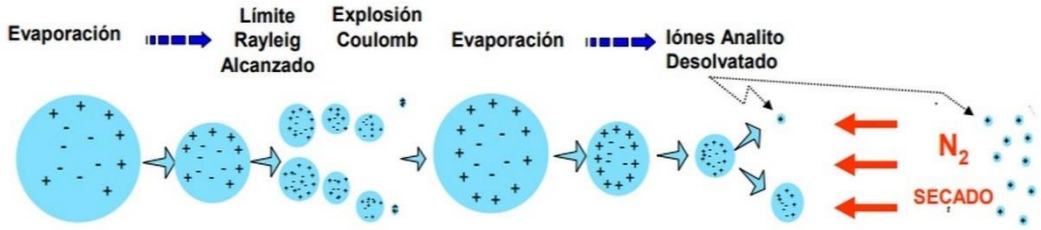


Figura 27. Esquema del proceso de formación del electrospray.

Como consecuencia, las gotas se encogen y los iones que se encuentran en la superficie se ven forzados a aproximarse entre sí debido al campo electrostático que se aplica entre la salida del capilar y la entrada al espectrómetro de masas (±2-5 kV). En cierto momento, la repulsión de los iones se hace mayor que la tensión de la superficie que mantiene unidas las gotas en forma esférica, y las pequeñas gotas se rompen. Debido a fuerzas de repulsión coulómbica aumenta la tensión superficial de las microgotas y estas acaban "explotando" ("explosiones de Coulomb"), formándose así una serie de pequeñas gotas cargadas que seguirán sufriendo procesos de evaporación y explosión sucesivos hasta que finalmente se forman iones cargados desnudos que pasan a fase gaseosa con una o más cargas y son atraídos hacia la entrada del espectrómetro de masas como consecuencia del voltaje aplicado.

La ionización se puede llevar a cabo en el modo positivo o negativo. En el modo positivo, se podrán formar iones múltiplemente protonados $[M+nH]^{n+}$ donde *n* es el número de protones cargados positivamente en la molécula. Del mismo modo, es posible también la formación de aductos con iones sodio, litio, potasio, amonio, etc. En el modo negativo, se observa normalmente la desprotonación de las moléculas, pudiéndose formar también iones múltiplemente desprotonados $[M-nH]^{n-}$.

En el caso de la EI (**Figura 28**) las moléculas de una sustancia volátil se convierten en iones positivos al interactuar con un haz de electrones generado en un filamento metálico de tungsteno o renio calentado a altas temperaturas. El impacto de los electrones sobre las moléculas volátiles puede dar lugar simplemente a la generación de un ion positivo o bien a una ruptura en fragmentos más pequeños, a su vez cargados positivamente. Dependiendo de la energía de los electrones que forman el haz, esta fragmentación se producirá en mayor o menor grado. Generalmente, se suelen usar haces de electrones con una energía de 70 eV, lo que da lugar a una fragmentación significativa. Esta energía de electrones bombardeantes de 70 eV supera a la necesaria para ionizar las moléculas orgánicas. Las energías de ionización (o potenciales de ionización) para las moléculas orgánicas están en el intervalo de 6 a 13 eV, y dependen de su estructura molecular. Una de las limitaciones de esta técnica reside en el hecho de que las moléculas, que se ionizan, deben estar en fase vapor, o sea, ser volatilizables y no experimentar descomposición térmica, previa a su ionización. Este tipo de ionización se ha utilizado en la GC del **Capítulo 10.**

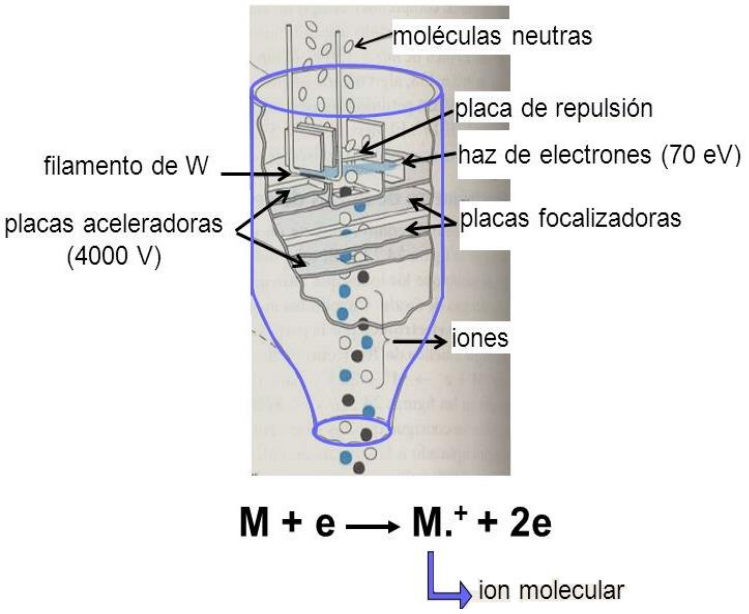


Figura 28. Esquema de la ionización por impacto electrónico.

Una vez que en la interfase se ha llevado a cabo la transferencia de los iones procedentes del capilar desde la fase líquida a la fase gaseosa, los iones son dirigidos hacia el analizador de masas. Los analizadores de masas permiten la separación, detección y cuantificación de los analitos en estudio con un grado de sensibilidad y selectividad muy elevado, proporcionando información sobre su masa molecular.

Existen diversos tipos de analizadores: cuadrupolos, trampas de iones, sectores magnéticos, analizadores de tiempo de vuelo, etc. Como se ha comentado anteriormente, en el desarrollo experimental de esta memoria se han utilizado **TOF** y **QTOF**.

El TOF (**Figura 29**) separa los iones según la distinta velocidad que adquieren en su interior en función de su relación *m/z*. En primer lugar, los iones son extraídos de la cámara de ionización y acelerados hacia el tubo de vuelo mediante un campo electrostático que les aporta una elevada energía cinética. Los iones de mayor *m/z* "volarán" a menor velocidad que los de menor *m/z*. La resolución entre los iones de diferente *m/z* será mejor cuanto mayor sea la longitud del tubo (habrá una mayor separación de los iones en el tiempo) y cuanto menor sea la dispersión en energías de los iones formados en la fuente.

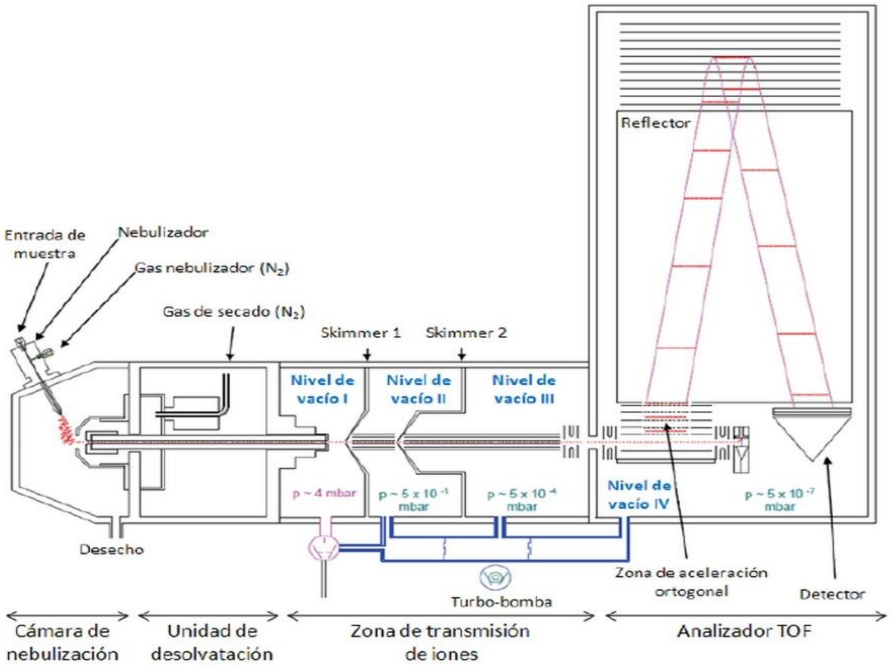


Figura 29. Esquema detallado de las partes de un espectrómetro de masas TOF.

La muestra disuelta entra en la cámara de nebulización donde tiene lugar la formación del spray. Los iones formados atraviesan la unidad de desolvatación, que separa las zonas a presión atmosférica de la primera zona de alto vacío, y que consta de un calentador del gas de secado y un capilar de cristal. Se llega a través de ella a la zona de transmisión o transferencia óptica que consta de tres módulos

que están a alto vacío, separados entre sí por varios skimmers. Los dos hexapolos son los que transfieren los iones hasta la zona de alto vacío, mientras que las lentes enfocan o dirigen dichos iones.

La zona de aceleración ortogonal contiene dos de las últimas lentes mencionadas anteriormente y acelera los iones hacia el tubo de vuelo aplicando un campo eléctrico intermitente.

En función de su masa, los iones se introducen en mayor o menor medida en el reflector. Detrás del mismo hay zonas de tensión que repelen los iones que le llegan; lógicamente, se repelen los iones pequeños con más facilidad.

Este detector TOF es rápido y sensible, permite la determinación de masas exactas y la distribución isotópica, lo que da óptimos resultados en un rango muy amplio sin requerir tediosos procesos rutinarios de re-calibración. Es decir, aporta una mayor fiabilidad de los resultados aplicando un método analítico casi bidimensional: combinando la determinación de masas exactas con el análisis de la distribución isotópica.

Algunas de las especificaciones de los analizadores TOF comerciales son: un rango de masas de 50-3000 *m/z*, una resolución de 10.000-15.000 FWHM (Full Width at Half Maximum, anchura a media altura), y una exactitud de 3 ppm si se realiza calibración interna y 5 ppm cuando es externa.

El analizador de masas QTOF es muy parecido al TOF con la principal diferencia de que a los componentes básicos de un TOF se les suma un cuadrupolo y una celda de colisión. Los skimmers que separaban los distintos módulos de la zona de transmisión en el TOF se sustituyen por funnels, que son anillos concéntricos apilados en forma de embudo. Estos evitan la pérdida de iones, mejorando la transmisión óptica y, como consecuencia, la sensibilidad. El cuadrupolo tiene la misión de seleccionar determinados iones para posteriormente fragmentarlos en la celda de colisión con la ayuda de un gas de colisión, que generalmente es nitrógeno. Los iones fragmentados se separan de la misma manera que en un analizador TOF, en función de su relación *m/z*. En la **Figura 30** se muestra el esquema básico de un QTOF.

La principal ventaja de este tipo de analizadores es que añade un tercer nivel de información para la información de compuestos con la posibilidad de hacer MS/MS, para estudiar el patrón de fragmentación de los compuestos, con una exactitud similar a la del TOF tanto en iones precursores

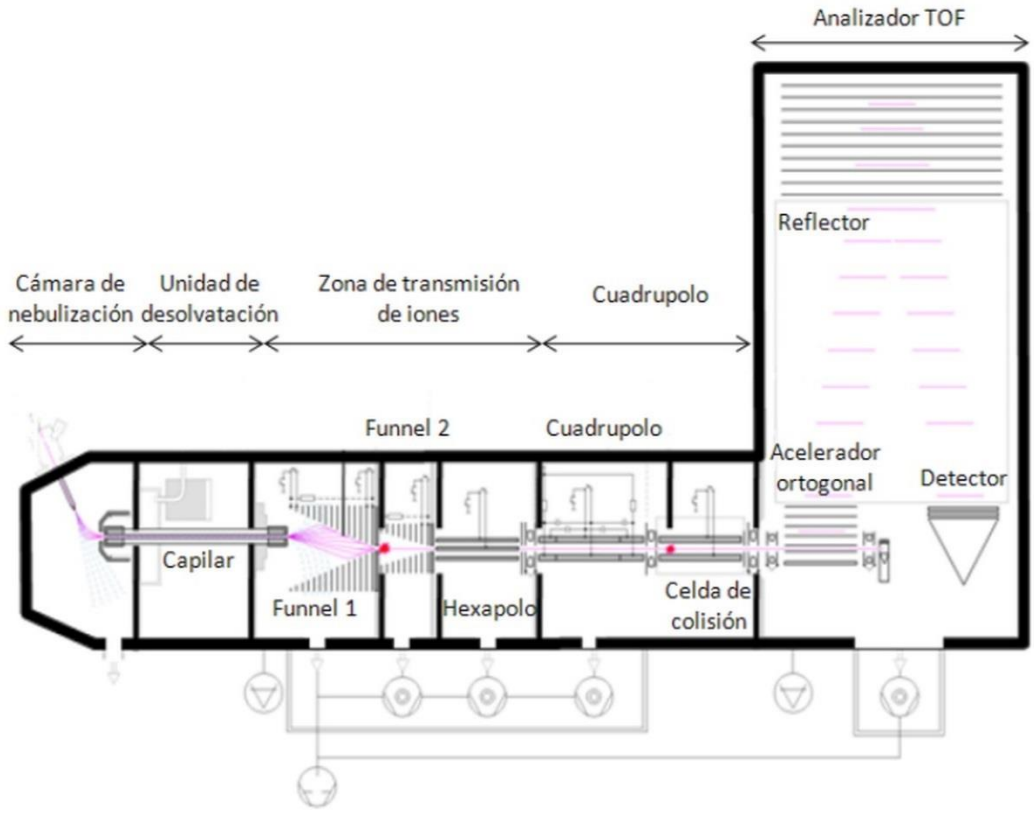


Figura 30. Esquema detallado de las partes de un espectrómetro de masas QTOF.

como de fragmentos. Todo esto se suma a la exactitud de masas y a la distribución isotópica que ya proporcionaba el TOF. Por ello, el análisis de masas inicial de las matrices objeto de estudio se han realizado mediante el analizador QTOF, con el propósito de obtener la mayor información posible con respecto a su composición (**Capítulos 1 - 4**). Una vez obtenida la caracterización pormenorizada, se ha hecho uso del TOF para el análisis de las fracciones purificadas.

5.2.3. Resonancia magnética nuclear

La **resonancia magnética nuclear** (RMN) es la técnica analítica que proporciona mayor información estructural y estereoquímica en un tiempo asequible. La técnica no es destructiva y tiene aplicaciones en todas las áreas de la Química y en algunas de la Biología. Es una espectroscopía de absorción cuyo

fundamento es la absorción de energía (radiofrecuencias) por un núcleo magnéticamente activo, que está orientado en el seno de un campo magnético, y que por efecto de esa energía cambia su orientación del espín nuclear. Con esta técnica se pueden identificar moléculas, determinar su estructura o estudiar procesos dinámicos. Por ejemplo, ha sido clave en la determinación de la estructura de proteínas en disolución y, por otro lado, las técnicas de imagen de RMN son una herramienta indispensable en el diagnostico en medicina.

Las partes fundamentales de un espectrómetro de RMN son (**Figura 31**): un imán, actualmente una bobina superconductora, que suministra el campo magnético principal, un oscilador de radiofrecuencias que suministra la energía necesaria para cambiar la orientación de los núcleos, una bobina detectora que recibe las señales y un sistema informatizado que gobierna todo el aparato y que incluye un sistema de amplificación y registro.

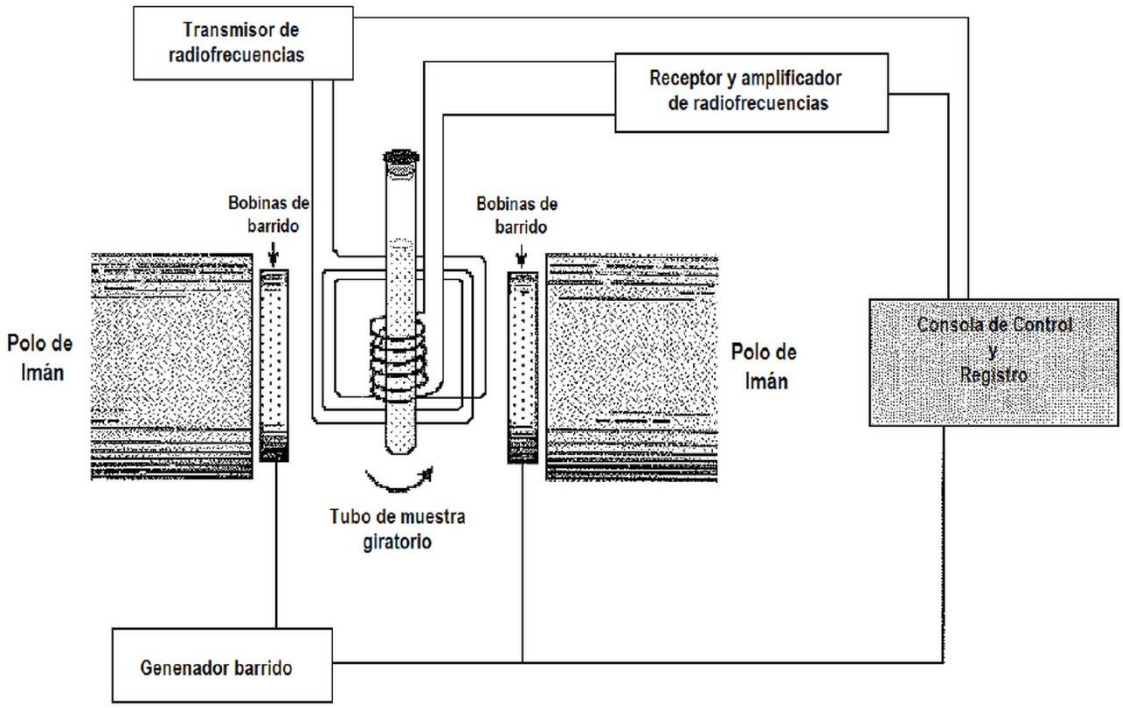


Figura 31. Esquema de un equipo de RMN.

Entre los núcleos más frecuentes en los compuestos orgánicos son magnéticamente activos el protón (^{1}H), carbono (^{13}C), nitrógeno (^{15}N), fósforo (^{31}P) y flúor (^{19}F). Las muestras, generalmente, son disoluciones en disolventes que no tengan átomos de protio (^{1}H). Frecuentemente se usan el

deuterocloroformo, hexadeuterodimetilsulfóxido, óxido de deuterio, deuterobenceno, deuteropiridina y otros. Los espectros más comunes son representaciones de la intensidad de absorción frente a la frecuencia de resonancia (generalmente a través del parámetro δ) y presentan señales cuya posición, forma y tamaño están íntimamente relacionadas con la estructura molecular. El análisis detallado de estos espectros proporciona valiosa información estructural y estereoquímica. Espectros bidimensionales permiten relaciones entre distintos núcleos o distintas magnitudes del mismo núcleo.

Esta técnica aporta una información complementaria a otras más convencionales en la caracterización de compuestos fenólicos, sobre todo a la hora de dilucidar entre formas isoméricas, por lo que se ha requerido en el **capítulo 11** de esta tesis doctoral.

6. Bioactividad de compuestos fenólicos

El creciente interés que despiertan los compuestos fenólicos se debe a diversos factores. En primer lugar, debido a su **actividad antioxidante** (139–141), así como por jugar un importante papel en el crecimiento y metabolismo de la planta. Por otro lado, tienen un fuerte impacto en las cualidades organolépticas y nutricionales de frutas y vegetales. En los alimentos derivados de fuentes vegetales, los compuestos fenólicos están íntimamente relacionados con sus cualidades sensoriales y nutricionales, de forma que pueden contribuir al amargor, astringencia, color, olor y estabilidad oxidativa del alimento. Y, por último, poseen una demostrada actividad fisiológica en humanos (142).

139. Rice-Evans C, Miller N, Paganga G. Antioxidant properties of phenolic compounds. *Trends Plant Sci*. 1997;2(4):152–9.

140. Sengul M, Yildiz H, Gungor N, Cetin B, Eser Z, Ercisli S. Total phenolic content, antioxidant and antimicrobial activities of some medicinal plants. *Pak J Pharm Sci*. 2009;22(1):102–6.

141. Zheng W, Wang SY. Antioxidant Activity and Phenolic Compounds in Selected Herbs. *J Agric Food Chem*. 2001;49(11):5165–70.

142. Cheynier V. Polyphenols in foods are more complex than often thought. Vol. 81, *The American journal of clinical nutrition*. 2005.

Además, diversos estudios han demostrado que los compuestos fenólicos exhiben un amplio rango de propiedades fisiológicas, como antialergénicas, cardioprotectoras, antiaterogénicas, antiinflamatorias, antimicrobianas, antihiperlipémicas y antioxidantes (143–145). Esta demostrada bioactividad de los polifenoles los convierte en importantes compuestos funcionales y, por tanto, es de gran interés su determinación en matrices tanto vegetales como biológicas.

En el caso de los compuestos fenólicos contenidos en las matrices vegetales objeto de estudio en la presente memoria, estos han demostrado poseer unas actividades biológicas destacables como antioxidantes y antiinflamatorias, y efectos beneficiosos en patologías como la obesidad y enfermedades raras de tipo esquelético. Es por ello que a lo largo de este bloque se detallan aspectos particulares de estos efectos y el papel de los polifenoles en ellos. Además, en los **Capítulos 7** y **9** se realiza un estudio exhaustivo de los efectos que poseen las matrices incluidas y los compuestos fenólicos responsables en la salud.

Por otro lado, también se hará mención de las técnicas más relevantes a la hora de determinar la **bioactividad** de un extracto vegetal o de los compuestos que lo componen en relación con las capacidades que se les atribuyen. Para ello es necesario evaluar su eficacia y mecanismo de acción en las diferentes patologías que se pretenden abordar, mediante el uso de diferentes ensayos *in vitro* e *in vivo* que proporcionen los resultados necesarios para comprender cómo actuarán en el organismo. En esta línea, los ensayos ***in vitro*** (del latín “dentro del vidrio”) son aquellos que se realizan fuera de un sistema vivo en un ambiente controlado y los ensayos ***in vivo*** (del latín “dentro de lo vivo”) son los que se realizan en organismos vivos. Ambos tipos de ensayos tienen sus ventajas e inconvenientes.

143. Rajendran P, Nandakumar N, Rengarajan T, Palaniswami R, Gnanadhas EN, Lakshminarasaiah U, et al. Antioxidants and human diseases. *Clin Chim Acta*. 2014;436:332–47.

144. Han X, Shen T, Lou H. Dietary Polyphenols and Their Biological Significance. *Int J Mol Sci*. 2007;8(9):950–88.

145. Shahidi F, Ambigaipalan P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects - A review. *J Funct Foods*. 2015;18:820–97.

La mayor desventaja de la experimentación *in vivo* comparada con la *in vitro* pueden ser las cuestiones éticas y morales que la experimentación animal suscita en la sociedad e incluso en los propios investigadores. Con respecto a las desventajas económicas de la experimentación *in vivo*, el mantenimiento y utilización de los animales de experimentación tiene costes mucho más elevados que los ensayos *in vitro*. Por otro lado, la experimentación *in vivo* también presenta ciertas limitaciones científicas ya que los ensayos *in vitro* permiten una gran versatilidad en el diseño experimental y un número elevado de réplicas, permitiendo la monitorización y automatización y obteniendo resultados de forma rápida y fiable. Esto es más difícil cuando se utilizan animales de experimentación ya que la variación biológica existente entre ellos hace que los resultados no siempre sean totalmente reproducibles, además de que se necesita de personal muy cualificado para planificar y llevar a cabo los experimentos. Pero, por otro lado, los ensayos *in vitro* necesitan de la validación de los métodos y presentan, en muchas ocasiones, la imposibilidad de extrapolar los resultados, ya que no se tiene en cuenta el organismo vivo al completo. Además, hay que añadir que los pacientes desarrollan patologías paralelas y otras complicaciones que solo pueden estudiarse en un organismo vivo. Por último, el estudio y desarrollo de nuevos compuestos bioactivos solo es posible utilizando animales de experimentación, con el fin de establecer la dosis y posología, la cinética y el metabolismo y estudiar las incompatibilidades y los efectos secundarios de los compuestos bioactivos; también son necesarios animales para la producción de anticuerpos mono y policlonales, para el estudio de respuestas conductuales a psicofármacos, establecer la eficacia de vacunas, estudiar la respuesta a implantes, etc. Por otro lado, se presentan una serie de inconvenientes legales que impiden el uso de animales de experimentación en los casos que se recogen en la Directiva 2010/63/UE del Parlamento Europeo y del Consejo y en el Real Decreto 53/2013, que establece en el "Artículo 24. Elección de los métodos" que:

1) No deberá realizarse un procedimiento en animales si la normativa de la Unión Europea reconoce otro método u otra estrategia de ensayo para obtener el resultado perseguido que no implique la utilización de animales vivos.

2) Si se deben realizar experimentos en animales, se optará por los procedimientos que tengan las mayores probabilidades de proporcionar resultados satisfactorios y que cumplan el mayor número de los siguientes requisitos:
    a) que utilicen el menor número de animales,
    b) que afecten a animales con la menor capacidad de sentir dolor, sufrimiento, angustia o daño duradero (los menos evolucionados),
    c) que causen menor dolor, sufrimiento, angustia o daño duradero.

A continuación, se abordarán cada una de las actividades beneficiosas que los compuestos fenólicos contenidos en las matrices incluidas en la presente tesis doctoral han demostrado poseer.

6.1. Capacidad antioxidante de compuestos fenólicos

El oxígeno, asociado a las condiciones de vida aerobia, representa la fuerza motriz para el mantenimiento del metabolismo y viabilidad celular, al mismo tiempo que entraña un peligro potencial debido a sus especiales características paramagnéticas, responsables de la formación de intermediarios dotados de una alta reactividad, conocidas como **especies reactivas del oxígeno** (ROS, del inglés "Reactive Oxygen Species")(146).

En 1985, *H. Sies* propuso el concepto de «**daño o estrés oxidativo**» como un desbalance, en el que hay un aumento de oxidantes o una disminución de antioxidantes, en comparación con una situación de homeostasis. Como consecuencia de esto, se pueden producir alteraciones de la relación estructura-función en cualquier órgano, sistema o grupo celular especializado, por lo que se reconoce como mecanismo general de daño celular, asociado con la fisiopatología primaria o la evolución de un número creciente de entidades y síndromes de interés médico-social, involucrado en la génesis y en las consecuencias de dichos eventos. Se considera que los radicales libres producen al azar un daño acumulativo en las macromoléculas biológicas, que conduce a una disminución de las funciones vitales y al envejecimiento (147).

146. Davies KJA. Oxidative stress: the paradox of aerobic life. *Biochem Soc Symp*. 1995;61:1–31.

147. Sies H, Cadenas E. Oxidative Stress: Damage to Intact Cells and Organs. *Philos Trans R Soc B Biol Sci*. 1985;311(1152):617–31.

Los **radicales libres** son especies moleculares activadas, dotadas de un electrón desapareado en un nivel energético superior, y por tanto, con propiedades paramagnéticas, lo que les confiere una alta e indiscriminada reactividad, ya que, al ser inestables, tienen tendencia a ceder o a captar un electrón de otra molécula o radical para conseguir una configuración electrónica estable. Esta elevada reactividad implica una baja especificidad química, de manera que pueden reaccionar con todo tipo de moléculas vecinas, ya sean lípidos, proteínas, glúcidos o ácidos nucleicos. Esta elevada reactividad también hace que estos radicales no sobrevivan más de unos cuantos microsegundos en el medio, y en unas concentraciones muy bajas. También hay algunas moléculas que tienen átomos de oxígeno reactivos pero no en su forma radical y que participan de forma activa en las reacciones de los radicales libres en los sistemas biológicos. Para englobar todos estos compuestos derivados del oxígeno, radicales y no radicales, se creó el término "ROS" (148). Así pues, entre las ROS se encuentran:

- Radicales: El ion-radical superóxido ($O_2^{\bullet -}$), y los radicales hidroxilo ($^{\bullet}OH$), alcoxilo ($RO^{\bullet}$), peroxilo ($ROO^{\bullet}$) y óxido nítrico ($NO^{\bullet}$).
- No radicales: Peróxido de hidrógeno (H_2O_2), ozono (O_3), hidroperóxido (ROOH), ácido hipocloroso (HOCl), oxígeno singlete ($^1O_2$) y peroxinitrilo ($ONOO^-$).

Ante el estrés oxidativo, debido a la presencia de estas especies, el organismo responde con la defensa antioxidante endógena mediante enzimas antioxidantes como la catalasa o la superóxido dismutasa, pero en determinadas ocasiones puede ser insuficiente. Durante los últimos años se ha puesto de manifiesto la existencia de una estrecha relación entre esta situación de desequilibrio y una serie de procesos fisiológicos y patológicos que limitan la calidad y la expectativa de vida de muchas personas, como enfermedades crónicas y relacionadas con el envejecimiento, entre las que se encuentran dos de las mayores causas de mortalidad en las sociedades occidentales como son el cáncer y las cardiopatías (149).

148. Halliwell B. Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. *Plant Physiol*. 2006;141(2):312–22.

149. Lee J, Koo N, Min DB. Reactive Oxygen Species, Aging, and Antioxidative Nutraceuticals. *Compr Rev Food Sci Food Saf*. 2004;3(1):21–33.

La actividad antioxidante que los compuestos fenólicos han demostrado se debe a su habilidad captadora de radicales libres, a la capacidad de donar átomos de hidrógeno o electrones, o a la quelación de cationes metálicos que intervienen en las reacciones de formación de ROS, modulación de la expresión de genes responsables de la síntesis, activación e inhibición de enzimas antioxidantes o relacionadas con ellas e interacción con señales celulares (47,150–152). Su estructura es clave para la eliminación de estos radicales y para la actividad quelante, y es lo que se conoce como **relación estructura-actividad** (SAR).

En el caso de los ácidos fenólicos, por ejemplo, la actividad antioxidante depende del número y posiciones de los grupos hidroxilo en relación al grupo funcional carboxilo (153). Los ácidos monohidroxibenzoicos con un grupo funcional OH en posición *orto* o *para* con relación al grupo COOH, no muestran actividad antioxidante; en cambio, sí que son activos cuando este grupo hidroxilo se presenta en la posición *meta*, como es el caso del ácido *m*-hidroxibenzoico. Esta actividad antioxidante aumenta con un mayor grado de hidroxilación, como es el caso del ácido gálico (trihidroxilado). Sin embargo, la sustitución de los grupos hidroxilo con grupos metoxilo en las posiciones 3 y 5, como en el ácido siríngico, reduce la actividad. Por su parte, los ácidos hidroxicinámicos presentan mayor actividad antioxidante en comparación con los hidroxibenzoicos (154). El incremento de esta actividad podría ser debido a la presencia del doble enlace conjugado del grupo CH=CH-COOH, que garantiza una mayor capacidad de donar protones y estabilizar radicales libres en comparación con el grupo COOH en los ácidos hidroxibenzoicos.

150. Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem*. 2004;84(4):551–62.

151. Huang D, Ou B, Prior RL. The Chemistry behind Antioxidant Capacity Assays. *J Agric Food Chem*. 2005;53(6):1841–56.

152. Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun T. Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. *Mutat Res Mol Mech Mutagen*. 2005;579(1–2):200–13.

153. Robards K, Prenzler P, Tucker G. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem*. 1999;66(4):401–36.

154. Andreasen MF, Landbo A-K, Christensen LP, Hansen Å, Meyer AS. Antioxidant Effects of Phenolic Rye (Secale cereale L.) Extracts, Monomeric Hydroxycinnamates, and Ferulic Acid Dehydrodimers on Human Low-Density Lipoproteins. *J Agric Food Chem*. 2001;49(8):4090–6.

Las SAR han demostrado, además, que el grupo catecol es el principal determinante de la actividad antioxidante de los fenoles, hecho que justifica que el ácido clorogénico y el ácido cafeico sean más efectivos que los antioxidantes clásicos como el ácido ascórbico (155).

La SAR de los flavonoides, en general, es más complicada que en los ácidos anteriormente descritos debido a la relativa complejidad de sus moléculas. Algunas características estructurales y la naturaleza de las sustituciones en los anillos B y C que determinan la actividad de estos compuestos son las siguientes (156) (**Figura 32**):

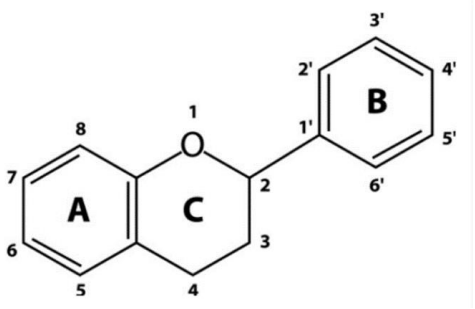


Figura 32. Esqueleto básico de flavonoides

1) El grado de hidroxilación y la posición de los grupos OH en el anillo B, en particular, una estructura *orto*-dihidroxilo (grupo catecol) se traduce en una mayor actividad, ya que confiere mayor estabilidad al radical por deslocalización electrónica (157), actuando además como sitio de fijación preferida para trazas de metales (158).

2) La presencia de los grupos hidroxilos en las posiciones 3', 4' y 5' del anillo B (grupo pirogalol) ha sido reportada por incrementar la actividad antioxidante, en comparación con la de flavonoides que tienen un simple grupo hidroxilo. Sin embargo, bajo las mismas condiciones, tales compuestos pueden actuar como pro-oxidantes, neutralizando el efecto antioxidante (157).

155. Rice-Evans CA, Miller NNJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med*. 1996;20(7):933–56.

156. Amic D, Davidovic-Amic D, Beslo D, Rastija V, Lucic B, Trinajstic N. SAR and QSAR of the Antioxidant Activity of Flavonoids. *Curr Med Chem*. 2007;14(7):827–45.

157. Van Acker SABE, Van Den Berg D, Tromp MNJL, Griffioen DH, Van Bennekom WP, Van Der Vijgh WJF, et al. Structural aspects of antioxidant activity of flavonoids. *Free Radic Biol Med*. 1996;20(3):331–42.

158. Pietta PG. Flavonoids as antioxidants. J Nat Prod. 2000;63(7):1035–42.

3) Un doble enlace entre los carbonos C-2 y C-3, conjugado con el grupo 4-oxo, en el anillo C, incrementa la capacidad captadora de radicales de los flavonoides (158).

4) Un doble enlace entre C-2 y C-3, combinado con un 3-OH, en el anillo C, incrementa también la capacidad captadora de radicales, como es el caso del kampferol (157). Una sustitución del grupo 3-OH produce un incremento del ángulo de torsión y una pérdida de coplanaridad y, subsecuentemente, una reducción de la actividad antioxidante (159).

5) La sustitución de grupos hidroxilo en el anillo B por grupos metoxilos altera el potencial redox y la capacidad captadora de radicales de los flavonoides (158,159).

6) Los 3- y 7-O-glicósidos tienen menor actividad que sus agliconas frente a radicales libres (155).

6.1.1. Determinación de la capacidad antioxidante

Debido a la complejidad de los procesos de oxidación, no existe un método que refleje de forma completa el perfil antioxidante de una muestra, por tanto, es importante trabajar con varios métodos para facilitar la comparación e interpretación de los resultados. Por esto se han planteado una serie de condiciones que debería reunir un procedimiento estandarizado de medida de capacidad antioxidante (160):

- Evaluar reacciones de transferencia de electrones y de átomo de hidrógeno.
- Especificar el sustrato de oxidación.
- Medir reacciones químicas que de hecho ocurran en reacciones potenciales, es decir, asegurar que el sustrato y el modo de inducir la oxidación son relevantes como fuentes de daño oxidativo.
- Ser sencillo.
- Tener un mecanismo y un punto final definido.

159. Seeram NP, Nair MG. Inhibition of Lipid Peroxidation and Structure−Activity-Related Studies of the Dietary Constituents Anthocyanins, Anthocyanidins, and Catechins. *J Agric Food Chem*. 2002;50(19):5308–12.

160. Frankel EN, Meyer AS. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J Sci Food Agric*. 2000;80(13):1925–41.

- Poseer una instrumentación más o menos disponible.
- Tener una buena reproducibilidad.
- Ser adaptable para medir antioxidante hidrofílicos y lipofílicos.
- Usar distintas fuentes de radicales.
- Ser adaptable para análisis rutinarios a gran escala.

La realidad es que no existe ningún método en la actualidad que reúna tales características y es difícil que llegue a ser posible evaluar la capacidad antioxidante de una muestra por un solo método, en vez de por la combinación de varios, como se hace en la actualidad. Esto se debe a varias razones; en primer lugar, los antioxidantes pueden ejercer su acción mediante mecanismos muy diversos como ya se ha visto (pueden suprimir la generación de los primeros radicales que inician el daño oxidativo, capturar radicales libres, quelar metales, formar complejos, reducir algunos compuestos, inducir la actividad de sistemas biológicos antioxidantes...) y en un mismo alimento puede haber mezclas de diferentes antioxidantes con distintos mecanismos de acción y entre los que, además, se pueden establecer reacciones sinérgicas, por lo que serán necesarios distintos análisis para poder considerar los posibles mecanismos de acción de todos los antioxidantes presentes en un alimento.

Las medidas de la actividad antirradicalaria se pueden realizar mediante dos estrategias distintas, en función de la información que se desea obtener (151):

- Determinación directa: El radical se emplea como un factor de cuantificación (produce una señal analítica). La adición del antioxidante, antes o después de la generación del radical, provoca una disminución de la señal (métodos TEAC**,** DPPH, etc.)
- Determinación indirecta: La presencia de radicales libres produce la pérdida o aparición de un reactivo y, por tanto, en presencia de un antioxidante se provoca el aumento o disminución de la señal (métodos FRAP, ORAC, TBARS, etc.)

A continuación se describen los métodos que se han utilizado para determinar la capacidad antioxidante en las matrices de estudio.

6.1.1.1. FRAP (Ferric ion Reducing Antioxidant Power)

Este método de transferencia de electrones (SET) se basa en la reducción del complejo de la tripiridiltriazina férrica al complejo ferroso por un antioxidante en medio ácido (**Figura 33**) (161).

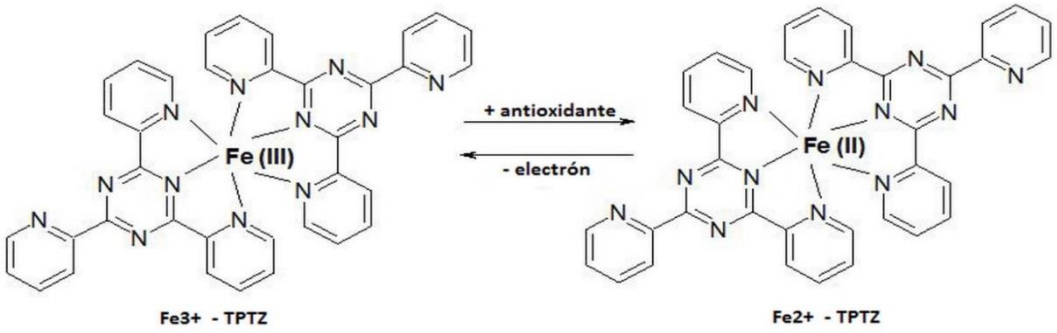


Figura 33. Reacción producida en el método FRAP.

Se trata de un método espectrofotométrico en el que se mide la absorbancia del Fe^{2+}. Así, cuanto más antioxidante es la sustancia objeto de estudio, mayor es la reducción y mayor la concentración de Fe^{2+} y, por tanto, más alta la señal de absorbancia.

6.1.1.2. TEAC (Trolox-Equivalent Antioxidant Capacity)

Se trata de una reacción SET, donde el radical $ABTS^{\bullet+}$ se genera a partir de su precursor, el ácido 2,2'-azinobis (3- etilbenzotiazolín)-6-sulfónico (ABTS) (**Figura 34**) (162). El radical catiónico obtenido es un compuesto de color verde-azulado, estable y con un espectro de absorción en el Visible.

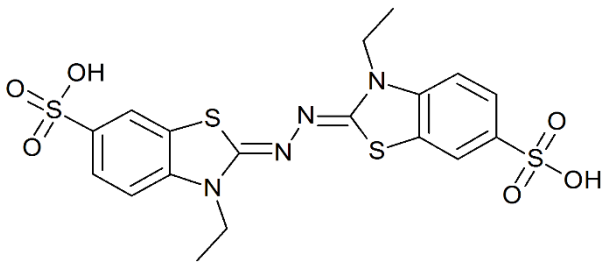


Figura 34. Estructura del ácido 2,2'-azinobis (3- etilbenzotiazolín)-6-sulfónico (ABTS).

161. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal Biochem*. 1996;239(1):70–6.

162. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med*. 1999;26(9–10):1231–7.

La ventaja de este ensayo es que puede realizarse tanto en muestras hidrosolubles como liposolubles, eligiendo el disolvente apropiado en cada caso. La forma más usual es generar el radical $ABTS^{\bullet+}$ químicamente utilizando persulfato potásico.

6.1.1.3. ORAC (Oxygen Radical Absorbance Capacity)

El fundamento del método ORAC se basa en la habilidad que tienen los compuestos antioxidantes para bloquear radicales libres por donación de un átomo de hidrógeno:

$$X + AH \rightarrow XH + A^{\bullet}$$

En este método se mide la capacidad de captación de un radical específico, el peroxilo, generado a partir de la molécula orgánica AAPH (2,2'-Azobis-(2-aminopropano)-dihidrocloruro). Estos radicales atacarán a la molécula de fluoresceína, produciendo fluoresceína oxidada, que ya no emite fluorescencia y, produciendo, por tanto, un descenso en la misma (**Figura 35**) (163).

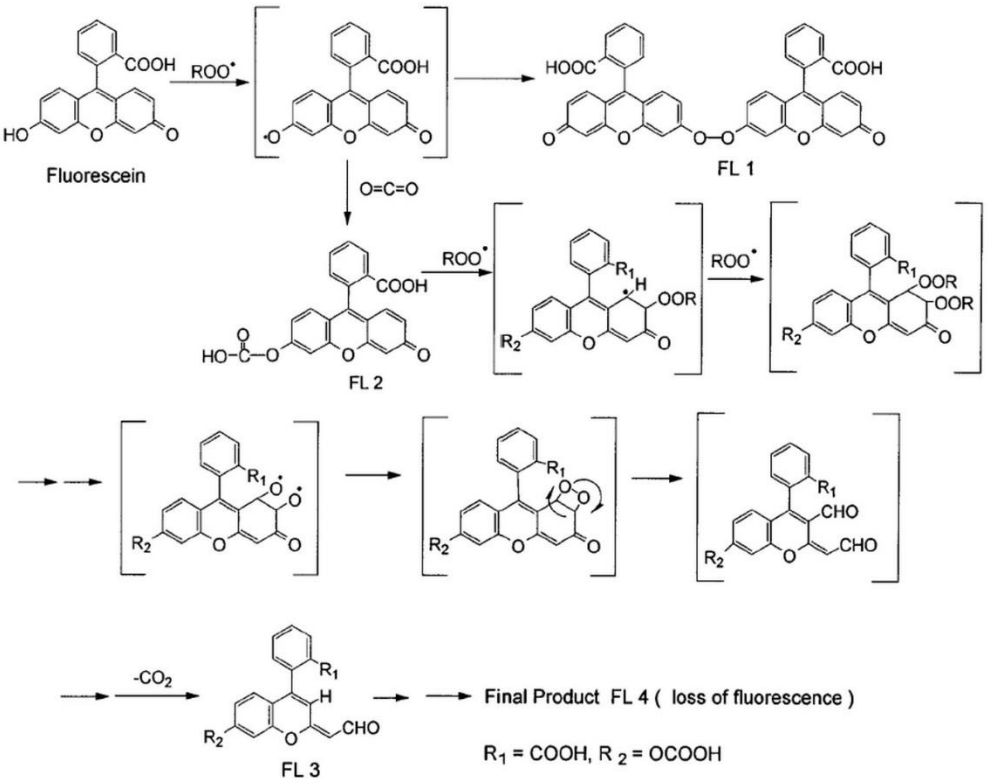


Figura 35. Esquema del mecanismo de reacción de la fluoresceína en presencia de AAPH. Los productos de oxidación generados presentan una menor fluorescencia.

163. Ou B, Hampsch-Woodill M, Prior RL. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J Agric Food Chem*. 2001;49(10):4619–26.

6.1.1.4. Ensayos antioxidantes en células

El **cultivo celular** tuvo su origen en el siglo XIX, como un método para el estudio del comportamiento de las células animales libres de las variaciones sistémicas ocurridas dentro del organismo y bajo el estrés de un experimento. En la actualidad, pueden cultivarse en el laboratorio células procedentes de una amplia gama de tejidos y organismos diferentes. Los cultivos de células *in vitro* consisten en un sistema formado por células provenientes de un órgano o un tejido, normal o tumoral, mantenidas en medios de cultivo de composición química definida y en condiciones de temperatura, pH, aireación y humedad controladas. De esta forma se aseguran su supervivencia y multiplicación.

La capacidad antioxidante de los extractos incluidos en la presente tesis doctoral también se ha determinado a nivel celular. Mediante la determinación de la producción de ROS en células humanas endoteliales de la vena umbilical (HUVEC) con la sonda 2', 7'-diacetato de diclorodihidrofluoresceína, que en presencia de ROS se convierte en su forma fluorescente (DCF) y es cuantificada por citometría de flujo (**Capítulos 1** y **4**).

6.1.2. Determinación de polifenoles totales y contenido en flavan-3-oles

El contenido de polifenoles totales y de flavan-3-oles se determinó en los extractos ricos en proantocianidinas: té verde, corteza de pino, semilla de uva y cacao. En el primer caso, se llevó a cabo por el método de **Folin-Ciocalteu** que se basa en la reducción de una mezcla de ácido fosfotúngstico ($H_3PW_{12}O_{10}$) y ácido fosfomolíbdico ($H_2PMo_{12}O_{40}$) por los polifenoles presentes en las muestras, a una mezcla de óxidos de tusgsteno (W_8O_{23}) y de molibdeno (Mo_8O_{23}), que presentan color azul. Esta coloración puede determinarse midiendo la absorción de radiación a una longitud de onda de 765 nm, la cual se relaciona de manera directa con la cantidad de polifenoles presentes.

Para el caso de la determinación del contenido de flavan-3-oles de estas mismas muestras se ha utilizado el método de la **Vainillina**. Este método se basa en la condensación de la vainillina con

proantocianinas en una solución acidificada. La vainillina protonada, un electrofílo débil, reacciona con el anillo del flavonoide en la posición 6 u 8. El producto de esta reacción se deshidrata fácilmente para dar un color rosa ligero a un intenso rojo cereza que es medido a 500 nm. La estabilidad del color del aducto vainillina-tanino puede incrementarse cuando la luz es excluída y la temperatura de reacción es controlada y entonces se obtienen resultados exactos y reproducibles. El ensayo es específico para flavan-3-ol, dihidrochalconas y proantocianinas, las cuales tienen un enlace simple en la posición 2,3 y poseen grupos hidroxilos en la posición meta del anillo B. La catequina es frecuentemente usada como estándar en el ensayo de la vainillina.

6.2. Capacidad antiinflamatoria de compuestos fenólicos

La **actividad antiinflamatoria** de los polifenoles ha despertado un gran interés científico en los últimos años debido al potencial que tienen para interferir en la evolución de ciertas enfermedades que cursan con procesos inflamatorios. La inflamación fue descrita en el año 10 a.C. por Celso "*rubor et tumor cum calore et dolore*", conocida como la Tétrada de Celsius. Posteriormente, Virchow (1821-1908) agregó un quinto signo, la "*functio laesa*" (pérdida de función). Estos cinco signos clínicos responden a los principales fenómenos vasculares y celulares que ocurren en las áreas inflamadas. El proceso inflamatorio involucra una serie de eventos inespecíficos que pueden ser provocados por numerosos estímulos o agresiones del medio como, por ejemplo: agentes biológicos, isquemia, interacciones antígeno-anticuerpo, traumatismos, lesiones térmicas o fisicoquímicas de otra índole, entre otros. Cada tipo de estímulo provoca una respuesta característica que constituye una variante relativamente menor del mismo fenómeno. A nivel macroscópico, la respuesta está usualmente acompañada por conocidos signos clínicos como tumefacción (edema), rubor, calor, dolor espontáneo a la palpación y desorden de la función tisular. Las manifestaciones clínicas generales más habituales son la leucocitosis y la neutrofilia, fiebre, anorexia, astenia, aumento de la velocidad de sedimentación globular, etc. (164). En el proceso global intervienen muchos mecanismos, algunos mediados por una variedad de moléculas de señalización, además de la activación de los factores del complemento

(165). Los mediadores pertenecen a diferentes clases químicas, tales como aminas biógenas (histamina, serotonina), proteínas y péptidos (enzimas hidrolíticas, citoquinas, factores de crecimiento, factores activadores de colonia, factores de complemento, anticuerpos, quininas), ROS y lípidos (factores activadores de plaquetas, prostanoides, leucotrienos). Estos mediadores inician, mantienen, agravan y modulan el curso de un gran número de enfermedades humanas como la artritis reumatoide, el asma, la inflamación intestinal, la EPOC, el cáncer y la obesidad, entre otras (166).

Existen numerosos trabajos sobre la evaluación de la actividad antiinflamatoria que se han realizado tanto en extractos vegetales como en metabolitos secundarios aislados de fuentes naturales (167). Dentro de los polifenoles, los flavonoides son los que alcanzan mayor relevancia por su capacidad para inhibir la activación de citoquinas proinflamatorias, factores nucleares y otras enzimas inflamatorias como: interleuquinas 2 y 6 (IL2, IL6), factor de necrosis tumoral alfa (TNF-α), factor nuclear kappa (NF-κB), proteína activadora 1 (AP-1), ciclooxigenasa 2 (COX-2), óxido nítrico sintasa inducible (iNOS), entre otras, y la producción de monóxido de nitrógeno (NO) en distintas líneas celulares (168,169).

La **Figura 36** muestra los puntos potenciales de acción de los polifenoles en la cascada inflamatoria (170).

164. Okin D, Medzhitov R. Evolution of Inflammatory Diseases. *Curr Biol*. 2012;22(17):R733–40.

165. Nathan C. Points of control in inflammation. *Nature*. 2002;420(6917):846–52.

166. Hunter P. The inflammation theory of disease. *EMBO Rep*. 2012;13(11):968–70.

167. Rahman I, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol*. 2006;72(11):1439–52.

168. González R, Ballester I, López-Posadas R, Suárez MD, Zarzuelo A, Martínez-Augustin O, et al. Effects of Flavonoids and other Polyphenols on Inflammation. *Crit Rev Food Sci Nutr*. 2011;51(4):331–62.

169. Guo W, Kong E, Meydani M. Dietary Polyphenols, Inflammation, and Cancer. *Nutr Cancer*. 2009;61(6):807–10.

170. Santangelo C, Varì R, Scazzocchio B, Di Benedetto R, Filesi C, Masella R. Polyphenols, intracellular signalling and inflammation. *Ann Ist Super Sanita*. 2007;43(4):394–405.

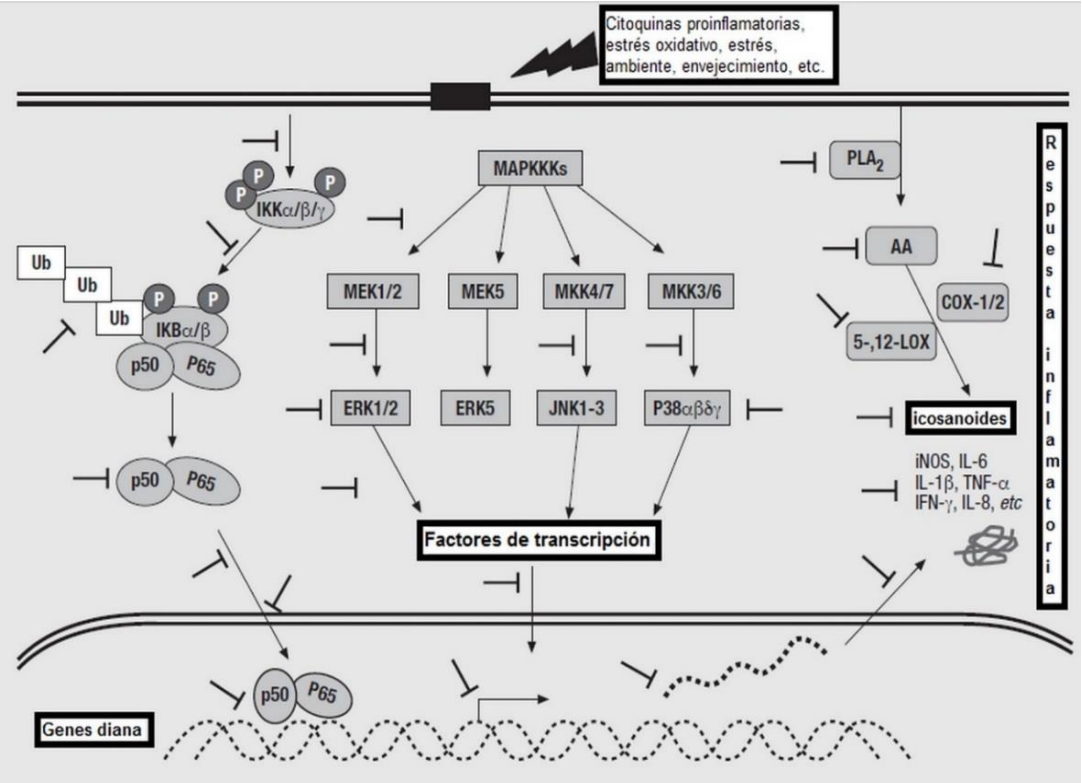


Figura 36. Puntos potenciales de acción de los polifenoles en la cascada inflamatoria (⊥). IKB, inhibidor kB; Ub, ubiquitina; IKK, IkB quinasa; ERK, quinasa reguladora de señales extracelulares; JNK, quinasa c-Jun amino terminal; p38 (p38-MAPK), p38 proteína quinasa activada por mitógenos; MEK (MKK), MAPK quinasa; MAPKKK, MAPK quinasa quinasa; IL-8, interleuquina 8; IFNγ, interferón-γ; TNF-α, factor de necrosis tumoral-α; IL-1β, interleuquina 1β; IL-6, interleuquina 6; iNOS, sintasa inducible de óxido nítrico; LOX, lipooxigenasa; COX, ciclooxigenasa; AA, ácido araquidónico; PLA_2, fosfolipasa A_2.

6.2.1. Determinación de la capacidad antiinflamatoria

En los últimos años ha sido creciente la cantidad de publicaciones e investigaciones alrededor de la comprensión de los mecanismos y de las moléculas involucradas en el **proceso inflamatorio**. El desarrollo de la biología molecular ha permitido estudiar numerosas enzimas y mediadores involucrados en el proceso, midiendo la expresión o evaluando las señales bioquímicas y fisiológicas que se activan en respuesta a un estímulo específico.

La actividad antiinflamatoria de los polifenoles está tomando cada vez más importancia, aunque por supuesto no es algo que debe sorprender dada la relación entre estrés oxidativo e inflamación. El

estrés oxidativo produce un aumento de enzimas tales como ciclooxigenasa (COX) y lipoxigenasa (LO), que están implicados en la liberación de factores como interleuquinas y quimioquinas.

Para determinar la actividad antiinflamatoria de un compuesto, se han desarrollados distintas técnicas tanto *in vitro* como *in vivo*. Entre las más frecuentes se encuentran: la determinación de actividad enzimática (inhibición de la 5-lipooxigenasa, de la xantina oxidasa o de la β–glucoronidasa), modelos celulares (macrófagos humanos de la línea celular de monocitos THP-1, macrófagos de ratón de la línea celular RAW 264.7 o células humanas endoteliales de la vena umbilical HUVEC) y modelos animales (inducción de edema, inducción de colitis o inducción de un estado inflamatorio general o localizado). A continuación, se describen las que se han llevado a cabo en la presente tesis doctoral.

6.2.1.1. Modelos celulares

Para el desarrollo de los ensayos antiinflamatorios de los compuestos fenólicos contenidos en las matrices de esta memoria (**Capítulos 1** y **4**) se han utilizado modelos celulares HUVEC. En el medio de cultivo de las células se incorpora habitualmente lipopolisacáridos de origen bacteriano (LPS) o TNF-α para simular una situación de inflamación, junto con el compuesto a estudiar. Transcurrido el tiempo de ensayo se determina tanto la secreción como la expresión génica de diversos mediadores implicados en el proceso inflamatorio como citoquinas, moléculas de adhesión o factores de transcripción.

En nuestro caso el intermediario a medir fue la proteína quimiotáctica de monocitos 1 (MCP-1), que es un mensajero químico que induce la quimiotaxis, migración e infiltración de los macrófagos al área inflamada. La cuantificación de la concentración de MCP-1 tras el tratamiento con los extractos se determina empleando el enzimo-inmunoensayo (ELISA) y la cuantificación de la expresión génica mediante la reacción en cadena de la polimerasa (PCR) a tiempo real.

6.2.1.2. Estudio de los intermediarios del metabolismo celular mediante GC-QTOF

Por otro lado, ya se ha mencionado anteriormente la estrecha relación entre el estrés oxidativo, la inflamación y ciertas patologías que son derivadas de desórdenes metabólicos. La formación de ROS es inherente al metabolismo aeróbico por medio del cual se obtiene energía de las diferentes moléculas en las células y en este sentido la glucosa no es la excepción; aún más, la glucosa es la principal molécula que se oxida para dar energía y la más abundante en la célula y en el organismo para fines metabólicos. En el metabolismo, las ROS son producto de una reducción parcial del oxígeno, y los electrones que este adquiere son donados anómalamente por reacciones óxido-reductoras que lo reducen parcialmente. La reducción del oxígeno en el metabolismo es un paso necesario, indispensable, pero esos electrones, siendo adecuadamente otorgados, deberían de reducir el oxígeno hasta agua con la combinación de dos protones en la reacción. Además, el catabolismo es

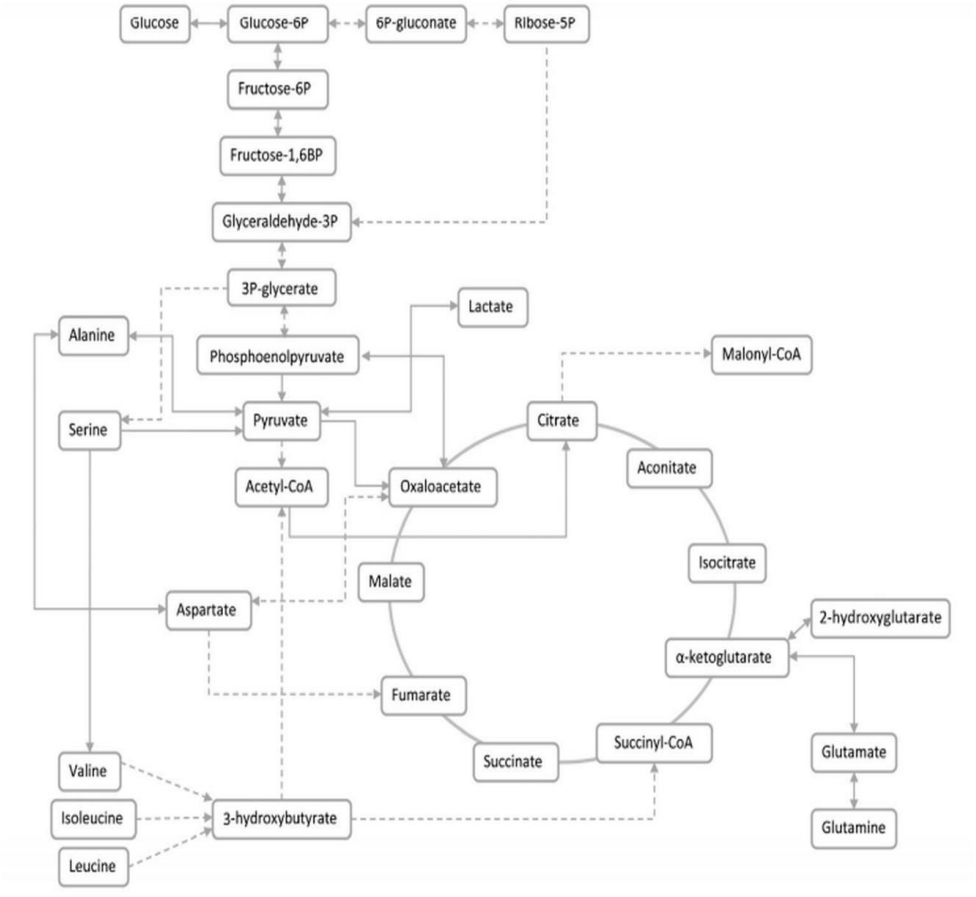


Figura 37. Intermediarios de las rutas del metabolismo energético.

eminentemente oxidativo y se basa en la pérdida de electrones de las moléculas metabolizadas. Es por ello que es posible obtener ROS en varias reacciones oxidativas tanto de la glucolisis como del ciclo de los ácidos tricarboxílicos. Evidentemente el incremento de la actividad metabólica a cualquier escala, viene aparejada con un incremento proporcional de la cantidad de ROS formadas de manera secundaria. Sin embargo, cuando esta actividad metabólica se incrementa muy por arriba de las condiciones fisiológicas (estrés metabólico) el incremento de la formación de ROS no se suma sino que se potencia, dado que en condiciones de un incremento notable del potencial de la membrana interna mitocondrial, debido a una acumulación excesiva de protones en el espacio intermembranal, se incrementa la permanencia, tiempo y posibilidad de reducir parcialmente el oxígeno en los diferentes puntos de la cadena respiratoria. En tal sentido el estrés metabólico, el energético, el respiratorio, el infeccioso, un incremento en la ingesta de nutrientes o una demanda metabólica y óxido-reductora inducida por moléculas endógenas o xenobióticas generarán un incremento en la demanda del metabolismo, incrementando la actividad de la cadena transportadora de electrones y la cantidad de especies reactivas de oxígeno, aumentando la posibilidad de tener daño oxidativo (171).

Dada la importancia del estrés oxidativo generado en el **metabolismo energético**, en la presente memoria se plantea una aproximación para demostrar el efecto beneficioso que los compuestos fenólicos pueden ejercer en las rutas metabólicas (**Figura 37**) (125), estudiando el comportamiento de metabolitos intermediarios de la glucolisis, de la ruta de las pentosas fosfato y del ciclo de Krebs como marcadores indirectos mediante GC-EI-QTOF-MS en dos modelos celulares, uno de oxidación y otro de inflamación (**Capítulo 10**).

6.2.1.3. Inmunofluorescencia

La **inmunofluorescencia** (IF) es una técnica de gran utilidad para la detección y localización de antígenos celulares mediante el uso de anticuerpos marcados con fluorocromos.

171. Calderón Salinas JV, Muñoz Reyes, Elvia Guadalupe Quintanar Escorza MA. Estrés oxidativo y diabetes mellitus. *Rev Educ bioquímica*. 2013;32(2):53–66.

Aunque el procedimiento es relativamente sencillo, incluyendo los pasos de fijación y permeabilización de las muestras, bloqueo e incubación con los anticuerpos marcados, en muchos casos el éxito del ensayo puede depender del correcto ajuste de determinadas variables durante el proceso. Como técnica de tinción, puede ser utilizada en cortes de tejidos, líneas celulares cultivadas, células individuales y secreciones que contengan células en suspensión con la finalidad de analizar la presencia y distribución de nuestras moléculas diana. Además, puede ser utilizada en combinación con otras técnicas de coloración fluorescente que no hagan uso de anticuerpos, como por ejemplo DAPI para marcar ADN (como en el caso de la tinción Hoechst).

Existen dos tipos de técnicas de IF; primaria (o directa), en la que solo se hace uso de un anticuerpo que se encuentra químicamente unido a un fluorocromo; y secundaria (o indirecta), en la que se hace uso de dos anticuerpos; el anticuerpo primario es el que reconoce y se une a la molécula diana, mientras que el secundario que es el que se encuentra marcado con el fluoróforo, reconoce al primario y se une a él. Esta última es la que se ha utilizado para la determinación de la modulación de la AMPK en adipocitos hipertrofiados (**Capítulo 8**).

6.3. Efecto de los compuestos fenólicos en la salud

6.3.1. Relación de los compuestos fenólicos y la obesidad

La **obesidad** es una patología ampliamente extendida, de elevada prevalencia en los países industrializados (en 2016, más de 1900 millones de adultos tenían sobrepeso, de los cuales, más de 650 millones eran obesos), y se considera uno de los principales problemas de salud de la sociedad moderna (172). Esta enfermedad se define como un exceso de almacenamiento de energía en forma de grasa dentro de los adipocitos que forman el tejido adiposo, y por lo tanto se acompaña de un aumento en el tamaño de estas células (hipertrofia) y del número de estas (hiperplasia).

172. Organización Mundial de la Salud (OMS). Obesidad y Sobrepeso. Nota Descr. 2017;2.

Además, tiene un origen multifactorial abarcando desde factores genéticos y/o neuroendocrinos, la dieta, el sedentarismo o el uso de algunos fármacos (antidepresivos, anticonceptivos o glucocorticoides), entre otros. La leptina, un neuropéptido, es secretada por los adipocitos y actúa a nivel hipotalámico, controlando el apetito de tal forma que la leptinemia es reflejo de las reservas grasas del cuerpo. Una expresión elevada de leptina conlleva una hiperfagia que dará lugar a la obesidad.

Estudios *in vitro* sugieren que factores liberados localmente por los adipocitos hipertrofiados, como el TNF-α y el factor de crecimiento insulínico 1 (IGF-1), estimulan la hiperplasia (173) y, por lo tanto, favorecen la diferenciación de los preadipocitos a adipocitos maduros. Por otro lado, el receptor gamma activador de la proliferación del peroxisoma (PPAR-γ) es uno de los receptores nucleares más importantes que estimula la hiperplasia del adipocito. Como es lógico, para suministrar oxígeno y nutrientes al tejido adiposo en expansión, se incrementa el número y el tamaño de los vasos sanguíneos. En consecuencia, la fase inicial de la adipogénesis se vincula de manera pronunciada con la angiogénesis. El factor de crecimiento vascular endotelial (VEGF) liberado por los adipocitos en crecimiento es el principal mediador de esta angiogénesis (174). Existen varias enfermedades y trastornos asociados con la obesidad, especialmente diabetes, cáncer, enfermedades cardiovasculares, hígado graso no alcohólico (NAFLD) e inflamación (175).

La relación de la **obesidad** y la **inflamación** (**Figura 38**) se pone de manifiesto al estudiar los niveles de expresión de adipoquinas como las IL-1 y 6, el TNF-α y la proteína quimioatrayente de monocitos 1 (MCP-1), todas ellas aumentadas en la obesidad (176).

173. Avram MM, Avram AS, James WD. Subcutaneous fat in normal and diseased states: 3. Adipogenesis: From stem cell to fat cell. *J Am Acad Dermatol*. 2007;56(3):472–92.

174. Hausman GJ, Richardson RL. Adipose tissue angiogenesis. *J Anim Sci*. 2004;82(3):925.

175. Bray GA. Medical consequences of obesity. *J Clin Endocrinol Metab*. 2004;89(6):2583–9.

176. Gómez Ambrosi J, Rodriguez A. Papel del tejido adiposo en la inflamación asociada a la obesidad. *Rev Española Obes*. 2008;6(5):264–79.

La elevada producción de estas proteínas va a potenciar el estado inflamatorio en el tejido adiposo favoreciendo la activación e infiltración de macrófagos maduros. De hecho, se ha observado que el incremento en la secreción de citoquinas como el TNF-α puede estimular a los preadipocitos y a las

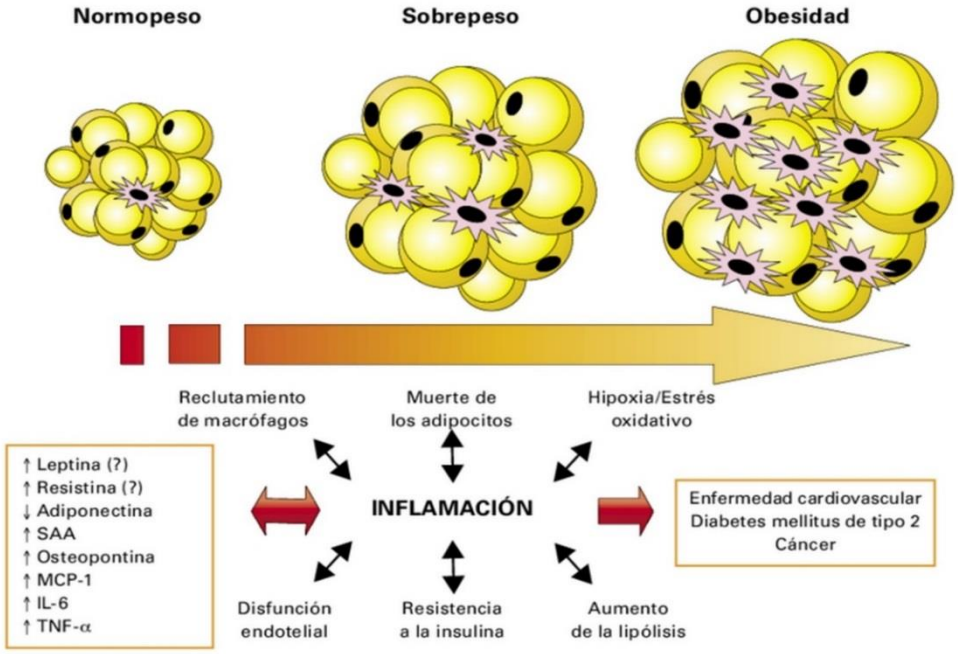


Figura 38. Relación entre la expansión del tejido adiposo y el incremento del estado proinflamatorio. A medida que aumenta el tejido adiposo, se modifica la producción de adipoquinas y se desencadenan una serie de procesos fisiopatológicos relacionados con la inflamación que van a conducir a un incremento del riesgo de sufrir enfermedad cardiovascular, diabetes mellitus de tipo 2 y cáncer, entre otras comorbilidades. IL-6: interleuquina 6; MCP-1: proteína quimioatrayente de monocitos-1; SAA: amiloide sérico A; TNF-α: factor de necrosis tumoral α.

células endoteliales a producir MCP-1, atrayendo a los macrófagos al tejido adiposo. Una vez infiltrados en el tejido adiposo, los macrófagos comienzan a secretar citoquinas y quimioquinas tales como TNF- α, IL-1, IL-6, y MCP-1.

Este patrón de secreción, junto con el producido por adipocitos y otros tipos celulares, puede perpetuar un círculo vicioso de reclutamiento de macrófagos y producción de citoquinas iinflamatorias, llevando a una inflamación primaria local en el tejido adiposo (173,177). La secreción de estos mediadores inflamatorios va a generar diferentes ROS como el anión superóxido ($O_2^{\bullet -}$), el radical hidroxilo ($^{\bullet}OH$), el peróxido de hidrógeno (H_2O_2) y el oxígeno singlete ($^1O_2$).

177. Bahceci G, Bahceci T. Atmaca, & Arikan (2007). The correlation between adiposity and adiponectin, tumor necrosis factor alpha, interleukin-6 and high sensitivity C-reactive. *J Endocrinol Invest*. 2007;30:210–4.

Estas ROS aumentarán el daño celular y tisular activando la producción de todas las citoquinas implicadas en la inflamación y por consiguiente prolongarán esta respuesta inflamatoria.

Por otra parte, en mamíferos la serina/treonina quinasa activada por AMP (**AMPK**) es un sensor energético y regulador clave del metabolismo que se activa en respuesta a un aumento en la relación AMP:ATP en la célula. AMPK controla el metabolismo de la glucosa y de los lípidos, e integra circuitos de señalización entre tejidos periféricos y el hipotálamo, regulando la ingesta y el gasto energético de todo el organismo (178,179). AMPK se activa por un amplio abanico de estreses metabólicos que incluyen ausencia de glucosa, ejercicio, hipoxia, isquemia, estrés oxidativo y estrés hiperosmótico. A excepción del último, todos los demás están relacionados con un aumento en la relación AMP:ATP celular. Una vez AMPK es activada, inhibe rutas anabólicas (biosintéticas) y otros procesos que consuman ATP, mientras que activa aquellas rutas catabólicas que generan ATP, todo ello con el objetivo de restablecer y mantener el balance energético celular.

Entre las muchas funciones que se le atribuyen a AMPK, su papel en el control de la homeostasis de la glucosa es de particular importancia para la supervivencia porque, además de ser la principal fuente de energía para la mayoría de los organismos, la glucosa puede ejercer efectos reguladores de tipo “hormonal”, participando en el crecimiento, metabolismo y desarrollo celular (180). Es por ello que en la presente tesis doctoral se va a estudiar el efecto de distintos compuestos fenólicos en la modulación de AMPK como un parámetro directamente relacionado con la obesidad (**Capítulo 8**).

Se han llevado a cabo numerosos estudios utilizando extractos vegetales y compuestos fenólicos purificados a partir de ellos, con el objetivo de prevenir o disminuir la obesidad.

178. Carling D. The AMP-activated protein kinase cascade – a unifying system for energy control. *Trends Biochem Sci*. 2004;29(1):18–24.

179. Hardie DG, Sakamoto K. AMPK: A Key Sensor of Fuel and Energy Status in Skeletal Muscle. *Physiology*. 2006;21(1):48–60.

180. Rolland F, Winderickx J, Thevelein JM. Glucose-sensing mechanisms in eukaryotic cells. *Trends Biochem Sci*. 2001;26(5):310–7.

Cabe destacar los que se han centrado en el té verde y las proantocianidinas que lo componen, principalmente la epigallocatequina gallato (181–183), así como en la semilla de uva (184–186). Además, otros polifenoles que han sido destacados por su demostrada actividad mejorando la obesidad son la curcumina (187,188) y la quercetina (189,190).

6.3.2. Relación de los compuestos fenólicos y las enfermedades raras

Las **enfermedades raras** son enfermedades con una baja prevalencia que se definieron por primera vez en la regulación de la Unión Europea (UE) en 1999 (EC) N°141/2000 (Regulation (EC) Nº 141/2000 of the European Parlament and of the Council of 16 December 1999 on orphan medicinal products). La UE las considera como tal cuando afectan a no más de 5 de cada 10 mil personas (191), como ejemplo, la acondroplasia presenta una prevalencia estimada de 2.6 por cada 100 mil personas en EU (192). No obstante, aunque estas enfermedades tengan una baja prevalencia, el total de personas afectadas en la UE alcanza un valor considerable, en torno al 8% de la población.

181. Wolfram S, Wang Y, Thielecke F. Anti-obesity effects of green tea: From bedside to bench. *Mol Nutr Food Res*. 2006;50(2):176–87.

182. Lin J-K, Lin-Shiau S-Y. Mechanisms of hypolipidemic and anti-obesity effects of tea and tea polyphenols. *Mol Nutr Food Res*. 2006;50(2):211–7.

183. Kao YH, Hiipakka RA, Liao S, Dulloo AG. Modulation of obesity by a green tea catechin. *Am J Clin Nutr*. 2000;72(5):1232–4.

184. Moreno DA, Ilic N, Poulev A, Brasaemle DL, Fried SK, Raskin I. Inhibitory effects of grape seed extract on lipases. *Nutrition*. 2003;19(10):876–9.

185. Charradi K, Sebai H, Elkahoui S, Ben Hassine F, Limam F, Aouani E. Grape Seed Extract Alleviates High-Fat Diet-Induced Obesity and Heart Dysfunction by Preventing Cardiac Siderosis. *Cardiovasc Toxicol*. 2011;11(1):28–37.

186. Terra X, Pallarés V, Ardèvol A, Bladé C, Fernández-Larrea J, Pujadas G, et al. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *J Nutr Biochem*. 2011;22(4):380–7.

187. Ejaz A, Wu D, Kwan P, Meydani M. Curcumin Inhibits Adipogenesis in 3T3-L1 Adipocytes and Angiogenesis and Obesity in C57/BL Mice. *J Nutr.* 2009;139(5):919–25.

188. Weisberg SP, Leibel R, Tortoriello D V. Dietary Curcumin Significantly Improves Obesity-Associated Inflammation and Diabetes in Mouse Models of Diabesity. *Endocrinology*. 2008;149(7):3549–58.

189. Nabavi SF, Russo GL, Daglia M, Nabavi SM. Role of quercetin as an alternative for obesity treatment: You are what you eat! Food Chem. 2015;179:305–10.

190. Ahn J, Lee H, Kim S, Park J, Ha T. The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways. *Biochem Biophys Res Commun*. 2008;373(4):545–9.

191. Rodwell C, Ayme S. 2014 Report on the State of the Art of Rare Disease Activities in Europe: 2014 Report on the State of the Art of Rare Disease Activities in Europe. 2014;1–68.

192. Orphanet. Prevalence of rare diseases : Bibliographic data. Orphanet Rep Ser. 2014;(1):1–29.

En general, los pacientes afectados por este tipo de enfermedades son particularmente vulnerables psicológica, social, económica y culturalmente hablando. Entre los principales problemas que deben afrontar se encuentran la inexistencia de tratamiento farmacológico o, en aquellas que sí lo tienen, la reticencia de las industrias farmacéuticas a comercializarlos. Además, a todo esto se le une el retraso en los diagnósticos o la inexistencia de estos hasta estadíos avanzados de la enfermedad, ya sea por la escasez de conocimientos especializados, por un diagnóstico inicial equivocado o por dificultades de acceso a la asistencia (191,193). Por todo ello se plantea una serie de nuevas estrategias encaminadas al tratamiento de enfermedades raras mediante la utilización de extractos vegetales como fuente natural de compuestos bioactivos dirigidos a dianas específicas.

Dentro de estas enfermedades, las condrodisplasias o enfermedades óseas son trastornos esqueléticos hereditarios producidos por un desorden genético que se manifiesta con deformidades de las extremidades y otras partes del cuerpo. Como es sabido, el crecimiento de los huesos largos depende de la proliferación y maduración de las células que constituyen el cartílago, los condrocitos. Estas células, en su proceso de maduración, pasan por una primera etapa en la que se hipertrofian para posteriormente morir en un proceso de tipo apoptótico. El crecimiento y desarrollo de los condrocitos en los cartílagos que flanquean los huesos está controlado por los factores de crecimiento de fibroblastos (FGF). Estos factores activan cuatro tipos diferentes de receptores denominados FGFR1, FGFR2, FGFR3 y FGFR4 (194), estando los tres primeros implicados en enfermedades congénitas esqueléticas y craneales (195).

Concretamente la **acondroplasia** es la forma más común de enanismo congénito, y está originada por mutaciones en el FGFR3 (196).

193. Orphanet. About Rare Diseases What is a rare disease ? www.orpha.net/consor/cgi-bin/Education_AboutRareDiseases.php?lng=EN#

194. Provot S, Schipani E. Molecular mechanisms of endochondral bone development. BiochemBiophysResCommun. 2005;328(3):658–65.

195. UniProt. UniProtKB - P22607 (FGFR3_HUMAN).www.uniprot.org/uniprot/P22607

196. Borrego E, Farrington DM, Downey FJ. Novedades en displasias óseas. Vol. 58, Revista Espanola de Cirugia Ortopedica y Traumatologia. 2014. p. 171–81.

Cuando el ligando FGF se une al receptor FGFR3, este parece limitar la osificación endocondral, por lo que su mutación incrementaría esta inhibición. A nivel molecular, la mutación del receptor FGFR3 produce su constante activación, produciendo cambios importantes a nivel de señalización intracelular. La mutación más común implica un cambio en el aminoácido Gly380 por Arg (G380R) con una prenetrancia del 100% (197). La activación y posterior dimerización del FGFR3 provocan la autofosforilación de residuos de tirosina (198), lo que sirve de sitio de unión de proteínas y efectores que propagan las señales de este receptor, como las proteínas quinasas reguladoras de la señal extracelular 1 y 2 (ERK1/2) (199). Una vez producida esta activación sostenida del receptor, se ocasiona una fosforilación continuada de las proteínas ERK1/2 (p-ERK1/2) (200,201).

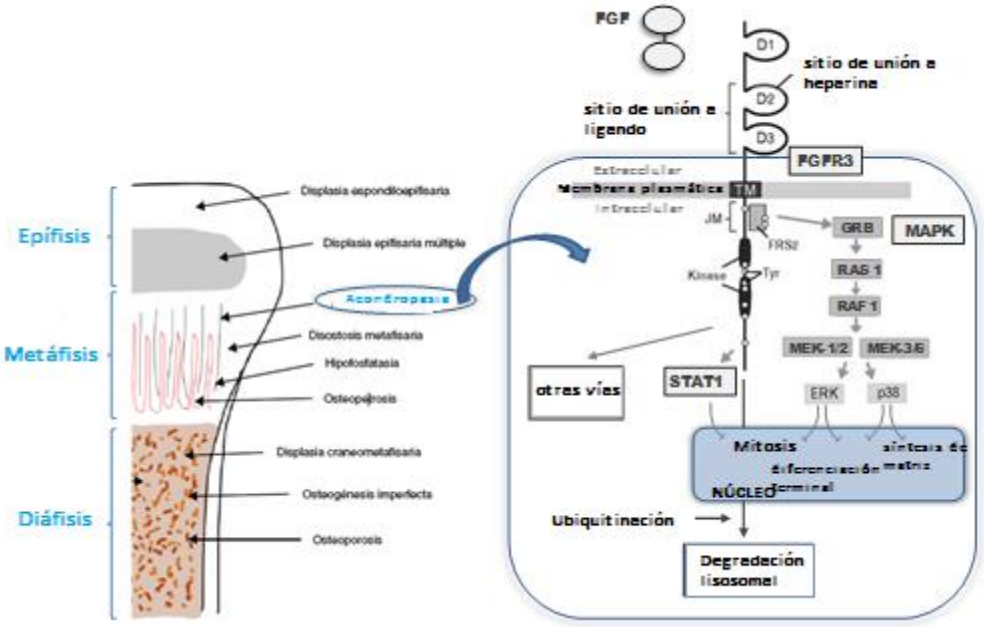


Figura 39. Rutas diana que podrían ser modificadas para inhibir la acción de FGFR3.

197. Horton WA, Hall JG, Hecht JT. Achondroplasia. *Lancet*. 2007;370(9582):162–72.

198. L.Nelson D, M.Cox M. Lehninger - Principios de Bioquímica (4a Edición). Barcelona (Spain): Omega; 2004.

199. Huete F, Guzman-Aranguez A, Ortín J, Hoyle CH V, Pintor J. Effects of diadenosine tetraphosphate on FGF9-induced chloride flux changes in achondroplastic chondrocytes. *Purinergic Signal*. 2011;7(2):243–9.

200. Hou DX, Kumamoto T. Flavonoids as protein kinase inhibitors for cancer chemoprevention: direct binding and molecular modeling. Antioxid Redox Signal. 2010;13(5):691–719.

201. BRENDA. Information on EC 2.7.10.1 - receptor protein-tyrosine kinase.www.brenda-enzymes.org/enzyme.php?ecno=2.7.10.1

La activación de esta proteína por fosforilación es la principal responsable de toda la cascada de señalización que promueve el receptor FGFR3 mutado. Las cuatro cascadas de señalización principales que podrían verse implicadas en este proceso son: activador de la transcripción 1 (STAT1), MAPK, fosfolipasa C gamma (PLCγ) y fosfatidilinositol-3-quinasa/proteína quinasa B (PI3K/AKT) (197) (**Figura 39**).

Por otra parte, las terapias actuales para la acondroplasia incluyen tratamientos hormonales o quirúrgicos. Los primeros no son recomendados por muchos expertos, debido a que no están claros los beneficios que puedan proporcionar a largo plazo, y con respecto a los segundos, son procedimientos que necesitarían ser repetidos a lo largo del tiempo y que conllevarían posibles complicaciones. Otras estrategias terapéuticas se han centrado en la reducción de las señales procedentes del FGFR3 mediante fármacos experimentales y que todavía se encuentran en fase preclínica (202). Por todo esto, la búsqueda de nuevas moléculas con baja toxicidad para tratamientos a largo plazo se hace necesaria en este tipo de enfermedades. Estudios recientes sugieren que los flavonoides pueden unirse directamente a algunas proteínas quinasas, alterar su estado de fosforilación y regular múltiples vías de señalización celular (203). En esta línea, el cacao es una importante fuente de flavonoides que podrían ser clave para el tratamiento de enfermedades (204) por lo que es objeto del **Capítulo 11**.

6.3.3. Evaluación del efecto de los compuestos fenólicos en la salud

Debido a la relación tan estrecha entre los compuestos fenólicos y diversas patologías, como las mencionadas anteriormente, la búsqueda de las bases moleculares y procesos implicados en cualquier patología se hacen necesarios.

202. Laederich MB, Horton WA. Achondroplasia: Pathogenesis and implications for future treatment. Vol. 22, *Current Opinion in Pediatrics*. 2010. p. 516–23.

203. Nam JK, Ki WL, Dong EL, Rogozin EA, Bode AM, Hyong JL, et al. Cocoa procyanidins suppress transformation by inhibiting mitogen-activated protein kinase kinase. *J Biol Chem*. 2008;283(30):20664–73.

204. Ellam S, Williamson G. Cocoa and human health. Annu Rev Nutr. 2013;33:105–28.

Estos dependen de la disponibilidad de modelos experimentales con los que compartan similitudes fisiológicas, anatómicas y metabólicas con los humanos.

Los modelos animales más comúnmente utilizados son la rata (*Rattus norvegicus*) y el ratón (*Mus musculus*), ya que presentan la ventaja de poder ser manipulados genéticamente y con precisión, tienen un coste relativamente bajo con respecto a otros modelos animales y ofrecen la posibilidad de poder controlar los factores ambientales que pueden influir en la progresión de las enfermedades que se quieren estudiar. Dentro de estos ensayos, el término ***in vivo***, se refiere al procedimiento diagnóstico o experimento que se realiza en el organismo mientras vive. En cambio, el término ***ex vivo***, hace referencia a procedimientos, mediciones o experimentos en órganos, tejidos o células vivas, que no se encuentran en, o han sido extraídas del organismo, pero que se mantienen vivas en un medio controlado.

A continuación se van a tratar las metodologías que han sido utilizadas en la tesis doctoral para evaluar el efecto de los compuestos fenólicos en la salud.

6.3.3.1. Viabilidad celular

La **viabilidad celular** es un fiel indicador de la citotoxicidad del tratamiento o condiciones a los que se somete a las células. Puede determinarse mediante citometría de flujo o mediante ensayos colorimétricos. La primera consiste en obligar a las células a pasar alineadas una a una frente a un haz láser mediante un flujo continuo. Cada célula, a la vez que dispersa la luz, emite luz fluorescente como consecuencia de la excitación láser a la que es sometida. También se puede determinar mediante la condensación nuclear con la tinción de Hoechst. Los marcadores de Hoechst son parte de una familia de colorantes fluorescentes azules utilizado para teñir el ADN. Este tipo de tinción es útil en experimentos en los que se usan múltiples fluoróforos, ya que existe una amplia diferencia entre las bandas máximas de excitación y sus espectros de emisión en los diferentes tintes de Hoechst. Otra ventaja de estos tintes es que son permeables a la membrana celular y pueden teñir tanto células vivas como fijadas. Dentro de los ensayos colorimétricos, uno de los más usados es el MTT (Bromuro

de 3-(4,5- di-metiltiazol-2-ilo)-2,5-difeniltetrazol) que al reducirse mediante la succinato deshidrogenasa de las mitocondrias funcionales dará lugar al formazán, de color azul intenso, permitiendo determinar la funcionabilidad mitocondrial de las células tratadas. El MTT junto con la determinación de la condensación nuclear por medio de la tinción de Hoescht, son los ensayos utilizados para determinar la toxicidad que los extractos objeto de estudio puedan presentar en los modelos celulares (**Capítulos 5**, **8** y **11**).

6.3.3.2. Modelos animales

Figura 40. Rata Wistar.

Muchas cepas silvestres son utilizadas en experimentación animal. Las ratas de la cepa Wistar (ratas albinas creadas en 1906 por el Instituto Wistar) (**Figura 40**) son las más usadas en estudios de fisiología, etología, farmacología y toxicología. Estas ratas son el primer organismo modelo utilizado en investigaciones biomédicas por ser fáciles de criar, de rápida reproducción, de fácil manejo y docilidad y por presentar una fisiología parecida al humano. Sin embargo, cuando se quiere estudiar una patología específica, se necesitan modelos animales especialmente diseñados para tal fin. La generación de nuevos animales de experimentación facilita el estudio de condiciones específicas que se encuentran en patologías humanas, y se basan en la creación de modelos mediante técnicas de manipulación genética. Entre estos modelos se encuentran los *knock-out* y *knock-in* con una mutación dirigida basada en la eliminación (*knock-out*) o inserción (*knock-in*) de un gen de interés, y los modelos transgénicos que contienen un segmento de ADN recombinante portador de información genética específica. Para la realización de los ensayos *ex vivo* de esta tesis doctoral (**Capítulo 11**) se ha utilizado un modelo animal de condrodispasias (ratón FGFR3 $^{Y367C/+}$) (**Figura 41**) (205).

205. Pannier S, Couloigner V, Messaddeq N, Elmaleh-Bergès M, Munnich A, Romand R, et al. Activating Fgfr3 Y367C mutation causes hearing loss and inner ear defect in a mouse model of chondrodysplasia. *Biochim Biophys Acta - Mol Basis Dis*. 2009;1792(2):140–7.

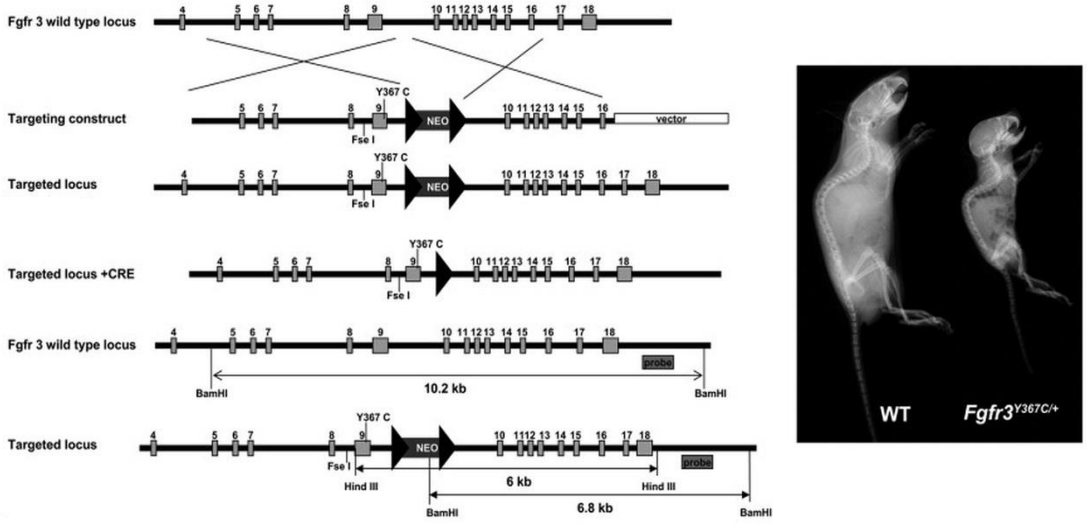


Figura 41. Modelo de ratón FGFR3 $^{Y367C/+}$: detalle de la mutación (izquierda) y radiografía a las 3 semanas del modelo wild type y del mutado (derecha).

6.3.3.3. Inmunohistoquímica

Los **estudios inmunohistoquímicos** (EI) son estudios histopagológicos que se basan en reacciones antígeno-anticuerpo. El proceso consiste en poner un corte histológico del tejido en estudio en incubación con una sonda que reconoce al antígeno y se fija a él. Luego revelamos la reacción con un cromógeno, que le da un color específico al tejido y podemos reconocerla al microscopio. Los métodos inmunohistoquímicos se desarrollaron a partir de 1941, año en el que Albert Hewett Coons describe un método de inmunofluorescencia para detectar antígenos celulares en secciones de tejido. 25 años después Nakane, Pierce y Avrameus, revolucionaron la técnica hasta hacerla práctica, útil, de fácil realización y aplicabilidad clínica general, llegando a la inmunohistoquímica como es hoy.

Esta técnica permite identificar la localización de una sustancia a nivel tisular o citológico, identificando los marcadores antigénicos característicos de una línea celular, células que secretan una proteína, receptores de membrana, gradientes de concentración tisulares o células que han respondido a una hormona (con anticuerpos específicos para las vías de señalización intracelular).

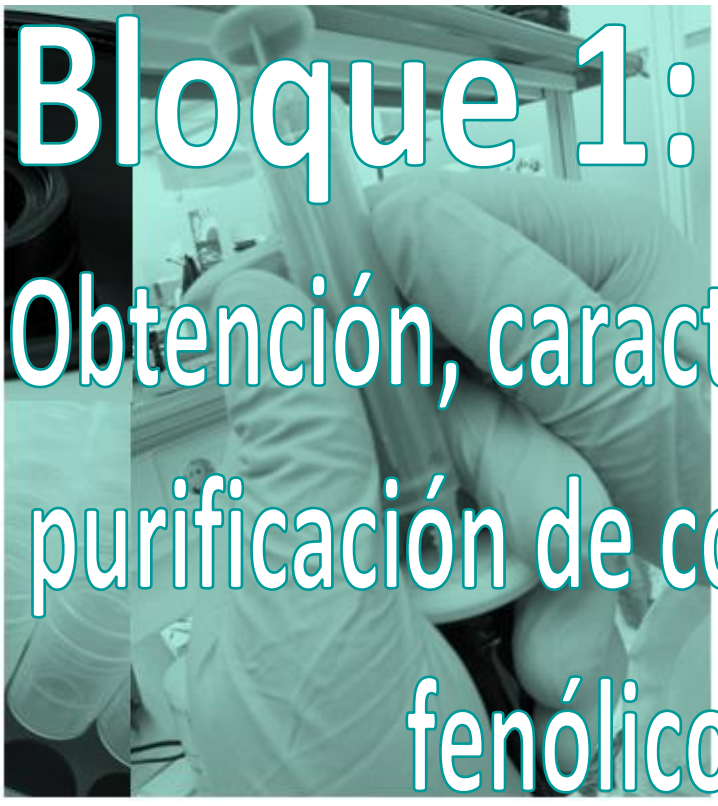
Estos marcadores se aplican para estudiar la capacidad de responder a tratamientos o, en nuestro caso, a compuestos fenólicos (**Capítulo 11**).

6.3.3.4. Western Blot

El **Western Blot** es una técnica utilizada para la detección de proteínas en una muestra celular o tisular. Se basa en la separación (ya sea por tamaño, hidrofobicidad, etc) de las proteínas desnaturalizadas mediante una electroforesis (generalmente usando un gel de poliacrilamida). Las proteínas son trasferidas desde el gel hacia una membrana (normalmente de nitrocelulosa o acetato), donde son detectadas utilizando anticuerpos específicos para la proteína. De esta forma se podrá comparar la cantidad de proteína presente en distintas muestras. Las aplicaciones del Western Blot son innumerables. A *groso modo*, dentro del campo de la biomedicina, tiene aplicaciones diagnósticas en enfermedades infecciosas, enfermedades hereditarias y congénitas, enfermedades autoinmunitarias, y cada vez es más utilizada en el cáncer, tanto para el diagnóstico precoz y el tratamiento como en investigación básica y aplicada. Otros campos en los que se utiliza esta técnica, son la inmunología y áreas relacionadas con esta, como la inflamación y el envejecimiento celular, y también en el ámbito de la microbiología, la genética y la genómica. En nuestro caso, se ha hecho uso de esta técnica para conocer el mecanismo de acción de ciertos compuestos fenólicos en vías directas o indirectas relacionadas con la acondroplasia (**Capitulo 11**).

Experimental


better.



Bloque 1:

Obtención, caracterización y purificación de compuestos fenólicos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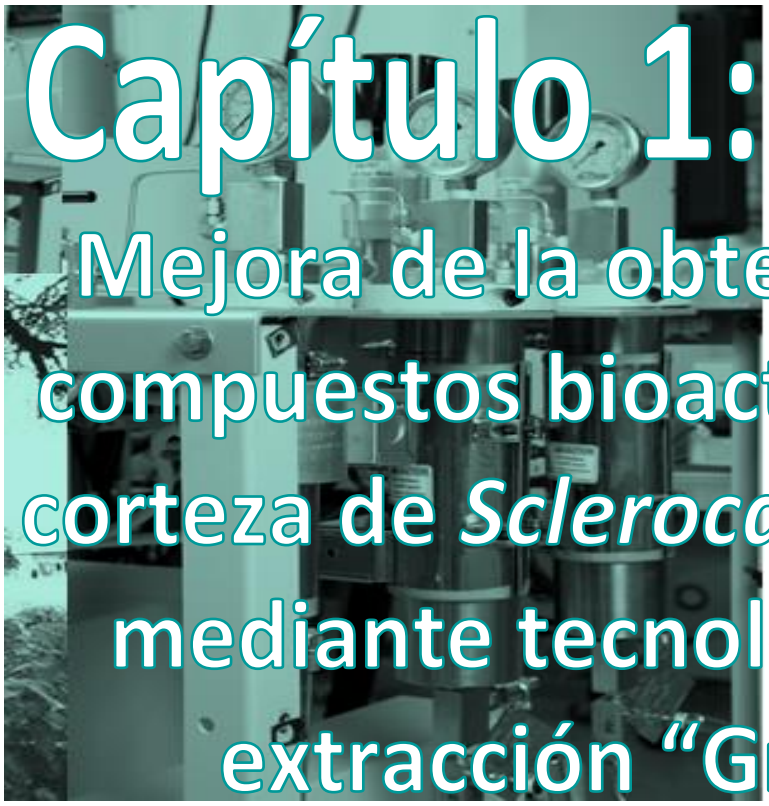

Capítulo 1:

Mejora de la obtención de compuestos bioactivos de la corteza de *Sclerocarya birrea* mediante tecnologías de extracción "Green"

Food Research International. FOODRES-D-18-00386. 2018
Special Issue "Conventional, Non-Conventional Extraction Techniques and New Strategies for the Recovery of Bioactive Compounds from Plant Material for Human Nutrition"
(Non-published - Guest Editor Dr. Domenico Montesano)

Enhancing obtainment of bioactive compounds from *Sclerocarya birrea* bark by "green" extraction approaches

ABSTRACT

Sclerocarya birrea (marula), a medium-sized to large tree indigenous to Southern Africa, is commonly a community dominant with significant importance in rural livelihoods for food, medicine, and carving. The bark, which contains 10-20% tannin as well as traces of alkaloids, provides fiber and gum, which is mixed with salt and water to produce ink or red dye, and provides several pharmacological benefits as antidiabetic, anti-inflammatory, antimicrobial, antoatherogenic and antioxidant, among others. In order to study the composition of marula bark, this study shows the comparison of different extraction methodologies as conventional extraction by solid-liquid method, supercritical fluid extraction and pressurized liquid extraction, performing under selected conditions and using only "food grade" solvents. In this way, a comprehensively characterization of the phenolic profile contained in the obtained extracts, a method based on HPLC-ESI-TOF-MS was used. A total of 71 compounds with a significant large percentage of them as a galloyl form distributed in five major categories. "Green" extractions as PLE represent a powerful tool to obtain bioactive compounds from *S. birrea* bark for using as supplements or food ingredients allowing the appreciation of this crop.

1. INTRODUCTION

Sclerocarya birrea, also known as marula, is a medium-sized to large deciduous Savannah tree indigenous to Southern Africa belonging to the family Anacardiaceae. It is commonly a community dominant, favored by wildlife in conservation areas and of significant importance in rural livelihoods for food, medicine, and carving (Helm et al., 2011). In addition, it is one of the plants that played a role in feeding people in ancient times and dubbed "food of kings". In recent year, the amounts produced are surprising (at least considering that this is a "lost" crop and as-yet is little grown in organized production). For instance, about 500 tons of marula were commercially processed for juice and 2000 tons for liqueur just in South Africa (Council, 2008). The bark, which contains 10-20% tannin as well as traces of alkaloids, provides fiber and gum, which is mixed with salt and water to produce ink or red dye (Kutama et al., 2013). A decoction of the bark has been used to treat dysentery, diarrhea, rheumatism and as a prophylactic remedy against malaria (Kpoviessi et al., 2011). Moreover, several studies about *S. birrea* steam bark extracts have reported their pharmacological properties such as anti-diabetic (Gondwe et al., 2008; Ojewole, 2004), anti-inflammatory (Fotio et al., 2009; Ojewole, 2003), anti-microbial (Eloff, 2001; Masoko et al., 2008), anti-atherogenic (Borochov-Neori et al., 2008; Ojewole, 2006) and antioxidant (Mariod et al., 2008, 2006), among others.

A comprehensive phytochemical analysis of non-selective *S. birrea* bark extractions using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to mass spectrometry (MS) and tandem MS throw a hybrid mass analyzer quadrupole-time-of-flight (QTOF) and with atmospheric-pressure ionization techniques as electrospray ionization (ESI) has been previously reported by *Jiménez-Sánchez et al.* This methodology successfully detected from monomers up to dimers of (epi)catechin, (epi)gallocatechin, and (epi)afzelechin units with one or two galloyl residues. In addition, a very high degree of galloylation was observed, which may have a role in the bioactivity attributed to these extracts (Jiménez-Sánchez et al., 2015). Recently, proanthocyanidins, a group of naturally occurring polyphenolic bioflavonoids, have attracted great interest on their potential

benefits as vasodilator, anti-carcinogenic, anti-allergic, anti-inflammatory, anti-bacterial, cardioprotective, immune-stimulating, anti-viral and estrogenic activities (Cádiz-Gurrea et al., 2015).

Since matrixes of plant origin contain thousands of diverse metabolites of varying polarities and concentrations, it is difficult to develop a single method for optimum extraction of all metabolites. Within this context, different extraction techniques have been used to obtain phenolic compounds as containing in barks being conventional extractions as solid-liquid extraction (SLE) (Kim et al., 2010; Mariod et al., 2008; Ojewole, 2007) the most reported. Nowadays, the search for an extraction methodology to obtain phenolic compounds is the focus of numerous studies, which have the objective to find out more cost-effective and greener techniques to obtain extracts with large amount of bioactive compounds. Among such technologies, supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) are the most widely employed in obtaining bioactive components from natural sources being a possible tool not only from a laboratory scale but also for agri-food industries (Chemat et al., 2012; Herrero et al., 2010, 2006; Taamalli et al., 2012).

Therefore, the main objective of this study was to compare different extraction methodologies as conventional extraction by solid-liquid extraction, SFE and PLE, performing under selected conditions and using only “food grade” solvents as water and ethanol, towards the extraction of phenolic compounds from *S. birrea* bark. In order to do a comprehensively characterization of the phenolic profile contained in the obtained extracts, a method based on HPLC-ESI-TOF-MS was used.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

All chemicals were of analytical or MS grade and used as received. For extraction, ethanol, sea sand and glass wood were purchased from Fisher Scientic (Leicestershire, UK). Carbon dioxide was supplied by Carburos Metalicos Grupo Air Products (Carburos Metálicos, Cornellá de Llobregat, Barcelona). Acetic acid and methanol for HPLC were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany)

and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Luteolin was purchased from Fluka (Sigma-Aldrich, Steinheim, Germany). Double-deionized water with conductivity lower than 18.2 MΩ was obtained with a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Instrumentation

Supercritical carbon dioxide extraction was carried out with a Waters Prep Supercritical Fluid Extraction systems (SFE-100 Waters®, TharSFC, Thar Technologies, Inc., Pittsburgh, PA, USA) equipped with CO_2 and co-solvent pumps (models P-50), an automated back pressure regulator, low and high pressure heating exchangers, a pressurized extraction vessel and pressurized collection vessels. SFE system was connected to an Accel 500 LC chiller by Thermo Scientific (TharSFC, Thar Technologies, Inc., Pittsburgh, PA, USA)

Accelerated solvent extraction (ASE) was performed in a Dionex™ ASE 350 extractor (Dionex Corp., Sunnyvale, CA, USA) using a stainless-steel cell of 34 mL volume and 200 mL vials for extracts collection.

LC analysis of *S. birrea* extracts were made with an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode array detector (DAD). The HPLC system was coupled to a TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI interface (model G1607 from Agilent Technologies, Palo Alto, CA, USA). Separation was carried out with a Zorbax Eclipse Plus C18 (1.8 µm, 150×4.6 mm) (Agilent Technologies, Palo Alto, CA, USA).

2.3. Sample preparation

S. birrea stem barks were provided by Herbafor S.L. (Murcia, Spain)). Stem barks were air-dried and then grounded into uniform powder using an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany). The resulting *S. birrea* stem bark powder was kept in darkness until used.

2.4. Extraction methods and conditions

2.4.1. Conventional solid–liquid extraction (SLE)

Phytochemical extraction from *S. birrea* stem bark powder was performed using five different proportions of H_2O:EtOH (SLE-A, 100:0; SLE-B, 75:25; SLE-C, 50:50; SLE-D, 25:75; and SLE-E, 0:100 (v/v).) To determine the best SLE condition, 6 g of sample was shaken, in dark and at room temperature, for 60 min with 30 mL of the different H_2O:EtOH mixtures described above. After that, the samples were centrifuged at 13000 *g* for 10 min in a centrifuge (Sorvall ST 16 R, Thermo Scientific, Leicestershire, UK) and the supernatants were collected and filtered through a 0.45 µm filter. The solvent was evaporated at 35 °C under vacuum in a Savant™ SpeedVac Concentrator SC250 EXP (Thermo Scientific, Sunnyvale, CA, USA). Each procedure was carried out in triplicate. The extracts were reconstituted and filtered through a 0.22 µm syringe filter and stored at -20 °C until HPLC analysis. Dried extracts were reconstituted in the extraction solvent up to a concentration of 5 mg/L and filtered through a 0.2 µm nylon syringe filters.

2.4.2. Supercritical Fluid Extraction (SFE)

SFE experiments were performed according to *Ghoreishi and Heidari* (2012) (Ghoreishi and Heidari, 2012) with some modifications. Briefly, the extractions were carried out in a 100 mL extraction column fitted with glass wool at the inlet and outlet. This column was charged with 15 g of plant powder (SFE3) plus 10 mL of ethanol (SFE1 and SFE2) and mixed with sea sand in a ratio 1:1. All SFE extractions were performed at 50°C and 200 bars in a dynamic mode with a total flow rate of 23 g/min of different solvent combinations (SFE1, CO_2; SFE2 and SFE3, CO_2 plus ethanol at 15%). All extractions were done in triplicate. The obtained extracts were evaporated and stored at -20 °C until HPLC analysis. For further analysis, dried extracts were reconstituted in the extraction solvent up to a concentration of 5 mg/L and filtered through a 0.2 µm nylon syringe filters.

2.4.3. Pressurized liquid extraction (PLE)

PLE experiments were performed in a static mode (11 MPa and 20 min as the pressure and extraction time) with combinations of different proportions of solvent composition (H_2O:EtOH from 0% to 100%, v/v) and temperatures in the range from 40 to 200 °C (see Table 1). Prior to use, extraction solvents were degassed for 10 min by using an ultrasonic bath. All extractions were done using 6 g of plant powder previously mixed with sea sand in a ratio 1:2 and loaded onto 34 mL stainless-steel extraction vessels. In order to prevent clogging of the metal frits, cellulose filters were placed at each end of the extraction vessel and two portions of sand (5g) were placed between sample and cellulose filters. The extraction conditions described above were applied and the extracts were collected in vials and immediately cooled in ice until room temperature. After that, extracts were centrifuged at 13000 rpm for 10 min in a centrifuge (Sorvall ST 16 R, Thermo Scientific, Leicestershire, UK). The extracts were dried at 35 °C under vacuum in a Savant™ SpeedVac Concentrator SC250 EXP (Thermo Scientific, Sunnyvale, CA, USA) and stored at -20ºC until HPLC analysis. All extractions were done in triplicate. For further analysis, dried extracts were reconstituted in the extraction solvent up to a concentration of 5 mg/L and filtered through a 0.2 µm nylon syringe filters.

Table 1. Extraction parameters of PLE.

PLE	P (psi)	T (min)	T (º C)	EtOH (%)	H_2O (%)	Dielectric Const.
A	1500	20	40	50	50	48,02
B	1500	20	63	85	15	31,02
C	1500	20	63	15	85	59,09
D	1500	20	120	100	0	19,00
E	1500	20	120	50	50	34,71
F	1500	20	120	0	100	50,41
G	1500	20	176	85	15	21,55
H	1500	20	176	15	85	33,43
I	1500	20	200	50	50	26,00

2.5. HPLC-ESI-TOF-MS analysis

To carry out the chemical characterization of phenolic extracts HPLC-ESI-TOF-MS analysis was applied following a previously described method by *Cádiz-Gurrea et al.* with some modifications (Cádiz-Gurrea et al., 2014). The injection volume was 20 µL and the chromatographic separation was carried out at room temperature. The mobile phase used was water with 0.5% acetic acid as eluent A and methanol as eluent B. The total run time was 57 min using a previously reported multistep linear gradient (Cádiz-Gurrea et al., 2014). The flow rate was 0.3 mL/min. The HPLC system was coupled to a TOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +4.5 kV. The other optimum values of the source parameters were: drying gas temperature, 210 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 2.3 bar. The detection was performed considering a mass range of 50–1200 *m/z*.

External mass spectrometer calibration was performed with sodium acetate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% of acetic) in quadratic þ high precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to polar compounds identification. The detection was performed considering a mass range of 50–1200 *m/z*. The optimum values of the source and transfer parameters were get for a good sensitivity and reasonable resolution within the mass range described above (Cádiz-Gurrea et al., 2014).

2.6. Statistical analysis

Origin (Version Origin Pro 8 SR0, Northampton, MA, USA) was employed to perform one-way analysis of variance (ANOVA) at a 95% confidence level ($p \leq 0.05$) in order to analyze statistically significant differences among the total phenolic content of extracts obtained under different extraction conditions.

3. RESULTS AND DISCUSSION

3.1. Characterization bioactive compounds from *S. birrea* by HPLC-ESI-TOF-MS.

Figure 1 shows the base peak chromatograms (BPCs) obtained in negative polarity for representative SLE, SFE and PLE extracts. Besides, all the identified compounds are presented in Table 2, numbered according to their elution order. This table includes all information reported by HPLC-ESI-TOF/MS analysis (retention time, experimental *m/z*, molecular formula, errors and σ values) and a list of extracts in which the proposed compounds have been detected. All of these compounds were characterized by the interpretation of their mass spectra provided by the TOF mass analyzer and the information previously reported (most of this compounds have previously described in S*. Birrea* samples).

In the present study, 71 compounds distributed in five major categories (gallic acid and derivatives, monomers from (epi)catechin and derivatives, dimers from (epi)catechin and derivatives, flavonoids and other compounds) were tentatively identified. The reported results pointed out that a significant large percentage of them appeared as a galloyl form. A number of four compounds remained unknown, as indicated in Table 2.

3.1.1. Gallic acid and derivatives

Concerning gallic acid and derivatives group, seven compounds were found in the extracts. These compounds corresponded to galloyl glucose isomers (peaks 4 and 5) at *m/z* 331, which eluted earlier than gallic acid (peak 7) at *m/z* 169 due to the presence of a sugar molecule in their structures. In addition, four galloyl derivatives were also detected at different retention times depending on their apolar substituents as methoxy groups: dimethoxy-hydroxyphenyl-O-galloyl glucopyranoside, hydroxyl-methoxyphenyl-O-galloyl glucopyranoside, galloyl-glucosyl dihydroxymethoxyacetophenone and trihydroxystilbene glucosyl-O-gallate at *m/z* 483, 453, 495 and 541, respectively (peaks 28, 32, 39 and 56). Among these compounds, dimethoxy-hydroxyphenyl-O-

galloyl glucopyranoside and hydroxyl-methoxyphenyl-O-galloyl glucopyranoside have previously been mentioned in the literature for other barks (Lampire et al., 1998; Santos et al., 2017; Shi et al., 2010) but they have been detected for the first time in *S. birrea*.

Gallic acid and its (epi)catechin derivatives are widely distributed in fruits and plants. These flavan-3-ol subunits can be esterified with gallic acid to form gallate derivatives. The esterified gallic acid core of these molecules supplies a diverse range of industrial uses, as antioxidants in food, in cosmetics and in the pharmaceutical industry. Several studies about these compounds have reported many potential therapeutic properties including anti-cancer and antimicrobial properties (Ow and Stupans, 2003). According to *Lu et al.,* (epi)gallocatechins gallate and (epi)catechin gallate have good scavenging effects and relatively high hydrophobic properties and thus both compounds showed much higher protective efficiency than, (−)-epicatechin, (+)-catechin or (−)-epigallocatechin. As for this latter case, its poor antioxidant activity and low hydrophobicity may prevent its passage through the cell membranes; thus, this compound confers the less antioxidant protection (Lu et al., 2006).

3.1.2. Monomers and dimers from (epi)catechin and derivatives

Twelve compounds were detected as monomers from (epi)catechin and derivatives. Among them, peaks 23 and 34 yielded deprotonated molecules at *m/z* 289, being identified as catechin and epicatechin, respectively. Moreover, different gallate derivatives were identified as (epi)gallocatechin isomers with *m/z* 305 (peaks 10 and 22), (epi)gallocatechin gallate isomers at *m/z* 457(peaks 19, 29 and 35), (epi)catechin gallate isomers at *m/z* 441(peaks 44, 45 and 48), (epi)catechin glucoside gallate at *m/z* 603 (peak 46) and (epi)afzelechin gallate at *m/z* 425 (peak 52).

Proanthocyanidins are a mixture of (epi)catechin monomers commonly bonded through C4-C8 or C4-C6 linkages (B-type). The largest class of compounds in all the extracts from *S. birrea* was one in which the compounds were mainly esterified with gallic acid and composed of dimers from (epi)catechin. When procyanidins incorporate gallate in their structure, the trend is an increase of antiradical power until polymerization degree equal to 3 (Jerez et al., 2007). In *S. birrea* extracts, these compounds were

identified as procyanidin B-type isomers at *m/z* 577 (peaks 14 and 47) and other gallate derivatives as procyanidin dimer gallate isomers at *m/z* 729 (peaks 25, 26 and 51). Among others gallate derivatives we could also detected a gallo(epi)catechin dimer at *m/z* 609 (peak 6) and two isomers from bis(epi)gallocatechin monogallate (peaks 9 and 11) with *m/z* 761. A dimeric proanthocyanidin with two (epi)gallocatechin units and two galloyl residues (peak 17) was found at *m/z* 913. Moreover, two isomers at *m/z* 897 from (epi)gallocatechin gallate (epi)catechin gallate (peaks 24 and 42), six isomers with different elution behavior at *m/z* 745 from (epi)gallocatechin gallate (epi)catechin (peaks 12, 16, 18, 20, 21 and 38) and (epi)gallocatechin (epi)catechin (peak 15) at *m/z* 593 were identified in the extracts. These two last isomers correspond to a loss of a gallic acid residue (− 152 Da) from the earlier one, respectively. An (epi)gallocatechin (epi)catechin gallate (peak 41) was also detected at *m/z* 743. At the end, the HPLC-ESI-TOF/MS method allowed to tentatively identify four isomers of (epi)catechin gallate dimer at *m/z* 881 (peaks 30, 31, 37 and 50).

3.1.3. Flavonoids

Regarding flavonoids (non-derivated from (epi)catechin), thirteen compounds have been detected in *S. birrea* extracts belonging to flavanols, flavanones, flavanonols, and chalcones. Among these, flavanol subclass was the most abundant one. Phenolic compounds belonging to this chemical group were dihydromyricetin isomers at *m/z* 319 (peaks 49 and 57), myricetin glucoside at *m/z* 479 (peak 53), jaceidin triacetate at *m/z* 485 (peak 54), rhamnetin at *m/z* 315 (peak 61), and dihydroquercetin and quercetin glucoside at *m/z* 303 and 463 (peaks 62 and 64, respectively). Concerning flavanones, three compounds were also detected, two of them at *m/z* 271 (peaks 68 and 71) and other one identified as eriodictyol glucoside at *m/z* 449 (peak 36). With regard to flavanonol, compound 69 had a deprotonated molecule at *m/z* 303 and was tentatively characterized as taxifolin. Among the chalcone sub-class, two chalcones, which were assigned as phloretin-C-glucoside and di-C-glucoside were found at *m/z* 435 and 597 (peaks 60 and 55). This class of flavonoids lacks a heterocyclic C ring, while the A-rings are derived from the acetate pathway and the B-rings from the shikimate pathway.

In contrast to the ubiquitously present flavonoids, dihydrochalcones seem to be restricted to about 30 plant families (Barreca et al., 2014).

3.1.4. Other compounds

Other phenolic compounds not belonging to the previous groups were tentatively identified as hydroxylbenzoic acids derivatives (peaks 13, 27 and 65), homaloside D (peak 59) and ellagic acid (peak 67).

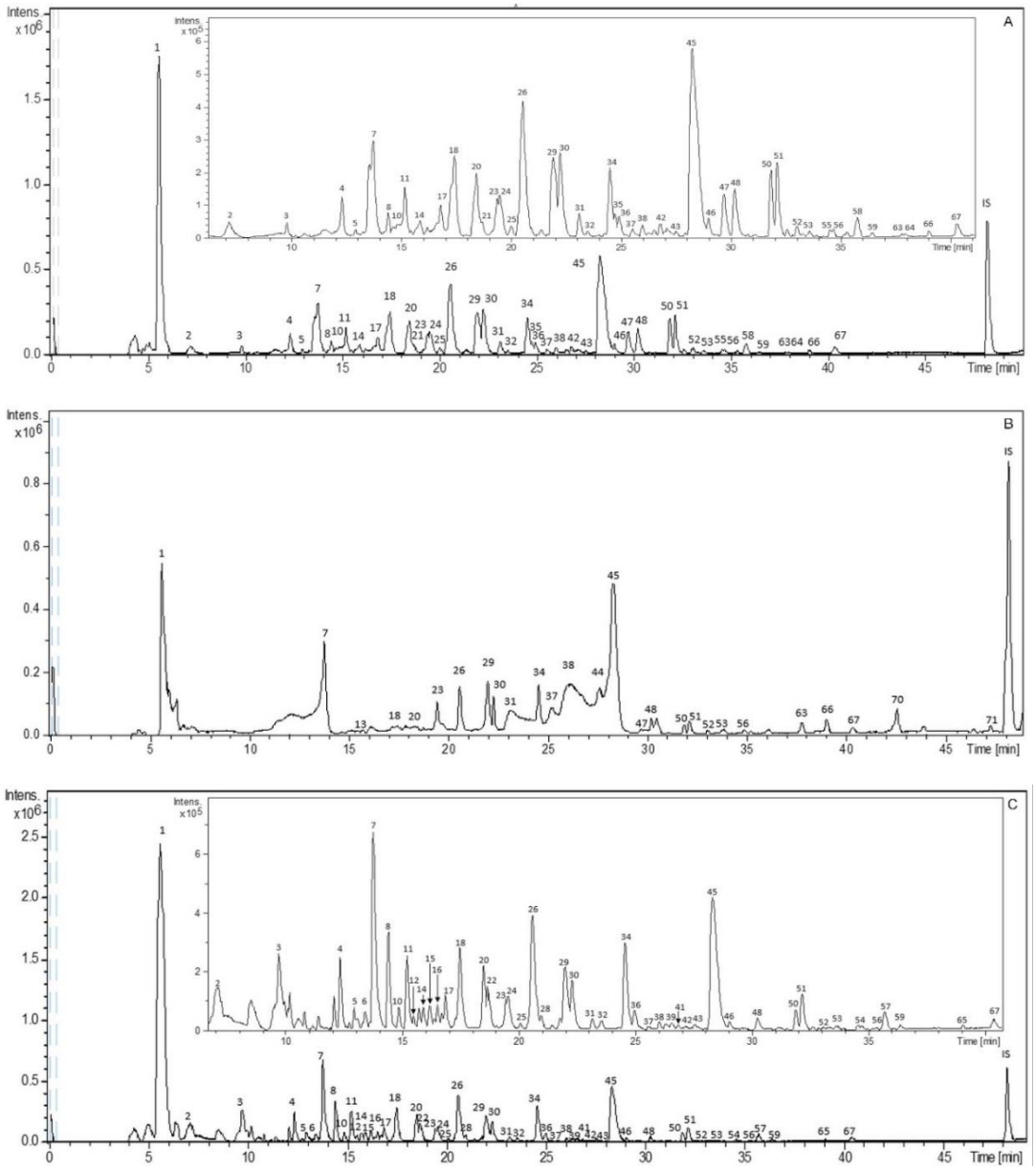

Figure 1. Base Peak Chromatograms (BPCs) of representative *S. birrea* extracts obtained under different extraction techniques: SLE (a), SFE (b) and PLE(c). Peak numbers correspond to those of Table 2.

Table 2. Identified compounds in the *S. birrea* extracts by HPLC-ESI-TOF-MS.

Peak	Proposed compound	RT	m/z calc.	m/z meas.	Err [ppm]	mSigma	Mol. Formula	SLE	SFE	PLE
1	Quinic acid	5,6	191,0561	191,0565	2.1	1.6	$C_7H_{12}O_6$	A-E	1, 3	A*-I
2	Sucrose	7,2	341,1089	341,1095	1.6	0.8	$C_{12}H_{22}O_{11}$	A-C	nd	A*,B,D-I
3	D-Raffinose	9,9	503,1618	503,1607	2.1	2.7	$C_{18}H_{32}O_{16}$	A, B	nd	A*-H
4	Galloyl glucose isomer 1	12,4	331,0671	331,0678	2.1	1.5	$C_{13}H_{16}O_{10}$	A-C	nd	A*-I
5	Galloyl glucose isomer 2	13,1	331,0671	331,0677	1.9	12.6	$C_{13}H_{16}O_{10}$	A,B	nd	A*,B,G,I
6	Gallo(epi)catechin dimer	13,4	609,125	609,1228	3.6	3.9	$C_{30}H_{26}O_{14}$	A*, B	nd	nd
7	Gallic acid	13,7	169,0142	169,0149	3.7	1.8	$C_7H_6O_5$	A-E	1, 3	A*-I
8	UK1	14,4	411,0259	411,0239	-5.0	37.5	$C_{17}H_8N_4O_9$	A*-C	nd	A, B, I
9	Bis(epi)gallocatechin monogallate 1	14,8	761,1359	761,1359	0.1	5.3	$C_{37}H_{30}O_{18}$	C*, D	nd	nd
10	(Epi)gallocatechin isomer 1	14,8	305,0667	305,067	1.0	0.9	$C_{15}H_{14}O_7$	A, B	nd	A*-I
11	Bis(epi)gallocatechin monogallate 2	15,2	761,1359	761,1365	0.7	4.6	$C_{37}H_{30}O_{18}$	A-C, E	nd	A*-I
12	(Epi)gallocatechin gallate (epi)catechin isomer 1	15,5	745,141	745,1414	-0.5	8.7	$C_{37}H_{30}O_{17}$	A*-C	nd	nd
13	Protocatechuic acid	15,7	153,0193	153,0192	1.0	5.9	$C_7H_6O_4$	nd	1, 3	G*-I
14	Procyanidin B dimer isomer 1	15,9	577,1351	577,1335	2.8	2.6	$C_30H_{26}O_{12}$	A*-C	nd	A-F
15	(Epi)catechin-(epi)gallocatechin	16,5	593,1301	593,1311	-1.8	2.7	$C_{30}H_{26}O_{13}$	A*, B	nd	nd
16	(Epi)gallocatechin gallate (epi)catechin isomer 2	16,7	745,141	745,1402	1.2	6.8	$C_{37}H_{30}O_{17}$	A*-C	nd	nd
17	Bis(epi)gallocatechin digallate	16,8	913,1469	913,1493	2.6	2.7	$C_{44}H_{34}O_{22}$	A-E	nd	A*-I
18	(Epi)gallocatechin gallate (epi)catechin isomer 3	17,4	745,141	745,1402	1.1	2.7	$C_{37}H_{30}O_{17}$	A-E	2, 3	A*-G, I
19	(Epi)gallocatechin gallate isomer 1	17,8	457,0776	457,0769	1.7	1.7	$C_{22}H_{18}O_{11}$	nd	nd	E*-G
20	(Epi)gallocatechin gallate (epi)catechin isomer 4	18,4	745,141	745,1418	1.0	0.7	$C_{37}H_{30}O_{17}$	A, E	2	A*-G, I
21	(Epi)gallocatechin gallate (epi)catechin isomer 5	18,6	745,141	745,1414	0.5	3.7	$C_{37}H_{30}O_{17}$	C	nd	A*-G
22	(Epi)gallocatechin isomer 2	18,7	305,0667	305,0669	0.8	1.7	$C_{15}H_{14}O_7$	A*-C	nd	nd
23	Catechin	19,4	289,0718	289,0725	2.7	3.2	$C_{15}H_{14}O_6$	A-E	01-mar	A*-I
24	(Epi)gallocatechin gallate (epi)catechin gallate isomer 1	19,5	897,152	897,1543	2.6	7.2	$C_{44}H_{34}O_{21}$	A-E	nd	A*, B,D-I
25	(Epi)catechin gallate (epi)catechin isomer 1	20	729,1461	729,1471	-1.0	9.8	$C_{37}H_{30}O_{16}$	B, C	nd	A*, B,D-I
26	(Epi)catechin gallate (epi)catechin isomer 2	20,5	729,1461	729,1476	2.0	3.1	$C_{37}H_{30}O_{16}$	A-E	2, 3	A*-I
27	Protocatechuic acid aldehide	20,5	137,0244	137,0245	-0.5	4.2	$C_7H_6O_3$	nd	1*	G-I

Table 2. Continued

28	Dimethoxy-hydroxyphenyl-O-galloyl-glucopyranoside	20,8	483,1144	483,1134	2.2	4.1	$C_{21}H_{24}O_{13}$	A*-C	nd	nd
29	(Epi)gallocatechin gallate isomer 2	21,9	457,0776	457,0783	1.5	5.7	$C_{22}H_{18}O_{11}$	A-E	1, 3	A*-I
30	(Epi)catechin gallate (epi)catechin gallate isomer 1	22,2	881,1571	881,1593	-2.6	3.4	$C_{44}H_{33}O_{20}$	A-E	2, 3	A*-G, I
31	(Epi)catechin gallate (epi)catechin gallate isomer 2	23,1	881,1571	881,1586	-1.7	11.8	$C_{44}H_{33}O_{20}$	A-E	2, 3	A*-I
32	Hydroxy-methoxyphenyl-O-galloyl-glucopyranoside	23,5	453,1038	453,1042	0.8	2.3	$C_{20}H_{22}O_{12}$	A-C	nd	A*-G
33	UK2 isomer 1	23,6	439,0671	439,0668	0.5	7.6	$C_{22}H_{16}O_{10}$	nd	nd	G*-I
34	Epicatechin	24,5	289,0718	289,0722	1.7	2.0	$C_{15}H_{14}O_{6}$	A-D	1, 3	A*-I
35	(Epi)gallocatechin gallate isomer 3	24,7	457,0776	457,0779	0.6	6.0	$C_{22}H_{18}O_{11}$	C, D	nd	A*, B, E-I
36	Eriodictyol-O-glucoside	24,9	449,1089	449,1069	4.6	2.3	$C_{21}H_{22}O_{11}$	A*-D	nd	A-D
37	(Epi)catechin gallate (epi)catechin gallate isomer 3	25,5	881,1571	881,1586	-1.7	11.8	$C_{44}H_{34}O_{20}$	A-C	2, 3	A*-G, I
38	(Epi)gallocatechin gallate (epi)catechin isomer 6	25,9	745,141	745,1416	0.8	6.9	$C_{37}H_{30}O_{17}$	A-D	nd	A*-E, G
39	Galloyl glucosyl dihydroxy methoxyacetophenone	26,2	495,1144	495,1131	2.6	20.4	$C_{22}H_{24}O_{13}$	A*-C	nd	nd
40	UK2 isomer 2	26,5	439,0671	439,0664	1.5	43.6	$C_{22}H_{16}O_{10}$	nd	nd	F*-I
41	(Epi)gallocatechin-(epi)catechin-gallate	26,6	743,1254	743,1282	-2.8	50.5	$C_{37}H_{28}O_{17}$	A*-C	nd	nd
42	(Epi)gallocatechin gallate (epi)catechin gallate isomer 2	26,7	897,152	897,1516	0.4	6.1	$C_{44}H_{34}O_{21}$	A*-C	nd	A-C
43	Lyoniside	27,5	551,2134	551,2136	0.4	4.2	$C_{27}H_{36}O_{12}$	A*-C	nd	A-G
44	(Epi)catechin gallate isomer 1	27,9	441,0827	441,0836	2.1	2.3	$C_{22}H_{18}O_{10}$	nd	1*	G-I
45	(Epi)catechin gallate isomer 2	28,2	441,0827	441,0836	2.0	2.0	$C_{22}H_{18}O_{10}$	A-E	2, 3	A*-I
46	(Epi)catechin-3-O-glucoside-gallate	28,9	603,1355	603,1355	0.1	15.1	$C_{28}H_{28}O_{15}$	A*-C, E	nd	A-G
47	Procyanidin B dimer isomer 2	29,6	577,1351	577,1346	0.9	12.1	$C_{30}H_{26}O_{12}$	A-D	3	A*-E, G-I
48	(Epi)catechin gallate isomer 3	30,1	441,0827	441,0814	2.9	2.4	$C_{22}H_{18}O_{10}$	A-E	2, 3	A*-I
49	Dihydromyricetin isomer 1	31	319,0459	319,0465	1.7	0.3	$C_{15}H_{12}O_{8}$	nd	nd	G*-I
50	(Epi)catechin gallate (epi)catechin gallate isomer 4	31,8	881,1571	881,1602	-3.6	5.0	$C_{44}H_{34}O_{20}$	A-E	2, 3	A*-I
51	(Epi)catechin gallate (epi)catechin isomer 3	32,1	729,1461	729,1488	3.8	2.8	$C_{37}H_{30}O_{16}$	A-E	2, 3	A*-I
52	(Epi)afzelechin gallate	33	425,0878	425,0892	3.2	9.5	$C_{22}H_{18}O_{9}$	A-E	2, 3	A*-G
53	Myricetin glucoside	33,5	479,0831	479,0849	-3.8	14.0	$C_{21}H_{20}O_{13}$	A*-E	2	A-C, F
54	Jaceidin triacetate	33,6	485,1089	485,1093	0.8	8.3	$C_{24}H_{22}O_{11}$	nd	nd	D*, E,G-I
55	Phloretin-di-C-glucoside	34,6	597,1825	597,1807	3.0	13.1	$C_{27}H_{34}O_{15}$	A*-E	nd	A, B
56	Trihydroxystilbene glucosyl-O-gallate	34,7	541,1351	541,1362	-1.9	14.9	$C_{27}H_{26}O_{12}$	A*-E	2	A, B, D-F

Table 2. Continued

57	Dihydromyricetin isomer 2	35,6	319,0459	319,0465	1.7	0.3	$C_{15}H_{12}O_8$	nd	nd	E, G*-I
58	UK3	35,7	439,1093	439,1073	4.5	36.7	$C_{16}H_{24}O_{14}$	A*-C, E	2	A, B
59	Homaloside D	36,4	543,1508	543,1529	-3.8	19.2	$C_{27}H_{28}O_{12}$	A*-C	nd	A-C
60	Phloretin-C-glucoside (nothofagin)	36,4	435,1297	435,1281	3.6	8.8	$C_{21}H_{24}O_{10}$	C*, E	2	nd
61	Rhamnetin	36,6	315,051	315,051	-0.1	46.1	$C_{16}H_{12}O_7$	nd	nd	G*-I
62	Dihydroquercetin	37,6	303,051	303,0514	1.2	17.9	$C_{15}H_{12}O_7$	nd	nd	D*-I
63	Pentamethoxystilbene isomer 1	37,7	329,1394	329,1402	2.3	6.8	$C_{19}H_{22}O_5$	B*, C, E	1, 3	A, B, E
64	Quercetin glucoside	37,9	463,0882	463,0883	-0.3	0.7	$C_{21}H_{20}O_{12}$	C*, E	nd	A, B
65	Syringic aldehyde	38,7	181,0506	181,0503	1.7	0.8	$C_9H_{10}O_4$	nd	nd	G*-I
66	Pentamethoxystilbene isomer 2	38,9	329,1394	329,1387	2.4	5.5	$C_{19}H_{22}O_5$	A*-E	1, 3	A-F
67	Ellagic acid	40,2	300,999	301,0004	4.6	15.7	$C_{14}H_6O_8$	A-E	2, 3	A*-I
68	Naringenin	40,8	271,0612	271,0609	1.3	7.8	$C_{15}H_{12}O_5$	nd	nd	F, G*, I
69	Taxifolin	41,6	303,051	303,0519	2.8	0.9	$C_{15}H_{12}O_7$	nd	nd	E, G*-I
70	Nonanedioic acid (azelaic acid)	42,3	187,0981	187,0976	-2.8	5.1	$C_9H_{16}O_4$	nd	1*-3	nd
71	Flavanone	47,2	271,0612	271,0622	-3.5	8.3	$C_{15}H_{12}O_5$	nd	2*, 3	nd

* RT, calc. and meas. m/z, error and σ values are referred to this extract

nd Non detected

Finally, other identified compounds were a cyclic polyol was assigned as quinic acid (peak 1) at *m/z* 191 and sugars (peaks 2 and 3). In addition, a lignan glycoside identified as lyoniside was also detected at *m/z* 551 (peak 43). Concerning stilbenes, two isomers at *m/z* 329 were tentatively assigned to pentamethoxystilbene (peaks 63 and 66). These compounds have been reported to show a high anti-proliferative effect on different human cancer cell lines (Chimento et al., 2016; Horvath et al., 2007; Pan et al., 2010). Moreover, dicarboxylic fatty acid corresponding to deprotonated molecule at *m/z* 187 was identified as nonanedioic acid (peak 70). There were some compounds, for which the structure could not be identified when the MS experiment was performed (peaks 8, 33, 40 and 58).

3.2. Effect of conventional and new extraction techniques on bioactive compounds recovery.

3.2.1. Extraction yield.

Extraction yields for all experiments are reported in Table 3. As observed, it appears clear that PLE represents the best option for the extraction since this technique provides high extraction yield at all experimental conditions. In this sense, the highest yield values were obtained from PLE-I and -G, where the temperature was set at 200 and 176 °C, and the solvent was 50:50 and 85:15 of ethanol:water (v/v), respectively. These. These results pointed out that an increase in both, the temperature and the percentage of ethanol in the solvent, may favour a faster migration of components from sample to the solvent reaching highest yield values.

Table 3. Values of extraction yield (%) obtained under different extraction methodology.

SLE					SFE			PLE								
A	**B**	**C**	**D**	**E**	**1**	**2**	**3**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
6,16	11,62	12,08	9,27	7,30	0,56	2,20	0,57	23,02	19,68	25,15	20,21	28,92	21,25	30,50	20,92	41,90

Concerning SLE methodology, it gave lower results than PLE being the highest value when the proportion of ethanol:water was set at 50 % (SLE-C). These results are in agreement with previous reports which have established that the application of solvent mixtures enhance the extraction yields by improving the solubility and increasing interaction of the targeted analyte with the extraction

solvent (Mustafa and Turner, 2011). As regards SFE, this technique showed the worst efficient extraction yield.

3.3. Comparative study of phytochemical composition of *S. Birrea* extracts obtained under different extraction methodologies.

The differences in the chemical structure of the phenolic compounds confer differences to their physico-chemical properties such as solubility, thermal and chemical stability, and ability to link to other compounds. Moreover, the molecular weight and conformational flexibility of phenols have been reported to influence their tendency to be retained in the plant cell wall matrix when a extraction approach is applied (Pinelo et al., 2008). In this scenario, the technological processing used to *S. birrea* bark was evaluated in order to determine the compositional variations. The base peak area of each signal in HPLC-MS chromatograms was used to provide semi-quantitative information for comparison purposes. These differences are shown in Figure 2 and 3 by abundance as means of comparison of the corrected area of each individual compound (analyte peak area/internal standard area). Phenolic contents showed a large variation among the different extracts according to the extraction technique employed and the different conditions within each extraction system.

Figure 2A,B,C showed the recovery of the gallic acid and derivatives group by the extraction techniques and experimental conditions. In this group, the most abundant extracted compound in the three applied methodologies was gallic acid (peak 7) being the abundance values of “green” technologies (SFE and PLE) significantly higher than values reported by SLE. On the contrary, this conventional technique showed the greatest number of different compounds belonging to this sub-class. It is noted that the dimethoxy-hydroxyphenyl-O-galloyl-glucopyranoside and hydroxyl-methoxyphenyl-O-galloyl-glucopyranoside (peaks 28 and 32 respectively), which have been reported to have high antioxidant capacity (Shi et al., 2010), were only detected in SLE extracts . Almost all SLE-extracted compounds showed a positive recovery trend over the water percentage increase, that is, the more water percentage, the more abundance of the compound, except for trihydroxystilbene

glucosyl-O-gallate (peak 56). For the latter compound, the most efficient SLE condition was set at 50:50 EtOH:H_2O (v/v).

Regarding SFE extracts, gallic acid (peak 7) was obtained under the three experimental conditions, however there were significant differences between SFE3 (lower values) and the other experiments developed using this technique (S. I. Table 4). Furthermore, trihydroxystilbene glucosyl-O-gallate (peak 56) was only extracted under SFE2 condition. This condition differs from SFE1 in the use of ethanol as supercritical fluid co-solvent and from SFE3 in the use of ethanol in the sample preparation. The combination of both factors could be responsible of the presence of this compound in SFE2 extracts.

In case of PLE technique, only galloyl glucose isomer and gallic acid (peaks 4 and 7, respectively) were detected in all conditions but with a different behavior. Galloyl glucose isomer showed high abundance values in conditions set at low temperature. With regard to gallic acid, this compound showed the opposite performance being the glucose unit the difference between these compounds. Moreover, the other glycosylated compounds (peaks 5, 32 and 56) were detected in increased amount in PLE conditions developed at low temperatures.

On the other hand, a large number of compounds belonging to monomers and derivatives group appeared in PLE extracts. In this technique, the behavior of this sub-class was similar to gallic acid and derivatives. Indeed, it was observed that the lower temperature, the more abundance of these compounds. The differences between some isomers, which were found at higher temperatures, may be explained by the monomer subunit since there was a different extraction response of (+)-catechin (peak 23). This compound showed a similar extraction trend in all temperatures, being increased by the high percentage of ethanol. On the other hand, (-)-epicatechin (peak 34), which was more abundant, reached high abundance values at low temperatures (Figure 2F). With regard to SLE technique, the most of compounds were detected with high intensity when the water percentage was higher than ethanol, except minor isomers ((epi)catechin gallate isomers, peaks 45 and 48). This could be due to the fact that (+)-catechin was only observed at high percentages of ethanol and there was

no significant differences at high percentages of water. Concerning SFE technology (Figure 2E), it is worth stressing that almost no compound was extracted with SFE1 condition or with very low content being SFE2 the best condition for obtaining monomers and gallate derivatives. In particular, (epi)catechin gallate isomer (peak 44) was only detected in SFE1 extract and in the highest temperature of PLE conditions (PLE-G, -H, -I). Concerning (epi)catechin gallate isomer (peak 45), this derivative was extracted by the three techniques appearing with large intensity in advanced extraction systems(SFE and PLE). Moreover, SFE2 condition applied the best extraction parameters for obtaining different gallate derivatives, which are compounds with reported biological properties (Gan et al., 2017; Hayatsu et al., 1992; Lao et al., 2015; Lin et al., 2005). Nevertheless, the results also pointed out that several compounds like galloyl-flavan-3-ols showed an increase in the extraction recovery linked to the increase of the ethanol levels applied in the three technologies.

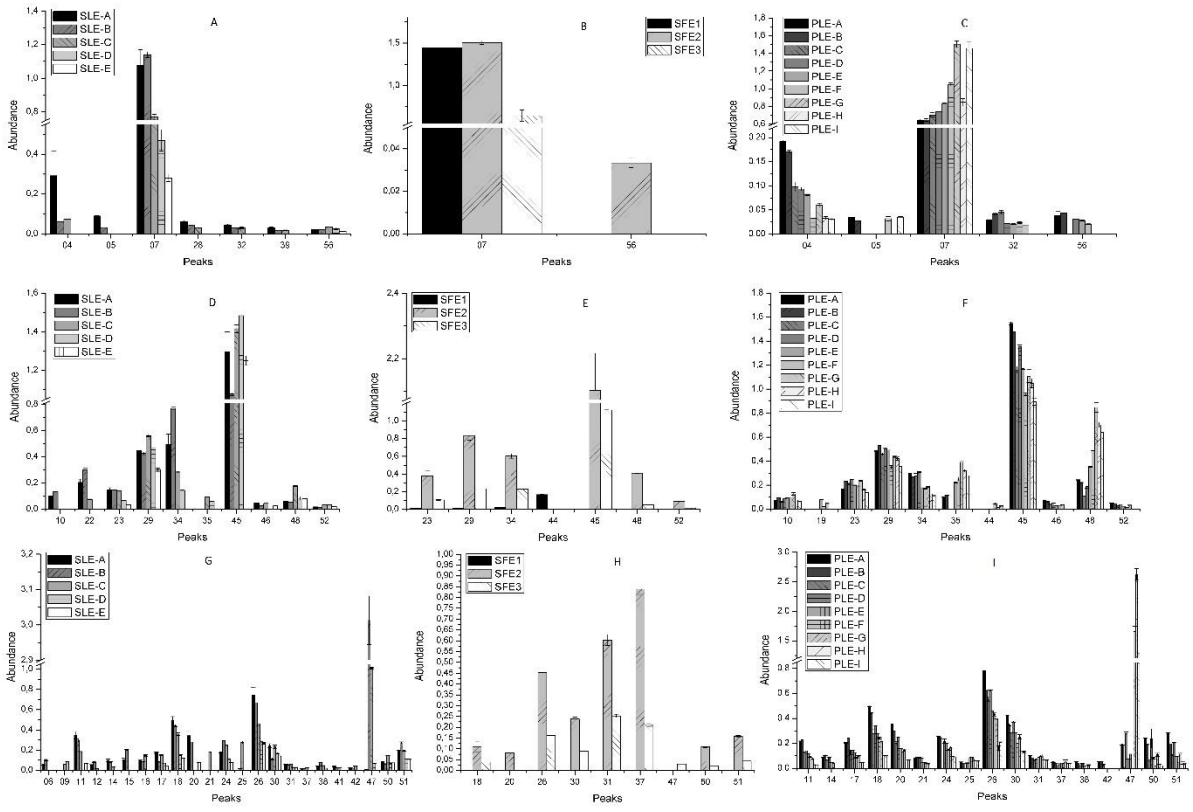

Figure 2. Abundance* of the different groups of compounds: gallic acid and derivatives (A, B, C), monomers of flavan-3-ols and derivatives (D, E, F), dimers of flavan-3-ols and derivatives (G, H, I) extracted from *S. birrea* by SLE (A, D, G), SFE (B, E, H) and PLE (C, F, I).

*means of comparison of the ratio of analyte peak area to internal standard peak area

Dimers from flavan-3-ols and derivatives group presented the large number of extracted compounds in the three strategies (Figure 2G,H,I). In SLE, the best conditions were those that contained high proportion of water except in peaks 9, 21, 25, 37, 38, 42, 47, 50 and 51 that obtained the most abundance when the solvent proportion was 50 % of H_2O:Ethanol (v:v). Within this chemical group, the most intense peak was an isomer of procyanidin B dimer (peak 47). This compound reached the maximum abundance at 75 % of water. In this way, peak 47 was also found at high amount in PLE experiments developed at high temperature, reaching the highest level in PLE-H (15:85, H_2O:Ethanol (v:v)). On the contrary, the most of compounds in this methodology showed the same behavior that monomers and derivatives group, they got the most intense area when the temperature was low. This could be due to galloyl groups in their structures. Concerning SFE, procyanidin B dimer was only detected in SFE3. This condition differs from SFE2 in that the sample does not contain ethanol. Moreover, any compound in this class could be extracted with SFE1, including SFE2 experiments the best condition to obtain them.

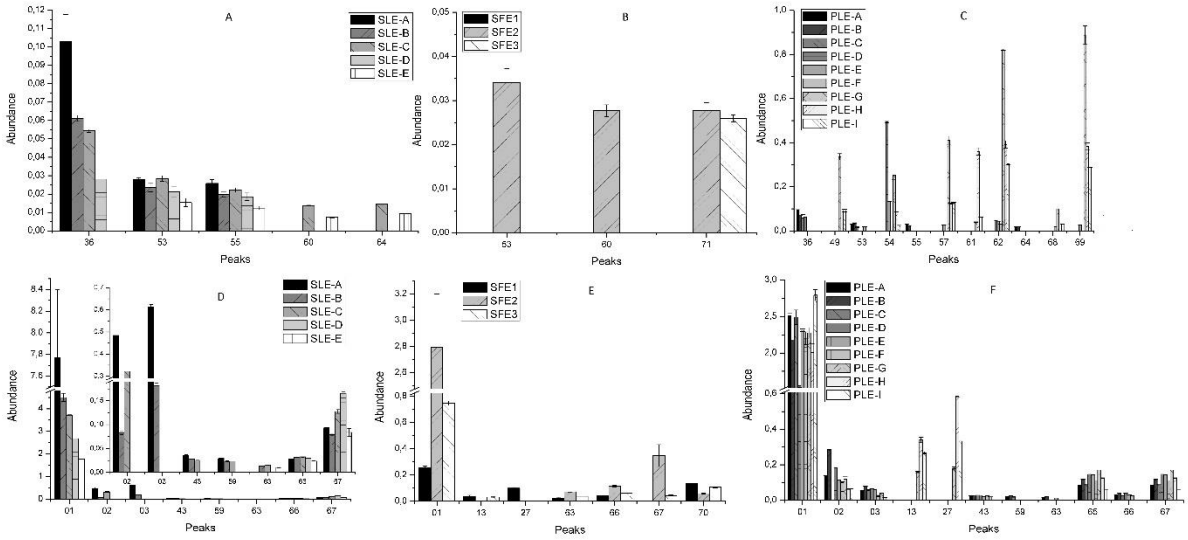

Figure 3. Abundance* of the different groups of compounds: non-derivatives flavan-3-ols flavonoids (a, b, c) and other compounds (d, e, f) extracted from *S. birrea* by SLE (A, D), SFE (B, E) and PLE (C, F).

*means of comparison of the ratio of analyte peak area to internal standard peak area

Regarding flavonoids group, which are non-derivatives of flavan-3-ols (Figure 3A,B,C), the abundance was generally quite low in the three technologies. PLE covered the extractions conditions that obtained better results in terms of number of compounds and of abundance. In addition, most of them belonging to flavonol subclass were extracted when the temperature had the highest value except peaks 36, 53, 55 and 64. These peaks were characterized as chemical compounds with a glucose unit in their structures. In fact, these four compounds also appeared in SLE with similar intensity but aglycons were not available in this conventional extraction at room temperature. In case of SFE, only two glycosylated flavonoids were detected in condition 2 and a flavanone in SFE2 and 3.

The last category includes other phenolic compounds with different chemical structures, sugar and others. Comparing the number of compounds in the three techniques, PLE was the best option for the extraction of the compounds in this group (Figure 3F) although the abundance of peaks 1, 2 and 3 was higher in SLE above all at high water percentage conditions (Figure 3d). On the other hand, compounds as ellagic acid (peak 67) appeared with major intensity when the percentage of ethanol was about 75 % in all methodologies being SFE2 significantly better than the other conditions.

4. CONCLUSIONS

In the present work, a comprehensive characterization by HPLC-ESI-TOF/MS provided a total of 71 compounds with a significant large percentage of them as a galloyl form distributed in five major categories. Notably, two gallic acid derivatives, dimethoxy-hydroxyphenyl-O-galloyl glucopyranoside and hydroxyl-methoxyphenyl-O-galloyl glucopyranoside, have been described for first time in *S. birrea*. Moreover, the main goal of this study has been to compare different extraction approaches such as conventional (SLE) and non-conventional or “green” techniques (SFE and PLE) using GRAS solvents. In terms of yield, PLE represents the best option since this methodology got the highest values for the conditions where the temperature and the percentage of ethanol were set over 176 °C and 50 % (ethanol:water).

The extraction processes are directly involved in the obtainment of different phenolic compounds due to the large types of chemical structures of them. For this reason, the extraction technologies were evaluated in order to determine the compositional variations of *S. birrea* bark. Our work concludes on a positive note that, the largest number of compounds were extracted with PLE, generally when the temperature achieves low values above all flavonoid aglycones, but not in case of gallic acid and minor (epi)catechin derivatives. In addition, the high percentage of water in SLE methodology provided a major number of gallic acid derivatives and flavonoids except in case of some (epi)catequin derivatives that are obtained at around 50 % of water:ethanol. In the end, SFE2 did prove effective as a way of extracting proanthocyanidins with galloyl residues.

5. ACKNOWLEGDMENTS

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Table 4. Statistical data (ANOVA) of best extraction condition for compounds which appear in all methodologies

Peak		1	**Peak**		7	**Peak**		18	**Peak**		20	**Peak**		23	**Peak**		26	**Peak**		29	**Peak**		30
m/z		191	**m/z**		169	**m/z**		745	**m/z**		745	**m/z**		289	**m/z**		729	**m/z**		457	**m/z**		881
PLE-G	SFE2	0	PLE-G	SFE2	0	PLE-A	SFE2	1	PLE-A	SFE2	1	PLE-D	SFE2	1	PLE-A	SFE2	1	PLE-B	SFE2	0	PLE-A	SFE2	1
	SLE-A	1		SLE-B	1		SLE-A	0		SLE-A	0		SLE-A	1		SLE-A	0		SLE-C	1		SLE-A	1
SFE2	PLE-G	0	SFE2	PLE-G	0	SFE2	PLE-A	1	SFE2	PLE-A	1	SFE2	PLE-D	1	SFE2	PLE-A	1	SFE2	PLE-B	0	SFE2	PLE-A	1
	SLE-A	1		SLE-B	1		SLE-A	1		SLE-A	1		SLE-A	1		SLE-A	1		SLE-C	1		SLE-A	0
SLE-A	PLE-G	1	SLE-B	PLE-G	1	SLE-A	PLE-A	0	SLE-A	PLE-A	0	SLE-A	PLE-D	1	SLE-A	PLE-A	0	SLE-C	PLE-B	1	SLE-A	PLE-A	1
	SFE2	1		SFE2	1		SFE2	1		SFE2	1		SFE2	1		SFE2	1		SFE2	1		SFE2	0
Peak		31	**Peak**		34	**Peak**		37	**Peak**		44	**Peak**		45	**Peak**		47	**Peak**		48	**Peak**		50
m/z		881	**m/z**		289	**m/z**		881	**m/z**		441	**m/z**		441	**m/z**		577	**m/z**		441	**m/z**		881
PLE-A	SFE2	1	PLE-D	SFE2	1	PLE-G	SFE2	1	PLE-G	SFE2	1	PLE-A	SFE3	1	PLE-H	SFE3	1	PLE-G	SFE3	1	PLE-A	SFE2	1
	SLE-C	0		SLE-B	1		SLE-C	0		SLE-D	1		SLE-C	1		SLE-B	1		SLE-C	1		SLE-C	1
SFE2	PLE-A	1	SFE2	PLE-D	1	SFE2	PLE-G	1	SFE2	PLE-G	1	SFE3	PLE-A	1	SFE3	PLE-H	1	SFE3	PLE-G	1	SFE2	PLE-A	1
	SLE-C	1		SLE-B	1		SLE-C	1		SLE-D	1		SLE-C	1		SLE-B	1		SLE-C	1		SLE-C	1
SLE-C	PLE-A	0	SLE-B	PLE-D	1	SLE-C	PLE-G	0	SLE-D	PLE-G	1	SLE-C	PLE-A	1	SLE-B	PLE-H	1	SLE-C	PLE-G	1	SLE-C	PLE-A	1
	SFE2	1		SFE2	1		SFE2	1		SFE2	1		SFE3	1		SFE3	1		SFE3	1		SFE2	1
Peak		51	**Peak**		52	**Peak**		53	**Peak**		56	**Peak**		58	**Peak**		63	**Peak**		66	**Peak**		67
m/z		729	**m/z**		425	**m/z**		479	**m/z**		541	**m/z**		439	**m/z**		329	**m/z**		329	**m/z**		300
PLE-A	SFE2	1	PLE-A	SFE2	1	PLE-B	SFE2	0	PLE-B	SFE2	1	PLE-B	SFE2	1	PLE-B	SFE2	1	PLE-D	SFE2	1	PLE-G	SFE2	1
	SLE-B	0		SLE-C	1		SLE-C	1		SLE-C	1		SLE-A	1		SLE-C	0		SLE-C	0		SLE-D	0
SFE2	PLE-A	1	SFE2	PLE-A	1	SFE2	PLE-B	0	SFE2	PLE-B	1	SFE2	PLE-B	1	SFE2	PLE-B	1	SFE2	PLE-D	1	SFE2	PLE-G	1
	SLE-B	1		SLE-C	1		SLE-C	0		SLE-C	0		SLE-A	1		SLE-C	1		SLE-C	1		SLE-D	1
SLE-B	PLE-A	0	SLE-C	PLE-A	1	SLE-C	PLE-B	1	SLE-C	PLE-B	1	SLE-A	PLE-B	1	SLE-C	PLE-B	0	SLE-C	PLE-D	0	SLE-D	PLE-G	0
	SFE2	1		SFE2	1		SFE2	0		SFE2	0		SFE2	1		SFE2	1		SFE2	1		SFE2	1

1 There are significant differences

0 There are not significant differences

Table 5. Means of comparison of the ratio of analyte peak area to internal standard peak area for compounds extracted by SLE.

Peak	SLE-A	SD	SLE-B	SD	SLE-C	SD	SLE-D	SD	SLE-E	SD
1	7,772	0,629	4,504	0,176	3,724	0,029	2,689	0,041	1,781	0,023
2	0,485	0,046	0,083	0,003	0,323	0,019				
3	0,612	0,013	0,182	0,005						
4	0,291	0,124	0,060	0,002	0,074	0,002				
5	0,089	0,003	0,032	0,001						
6	0,058	0,007	0,105	0,009						
7	1,078	0,093	1,140	0,016	0,770	0,016	0,469	0,053	0,281	0,019
8	0,364	0,032	0,100	0,014	0,051	0,004				
9					0,060	0,004	0,085	0,003		
10	0,102	0,009	0,132	0,003						
11	0,347	0,037	0,297	0,017	0,186	0,008			0,072	0,002
12	0,054	0,008	0,083	0,005	0,018	0,002				
14	0,099	0,014	0,079	0,006	0,036	0,003				
15	0,108	0,011	0,200	0,007						
16	0,092	0,010	0,087	0,002	0,146	0,008				
17	0,174	0,008	0,085	0,006	0,151	0,003	0,073	0,001	0,046	0,001
18	0,494	0,039	0,442	0,010	0,358	0,012	0,148	0,006	0,119	0,003
20	0,342	0,044	0,280	0,007					0,077	0,001
21					0,181	0,006				
22	0,204	0,024	0,301	0,011	0,075	0,003				
23	0,151	0,014	0,146	0,003	0,142	0,001	0,067	0,002	0,036	0,001
24	0,182	0,010	0,293	0,014	0,250	0,005	0,115	0,009	0,080	0,002
25			0,019	0,001	0,276	0,012				
26	0,743	0,075	0,668	0,019	0,461	0,018	0,287	0,009	0,268	0,007
28	0,063	0,005	0,045	0,001	0,029	0,003				
29	0,448	0,028	0,426	0,012	0,561	0,009	0,453	0,012	0,301	0,013
30	0,244	0,021	0,114	0,010	0,233	0,019	0,170	0,005	0,142	0,008
31	0,054	0,007	0,051	0,003	0,061	0,003	0,029	0,003	0,028	0,003
32	0,044	0,005	0,030	0,001	0,030	0,004				
34	0,495	0,077	0,769	0,009	0,283	0,005	0,144	0,002		
35					0,095	0,002	0,060	0,001		
36	0,103	0,015	0,061	0,002	0,054	0,001	0,028	0,006		
37	0,018	0,002	0,014	0,001	0,024	0,002				
38	0,037	0,006	0,076	0,003	0,047	0,002	0,022	0,008		
39	0,030	0,004	0,018	0,001	0,018	0,002				
41	0,040	0,005	0,026	0,001	0,024	0,002				
42	0,029	0,002	0,021	0,001	0,045	0,003				
43	0,036	0,001	0,028	0,001	0,025	0,001				
45	1,297	0,102	1,077	0,008	1,415	0,020	1,483	0,036	1,251	0,025
46	0,046	0,000	0,027	0,002	0,044	0,001			0,025	0,002

Table Cont.										
47	0,008	0,001	3,013	0,068	1,009	0,009	0,070	0,001		
48	0,063	0,005	0,054	0,001	0,176	0,004	0,083	0,012	0,081	0,003
50	0,090	0,001	0,062	0,005	0,150	0,001	0,069	0,007	0,075	0,001
51	0,188	0,012	0,272	0,010	0,197	0,007	0,112	0,002	0,108	0,002
52	0,018	0,000	0,015	0,000	0,036	0,001	0,034	0,000	0,022	0,001
53	0,028	0,001	0,024	0,002	0,028	0,001	0,021	0,003	0,015	0,002
55	0,026	0,002	0,020	0,001	0,022	0,001	0,019	0,002	0,012	0,001
56	0,021	0,001	0,021	0,001	0,035	0,002	0,026	0,005	0,013	0,001
58	0,102	0,008	0,079	0,000	0,079	0,001			0,029	0,000
59	0,030	0,000	0,023	0,001	0,023	0,001				
60					0,014	0,000			0,007	0,000
63			0,014	0,001	0,016	0,001			0,010	0,001
64					0,015	0,001			0,009	0,001
66	0,028	0,000	0,032	0,000	0,033	0,001	0,030	0,000	0,024	0,000
67	0,093	0,001	0,079	0,002	0,127	0,004	0,166	0,003	0,084	0,008

Table 6. Statistical data (ANOVA) of all compounds in each extraction methodologies.

			Peak	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
			m/z	191	341	503	331	331	609	169	411	761	305	761	745	153	577	593	745	913	745	457	745	745	305	289	897	729	729	137	483	457	881	881	453	439	289	457
DIFFERENT EXTRACTION CONDITIONS	SLE	A	B	1	1	1	1	1	1	0	1	*	1	0	1	nd	1	1	0	1	1	nd	1	nd	1	0	1	*	0	nd	1	0	1	0	1	nd	1	*
			C	1	1	*	1	*	*	1	1	*	*	1	1	nd	1	*	1	1	1	nd	1	nd	1	0	1	*	1	nd	1	1	0	0	1	nd	1	*
			D	1	*	*	*	*	*	1	*	*	*	*	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	0	1	1	*	nd	1	*
			E	1	*	*	*	*	*	1	*	*	*	1	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	1	1	1	*	nd	*	*
		B	A	1	1	1	1	1	1	0	1	*	1	0	1	nd	1	1	0	1	1	nd	1	nd	1	0	1	*	0	nd	1	0	1	0	1	nd	1	*
			C	0	1	*	0	*	*	1	1	*	*	1	1	nd	1	*	1	1	1	nd	1	nd	1	0	1	1	1	nd	1	1	1	0	0	nd	1	*
			D	1	*	*	*	*	*	1	*	*	*	*	*	nd	*	*	0	1	nd	1	nd	*	1	1	*	1	nd	*	0	1	1	*	nd	1	*
			E	1	*	*	*	*	*	1	*	*	*	1	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	1	0	1	*	nd	*	*
		C	A	1	1	*	1	*	*	1	1	*	*	1	1	nd	1	*	1	1	1	nd	1	nd	1	0	1	*	1	nd	1	1	0	0	1	nd	1	*
			B	0	1	*	0	*	*	1	1	*	*	1	1	nd	1	*	1	1	1	nd	1	nd	1	0	1	1	1	nd	1	1	1	0	0	nd	1	*
			D	1	*	*	*	*	*	1	*	1	*	*	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	1	1	1	*	nd	1	1
			E	1	*	*	*	*	*	1	*	*	*	*	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	1	1	1	*	nd	*	*
		D	A	1	*	*	*	*	*	1	*	*	*	*	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	0	1	1	*	nd	1	*
			B	1	*	*	*	*	*	1	*	*	*	*	*	nd	*	*	*	0	1	nd	1	nd	*	1	1	*	1	nd	*	0	1	1	*	nd	1	*
			C	1	*	*	*	*	*	1	*	1	*	*	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	1	1	1	*	nd	1	1
			D	1	*	*	*	*	*	1	*	*	*	*	*	nd	*	*	*	1	0	nd	1	nd	*	1	1	*	0	nd	*	1	0	0	*	nd	*	*
		E	A	1	*	*	*	*	*	1	*	*	*	1	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	1	1	1	*	nd	*	*
			B	1	*	*	*	*	*	1	*	*	*	1	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	1	0	1	*	nd	*	*
			C	1	*	*	*	*	*	1	*	*	*	1	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	1	nd	*	1	1	1	*	nd	*	*
			D	1	*	*	*	*	*	1	*	*	*	*	*	nd	*	*	*	1	1	nd	1	nd	*	1	1	*	0	nd	*	1	0	0	*	nd	*	*
	SFE	1	2	1	nd	nd	nd	nd	nd	0	nd	nd	nd	nd	nd	*	nd	nd	nd	nd	*	nd	nd	nd	nd	1	nd	nd	*	nd	nd	1	*	*	nd	nd	1	nd
			3	0	nd	nd	nd	nd	nd	1	nd	nd	nd	nd	nd	0	nd	nd	nd	nd	*	nd	nd	nd	nd	1	nd	nd	*	nd	nd	1	*	*	nd	nd	1	nd
		2	1	1	nd	nd	nd	nd	nd	0	nd	nd	nd	nd	nd	*	nd	nd	nd	nd	*	nd	nd	nd	nd	1	nd	nd	*	nd	nd	1	*	*	nd	nd	1	nd
			3	1	nd	nd	nd	nd	nd	1	nd	nd	nd	nd	nd	*	nd	nd	nd	nd	1	nd	nd	nd	nd	1	nd	nd	1	nd	nd	1	1	0	nd	nd	1	nd
		3	1	0	nd	nd	nd	nd	nd	1	nd	nd	nd	nd	nd	0	nd	nd	nd	nd	*	nd	nd	nd	nd	1	nd	nd	*	nd	nd	1	*	*	nd	nd	1	nd
			2	1	nd	nd	nd	nd	nd	1	nd	nd	nd	nd	nd	*	nd	nd	nd	nd	1	nd	nd	nd	nd	1	nd	nd	1	nd	nd	1	1	0	nd	nd	1	nd
	PLE	A	B	1	1	1	1	1	nd	0	1	nd	1	0	nd	*	1	nd	nd	1	1	*	1	0	nd	1	0	1	1	*	nd	1	1	0	1	*	1	0
			C	0	*	0	1	*	nd	0	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	0	nd	1	*	*	1	*	nd	1	1	1	1	*	1	*
			D	1	1	1	1	*	nd	0	*	nd	1	1	nd	*	0	nd	nd	1	1	*	1	0	nd	1	0	1	1	*	nd	0	1	0	1	*	1	1
			E	0	1	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	0	1	0	1	*	1	1
			F	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
			G	1	1	1	1	0	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
			H	1	1	1	1	*	nd	1	*	nd	0	1	nd	*	*	nd	nd	1	*	*	*	*	nd	0	1	0	1	*	nd	1	*	1	*	*	1	1
			I	1	1	*	1	0	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	0	1	1	*	nd	1	1	0	*	*	1	1
		B	A	1	1	1	1	1	nd	0	1	nd	1	0	nd	*	1	nd	nd	1	1	*	1	0	nd	1	0	1	1	*	nd	1	1	0	1	*	1	0
			C	1	*	1	1	*	nd	0	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	0	nd	1	*	*	1	*	nd	1	1	0	0	*	0	*
			D	1	1	1	1	*	nd	0	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	0	nd	1	1	0	0	*	nd	1	1	0	1	*	1	*
			E	0	1	1	1	*	nd	1	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	0	1	0	1	*	1	1
			F	0	1	1	1	*	nd	1	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
			G	0	1	1	1	0	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	1	nd	0	1	1	1	*	nd	1	1	0	1	*	1	1
			H	0	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	*	nd	1	*	1	*	*	1	0
			I	1	1	*	1	1	nd	1	1	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	0	*	*	1	1
		C	A	0	*	0	1	*	nd	0	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	0	nd	1	*	*	1	*	nd	1	1	1	1	*	1	*
			B	1	*	1	1	*	nd	0	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	0	nd	1	*	*	1	*	nd	1	1	0	0	*	0	*
			D	1	*	1	0	*	nd	0	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	0	nd	1	*	*	1	*	nd	1	1	0	1	*	1	*
			E	0	*	0	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	0	0	*	1	1	nd	0	*	*	1	*	nd	1	0	1	1	*	1	*
			F	1	*	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	0	*	1	1	nd	1	*	*	1	*	nd	1	1	1	1	*	1	*
			G	1	*	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	1	nd	1	*	*	1	*	nd	0	1	1	1	*	1	*
			H	1	*	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	*	*	1	*	nd	1	*	0	*	*	1	*
			I	1	*	*	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	0	*	*	1	*
		D	A	1	1	1	1	*	nd	0	*	nd	1	1	nd	*	0	nd	nd	1	1	*	1	0	nd	1	1	1	1	*	nd	0	1	1	1	*	0	*
			B	1	1	1	1	*	nd	0	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	0	nd	1	1	0	0	*	nd	1	1	0	1	*	1	*
			C	1	*	1	0	*	nd	0	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	0	nd	1	*	*	1	*	nd	1	1	0	1	*	1	*
			D	1	1	0	0	*	nd	0	*	nd	0	1	nd	*	1	nd	nd	0	1	*	1	1	nd	1	0	1	1	*	nd	0	1	0	0	*	1	*
			E	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	*
			F	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	*
			G	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	1	0	*	1	*
			H	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	*	nd	1	*	0	*	*	1	*
			I	1	1	*	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	0	*	*	1	*
		E	A	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
			B	0	1	1	1	*	nd	1	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
			C	1	*	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	0	*	1	1	nd	1	*	*	1	*	nd	1	1	0	1	*	1	1
			D	1	1	1	1	*	nd	1	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	1	0	*	1	*
			E	0	0	1	1	*	nd	1	*	nd	0	1	nd	*	0	nd	nd	1	0	1	1	0	nd	0	1	0	0	*	nd	1	1	0	0	*	0	1
			F	0	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	1	0	1	nd	1	0	1	0	*	nd	1	1	0	1	*	0	1
			G	0	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	1	0	1	nd	1	1	0	1	*	nd	1	1	0	0	*	0	1
			H	1	1	1	1	*	nd	0	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	*	nd	1	*	1	*	*	1	1
			I	1	1	*	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
		F	A	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
			B	0	1	1	1	*	nd	1	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
			C	1	*	1	1	*	nd	1	*	nd	1	1	nd	*	1	nd	nd	1	0	*	1	1	nd	1	*	*	1	*	nd	1	1	0	0	*	1	1
			D	1	1	1	1	*	nd	1	*	nd	0	1	nd	*	1	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	1	0	*	1	*
			E	0	0	1	1	*	nd	1	*	nd	0	1	nd	*	0	nd	nd	1	0	1	1	0	nd	0	1	0	0	*	nd	1	1	0	0	*	0	1
			F	0	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	1	0	1	nd	1	0	1	0	*	nd	1	1	0	1	*	0	1
			G	0	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	1	0	1	nd	1	0	1	0	*	nd	1	1	0	1	*	0	1
			H	0	1	1	0	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	*	nd	1	*	1	*	*	1	1
			I	1	1	*	0	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	0	1	1	*	*	1	1
		G	A	1	1	1	1	0	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	0	1	*	1	1
			B	0	1	1	1	0	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	1	nd	0	1	1	1	*	nd	1	1	0	1	*	1	1
			C	1	*	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	1	nd	1	*	*	1	*	nd	0	1	1	1	*	1	1
			D	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	1	nd	1	1	1	1	*	nd	1	1	1	0	*	1	*
			E	0	0	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	1	0	1	nd	1	1	0	1	*	nd	1	1	0	0	*	0	1
			F	0	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	0	0	1	0	1	nd	1	0	1	0	*	nd	1	1	0	1	*	0	1
			G	1	1	1	1	*	nd	1	*	nd	1	1	nd	1	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	1	nd	0	*	1	*	1	1	1
			H	1	1	*	1	0	nd	0	*	nd	1	1	nd	1	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	1	nd	1	1	1	*	1	1	1
			I	1	1	*	1	0	nd	1	*	nd	1	1	nd	1	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	1	nd	1	1	1	*	1	1	1
		H	A	1	1	1	1	*	nd	1	*	nd	0	1	nd	*	*	nd	nd	1	*	*	*	*	nd	0	1	0	1	*	nd	1	*	1	*	*	1	1
			B	0	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	*	nd	1	*	1	*	*	1	0
			C	1	*	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	*	*	1	*	nd	1	*	0	*	*	1	*
			D	1	1	1	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	*	nd	1	*	0	*	*	1	*
			E	1	1	1	1	*	nd	0	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	*	nd	1	*	1	*	*	1	1
			F	0	1	1	0	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	*	nd	1	*	1	*	*	1	1
			G	1	1	1	1	*	nd	1	*	nd	1	1	nd	1	*	nd	nd	1	*	*	*	*	nd	1	1	1	1	1	nd	0	*	1	*	1	1	1
			H	1	0	*	0	*	nd	1	*	nd	1	0	nd	1	*	nd	nd	0	*	*	*	*	nd	1	0	0	1	1	nd	1	*	0	*	1	0	1
			I	1	1	*	1	0	nd	1	1	nd	0	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	0	*	*	1	1
		I	A	1	1	*	1	0	nd	1	1	nd	0	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	1	*	*	1	1
			B	1	1	*	1	1	nd	1	1	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	1	*	*	1	1
			C	1	*	*	1	*	nd	1	*	nd	0	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	*	*	1	*	nd	1	1	0	*	*	1	*
			D	1	1	*	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	0	*	*	1	*
			E	1	1	*	1	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	1	1	1	1	*	1	1
			F	1	1	*	0	*	nd	1	*	nd	1	1	nd	*	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	*	nd	0	1	1	*	*	1	1
			G	1	1	*	1	0	nd	0	*	nd	1	1	nd	1	*	nd	nd	1	1	*	1	*	nd	1	1	1	1	1	nd	1	1	1	*	1	1	1
			H	1	0	*	0	*	nd	1	*	nd	1	0	nd	1	*	nd	nd	0	*	*	*	*	nd	1	0	0	1	1	nd	1	*	0	*	1	0	1

1 - There are significant differences
0 - There are not significant differences
nd - compound non detected in the tecnique or only in one condition
* - compound non detected in some condition

Table 6. Statistical data (ANOVA) of all compounds in each extraction methodologies.

			Peak	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
			m/z	449	881	745	495	439	743	897	551	441	441	603	577	441	319	881	729	425	479	485	597	541	319	439	543	435	315	303	329	463	181	329	300	271	303	187	271
DIFFERENT EXTRACTION CONDITIONS	SLE	A	B	1	1	1	1	nd	1	1	1	nd	1	1	1	0	nd	1	1	1	0	nd	1	0	nd	1	1	*	nd	nd	*	*	nd	1	1	nd	nd	1	1
			C	1	1	0	1	nd	1	1	1	nd	0	0	1	1	nd	1	0	1	0	nd	0	1	nd	1	1	*	nd	nd	*	*	nd	1	1	nd	nd	1	1
			D	1	*	1	*	nd	*	*	*	nd	1	*	0	1	nd	1	1	1	1	nd	1	0	nd	*	*	*	nd	nd	*	*	nd	0	1	nd	nd	1	0
			E	*	*	*	*	nd	*	*	*	nd	0	1	*	1	nd	1	1	1	1	nd	1	1	nd	1	*	*	nd	nd	*	*	nd	1	0	nd	nd	1	0
		B	A	1	1	1	1	nd	1	1	1	nd	1	1	1	0	nd	1	1	1	0	nd	1	0	nd	1	1	*	nd	nd	*	*	nd	1	1	nd	nd	1	1
			C	0	1	1	0	nd	0	1	1	nd	1	1	1	1	nd	1	1	1	0	nd	0	1	nd	0	0	*	nd	nd	1	*	nd	0	1	nd	nd	1	0
			D	1	*	1	*	nd	*	*	*	nd	1	*	1	1	nd	0	1	1	0	nd	0	0	nd	*	*	*	nd	nd	*	*	nd	1	1	nd	nd	1	1
			E	*	*	*	*	nd	*	*	*	nd	1	0	*	1	nd	1	1	1	1	nd	1	1	nd	1	*	*	nd	nd	1	*	nd	1	0	nd	nd	1	1
		C	A	1	1	0	1	nd	1	1	1	nd	0	0	1	1	nd	1	0	1	0	nd	0	1	nd	1	1	*	nd	nd	*	*	nd	1	1	nd	nd	1	1
			B	0	1	1	0	nd	0	1	1	nd	1	1	1	1	nd	1	1	1	0	nd	0	1	nd	0	0	*	nd	nd	1	*	nd	0	1	nd	nd	1	0
			D	1	*	1	*	nd	*	*	*	nd	0	*	1	1	nd	1	1	1	1	nd	0	1	nd	*	*	1	nd	nd	*	*	nd	1	1	nd	nd	1	1
			E	*	*	*	*	nd	*	*	*	nd	1	*	*	0	nd	0	0	1	1	nd	1	1	nd	*	*	*	nd	nd	1	1	nd	1	1	nd	nd	1	1
		D	A	1	*	1	*	nd	*	*	*	nd	1	*	0	1	nd	1	1	1	1	nd	1	0	nd	*	*	*	nd	nd	*	*	nd	0	1	nd	nd	1	0
			B	1	*	1	*	nd	*	*	*	nd	1	*	1	1	nd	0	1	1	0	nd	0	0	nd	*	*	*	nd	nd	*	*	nd	1	1	nd	nd	1	1
			C	1	*	1	*	nd	*	*	*	nd	0	*	1	1	nd	1	1	1	1	nd	0	1	nd	*	*	1	nd	nd	*	*	nd	1	1	nd	nd	1	1
			E	*	*	*	*	nd	*	*	*	nd	1	*	*	0	nd	0	0	1	1	nd	1	1	nd	*	*	*	nd	nd	*	*	nd	1	1	nd	nd	0	0
		E	A	*	*	*	*	nd	*	*	*	nd	0	1	*	1	nd	1	1	1	1	nd	1	1	nd	1	*	*	nd	nd	*	*	nd	1	0	nd	nd	1	0
			B	*	*	*	*	nd	*	*	*	nd	1	0	*	1	nd	1	1	1	1	nd	1	1	nd	1	*	*	nd	nd	1	*	nd	1	0	nd	nd	1	1
			C	*	*	*	*	nd	*	*	*	nd	1	1	*	1	nd	1	1	1	1	nd	1	1	nd	1	*	*	nd	nd	1	1	nd	1	1	nd	nd	1	1
			D	*	*	*	*	nd	*	*	*	nd	1	*	*	0	nd	0	0	1	1	nd	1	1	nd	*	*	*	nd	nd	*	*	nd	1	1	nd	nd	0	0
	SFE	1	2	nd	*	nd	nd	nd	nd	nd	nd	nd	*	nd	nd	*	nd	*	*	*	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	nd	nd	1	*	nd	nd	1	*
			3	nd	*	nd	nd	nd	nd	nd	nd	nd	*	nd	nd	*	nd	*	*	*	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	nd	nd	1	*	nd	nd	1	*
		2	1	nd	*	nd	nd	nd	nd	nd	nd	nd	*	nd	nd	*	nd	*	*	*	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	nd	nd	1	*	nd	nd	1	*
			3	nd	1	nd	nd	nd	nd	nd	nd	nd	1	nd	nd	1	nd	1	1	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	nd	nd	1	1	nd	nd	1	0
		3	1	nd	*	nd	nd	nd	nd	nd	nd	nd	*	nd	nd	*	nd	*	*	*	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	nd	nd	1	*	nd	nd	1	*
			2	nd	1	nd	nd	nd	nd	nd	nd	nd	1	nd	nd	1	nd	1	1	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	1	nd	nd	1	1	nd	nd	1	0
	PLE	A	B	1	0	1	nd	*	nd	0	0	*	0	0	1	0	*	0	1	1	1	*	1	0	*	0	1	nd	*	*	1	0	*	1	1	*	*	nd	nd
			C	1	1	1	nd	*	nd	1	1	*	1	1	0	1	*	1	1	1	1	*	*	*	0	*	*	1	nd	*	*	*	*	*	*	1	1	*	nd	nd
			D	1	0	1	nd	*	nd	*	0	*	1	1	0	1	*	0	1	1	1	*	*	*	1	*	*	*	nd	*	*	*	*	*	*	1	1	*	nd	nd
			E	*	1	1	nd	*	nd	*	1	*	1	1	0	1	*	1	1	1	*	*	*	1	*	*	*	nd	*	*	1	*	*	0	1	*	*	nd	nd
			F	*	0	*	nd	*	nd	*	1	*	1	1	0	1	*	1	1	1	1	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	1	*	*	nd	nd
			G	*	0	1	nd	*	nd	*	1	*	1	1	1	1	*	1	1	1	*	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	1	*	*	nd	nd
			H	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	*	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	1	*	*	nd	nd
			I	*	0	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	*	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	1	*	*	nd	nd
		C	A	1	1	1	nd	*	nd	1	1	*	1	1	0	1	*	1	1	1	1	*	*	*	*	*	1	nd	*	*	*	*	*	1	1	1	*	*	nd	nd
			B	0	0	1	nd	*	nd	1	0	*	1	1	1	1	*	1	1	1	1	*	*	*	*	*	1	nd	*	*	*	*	*	1	1	1	*	*	nd	nd
			D	0	0	1	nd	*	nd	*	0	*	1	0	1	1	*	1	1	1	1	*	*	*	*	*	*	nd	*	*	*	*	*	1	1	1	*	*	nd	nd
			E	*	0	1	nd	*	nd	*	1	*	0	0	1	1	*	0	0	1	*	*	*	*	*	*	*	nd	*	*	*	*	*	1	1	*	*	nd	nd
			F	*	1	*	nd	*	nd	*	1	*	1	0	*	1	*	0	1	1	1	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	1	*	*	nd	nd
			G	*	1	1	nd	*	nd	*	1	*	0	0	1	1	*	0	1	1	1	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	1	*	*	nd	nd
			H	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	1	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	1	*	*	nd	nd
			I	*	1	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	1	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	1	*	*	nd	nd
		D	A	1	0	1	nd	*	nd	*	0	*	1	1	0	1	*	0	1	1	*	*	*	0	*	*	*	nd	*	*	*	*	*	1	1	*	*	nd	nd
			B	0	0	1	nd	*	nd	*	0	*	1	1	0	0	*	0	0	1	*	*	*	*	*	*	*	nd	*	*	*	*	*	0	1	*	*	nd	nd
			C	0	0	1	nd	*	nd	*	0	*	1	0	1	1	*	1	1	1	*	*	*	*	*	*	*	nd	*	*	*	*	*	1	1	*	*	nd	nd
			E	*	1	1	nd	*	nd	*	1	*	1	0	0	1	*	1	1	1	*	1	*	0	*	*	*	nd	*	0	*	*	*	1	0	*	*	nd	nd
			F	*	0	*	nd	*	nd	*	1	*	1	0	*	1	*	1	1	1	*	*	*	1	*	*	*	nd	*	0	*	*	*	1	1	*	*	nd	nd
			G	*	0	1	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	*	1	*	*	*	*	*	nd	*	1	*	*	*	*	1	*	*	nd	nd
			H	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	*	1	*	*	*	*	*	nd	*	1	*	*	*	*	1	*	*	nd	nd
			I	*	0	*	nd	*	nd	*	1	*	1	1	1	1	*	1	1	1	*	1	*	*	*	*	*	nd	*	1	*	*	*	*	1	*	*	nd	nd
		E	A	*	1	1	nd	*	nd	*	1	*	1	1	0	1	*	1	1	1	*	*	*	1	*	*	*	nd	*	*	1	*	*	0	1	*	*	nd	nd
			B	*	1	1	nd	*	nd	*	1	*	1	1	0	1	*	1	1	1	*	*	*	1	*	*	*	nd	*	*	1	*	*	1	1	*	*	nd	nd
			C	*	0	1	nd	*	nd	*	1	*	0	0	1	1	*	0	0	1	*	*	*	*	*	*	*	nd	*	*	*	*	*	1	1	*	*	nd	nd
			D	*	1	1	nd	*	nd	*	1	*	1	0	1	1	*	1	1	1	*	1	*	0	*	*	*	nd	*	0	*	*	*	1	0	*	*	nd	nd
			F	*	1	*	nd	*	nd	*	1	*	1	0	*	1	*	0	1	1	*	*	*	0	*	*	*	nd	*	0	*	*	*	1	1	*	*	nd	nd
			G	*	1	1	nd	*	nd	*	1	*	0	0	1	1	*	0	1	1	*	1	*	*	1	*	*	nd	*	1	*	*	*	*	1	*	1	nd	nd
			H	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	0	1	1	*	1	*	*	1	*	*	nd	*	1	*	*	*	*	1	*	1	nd	nd
			I	*	1	*	nd	*	nd	*	*	*	1	*	1	1	*	0	1	*	*	1	*	*	1	*	*	nd	*	1	*	*	*	*	1	*	1	nd	nd
		F	A	*	0	*	nd	*	nd	*	1	*	1	1	*	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	*	*	*	*	1	1	*	*	nd	nd
			B	*	0	*	nd	*	nd	*	1	*	1	1	*	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	*	*	*	*	1	1	*	*	nd	nd
			C	*	1	*	nd	*	nd	*	1	*	1	0	*	1	*	0	1	1	1	*	*	*	*	*	*	nd	*	*	*	*	*	1	1	*	*	nd	nd
			D	*	0	*	nd	*	nd	*	1	*	1	1	*	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	0	*	*	*	1	1	*	*	nd	nd
			E	*	1	*	nd	*	nd	*	1	*	1	0	*	1	*	0	1	1	1	*	*	0	*	*	*	nd	*	0	*	*	*	1	1	*	*	nd	nd
			G	*	0	*	nd	1	nd	*	1	*	1	0	*	1	*	1	1	1	*	*	*	*	*	*	*	nd	*	1	*	*	*	*	1	1	*	nd	nd
			H	*	*	*	nd	1	nd	*	*	*	1	*	1	1	*	0	0	1	*	*	*	*	*	*	*	nd	*	1	*	*	*	*	1	1	*	nd	nd
			I	*	0	*	nd	1	nd	*	*	*	0	*	*	1	*	0	0	*	*	*	*	*	*	*	*	nd	*	1	*	*	*	*	1	1	*	nd	nd
		G	A	*	0	1	nd	*	nd	*	1	*	1	1	1	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			B	*	0	1	nd	*	nd	*	1	*	1	1	1	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			C	*	1	1	nd	*	nd	*	1	*	0	0	1	1	*	0	0	1	0	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			D	*	0	1	nd	*	nd	*	1	*	1	0	1	1	*	1	1	1	*	1	*	1	*	*	*	nd	*	1	*	*	*	*	1	*	*	nd	nd
			E	*	1	*	nd	*	nd	*	1	*	0	0	1	1	*	0	1	1	*	1	*	1	1	*	*	nd	*	1	*	*	*	*	1	*	1	nd	nd
			F	*	0	1	nd	1	nd	*	1	*	1	0	*	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	1	*	*	*	*	1	1	*	nd	nd
			G	*	*	*	nd	1	nd	*	1	0	*	1	1	1	1	1	1	1	1	*	*	1	*	*	*	nd	1	1	*	*	1	*	1	1	*	nd	nd
			H	*	0	*	nd	1	nd	*	*	1	1	*	1	1	1	1	1	*	*	1	*	*	1	*	*	nd	1	1	*	*	1	*	1	1	1	nd	nd
			I	*	0	*	nd	1	nd	*	*	1	1	*	1	1	1	1	1	*	*	1	*	*	1	*	*	nd	1	1	*	*	1	*	1	1	1	nd	nd
		H	A	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			B	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			C	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	0	1	0	0	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			D	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	*	1	*	1	*	*	*	nd	*	1	*	*	*	*	1	*	*	nd	nd
			E	*	*	*	nd	*	nd	*	*	*	1	*	1	1	*	0	1	1	*	1	*	1	1	*	*	nd	*	1	*	*	*	*	1	*	1	nd	nd
			F	*	*	*	nd	1	nd	*	*	*	1	*	1	1	*	0	0	1	1	*	*	1	*	*	*	nd	*	1	*	*	*	*	1	*	*	nd	nd
			G	*	*	*	nd	1	nd	*	*	1	0	*	1	1	1	1	1	1	1	*	*	1	*	*	*	nd	1	1	*	*	1	*	1	1	*	nd	nd
			H	*	*	*	nd	1	nd	*	*	1	1	*	1	1	0	0	0	*	*	1	*	*	0	*	*	nd	1	1	*	*	1	*	1	*	1	nd	nd
		I	A	*	0	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			B	*	0	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	1	1	*	*	1	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			C	*	1	*	nd	*	nd	*	*	*	1	*	1	1	*	0	1	*	0	*	*	*	*	*	*	nd	*	*	*	*	*	*	1	*	*	nd	nd
			D	*	0	*	nd	*	nd	*	*	*	1	*	1	1	*	1	1	*	1	*	*	1	*	*	*	nd	*	1	*	*	*	*	1	*	*	nd	nd
			E	*	1	*	nd	*	nd	*	*	*	1	*	1	1	*	0	1	*	*	1	*	1	1	*	*	nd	*	1	*	*	*	*	1	*	1	nd	nd
			F	*	0	*	nd	1	nd	*	*	*	0	*	1	1	*	0	0	1	*	1	*	*	1	*	*	nd	*	1	*	*	*	*	1	1	*	nd	nd
			G	*	0	*	nd	1	nd	*	*	1	1	*	1	1	1	1	1	*	*	1	*	*	1	*	*	nd	1	1	*	*	1	*	1	1	1	nd	nd
			H	*	*	*	nd	1	nd	*	*	1	1	*	1	1	0	0	0	*	*	1	*	*	0	*	*	nd	1	1	*	*	1	*	1	*	1	nd	nd

1 - There are significant differences

0 - There are not significant differences

nd - compound non detected in the tecnique or only in one condition

* - compound non detected in some condition

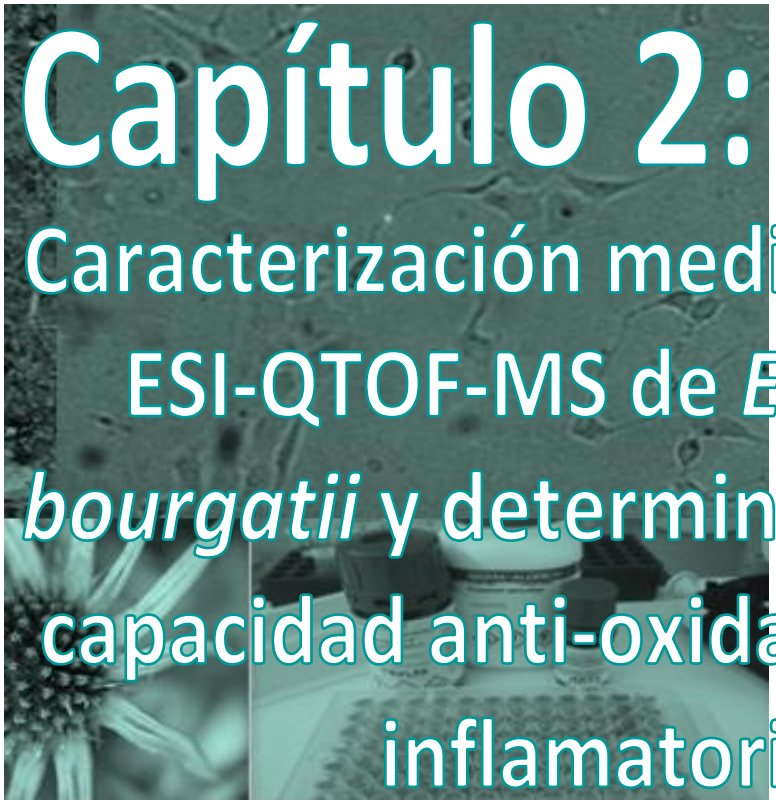
Table 7. Means of comparison of the ratio of analyte peak area to internal standard peak area for compounds extracted by SFE.

Peak	SFE1	SD	SFE2	SD	SFE3	SD
1	0,254	0,014	2,794	0,404	0,747	0,017
7	1,444	0,056	1,507	0,024	0,648	0,066
13	0,039	0,009			0,033	0,002
18			0,109	0,024	0,039	0,001
20			0,082	0,002		
23	0,018	0,002	0,381	0,055	0,107	0,004
26			0,454	0,006	0,162	0,001
27	0,100	0,007				
29	0,019	0,002	0,835	0,051	0,234	0,010
30			0,238	0,009	0,089	0,002
31			0,604	0,025	0,252	0,008
34	0,026	0,001	0,604	0,025	0,227	0,002
37			0,839	0,027	0,212	0,008
44	0,168	0,005				
45			2,102	0,114	1,123	0,008
47					0,030	0,001
48			0,411	0,003	0,050	0,001
50			0,109	0,003	0,022	0,000
51			0,158	0,004	0,043	0,001
52			0,090	0,002	0,017	0,001
53			0,034	0,003		
56			0,033	0,002		
58			0,024	0,001		
60			0,028	0,001		
63	0,026	0,004	0,073	0,005	0,039	0,001
66	0,040	0,002	0,116	0,006	0,061	0,001
67			0,348	0,085	0,045	0,004
70	0,135	0,001	0,060	0,003	0,107	0,004
71			0,028	0,002	0,026	0,001

Table 8. Means of comparison of the ratio of analyte peak area to internal standard peak area for compounds extracted by PLE.

Peak	PLE-A	SD	PLE-B	SD	PLE-C	SD	PLE-D	SD	PLE-E	SD	PLE-F	SD	PLE-G	SD	PLE-H	SD	PLE-I	SD
1	2,511	0,035	2,180	0,024	2,490	0,099	1,552	0,021	2,311	0,018	2,202	0,087	2,276	0,071	2,006	0,123	2,791	0,074
2	0,135	0,004	0,283	0,004			0,183	0,002	0,116	0,000	0,101	0,003	0,120	0,014	0,063	0,002	0,064	0,002
3	0,052	0,003	0,081	0,001	0,056	0,004	0,064	0,001	0,060	0,003	0,025	0,004	0,041	0,001	0,017	0,001		
4	0,191	0,004	0,171	0,003	0,098	0,010	0,093	0,004	0,081	0,002	0,033	0,000	0,060	0,004	0,033	0,003	0,031	0,003
5	0,035	0,000	0,028	0,001									0,032	0,004			0,035	0,001
7	0,644	0,010	0,646	0,015	0,704	0,024	0,739	0,002	0,835	0,004	1,049	0,017	1,504	0,036	0,847	0,042	1,455	0,077
8	0,102	0,008	0,055	0,005													0,043	0,003
10	0,071	0,000	0,095	0,002	0,062	0,002	0,089	0,001	0,096	0,001	0,098	0,000	0,128	0,011	0,076	0,001	0,063	0,002
11	0,219	0,001	0,229	0,003	0,133	0,010	0,142	0,001	0,116	0,003	0,095	0,004	0,078	0,007	0,031	0,000	0,029	0,001
13													0,158	0,006	0,340	0,015	0,267	0,006
14	0,088	0,002	0,108	0,005	0,066	0,003	0,084	0,005	0,048	0,001	0,041	0,002						
17	0,204	0,003	0,242	0,004	0,125	0,017	0,146	0,001	0,127	0,002	0,085	0,003	0,098	0,006	0,048	0,002	0,050	0,002
18	0,499	0,005	0,440	0,005	0,279	0,032	0,343	0,003	0,278	0,001	0,244	0,007	0,215	0,016			0,101	0,003
19									0,079	0,000	0,025	0,002	0,050	0,004				
20	0,356	0,005	0,300	0,004	0,201	0,015	0,252	0,001	0,161	0,001	0,131	0,005	0,144	0,010			0,066	0,002
21	0,086	0,001	0,086	0,002	0,087	0,002	0,082	0,001	0,047	0,001	0,047	0,002	0,037	0,005				
23	0,171	0,001	0,234	0,002	0,211	0,006	0,250	0,003	0,200	0,002	0,194	0,007	0,235	0,005	0,169	0,008	0,139	0,004
24	0,258	0,001	0,245	0,007			0,218	0,017	0,199	0,002	0,151	0,005	0,169	0,008	0,095	0,002	0,093	0,002
25	0,052	0,002	0,043	0,001			0,043	0,003	0,086	0,001	0,090	0,002	0,079	0,008	0,061	0,001	0,063	0,001
26	0,781	0,004	0,624	0,002	0,554	0,019	0,619	0,008	0,460	0,004	0,432	0,013	0,401	0,013	0,162	0,018	0,210	0,004
27													0,177	0,012	0,585	0,003	0,333	0,030
29	0,484	0,003	0,530	0,004	0,459	0,011	0,505	0,007	0,492	0,001	0,351	0,014	0,435	0,007	0,421	0,014	0,356	0,004
30	0,426	0,003	0,350	0,008	0,284	0,002	0,372	0,005	0,283	0,002	0,201	0,005	0,254	0,018			0,132	0,006
31	0,102	0,002	0,086	0,006	0,070	0,001	0,076	0,003	0,088	0,003	0,099	0,006	0,100	0,014	0,068	0,001	0,070	0,001
32	0,030	0,001	0,042	0,001	0,046	0,003	0,022	0,001	0,021	0,002	0,024	0,002	0,019	0,000				
33													0,136	0,009	0,108	0,005	0,033	0,003
34	0,301	0,001	0,266	0,002	0,276	0,024	0,309	0,004	0,178	0,002	0,170	0,004	0,187	0,004	0,128	0,007	0,113	0,005
35	0,102	0,001	0,114	0,002					0,224	0,005	0,252	0,014	0,389	0,009	0,320	0,012	0,279	0,007
36	0,096	0,000	0,069	0,000	0,059	0,014	0,062	0,002										
37	0,053	0,006	0,040	0,002	0,024	0,002	0,038	0,001	0,045	0,001	0,041	0,003	0,052	0,016			0,042	0,001
38	0,054	0,000	0,045	0,000	0,024	0,001	0,038	0,003	0,019	0,001			0,030	0,002				
40											0,034	0,006	0,148	0,008	0,087	0,001	0,067	0,004
42	0,055	0,001	0,057	0,001	0,029	0,005												
43	0,027	0,000	0,028	0,000	0,029	0,001	0,028	0,000	0,021	0,000	0,026	0,001	0,020	0,000				
44													0,049	0,005	0,017	0,000	0,027	0,003
45	1,547	0,011	1,476	0,012	1,160	0,028	1,354	0,014	1,173	0,009	0,956	0,010	1,108	0,054	1,049	0,038	0,896	0,030
46	0,077	0,001	0,060	0,004	0,041	0,002	0,034	0,018	0,031	0,003	0,026	0,002	0,036	0,001				
47	0,192	0,005	0,009	0,000	0,277	0,015	0,074	0,001	0,115	0,000			1,661	0,086	2,625	0,098	1,282	0,046
48	0,243	0,003	0,221	0,003	0,112	0,002	0,188	0,013	0,354	0,004	0,486	0,002	0,848	0,041	0,702	0,022	0,640	0,018
49													0,336	0,015	0,088	0,001	0,092	0,006
50	0,241	0,002	0,193	0,002	0,066	0,001	0,240	0,078	0,082	0,007	0,022	0,000	0,109	0,014	0,032	0,001	0,023	0,001
51	0,283	0,001	0,197	0,000	0,107	0,006	0,207	0,000	0,098	0,001	0,045	0,000	0,115	0,007	0,044	0,009	0,043	0,001
52	0,052	0,000	0,047	0,001	0,032	0,015	0,039	0,000	0,024	0,000	0,016	0,000	0,033	0,003				
53	0,031	0,001	0,036	0,002	0,017	0,002					0,019	0,001						
54							0,493	0,004	0,133	0,001			0,252	0,019	0,085	0,001	0,028	0,001
55	0,029	0,001	0,023	0,001														
56	0,038	0,009	0,044	0,001			0,032	0,002	0,028	0,001	0,021	0,001						
57									0,027	0,001			0,410	0,018	0,123	0,002	0,125	0,005
58	0,086	0,001	0,088	0,004														
59	0,022	0,000	0,026	0,001	0,020	0,003												
61													0,039	0,002	0,360	0,016	0,062	0,001
62							0,046	0,001	0,044	0,001	0,029	0,001	0,821	0,003	0,392	0,017	0,302	0,003
63	0,015	0,000	0,021	0,000					0,014	0,000								
64	0,018	0,001	0,018	0,001														
65													0,077	0,002	0,120	0,005	0,039	0,004
66	0,030	0,000	0,040	0,000	0,025	0,000	0,040	0,000	0,030	0,000	0,026	0,000						
67	0,082	0,002	0,116	0,003	0,092	0,001	0,147	0,000	0,143	0,002	0,112	0,005	0,170	0,000	0,125	0,003	0,062	0,001
68											0,017	0,000	0,100	0,012			0,030	0,001
69									0,027	0,001			0,888	0,042	0,383	0,015	0,289	0,001


micrOTOF-Q II



Capítulo 2:

Caracterización mediante HPLC-ESI-QTOF-MS de *Eryngium bourgatii* y determinación de su capacidad anti-oxidante y anti-inflamatoria

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Comprehensive characterization by HPLC-ESI-Q-TOF-MS from an *Eryngium bourgatii* extract and their antioxidant and anti-inflammatory activities

ABSTRACT

The genus Eryngium (family Apiaceae) consists of about 300 species. This genus is known to contain polyacetylenes, flavonoids and saponins, coumarins, and monoterpene glycosides. In folk medicine, various species are used for a wide range of foods; particularly roots are used against various inflammatory disorders, oedema, sinusitis, urinary infections, etc. The aim of the present study was to characterize phenolic compounds in the leaves and flowers of Eryngium bourgatii using HPLC-ESI-Q-TOF-MS. A total of 44 compounds were identified belonging to various structural classes such as flavonols (quercetin, kaempferol, isorhamnetin and derivatives), flavanones (naringenin rhamnoglucoside), cinnamic acids (chlorogenic, rosmarinic, ferulic and caffeic acids and derivatives), benzoic acids (p-hydroxybenzoic acid glucoside, arbutin, syringic acid, glucogallin, and gentisic acid and derivatives) and other organic acids (gluconic, citric, and quinic acids and derivatives). The method described simultaneously a wide range of phenolic compounds and a tentative characterization was made of the major compounds of this extract. In addition, E. bourgatii extract shows antioxidant activity (by TEAC, FRAP and ORAC methods) and anti-inflammatory properties, decreasing the generation of reactive oxygen species and inhibiting the production of MCP-1 and their transcripts, respectively, in TNF-α-induced HUVEC.

1. INTRODUCTION

The genus *Eryngium* comprises about 300 species distributed all around the world, mainly in Eurasia, North of Africa and South of America. It is the largest genus in the family *Apiaceae* and accounts for approximately three-quarters of the species diversity within *Saniculoideae*, the subfamily to which it belongs. *Eryngium* is extremely variable morphologically but it is easily distinguished from other members of *Apiaceae* by its capitate inflorescences and single bract per flower (Calvino, Martinez, & Downie, 2008).

Eryngium bourgatii is an herbaceous perennial to 30–50 cmtall, also known as Mediterranean sea-holly, with deeply lobed and silver-veined leaves, and vivid blue, branched stems bearing cone-like flower-heads. The traditional uses recorded for this genus are numerous. In Tropical America and the West Indies, where the plant is indigenous, the prevailing use of the plant is to treat fevers, colds and the flu. As a food, the leaves of *Eryngium foetidum* are added to curries, chutneys, stews and soups as a flavouring agent. It is cultivated in the urban gardens of Belém, Brazil as feeding (Paul, Seaforth, & Tikasingh, 2011). This specie is employed as a substitute or alternative for coriander (*Coriandrum sativum*) because of the similar aroma (Flamini, Tebano, & Cioni, 2008). Eryngium species but generally *Eryngium campestre* and *Eryngium maritimum* are known in Turkish folk-medicine as “Bogadikeni” and widely distributed in all parts of Turkey. Infusion of aerial and root parts of this species are used in folk remedies as antitussive, diuretic, appetizer, stimulant and aphrodisiac (Kupeli, Kartal, Aslan, & Yesilada, 2006).

Previous phytochemical investigations on the Eryngium genus had shown the presence of essential oils, acetylenes, coumarins, saponins, flavonoids, and rosmarinic acid derivatives (Lam, Christensen, & Thomasen, 1992; Zhang et al., 2008).

This paper presents the analysis of leaves and inflorescences of *E. bourgatii* using HPLC-ESI-Q-TOF-MS to characterize the polyphenolic fraction and other related compounds, to measure the antioxidant activity by several in vitro methods based on electron transfer mechanism (TEAC and FRAP) and on

hydrogen atom mechanism (ORAC) and also to measure the intracellular generation of reactive oxygen species (ROS) and expression of MCP-1 in human umbilical vein endothelial cells (HUVECs).

2. MATERIAL AND METHODS

2.1. Chemicals

All chemicals were of HPLC-MS and used as received. Acetic acid and acetonitrile for HPLC were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Sample preparation

Plants were harvested by the researchers in July–August 2011 in the Pyrenees Mountains, cleaned and prepared for extraction with hot water at 40 °C 1 h. The soluble part was loaded onto a 1.5×25 cm chromatography column containing Amberlite™ FPX66. The retained phenolic fraction was eluted with methanol, and subsequently lyophilized. For subsequent analyses, extracts were dissolved in distilled water to a concentration of 25 g/L and filtered through a 0.45 µm PVD filter.

2.3. Instrumentation

Analyses were made using an UHPLC of Waters ACQUITY. The UHPLC column used was a Zorbax Eclipse Plus C18 (1.8 µm, 150×4.6 mm). The UHPLC system was coupled to a Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI interface.

2.4. Chromatographic procedure

The compounds from leaves and flowers of *E. bourgatii* were separated at room temperature. The mobile phases consisted of acetic acid 0.5% (A) and acetonitrile (B). The following multi-step linear gradient was applied: 0 min, 0% B; 20 min, 30% B; 30 min, 60% B; 40 min, 80% B; 50 min, 100% B; 54

min, 100% B; 55 min, 0% B; 60 min, 40% B. The initial conditions were held for 10 min. The injection volume in the HPLC system was 15 µL.

2.5. ESI-Q-TOF-MS detection

The HPLC system was coupled to a Q-TOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +4.0 kV. The other optimum values of the source parameters were: drying gas temperature, 210 °C; drying gas flow, 8 L/min; and nebulizing gas pressure, 2 bar. The detection was performed considering a mass range of 50–1100 *m/z*.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonics), which provided a list of possible elemental formulas using Generate Molecular Formula Editor. This uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (σ value) for increased confidence in the suggested molecular formula (Bruker Daltonics Technical Note 008, 2004). The widely accepted accuracy threshold for confirmation of elemental compositions was established at 5 ppm (Bringmann et al., 2005). Even with very high mass accuracy (<3 ppm in most of the cases), many chemically possible formulae were determined depending on the mass regions considered. Therefore, high mass accuracy alone is not sufficient to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns as a single further constraint removes >95% of the false candidates. This orthogonal filter can reduce several thousand candidates to only a small number of molecular formulas.

During the development of the HPLC method, external instrument calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, with a sodium formate cluster solution passing through containing 5 mM sodium hydroxide and 0.2% acetic acid in water:isopropanol 1:1 v/v. The calibration solution was injected at the beginning of each run and all the spectrawere calibrated prior to the compound identification.

2.6. Measurement of the Trolox equivalent antioxidant capacity (TEAC)

The Trolox equivalent antioxidant capacity (TEAC) assay, which measures the reduction of the radical cation of 2,2'-azinobis-(3- ethylbenzothiazoline-6-sulphonate) (ABTS) by antioxidants, was performed by using a previously described method (Laporta, Perez-Fons, Mallavia, Caturla, & Micol, 2007). Briefly, ABTS radical cation (ABTS+·) was produced by reacting ABTS (Sigma-Aldrich, Europe) stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stay in the dark at room temperature for 12–24 h before use. To perform the antioxidant assay with *E. bourgatii* extracts, the ABTS+· solution was diluted with water up to reaching an absorbance value of 0.70 (±0.02) at 734 nm. For the photometric assay, 1 mL of the ABTS+· solution and 100 µL of the extract were mixed for 45 s and measured immediately after 5 min at 734 nm (absorbance did not change significantly up to 10 min).

2.7. Ferric-reducing ability power (FRAP)

The FRAP assay was carried out following the method described by Benzie and Strain (Benzie & Strain, 1996). Briefly, 40 µL of the extracts were mixed on a 96-well plate with 250 µL of freshly prepared FRAP reagent. Samples were incubated for 10 min at 37 °C, and then, absorbance at 593 nm was recorded during 4 min on a microplate reader (SPECTROstarOmega, BMG LabTech GmbH, Offenburg, Germany). FRAP values were calculated using $FeSO_4 \cdot 7H_2O$ as standard.

2.8. Assay of the oxygen radical absorbance capacity (ORAC)

To assay the capacity of the extracts to scavenge peroxyl radicals, a validated ORAC method was used (Ou, Hampsch-Woodill, & Prior, 2001) with the modifications developed by Laporta et al. (Laporta et al., 2007). Briefly, the automated ORAC assay was carried out on a Fluostar Galaxy spectrofluorometric analyzer (BMG Labtechnologies GmbH; Offenburg, Germany). In the final assay mixture (200 µL total volume), fluorescein (FL) and 2,2'-azobis-(2-methylpropionamine)- dihydrochloride (AAPH) were used at 90 nM and 12.8 mM, respectively. Several dilutions of Trolox (1–40 µM) were used to construct the

calibration curve. A freshly prepared AAPH solution was used for each experiment. The temperature of the incubator was set at 37 °C and the FL fluorescence was recorded every minute after the addition of AAPH. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area of the FL decay curve (area under curve, AUC).

2.9. Cell culture and methods for related measurement outcomes

Experiments with human cells followed procedures approved by the ethics committee of Hospital Universitari de Sant Joan (Reus, Spain). Viability of the cells was assessed using the CellTiter-Fluor™ Cell Viability Assay (Promega, Madrid, Spain) according to the manufacturers' instructions. Cell cultures with viability of more than 97% were used for the experiments. HUVECs, obtained from the American Type culture collection (Manassas, VA), were propagated using endothelial cell-growth medium containing recombinant human growth factors, fetal bovine serum (FBS, Gibco, Paisley, UK) to a final concentration of 10%, heparin and hydrocortisone. Cells were cultured in 6-well plates in a humidified chamber containing 5% CO_2 at 37 °C until confluent and used for experiments at passages 2–6.

After preparatory experiments, we used preconditioning medium (containing TNF-α [R&D Systems; Minneapolis, MN, USA], 5 ng/mL) for 6 h. This was discarded, cells washed twice and incubated for 48 h with fresh medium containing TNF-α 0.2 ng/mL and different concentrations (10–100 μg/mL) of the E. bourgatii extract. After incu- bation, the supernatants were collected for ELISA analysis. Total RNA was extracted from cells using the RNeasy Mini Kit using the protocol provided by the manufacturers (Qiagen, Chatsworth, CA, USA). High- capacity cDNA reverse transcription kit (Applied Biosystems) was used to obtain cDNA. Primers (for MCP-1 5′-ATGAAAGTCTCTGCCG CC-3′ and 5′-TTGCTTGTCCAGGTGGTC-3′ and 5′-TCATTGACCTCAACT ACATG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′ for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)) as well as other reagents were obtained from Applied Biosystems to be used on the 7900HT Real Time PCR system analysed with RQ documents and the RQ Manager Software for automated data analysis. The GAPDH gene is considered

constitutive in these cells. We have found under these conditions that both GAPDH mRNA and 18S ribosomal mRNA provide good loading controls. Of note, beta-actin mRNA was considered to be a less accurate internal standard. RNAGene-expression values were calculated as a comparison of measured threshold cycle numbers (Ct) for each reaction to the designated endogenous control via the $2^{-\Delta\Delta Ct}$ method.

To measure the intracellular generation of reactive oxygen species (ROS), the 2',7'-dichlorofluorescein diacetate (H2DCF-DA) that becomes fluorescent on oxidation to DCF was used essentially as described (Koziol, Zagulski, Bilinski, & Bartosz, 2005). Cells under established conditions were washed with PBS and then further incubated with 10 µmol/L of H2DCF-DA, for 60 min at 37 °C. After subsequent wash and gentle detachment of adherent cells, fluorescence was measured by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA) at the excitation 488 nm and wave emission 525 nm. Oxidation of H_2DCF-DA is currently used for assaying peroxides and/or peroxynitrites but requires catalysts (peroxidase or esterase) and this cannot be dismissed as a limitation in this particular model.

3. RESULTS AND DISCUSSION

3.1. Characterization of leaves and flowers extracts from *E. bourgatii* by HPLC-ESI-Q-TOF-MS

Fig. 1 shows the base peak chromatogram from E. bourgatii by HPLC-ESI-Q-TOF-MS. The compounds characterized are presented in Table 1, identified considering the elution order. All the compounds were characterized by the interpretation of their mass spectra obtained by the Q-TOF-MS and also taking into account the data provided by the literature.

In the present study, the compounds are classified into four groups: cinnamic acids and derivatives, hydroxybenzoic acids and derivatives, flavonoids and other compounds.

3.1.1. Cinnamic acids and derivatives

Among the cinnamic acids, caffeoylquinic acid and its derivatives merit special attention. Other members of the family, such as coumaric and ferulic acid are also important. Caffeic acid was mostly

esterified with quinic acid, the most widespread product being chlorogenic acid isomers. The quinic acid derivatives were identified based on the hierarchical key for identification by LC/MSn of quinic acid derivatives proposed elsewhere (Clifford, Johnston, Knight, & Kuhnert, 2003; Clifford, Knight, & Kuhnert, 2005).

Peaks 15, 17, 18, 23, and 26 displayed a $[M–H]^-$ at *m/z* 353 and were characterized as chlorogenic acid isomers. These peaks presented the same fragmentation pattern at *m/z* 191, corresponding to deprotonated quinic acid. The $[caffeic\ acid\text{-}H]^-$ ion at *m/z* 179 (more pronounced for 3-OH compounds) (Clifford et al., 2003; Clifford et al., 2005) was not observed, indicating that a quinic acid was replaced at the 1- or 5-position with the hydroxyl group.

Peaks 10 and 33 were identified as trihydroxycinnamoylquinic acid isomers (*m/z* 371) with the same MS/MS pattern as chlorogenic acid. Peak 30 showed an [M–H]− at *m/z* 337. Its MS2 spectra gave *m/z* at 191. It has been previously reported (Clifford et al., 2003; Clifford et al., 2005) that 5-O-p-coumaroylquinic acid gave the ion at *m/z* 191 in its MS^2 spectrum, while 3-O-p- and 4-O-p-coumaroylquinic acids gave the ions separately at *m/z* 163 and 173, respectively. Therefore, peak 30 was tentatively identified as 5-O-p-coumaroylquinic acid.

Peaks 32 and 37 with [M–H]− at *m/z* 367 corresponded to feruloylquinic acid isomers. Peak 32 showed fragment ions at *m/z* 191 [quinic acid-H]−, *m/z* 173 [quinic acid-H2O–H]−, and *m/z* 93 (unassigned). Peak 37 showed a main fragment ion at *m/z* 191. According to described previously, the first compound (32) was identified as 4-O-feruloylquinic acid and compound 37 was identified as 5-O-feruloylquinic acid (Clifford et al., 2003; Clifford et al., 2005).

Both peaks (45 and 47) gave the [M–H]− ions at *m/z* 515 and were characterized as dicaffeoylquinic acid isomers. The fragmentation patterns of these peaks significantly differed. Peak 45 showed an ion at *m/z* 335, and hence the compound was identified as 3,4-Odicaffeoylquinic acid (Clifford et al., 2003; Clifford et al., 2005). This ion was not detected in the MS2 spectrum of peak 47, but the ion at *m/z*

191 was observed. Based on the above-mentioned rules in the literature, compound 47 was identified as 3,5-O-dicaffeoylquinic acid.

Peak 53 with [M–H]− at *m/z* 529 was characterized as caffeoylferuloylquinic acid. It displayed fragments at *m/z* 353 [caffeoylquinic acid-H]−, 367 [feruloylquinic acid-H]−, 191 [quinic acid-H]−, and 179 [caffeoyl-H]−, indicating that it was 3-O-caffeoyl,5-O-feruloylquinic acid in accordance with previous reports (Clifford et al., 2003; Clifford et al., 2005).

Peak 54 was identified as dimethoxycaffeic acid with [M–H]− at *m/z* 207. Its fragmentation pattern produced an ion at *m/z* 147 corresponding to [M–H–H2O–CO–CH3]− (Pellati, Orlandini, Pinetti, & Benvenuti, 2011).

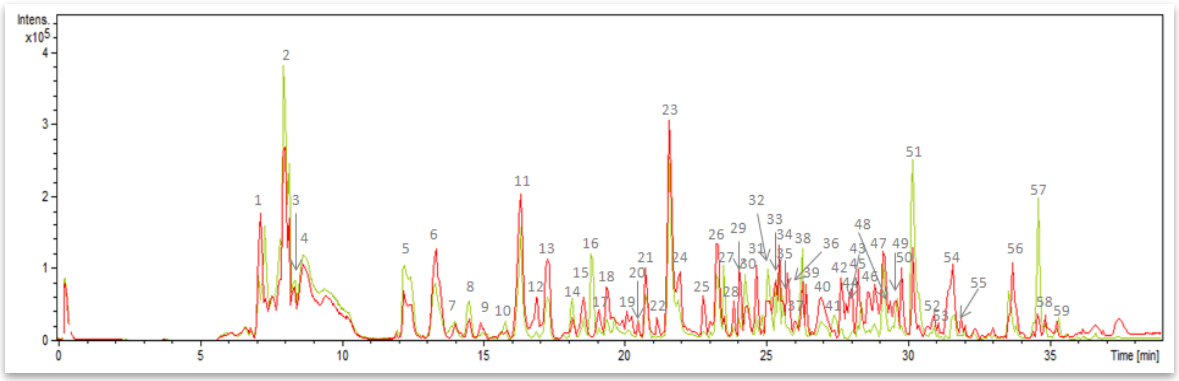

Figure 1. Base peak chromatogram of leaves (red) and flowers (green) of *Eryngium bourgatii* extracts.

Peaks 38, 42 and 51 were identified as rosmarinic acid derivatives (Le Claire, Schwaiger, Banaigs, Stuppner, & Gafner, 2005). Peak 38 was identified as glucopyranosyl rosmarinic acid with *m/z* 521. Peak 51 was characterized as rosmarinic acid with *m/z* 359. Their fragmentation pattern is the same *m/z* 197 [(M–H)–(C9H6O3)]− (−caffeoyl group) and *m/z* 161 ([M–H–2H2O]−) by losing two molecules of water. Also glucopyranosyl rosmarinic acid isomer showed an ion at 359 ([M–H–glucose]−) (fragment assignable to a rosmarinic acid moiety). Peak 42 was tentatively characterized as rosmarinic acid derivative (*m/z* 535). As shown in Fig. 2, its assignment was consistent with the presence of

fragments found at *m/z* 337 ([M–H–glucose–2H2O]−), 197 and 161 (the last two are the same ions as rosmarinic acid).

Table 1. Relevant analytical data for compounds isolated in leaves and flowers of *E. bourgatii* extracts.

Peak	Proposed compound	RT (min)	$[M-H]^-$	E (ppm)	mσ	MS/MS	Mol. formula
1	Gluconic acid	7.3	195.0503	3.9	2	177.0382; 159.0314; 129.0187	$C_6H_{12}O_7$
2	Quinic acid	7.97	191.0567	2.9	1.2	127.0393	$C_7H_{12}O_6$
3	Sucrose	8.36	341.1090	0.2	1.5	Non fragmented	$C_{12}H_{22}O_{11}$
4	Malic acid	8.63	133.0141	0.8	0.6	115.0038	$C_4H_6O_5$
5	Citric acid	12.2	191.0205	3.9	0.9	111.0081	$C_6H_8O_7$
6	Succinic acid	13.3	117.0191	1.6	3.6	Non fragmented	$C_4H_6O_4$
7	Arbutin	13.84	271.0825	0.7	5.3	108.0209	$C_{12}H_{16}O_7$
8	Hydroxymethylglutaric acid	14.49	161.045	3.2	8.1	117.0528	$C_6H_{10}O_5$
10	Trihydroxycinnamoylquinic acid (isomer)	15.69	371.0972	3.2	3.6	191.0558	$C_{16}H_{20}O_{10}$
11	Glycosyringic acid	16.3	359.0986	0.6	4.1	197.0464; 179.0351	$C_{15}H_{20}O_{10}$
12	Glycogallin	16.9	331.0673	0.8	10.9	168.0053	$C_{13}H_{16}O_{10}$
13	Protocatechuic acid hexoside (isomer)	17.24	315.0727	1.7	8.2	152.0115	$C_{13}H_{16}O_9$
14	Syringic acid	18.11	197.0456	0.2	5.8	179.0350; 135.0446	$C_9H_{10}O_5$
15	Chlorogenic acid (isomer)	18.51	353.0877	0.4	45.2	191.0561	$C_{16}H_{18}O_9$
17	Chlorogenic acid (isomer)	19.05	353.0847	8.8	62.2	191.0556	$C_{16}H_{18}O_9$
18	Chlorogenic acid (isomer)	19.31	353.0878	0.1	10.1	191.0558	$C_{16}H_{18}O_9$
19	Protocatechuic acid hexoside (isomer)	20.12	315.0722	0.2	4.4	153.0152; 109.0291	$C_{13}H_{16}O_9$
21	Hydroxybenzoic acid hexoside	20.72	299.0772	0	4	137.0236; 93.0337	$C_{13}H_{16}O_8$
23	Chlorogenic acid (isomer)	21.53	353.088	0.6	5.9	191.0561	$C_{16}H_{18}O_9$
25	Roseoside	22.74	385.1857	2.9	7.1	223.1344; 153.0919	$C_{19}H_{30}O_8$
26	Chlorogenic acid (isomer)	23.21	353.088	0.6	8.2	191.0565	$C_{16}H_{18}O_9$
29	Quercetin dihexoside	24.02	625.1411	0.1	6.7	301.0347	$C_{27}H_{30}O_{17}$
30	5-O-p-coumaroylquinic acid	24.29	337.0926	0.9	5.4	191.0564; 135.0441	$C_{16}H_{18}O_8$
32	4-O-feruloylquinic acid	25.02	367.1036	0.5	13.8	191.0562; 173.3469; 93.0335	$C_{17}H_{20}O_9$
33	Trihydroxycinnamoylquinic acid (isomer)	25.29	371.0983	0.2	10.5	191.056	$C_{16}H_{20}O_{10}$
34	Kaempferol dihexoside	25.43	609.146	0.2	11.9	285.0401	$C_{27}H_{30}O_{16}$
35	Isorhamnetin dihexoside	25.56	639.158	0.2	18.6	315.0509	$C_{28}H_{32}O_{17}$
37	5-O-feruloylquinic acid	26.03	367.1033	0.5	4.1	191.056	$C_{17}H_{20}O_9$
38	Glucopyranosyl rosmarinic acid	26.23	521.1295	1.2	7.7	359.0947; 161.239; 197.0415	$C_{24}H_{26}O_{13}$
40	Isoquercitrin	26.77	463.0872	2.2	6.1	300.0277	$C_{21}H_{20}O_{12}$
42	Rosmarinic acid derivative	27.57	535.1447	2	11.9	337.0927; 197.046; 161.0232	$C_{25}H_{28}O_{13}$
44	Isorhamnetin-(6-deoxyhexopyranosyl)-hexoside	27.98	623.1614	0.5	20.6	300.0609	$C_{28}H_{32}O_{16}$
45	3.4-O-dicaffeoylquinic acid (isomer)	28.18	515.1187	1.5	4.1	353.0873; 191.0529; 179; 173	$C_{25}H_{24}O_{12}$
46	Astragalin	28.32	447.0925	1.7	5.8	285.0443	$C_{21}H_{20}O_{11}$
47	3.5-O-dicaffeoylquinic acid (isomer)	29.06	515.1185	1.9	8.7	353.0876; 191.0559	$C_{25}H_{24}O_{12}$
49	Kaempferol malonylglycoside	29.59	533.0923	2.6	26.1	489.1014; 285.0392	$C_{24}H_{22}O_{14}$
51	Rosmarinic acid	30.13	359.0774	0.4	9.3	197.0456; 161.0241	$C_{18}H_{16}O_8$
52	Naringenin-7-O-rhamnoside-O-β-glucoside	30.87	579.1694	4.4	7.5	285.0769	$C_{27}H_{32}O_{14}$
53	3-O-caffeoyl-5-O-feruloylquinic acid	31.00	529.1339	2.3	10.2	367.1021; 353.0865; 191; 179	$C_{26}H_{26}O_{12}$
54	Dimethoxycaffeic acid	31.54	207.0671	3.9	2.2	165.0542;147.0444	$C_{11}H_{12}O_4$
55	Diosmetin acetylglycoside	31.81	503.1182	2.5	17.1	299.0563	$C_{24}H_{24}O_{12}$
56	Genistein acetyldiglycoside	33.62	575.1406	0	5.9	473.1083; 431.0965	$C_{27}H_{28}O_{14}$
58	Kaempferol-O-(2.6-di-O-p-coumaroyl)-hexoside (isomer)	34.76	739.1669	0	8.9	593.1257; 453.1180; 285; 163	$C_{39}H_{32}O_{15}$
59	Kaempferol-O-(2.6-di-O-p-coumaroyl)-hexoside (isomer)	35.17	739.1659	1.2	15.2	593.1244; 453.1176; 285; 163	$C_{39}H_{32}O_{15}$

3.1.2. Hydroxybenzoic acids and derivatives

Peaks 11 and 14 were identified as glycosyringic and syringic acids with [M–H]− at *m/z* 359 and *m/z* 197, respectively. The first gave MS2 ions at*m/z* 197, corresponding to loss of hexose, and*m/z* 179 by losing a H2O. Peak 14 produced anion radicals with *m/z* 179 ([M–H–18]−) and *m/z* 135 peak ([M–H–CO2]−).

Glycogallin (peak 12) with a [M–H]− at *m/z* 331 and fragmentation pattern at *m/z* 168 (corresponding to the deprotonated aglycon) due to the loss of the hexose moiety.

Peaks 13 and 19 presented an [M–H]− at *m/z* 315, corresponding to protocatechuic acid linked to a hexose, fragment ions [M–H]− at *m/z* 153 from protocatechuic acid and *m/z* 109 corresponding to the loss of CO2. The mass spectra of these peakswere consistentwith it being either protocatechuic acid hexoside or gentisic acid hexoside, but they were tentatively identified as protocatechuic acid hexoside because only this acid has been described in the genus Eryngium (Koleckar et al., 2008).

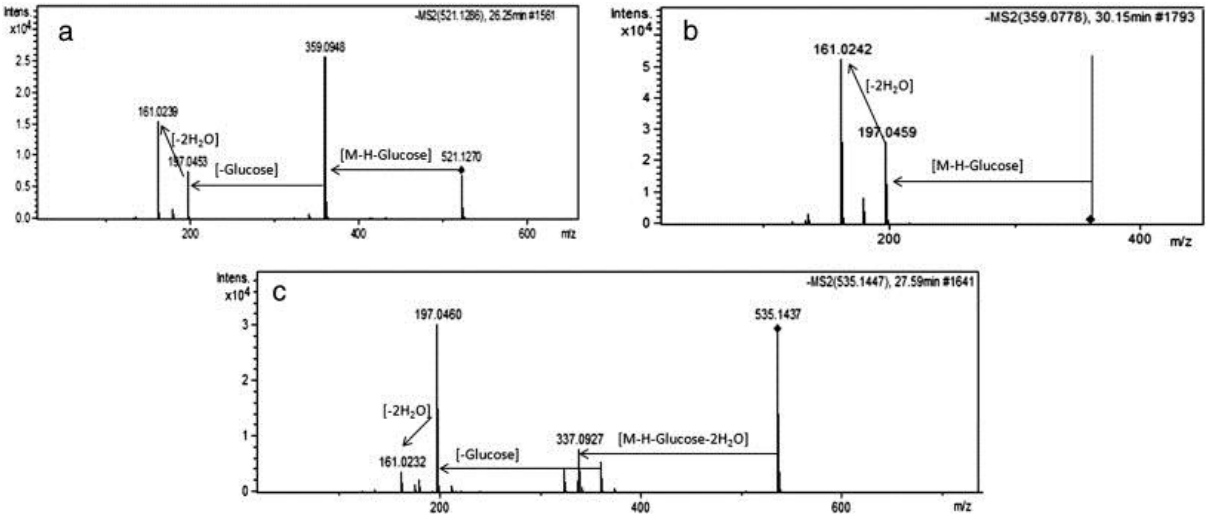

Figure 2. MS/MS spectra of rosmarinic acid derivatives. The spectra correspond to: (a) glucopyranosyl rosmarinic acid, (b) rosmarinic acid and (c) rosmarinic acid derivative.

A sugar conjugate of hydroxybenzoic acid showed [M–H]− ions at *m/z* 299 corresponding to hydroxybenzoic acid hexoside (Peak 21). Its MS/MS spectra revealed the loss of hexose moiety producing deprotonated hydroxybenzoic acid at *m/z* 137.

3.1.3. Flavonoids

Ten peaks were identified as flavonoids. These existed in the glycosylated form, the most abundant being kaempferol derivatives (Peaks 34, 46, 49, 58, and 59).

Peak 34 and 46 were characterized to kaempferol dihexoside (*m/z* 609) and astragalin (Wang, Su, Yuan, Deng, & Li, 2012) (kaempferol 3-O-β-D-glucopyranoside) (*m/z* 447), respectively. Both gave a main fragment at *m/z* 285, corresponding to kaempferol aglycone.

Peak 49 had [M–H]− at *m/z* 533, with fragments at *m/z* 489 (loss of CO2) and *m/z* 285 (loss of acetylhexose). This compound could thus be kaempferol malonylglycoside.

Peak 58 and 59 were identified as kaempferol-O-(2,6-di-O-pcoumaroyl)- β-hexoside isomers with *m/z* 739, according to the proposal by Zhang et al. (2008). The difference between both isomers consists of the position in which 2,6-di-O-p-coumaroyl-β-hexoside moiety are linked (3 or 3' position of kaempferol). Its fragmentation pattern is shown in Fig. 3. This compound gave fragment ions with *m/z* 593 corresponding to the loss of a coumaroyl unit, *m/z* 453 corresponding to the loss of kaempferol aglycone, *m/z* 285 (kaempferol aglycone moiety), and *m/z* 163 (coumaroyl moiety).

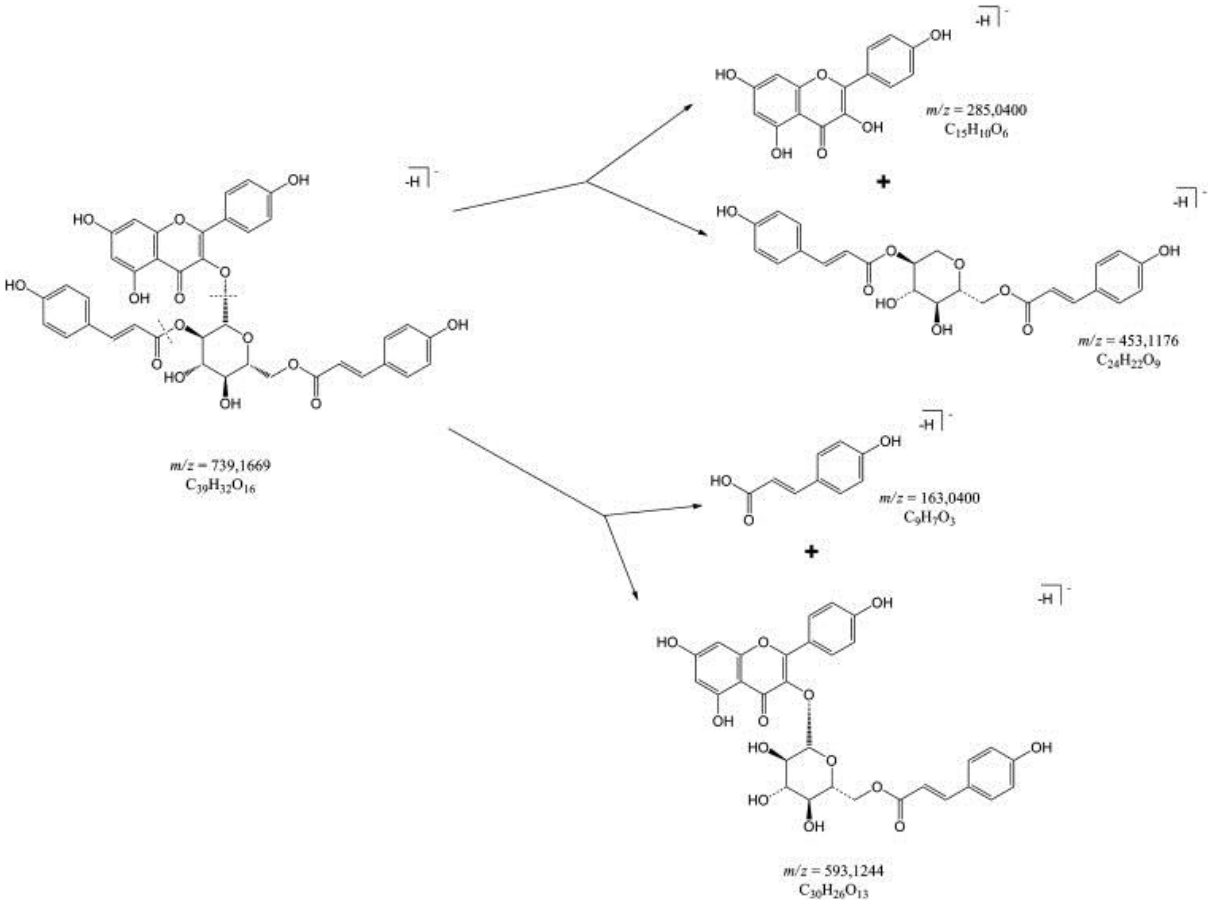

Figure 3. Fragmentation pattern of kaempferol-O-(2,6-di-O-p-coumaroyl)-hexoside.

Peak 29 was characterized as quercetin dihexoside. Its MS/MS spectrum showed a fragment at *m/z* 301 corresponding to the loss of two hexose moiety. Peak 40 was tentatively identified as isoquercitrin (Wang et al., 2012) with *m/z* 463. Product ion spectra of this peak showed a major fragment ion at

m/z 300. This could be due to the formation of the quinone anion (radical anion), obtained after hemolytic cleavage of the 0-glycosidic bond, and has been proposed as diagnostic for quercetin glycosides (Constant et al., 1997).

Peak 35 showed a [M–H]− at *m/z* 639 and was identified as a dihexoside of isorhamnetin according to MS/MS analysis of the [M–H]− ion, which yielded a fragment at *m/z* 315, corresponding to the loss of dihexoside moiety.

Peak 44 corresponded to isorhamnetin-(6-deoxyhexopyranosyl)-hexoside with *m/z* 623. Its MS2 ion at *m/z* 300 corresponded to [(M–H)–hexose–deoxyhexopyranose–CH3]−. This ionis themost prominent fragment in isorhamnetin glycosides corresponding to aglycone and loss of the methyl group, while rhamnetin shows an intense fragment at *m/z* 165 (Schieber, Keller, Streker, Klaiber, & Carle, 2002).

Peak 52 was identified as naringenin 7-O-rhamnoside-O-β-glucoside according to a previous study (Nacef, Ben Jannet, Hamza, & Mighri, 2008). This compound showed a [M–H]− at *m/z* 579 with a fragmentation pattern at*m/z* 285 corresponding to methylnaringenin (Ma, Vedernikova, Van den Heuvel, & Claeys, 2000).

Peak 55 (*m/z* 503) showed a fragment ion at *m/z* 299 (M–H–acetylglycoside)− corresponding to diosmetin aglycone, suggesting that this compound was diosmetin acetylglycoside.

Peak 56 showed a negative molecular ion [M–H]− at *m/z* 575, which corresponded to the genistein linked to acetyl and pentose and hexose. This compound produced two main fragment ions [M–H]−

at *m/z* 473, which corresponded to the genistein acetylglycoside and *m/z* 431 ([M–42]−, loss of acetyl residue). This was identified as genistein acetyldiglycoside.

3.1.4. Other compounds

Peaks 1, 2, 4, 5, 6 and 8 were identified as organic acids. Peak 1 (at *m/z* 195) corresponded to gluconic acid, with a fragmentation pattern at *m/z* 177 ([M–H–H2O]−), *m/z* 159 ([M–H–2H2O]−), and *m/z*

129 ([M–H–2H2O–CH2O]−). Peak 2 had a [M–H]− at *m/z* 191 and was identified as quinic acid, for which the most important fragment of quinic acid appeared at *m/z* 127 ([M–H–CO–H2O]−). Peak 4, characterized as malic acid, showed a [M–H]− at *m/z* 133, with a MS/MS pattern at *m/z* 115 corresponding to the loss of water. Citric acid (Peak 5) at *m/z* 191, showed an important fragment at *m/z* 111 ([M–H–CO2–2H2O]−). Peak 6 was tentatively identified as succinic acid, with a [M–H]− at *m/z* 117, but it was not fragmented. Peak 8, with a ([M–H]− at *m/z* 161, was identified as hydroxy-methylglutaric acid, and its MS2 spectrum showed one fragment at *m/z* 117 ([M–H–COO]−).

Peak 7 corresponded to arbutin ([M–H]− at *m/z* 271), which is a hydroquinone glycoside with a fragmentation pattern at *m/z* 108 (loss of glucose and H).

Peak 25, with [M–H]− at *m/z* 385, was characterized as roseoside, which is a sesquiterpene with a main MS/MS pattern at *m/z* 223, corresponding to the loss of glucose.

Finally, peak 3 are tentatively characterized as sucrose, with [M–H]− at *m/z* 341. However, its fragmentation pattern has not been obtained.

Table 2. Relevant analytical data for unknown compounds in leaves and flowers of *E. bourgatii* extracts.

Peak	RT (min)	$[M-H]^-$	Error (ppm)	mSigma	Fragmentation pattern	Mol. formula
9	15.02	295.1033	0.4	8.1	113.0251	$C_{11}H_{20}O_9$
16[a]	18.78	715.1716	1.5	11.9	313.0934; 203.0556; 109.0288	$C_{30}H_{36}O_{20}$
20	20.46	323.154	1.9	1.3	113.0229	$C_{13}H_{24}O_9$
22[b]	21.13	493.266	1.2	23.4	433.2443; 271.1915; 161.0453; 113.0233	$C_{23}H_{42}O_{11}$
24[b]	21.94	493.2661	1.3	15.8	433.2441; 161.0447	$C_{23}H_{42}O_{11}$
27	23.48	333.1191	0.5	2.2	247.1181; 161.0443; 113.0242	$C_{14}H_{22}O_9$
28[b]	23.82	475.2535	2.8	4.6	433.2440; 271.1935; 161.0442; 113.0244	$C_{23}H_{40}O_{10}$
31	24.62	443.119	1	5	267.0736; 193.0510; 113.0245	$C_{19}H_{24}O_{12}$
36[b]	25.7	475.2536	2.6	7.9	415.2332; 205.1230; 161.0447	$C_{23}H_{40}O_{10}$
39[b]	26.37	475.2536	2.6	7.9	415.2320; 161.0441	$C_{23}H_{40}O_{10}$
41	27.37	449.1649	3.4	6.7	387.1626; 249.0617; 199.0972	$C_{19}H_{30}O_{12}$
43[b]	27.78	501.233	2.3	9	457.2419; 421.2336; 161.0439; 113.0227	$C_{24}H_{38}O_{11}$
48[b]	29.32	501.2327	2.8	44.7	415.2324; 161.0437; 113.0228	$C_{24}H_{38}O_{11}$
50[b]	29.73	459.2588	2.6	4.5	417.2458; 399.2382; 161.0423; 113.0235	$C_{23}H_{40}O_9$
57	34.49	329.2335	0.4	6.3	211.1338	$C_{18}H_{34}O_5$

a Present only in flowers extract.

b Present only in leaves extract.

3.1.5. Unknown compounds

There were 15 compounds (Table 2), for which the structure could not be identified even when the MS/MS experiment was performed. There was not enough evidence to propose a structure. Table 2 includes the retention time, experimental *m/z*, MS/MS fragments, molecular formulas, errors, and σ values.

3.2. Antioxidant and anti-inflammatory activities

3.2.1. In vitro antioxidant capacity: TEAC, FRAP and ORAC assays

Three different in vitro methods were used in order to evaluate the antioxidant capacity of the E. bourgatii aqueous extract. TEAC and FRAP methods are based on electron transfer mechanisms (ET). Both have been used in a large variety of food (TEAC) and biological other hand, the ORAC method is based on hydrogen atom transfer (HAT) reactions, which scavenge the generation of peroxyl radicals through decomposition of azo compounds (Huang et al., 2005; Prior & Cao, 1999).

Table 3 shows the antioxidant values obtained for *E. bourgatii* extract. This extract was compared to another one belonging to the same family with proven antioxidant properties, i.e. coriander (*C. sativum*, Family Umbelliferae) (data not shown).

E. bourgatii showed high TEAC, FRAP and ORAC values. The TEAC value indicates a higher total reducing capacity of the extract. The FRAP value could be related to the presence of organic acids, specially, chlorogenic acids, which were identified in the extract. The higher reducing capacity of these compounds has been previously reported in the literature (De Leonardis, Pizzella, & Macciola, 2008; Wu, 2007). *E. bourgatii* also exhibited a significant ORAC value, which could be justified with the presence of hydrophilic compounds capable to inhibit the peroxyl radicals formation generated in this assay.

The results obtained in E. bourgatii extract were compared to those previously reported to Coriander extract (Surveswaran, Cai, Corke, & Sun, 2007). The TEAC and ORAC assays showed the highest values in *E. bourgatii* extract. Regarding the chemical composition, Coriander is rich in carotenoids, mainly in β-carotene and xanthophylls (Isabelle et al., 2010), that are highly lipophilic compounds and their antioxidant power in a water environment is poor, which could explain these results. However, FRAP value is similar to obtained for *E. bourgatii* (Wong, Leong, & Koh, 2006), explained through the reported efficacy of carotenoids, especially xanthophylls, as ferric reductors (Astrid Garzon et al., 2012; Mueller, Froehlich, & Boehm, 2011).

Table 3. *In vitro* antioxidant capacity by TEAC, FRAP and ORAC methods of *E. bourgatii* extract.

	TEAC[a]	FRAP[b]	ORAC[c]
E. bourgatii extract	0.915 ± 0.088	59.8 ± 2.4	259.6 ± 18.3

[a] Expressed in mmol Trolox equivalents/g extract (dw).
[b] Expressed in mmol Fe^{2+} equivalents/g extract (dw).
[c] Expressed in μmol Trolox equivalents/g extract (dw).

3.2.2. Reactive oxygen species and expression of MCP-1 in HUVEC

The effect of *E. bourgatii* extracts on the production of reactive oxygen species and expression of MCP-1 in HUVEC is shown in Fig. 4. All values are referred to those found in endothelial cells pre-incubated with TNF-α (5 ng/mL) for 6 h, washed, and further incubated with TNF-α, 0.2 ng/mL and the indicated amount of extracts for 48 h. A) The TNF-α-induced intracellular ROS generation was significantly inhibited by significant amounts of extract without altering cell viability (higher than 97% in all cases); B) the release of MCP-1 production was also inhibited and values were expressed as a percentage with respect to those measured under basal conditions; C) there was an 85-fold increase in MCP-1 transcripts in the experimental model with respect to non-stimulated cells (for clarity, data are not shown). The extract partially prevented the TNF-α-induced increase, and values are expressed as a relative decrease in MCP-1 transcripts normalized to GAPDH. All values were expressed as mean (SEM) of three individual experiments performed in triplicate; * pb0.05, pb0.01 with respect to TNF-α alone.

It was also established that an excess of ROS triggers inflammation (Han et al., 2010). *E. bourgatii* clearly possesses antioxidative and anti-inflammatory actions in HUVEC, especially at 80–100 μg/mL.

These properties might also have therapeutic implications, being major processes in inflammatory diseases. Once again, we highlight the importance of a particular formulation of phenolic compounds because *E. bourgatii* was particularly active in inhibiting the secretion of MCP-1, a key molecule that regulates the migration of non-resident macrophages to the tissues and overall systemic metabolism (Furukawa et al., 2004).

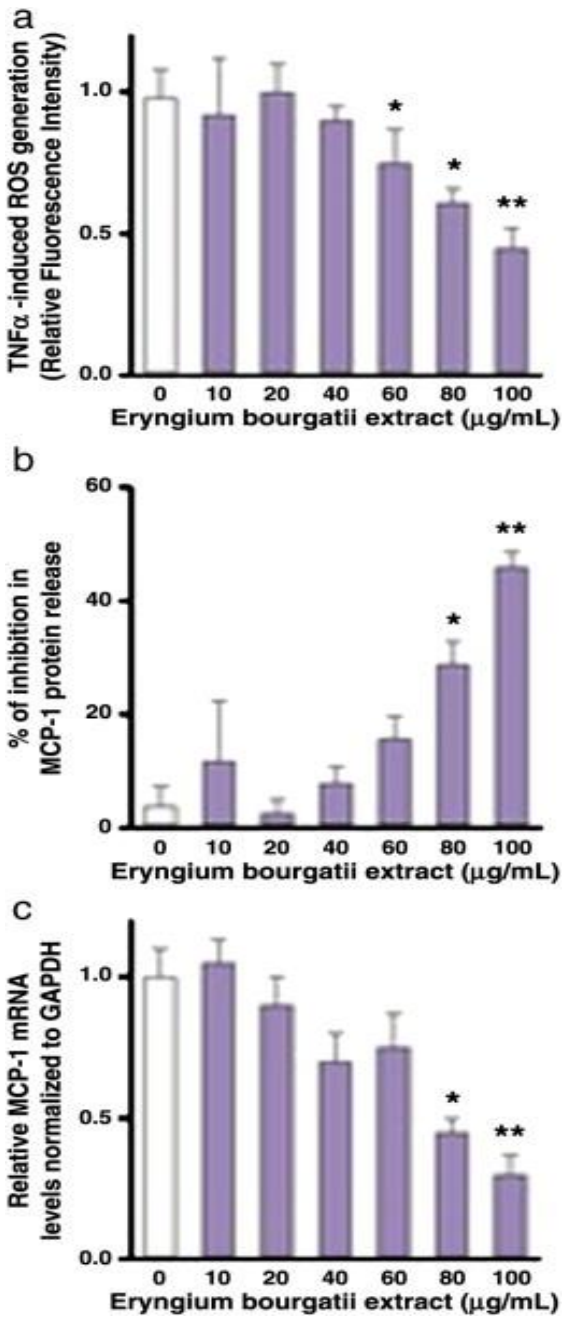

Figure 4. Effect of Eryngium bourgatii extracts on production of: (a) reactive oxygen species (ROS), (b) inhibition of MCP-1 and (c) relative MCP-1 mRNA levels in HUVEC.

4. CONCLUSIONS

A powerful analytical method has been used in the comprehensive characterization of leaf and inflorescence extracts from *E. bourgatii*. The combined use of HPLC separation with a small particle size column assisted by mass spectrometric detection with mass analyser, Q-TOF, proved to be a useful tool for identifying secondary metabolites produced by plants.

The method described separated a wide range of phenolic compounds and simultaneously provided the tentative characterization of the major compounds of this extract. It is important to

highlight that, to our knowledge, this is the first study available to characterize that the phenolic compounds from leaves and inflorescences of an *E. bourgatii* extract.

E. bourgatii extract showed antioxidant capacity by mechanisms based on electron transfer (TEAC, and FRAP) and hydrogen atom transfer (ORAC), and anti-inflammatory properties, decreasing reactive oxygen species generation as well as inhibiting MCP-1 and their transcripts production, respectively, in TNF-α-induced HUVEC cell line.

5. ACKNOWLEDGEMENS

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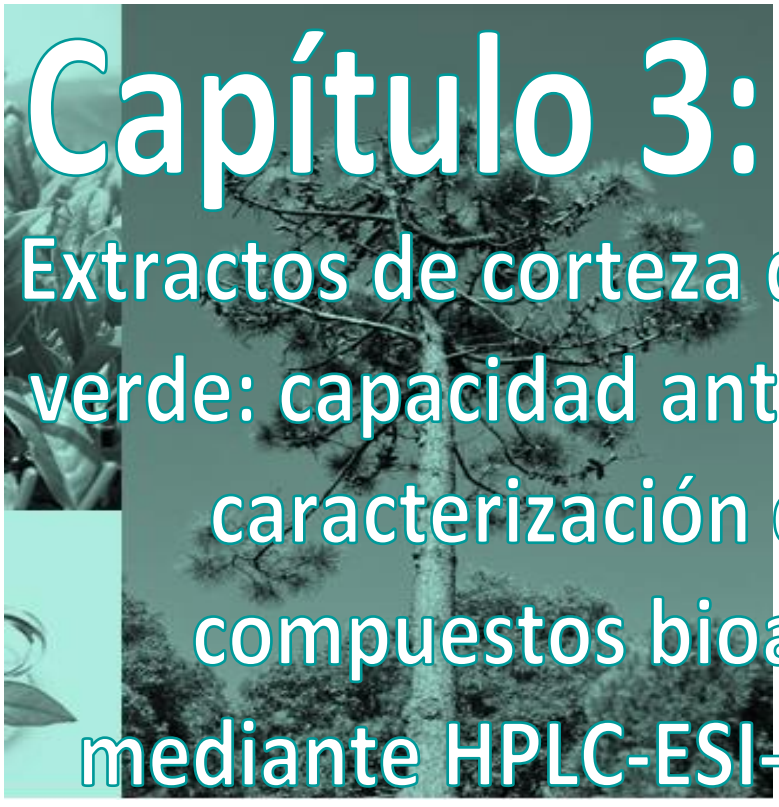

Capítulo 3:

Extractos de corteza de pino y té verde: capacidad antioxidante y caracterización de sus compuestos bioactivos mediante HPLC-ESI-QTOF-MS

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Pine Bark and Green Tea Concentrated Extracts: Antioxidant Activity and Comprehensive Characterization of Bioactive Compounds by HPLC–ESI-QTOF-MS

ABSTRACT

The consumption of polyphenols has frequently been associated with low incidence of degenerative diseases. Most of these natural antioxidants come from fruits, vegetables, spices, grains and herbs. For this reason, there has been increasing interest in identifying plant extract compounds. Polymeric tannins and monomeric flavonoids, such as catechin and epicatechin, in pine bark and green tea extracts could be responsible for the higher antioxidant activities of these extracts. The aim of the present study was to characterize the phenolic compounds in pine bark and green tea concentrated extracts using high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC–ESI-QTOF-MS). A total of 37 and 35 compounds from pine bark and green tea extracts, respectively, were identified as belonging to various structural classes, mainly flavan-3-ol and its derivatives (including procyanidins). The antioxidant capacity of both extracts was evaluated by three complementary antioxidant activity methods: Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC). Higher antioxidant activity values by each method were obtained. In addition, total polyphenol and flavan-3-ol contents, which were determined by Folin–Ciocalteu and vanillin assays, respectively, exhibited higher amounts of gallic acid and (+)-catechin equivalents.

1. INTRODUCTION

Medicinal and spice plants, which are well known for their pharmacological activity, contain many substances that exhibit radical-scavenging properties. Phenolic compounds are among the other substances included in this group. These compounds, which are secondary plant metabolites, are an essential part of the human diet. They are of considerable interest, due to their suggested advantageous health effects and possibility for use as natural food additives, since they influence the quality and stability of foods by acting as flavorants, colorants and antioxidants [1]. Great interest is currently centered on their potential benefits as complements to the organism's antioxidant defense system. Polyphenols are potent free radical-scavengers and are associated with multiple biological activities, including radioprotective, anti-inflammatory, anti-carcinogenic, antiviral and antibacterial properties, which are mainly attributed to their antioxidant and antiradical activity [2–4]. The *in vitro* antioxidant activity of foods and plants is generally studied by Trolox equivalent antioxidant capacity (TEAC), 2,2-diphenylpicrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC)-based methods [5–7]. Different methods are used for total phenolic and flavan-3-ol content determination, with the most common being the vanillin assay [8,9] and Folin–Ciocalteu assay [10].

Proanthocyanidins are found in many woody plants. The two most common sources of them are grape seeds (*Vitis vinifera*) and white pine (*Pinus maritima*, *Pinus pinaster*). Proanthocyanidins are also abundant in green tea (*Camellia sinensis*) and hawthorn (*Crataegus oxyacantha*), as well as in apples, berries, barley, bean hulls, cacao beans, rhubarb, rose hips and sorghum. These compounds are oligomers and polymers of flavan-3-ol monomer units most frequently linked either as C4→C6 or as C4→C8 (B-type proanthocyanidins). A-type proanthocyanidins possess a second interflavanoid bond, resulting in oxidative coupling between the C2→O7 positions (Figure 1). The most common classes are procyanidins consisting of catechin, epicatechin and/or their gallic acid esters and prodelphinidins containing gallocatechin and epigallocatechin and/or their galloylated derivatives [11,12].

Pine (*Pinus sylvestris* L.) tree bark is also valued medicinally for its rich content of proanthocyanidins. Pine bark extracts have been used as a folk medicine and are used as a dietary supplement and phytochemical remedy for several diseases (pycnogenol) [13,14]. They have also been shown to be a very powerful antioxidant and free radical-scavenger, even more powerful than either vitamin C or vitamin E.

Pine bark extract is used in cardiovascular and heart formulas and has also been shown to be beneficial to those with chronic venous insufficiency. Procyanidins occurring in pine bark consist mainly of the flavan-3-ol units of (+)-catechin [15,16].

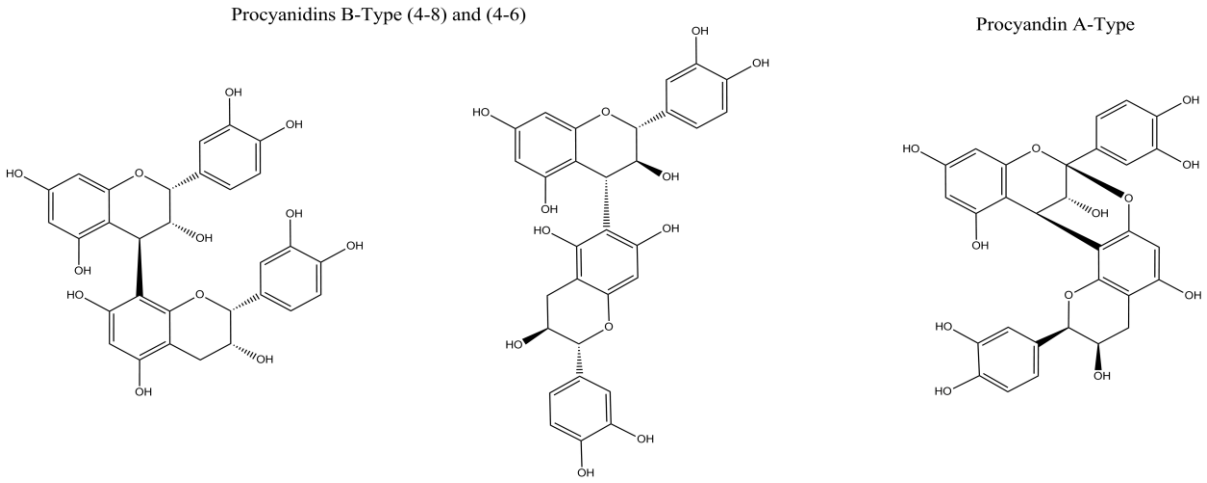

Figure 1. Structures of procyanidin (4β→8) and (4β→6)-dimers (B-type) and the (2β→7, 4β→8)-dimer (A-type).

Aside from water, tea (*Camellia sinensis*) is the most consumed beverage in the world. About 2.5 million tons of tea are produced worldwide every year. The most popular type of tea in the world is black tea, which is produced in India, Sri Lanka, Kenya and many other countries. Most of the tea produced in Japan is green tea, while China produces both green and black teas, as well as several other types of teas, such as oolong tea and Pu-er tea. In fresh tea leaves and green teas, catechins are the major polyphenols and are mainly composed of (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate [17,18].

Bioactive compounds have been analyzed by gas chromatography coupled to mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). HPLC and CE allow for efficient separation of flavonoids in different plant extracts. Electrospray ionization mass spectrometry (ESI-MS) allows for a softer ionization and permits structural information to be obtained using collisionally-induced dissociation (CID). Moreover, ESI-MS makes it possible

to discriminate between various flavonoid classes and gather information on the glycosylation position [19]. The negative ion ESI mass spectra show the presence of a series of non-galloylated and galloylated oligomeric procyanidins up to a trigalloylated octamer [20]. Reversed-phase high performance liquid chromatography (RP-HPLC) coupled to diode array detection (DAD) and/or MS are usually employed for analysis of these compounds [6,7,21,22]. Quadrupole time-of-flight mass spectrometry (QTOF-MS) combines high sensitivity and mass accuracy for both precursor and product ions, providing the elemental composition of the parent and fragment ions. This feature helps to identify compounds thoroughly and to differentiate between isobaric compounds. The potential of HPLC–ESI-QTOF-MS for qualitative purposes has been highlighted in several studies [23].

In this work, procyanidin-rich extracts from pine bark and green tea were analyzed and compared by HPLC coupled to a quadrupole time-of-flight (QTOF) mass spectrometer and equipped with an ESI interface. Additionally, we wanted to determinate the antioxidant potential present in both extracts by three complementary antioxidant activity methods: TEAC, FRAP and ORAC. We also wanted to evaluate the total phenolic and flavan-3-ol contents by Folin–Ciocalteu and vanillin assays.

2. MATERIAL AND METHODS

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Acetic acid and acetonitrile for HPLC were purchased from Fluka, Sigma–Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Solvents were filtered using a Solvent Filtration Apparatus 58061

(Supelco, Bellefonte, PA, USA). Dimethyl sulfoxide (DMSO) was purchased from Panreac (Barcelona, Spain).Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

The standards, procyanidin A2, (+)-catechin, (−)-epicatechin, gallic acid, quercetin and rutin, were purchase either from Fluka, Sigma-Aldrich (Steinheim, Germany) or Extrasynthese (Genay Cedex, France).

The reagents used to measure the antioxidant capacity and total phenolic/flavanol-3-ol content, AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride), TPTZ (1,3,5-triphenyltetrazolium chloride), ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate)), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein, potassium persulfate, ferric sulfate, Folin-Ciocalteu reagent, (+)-catechin and vanillin, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dehydrated sodium phosphate, trihydrated sodium acetate, sodium acetate, ferric chloride, hydrochloric acid, sodium carbonate and gallic acid were obtained from Panreac (Barcelona, Spain).

2.2. Sample Preparation

Concentrated pine bark and green tea extracts (Nutrafur, Spain) were used in this study. The polyphenols from whole extracts were analytically characterized using a 10 mg/mL solution of pine bark or green tea extracts. Briefly, 10 mg of these extracts were dissolved in 1 mL of DMSO. The sample was sonicated for 5 min, vortexed for 1 min, centrifuged for 5 min at 7700× *g* and then filtered through a 0.25 mm filter before the HPLC analysis.

2.3. Instrumentation

Analytical characterizations of pine bark and green tea extracts were performed using an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode array detector (DAD). The HPLC system was coupled to a quadrupole time-of-flight mass spectrometer (QTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ESI interface (model G1607A from Agilent

Technologies, Palo Alto, CA). Fluorescence (ORAC) and absorbance (Folin-Ciocalteu assay, vanillin assay, FRAP and TEAC) measurements were carried out on a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) using 96-well polystyrene microplates.

2.4. Chromatographic, UV and Spectrophotometric Conditions

The compounds from pine bark and green tea extracts were separated at room temperature using a Zorbax Eclipse Plus C18 column (1.8 µm, 150 mm × 4.6 mm). The mobile phases consisted of 0.5% acetic acid (A) and methanol (B). The following multi-step linear gradient was applied: 0 min, 0% B; 5 min, 25% B; 15 min, 35% B; 20 min, 39% B; 38 min, 60% B; 40 min, 70% B; 42 min, 80% B; 44 min, 100% B; 46 min, 0% B; and 48 min, 0% B. The initial conditions were held for 10 min. The injection volume was 10 µL, and the flow rate was 0.3 mL/min. For the spectrophotometric conditions for antioxidant assays, the excitation and emission wavelengths were 485 and 520 nm, respectively, for the ORAC assay. The absorbance wavelengths for Folin-Ciocalteu, vanillin, FRAP and TEAC assays were 760, 500, 593 and 734 nm, respectively.

2.5. ESI-QTOF-MS Detection

The HPLC system was coupled to a QTOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +3.5 kV. The other optimum values of the source parameters were: drying gas temperature, 220 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 2.5 bar. The detection was performed for a mass range of 50–1200 *m/z*.

The accurate mass data of the molecular ions were processed through the Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulas using Generate Molecular Formula Editor. This uses a CHNO algorithm, which provides standard functionalities, such as minimum/maximum elemental range, electron configuration and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured

isotope patterns (σ value) for increased confidence in the suggested molecular formula [60]. The widely accepted accuracy threshold for the confirmation of elemental compositions was established at 5 ppm [61]. Even with a very high mass accuracy (<3 ppm in most of the cases), many chemically possible formulae were determined depending on the mass regions considered.

Therefore, high mass accuracy alone is not sufficient to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns as a single further constraint removes >95% of the false candidates. This orthogonal filter can reduce several thousand candidates to only a small number of molecular formulas.

During the development of the HPLC method, external instrument calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, with a sodium acetate cluster solution passing through, containing 5 mM sodium hydroxide and 0.2% acetic acid in water:isopropanol (1:1, *v*/*v*). The calibration solution was injected at the beginning of each run, and all of the spectra were calibrated prior to compound identification.

2.6. Total Phenolic and Flavan-3-ol Contents

The total phenolic content was measured by the Folin–Ciocalteu method reported by [62], with some modifications. The extracts were dissolved in methanol (different concentrations of extracts were tested). Then, 10 µL aliquots were mixed with 50 µL of Folin-Ciocalteu reagent, 150 µL of 20% (*w*/*v*) sodium carbonate solution and 600 µL water. After 2 h of incubation at room temperature in the dark, 200 µL of the mixture was transferred into a well of the microplate, and the absorbance was read at 760 nm against a blank in a microplate spectrophotometer reader (BioTek). The phenol content was calculated based on the calibration curves of gallic acid and expressed as mg GAE/g of dry matter. Measurements were made in triplicate.

Both extracts were analyzed for total flavan-3-ol content using a method described in [63], with some modifications. For the analysis, a working solution of 1% vanillin in methanol and 10% HCl in methanol

(1:1, *v*/*v*) was prepared daily. The extract was dissolved in methanol (different concentrations were tested). Then, 100 µL aliquots were mixed with 1 mL of the previously prepared vanillin reagent. The mixture was allowed to react for 30 min at a room temperature. After that, 200 µL of the mixture were transferred into a well of the microplate, and the absorbance was read at 50 nm against a blank in a microplate spectrophotometer reader (BioTek). The blank was prepared by replacing the 100 µL samples or standard with methanol. Flavan-3-ol content was calculated based on the calibration curves of (+)-catechin and expressed as mg CE/g of dry matter. Measurements were made in triplicate.

2.7. Antioxidant Capacity Assays

The TEAC assay, which measures the reduction of the radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) by antioxidants, was performed by using a previously described method [6,7,64,65]. TEAC values were calculated using Trolox as the standard. The FRAP assay was carried out following the method described by Benzie and Strain, Cádiz-Gurrea *et al.* and Morales-Soto *et al.* [6,7,59,65]. FRAP values were calculated using $FeSO_4 \cdot 7H_2O$ as the standard. To assay the capacity of the extracts to scavenge peroxyl radicals, a validated ORAC method was used [66] with the modifications developed by Laporta *et al.*, Cádiz-Gurrea *et al.* and Morales-Soto *et al.* [6,7,64,65]. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area of the fluorescence decay curve (area under curve, AUC), as previously described in Laporta *et al.* [64]. Measurements were made in triplicate.

3. RESULTS AND DISCUSSION

3.1. Characterization of Polar Compounds by HPLC–ESI-QTOF-MS

A comprehensive characterization of phenolic compounds using advanced and powerful techniques is crucial. For this reason, suitable methods need to be established for their characterization in vegetable matrices. The use of QTOF technologies allows for the exact mass measurements of both MS and MS/MS ions to be achieved, which is essential for elemental composition assignment and, thus, for the characterization of small molecules [6,7].

2.1.1. Pine Bark Extract

A total of 37 compounds distributed in three major categories (flavan-3-ol and its derivatives, flavonols and other compounds) were analyzed in the present study. Figure 2a shows the base peak chromatogram (BPC) of the pine bark extract. The major peaks, which were identified based on elution order, are listed in Table 1. All of the compounds were characterized by interpretation of their mass spectra obtained by the QTOF-MS and also by taking into account previously reported data.

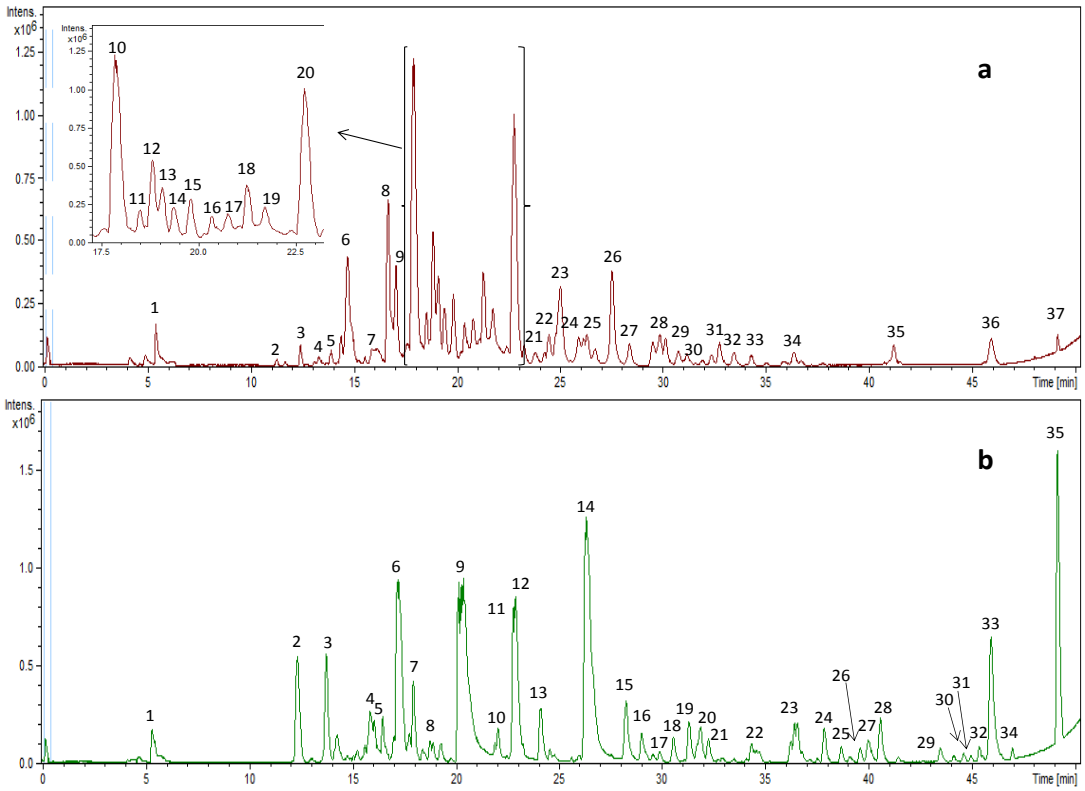

Figure 2. Base peak chromatogram of pine bark (**a**) and green tea (**b**) extracts.

Flavan-3-ol and Its Derivatives

Pine bark, which is valued medicinally for its rich content of proanthocyanidins, has been used as a folk medicine and is used as a dietary supplement. The main constituents of pine bark are known to be phenolic compounds, broadly divided into monomers (catechin, epicatechin) and condensed flavonoid (procyanidins) [13,24,25]. Procyanidins consist mainly of the flavan-3-ol units of (+)-catechin [15].

Monomeric Forms

The deprotonated ions (Peaks 10 and 20) at *m/z* 289 produced the MS^2 fragment ions at *m/z* 245, which correspond to the loss of one CO_2. These compounds were identified as (−)-epicatechin and (+)-catechin, respectively, based on the retention times and mass fragmentation comparison of $[M–H]^-$ ions with authentic standards.

B and A-Type Oligomeric Forms

Procyanidins were identified as the main phenolic components in pine bark [1,13,26]. In agreement with data published previously, B-type procyanidins are largely procyanidins in pine bark extracts, and they contain no or less than 10% prodelphinidins [27,32]. The chemical structure of B-type oligomers was based on the presence of (epi)catechin units, which are linked by a single bond. In our study, two dimers (Peaks 6 and 7) with $[M–H]^-$ ions at *m/z* 577 and one trimer (Peak 2) at *m/z* 865 have been detected. The major fragments were generated at the following *m/z*: *m/z* 289, which corresponds to deprotonated (epi)catechin; *m/z* 425, after the neutral loss of 152 amu ($C_8H_8O_3$) from retro-Diels–Alder (RDA) fission of the heterocyclic C ring; and *m/z* 577, which corresponds to the deprotonated dimer. A-type procyanidins, which are characterized by the existence of a doubly interflavanoid linkage, have not been reported in pine bark extracts. However, in this study, A-type oligomers have been detected. In this way, Peaks 4, 5, 18, 23, 25, 26, 29 and 31 with *m/z* 575 and Peaks 9 and 14 with *m/z* 863 were tentatively identified as A-type proanthocyanidin dimers and trimers, respectively. In MS^2, the main ions were at *m/z* 289, $[(epi)catechin–H]^-$, and 285, $[(epi)catechin–2H_2–H]^-$, both generated by the cleavage at the interflavanoid bonds.

Table 1. Retention time and mass spectral data of the compounds characterized in pine bark extract by HPLC–ESI-QTOF-MS and MS/MS

Peak	Proposed Compound	*RT* (min)	$[M–H]^-$ Measured	$[M–H]^-$ Calculated	Error (ppm)	mSigma	Fragmentation Pattern	Molecular Formula	Ref.
1	Sucrose	5.4	341,1098	341,1089	2.6	9	non fragmented	$C_{12}H_{22}O_{11}$	–
2	Procyanidin C	11.3	865,1981	865,1985	0.5	30	577, 289	$C_{45}H_{38}O_{18}$	[1,13,15,24–27]
3	Gardenoside	12.4	403,1257	403,1246	2.7	6.2	non fragmented	$C_{17}H_{24}O_{11}$	–
4	Procyanidin A (isomer 1)	13.3	575,1191	575,1195	0.6	22	289	$C_{30}H_{24}O_{12}$	–
5	Procyanidin A (isomer 2)	13.9	575,1189	575,1195	1	34.8	289	$C_{30}H_{24}O_{12}$	–
6	Procyanidin B (isomer 1)	14.7	577,1366	577,1351	2.6	5.3	425	$C_{30}H_{26}O_{12}$	[1,13,15,24–27]
7	Procyanidin B (isomer 2)	15.9	577,1347	577,1351	0.7	13.2	425, 289	$C_{30}H_{26}O_{12}$	[1,13,15,24–27]
8	Chalcan-flavan-3-ol dimer (isomer 1)	16.7	579,1532	579,1508	4.2	5.2	561	$C_{30}H_{28}O_{12}$	–
9	Procyanidin trimer A-type (isomer 1)	17	863,1842	863,1829	0.4	27.4	289, 285	$C_{45}H_{36}O_{18}$	–
10	(–)-epicatechin	17.9	289,0727	289,0718	3.4	4.9	245	$C_{15}H_{14}O_6$	[1,13,15,24–27]
11	Chalcan-flavan-3-ol dimer (isomer 2)	18.5	579,1512	579,1508	0.7	4.3	289	$C_{30}H_{28}O_{12}$	–
12	Chalcan-flavan-3-ol dimer (isomer 3)	18.8	579,152	579,1508	2.1	6.2	561, 289	$C_{30}H_{28}O_{12}$	–
13	Chalcan-flavan-3-ol dimer (isomer 4)	19.1	579,1528	579,1508	3.5	6.5	561	$C_{30}H_{28}O_{12}$	–
14	Procyanidin trimer A-type (isomer 2)	19.4	863,1869	863,1829	4.6	10.3	289	$C_{45}H_{36}O_{18}$	–
15	Chalcan-flavan-3-ol dimer (isomer 5)	19.8	579,1516	579,1508	1.3	5	561, 289	$C_{30}H_{28}O_{12}$	–
16	Chalcan-flavan-3-ol dimer (isomer 6)	20.4	579,152	579,1508	2	4	561	$C_{30}H_{28}O_{12}$	–
17	(Epi)fisetinidol-(epi)catechin (isomer 1)	20.8	561,1422	561,1402	3.4	5.9	273	$C_{30}H_{26}O_{11}$	–
18	Procyanidin A (isomer 3)	21.2	575,1195	575,1195	0.1	18.9	289	$C_{30}H_{24}O_{12}$	–
19	(Epi)fisetinidol-(epi)catechin (isomer 2)	21.7	561,1428	561,1402	4.7	6.5	289, 273	$C_{30}H_{26}O_{11}$	–
20	(+)-catechin	22.7	289,0729	289,0718	3.8	7.8	245	$C_{15}H_{14}O_6$	[1,13,15,24–27]
21	(Epi)fisetinidol-(epi)catechin (isomer 3)	23.8	561,1406	561,1402	0.6	38.5	289	$C_{30}H_{26}O_{11}$	–
22	(Epi)fisetinidol-(epi)catechin (isomer 4)	24.4	561,1409	561,1402	1.1	9.6	273	$C_{30}H_{26}O_{11}$	–
23	Procyanidin A (isomer 4)	25	575,1207	575,1195	2	8	423	$C_{30}H_{24}O_{12}$	–
24	(Epi)fisetinidol-(epi)catechin (isomer 5)	25.9	561,1413	561,1402	2	2.7	non fragmented	$C_{30}H_{26}O_{11}$	–
25	Procyanidin A (isomer 5)	26.3	575,1188	575,1195	1.3	12.7	289	$C_{30}H_{24}O_{12}$	–
26	Procyanidin A (isomer 6)	27.5	575,1221	575,1195	4.6	21.8	289	$C_{30}H_{24}O_{12}$	–
27	(Epi)fisetinidol-(epi)catechin (isomer 6)	28.3	561,1402	561,1402	0	5	non fragmented	$C_{30}H_{26}O_{11}$	–
28	(Epi)fisetinidol-(epi)catechin (isomer 7)	29.8	561,1416	561,1402	2.3	3	289, 273	$C_{30}H_{26}O_{11}$	–
29	Procyanidin A (isomer 7)	30.7	575,12	575,1195	0.8	14.3	289	$C_{30}H_{24}O_{12}$	–
30	(Epi)fisetinidol-(epi)catechin (isomer 8)	31.1	561,14	561,1402	0.5	11.8	245	$C_{30}H_{26}O_{11}$	–
31	Procyanidin A (isomer 8)	32.7	575,1205	575,1195	1.7	10.7	285	$C_{30}H_{24}O_{12}$	–
32	(Epi)fisetinidol-(epi)catechin (isomer 9)	33.4	561,1418	561,1402	2.8	7.9	289	$C_{30}H_{26}O_{11}$	–
33	Quercetin rhamnosylrutinoside	34,2	755,2041	755,204	0.2	11.9	301	$C_{33}H_{40}O_{20}$	[28]
34	Rutin	36.3	609,1476	609,1461	0.7	14.4	301	$C_{27}H_{30}O_{16}$	[28]
35	Isorhamnetin rutinoside	41.1	623,1614	623,1618	0.6	10.5	315	$C_{28}H_{32}O_{16}$	[25]
36	Quercetin	45.8	301,0357	301,0354	0.9	7.4	non fragmented	$C_{15}H_{10}O_7$	[28,29]
37	Kaempferol	49	285,041	285,0405	1.7	11.2	non fragmented	$C_{15}H_{10}O_6$	[30,31]

Nine isomers (Peaks 17, 19, 21, 22, 24, 27, 28, 30 and 32), with a $[M-H]^-$ at *m/z* 561, were detected and generated MS^2 fragment ions at *m/z* 289, 245 and 273, corresponding to deprotonated (epi)catechin, its loss of CO_2 and deprotonated fisetinidol (Figure 3). These compounds have been identified as (epi)fisetinidol–(epi)catechin for the first time in pine bark. According to several authors, they have been detected in different kinds of bark extracts, such as *Acacia mearnsii*, *Cotinus coggygria* wood and *Mimosa* [33–36] and as gambiriin B in *Uncaria gambir* extract [37,38].

Six isomers (Peaks 8, 11, 12, 13, 15 and 16) of chalcan-flavan3-ols dimer, with a $[M-H]^-$ at *m/z* 579, were detected in the pine bark extract. The MS^2 spectra showed major fragment ions at 561 and 289, corresponding to the loss of H_2O and deprotonated (epi)catechin. These compounds have also been identified in the literature as gambiriins A [37–39].

Flavonols

Peaks 33–37 were identified as flavonols and their derivatives. Peak 33, with a $[M-H]^-$ at *m/z* 755, was tentatively identified as quercetin rhamnosylrutinoside. It showed a major fragment ion at *m/z* 301, which corresponded to quercetin aglycone. Peaks 34 (*m/z* 609) and 36 (*m/z* 301) were characterized as rutin and quercetin. They were confirmed by comparison with the retention times of the standards. Peak 35 had a $[M-H]^-$ at *m/z* 623 and produced MS^2 fragment ions at *m/z* 315 (isorhamnetin aglycone). This compound was identified as isorhamnetin rutinoside. Peak 37, with a $[M-H]^-$ at *m/z* 285, was characterized as kaempferol on the basis of previously published data [30,31].

Other Compounds

Peak 1, which had a $[M-H]^-$ at *m/z* 341, was tentatively identified as sucrose. Peak 3 (*m/z* 403) was characterized as gardenoside (iridoid).

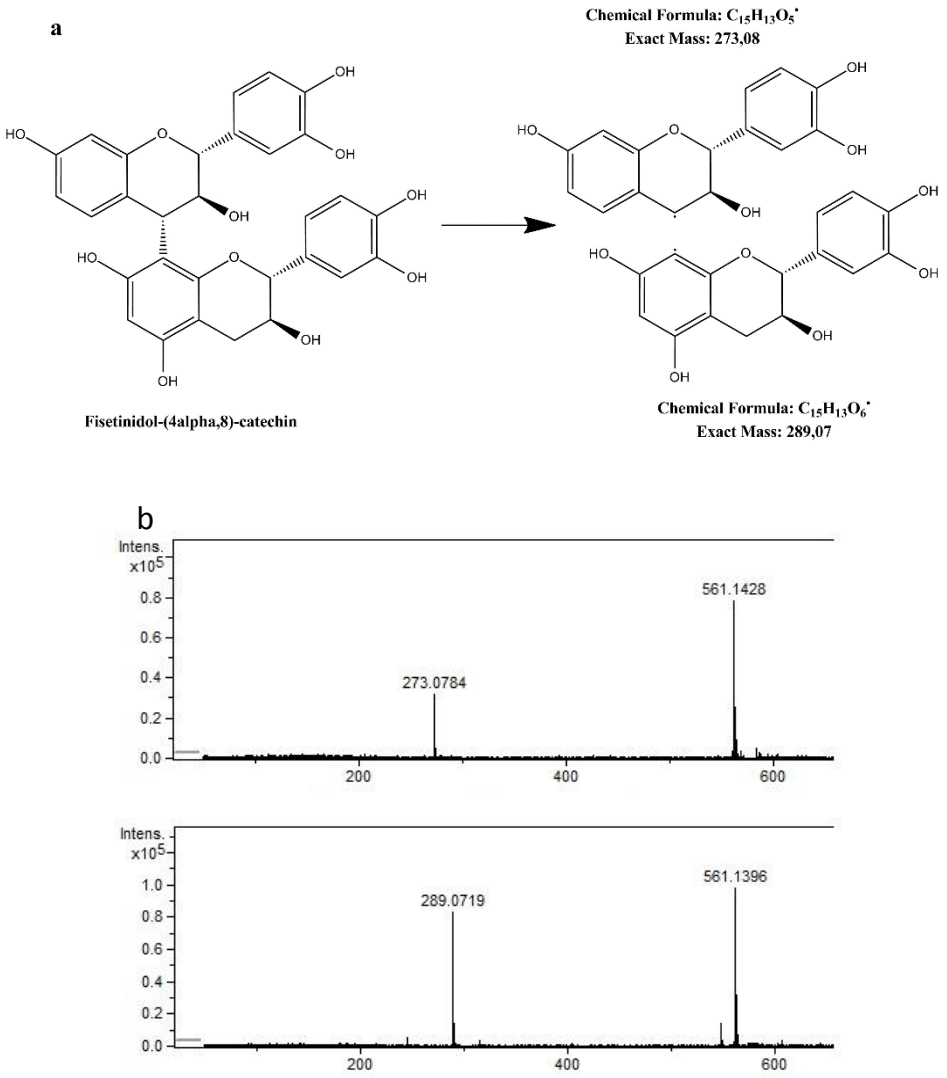

Figure 3. (**a**) Fragmentation pattern and (**b**) MS/MS spectra of fisetinidol-(4α,8)-catechin.

2.1.2. Green Tea Extract

A total of 35 compounds distributed in five major categories (flavan-3-ol and its derivatives, flavonols, flavanones, isoflavones and other compounds) were analyzed in the present study. Figure 2b shows the BPC of the green tea extract, and the major peaks, which were identified based on elution order, are assigned in Table 2. All of the compounds were characterized by interpreting the mass spectra obtained by QTOF-MS and also by taking into account previously reported data.

Table 2. Retention times and mass spectral data of the compounds characterized in green tea extract by HPLC–ESI-QTOF-MS and MS/MS

Peak	Proposed Compound	*RT* (min)	$[M-H]^-$ Measured	$[M-H]^-$ Calculated	Error (ppm)	mSigma	Fragmentation Pattern	Molecular Formula	Ref.
1	Quinic acid	5.3	191,0562	191,0561	0.4	7	127	$C_7H_{12}O_6$	[40,41]
2	Gallic acid	12.3	169,0143	169,0142	0.4	4.3	125	$C_7H_6O_5$	[17,42]
3	(Epi)gallocatechin (isomer 1)	13.7	305,0663	305,0667	1.1	6.6	169	$C_{15}H_{14}O_7$	[17,40,43,44]
4	(Epi)gallocatechin-(epi)gallocatechin gallate	15.8	745,1397	745,141	1.8	9.2	457, 169	$C_{37}H_{30}O_{17}$	[40]
5	(Epi)gallocatechin gallate glucoside	16.5	619,1308	619,1305	0.5	4.7	457, 305	$C_{28}H_{28}O_{16}$	–
6	(Epi)gallocatechin (isomer 2)	17.2	305,0677	305,0667	3.4	3	261, 219, 179, 165	$C_{15}H_{14}O_7$	[17,40,43,44]
7	(–)-epicatechin	17.9	289,0725	289,0718	2.4	2	245	$C_{15}H_{14}O_6$	[17,42,44]
8	Procyanidin B gallate (isomer 1)	18.9	729,1447	729,1461	2	15.8	577, 169	$C_{37}H_{30}O_{16}$	[43,45]
9	(Epi)gallocatechin gallate (isomer 1)	20.3	457,0789	457,0776	2.7	2.7	169	$C_{22}H_{18}O_{11}$	[17,40]
10	(Epi)gallocatechin digallate	22	609,0911	609,0886	4.1	5	457, 305, 169	$C_{29}H_{22}O_{15}$	[40,46]
11	(+)-catechin	22.8	289,0728	289,0718	3.5	1.3	245	$C_{15}H_{14}O_6$	[17,42,44]
12	(Epi)gallocatechin gallate (isomer 2)	22.9	457,0798	457,0776	4.8	1.4	289, 169	$C_{22}H_{18}O_{11}$	[17,40]
13	(Epi)gallocatechin methyl gallate	24.1	471,0938	471,0933	1.2	3.3	305, 183	$C_{23}H_{20}O_{11}$	[41,45,46]
14	(Epi)catechin gallate (isomer 1)	26.3	441,0843	441,0827	3.7	1.8	169	$C_{22}H_{18}O_{10}$	[17,40]
15	(Epi)catechin gallate (isomer 2)	28.2	441,0844	441,0827	3.8	4.7	289, 169	$C_{22}H_{18}O_{10}$	[17]
16	Procyanidin B gallate (isomer 2)	29	729,1464	729,1461	0.4	79.7	441, 289, 169	$C_{37}H_{30}O_{16}$	[43,45]
17	Eriodictyol	29.9	287,0565	287,0561	1.3	9.4	non fragmented	$C_{15}H_{12}O_6$	[17]
18	(Epi)catechin methyl gallate	30.5	455,0987	455,0984	0.6	15.8	289, 183	$C_{23}H_{20}O_{10}$	[47]
19	Epiafzelechin gallate	31.3	425,088	425,0878	1.1	8.4	169	$C_{22}H_{18}O_9$	[48]
20	Myricetin glucoside	31.8	479,0815	479,0815	3.2	5.8	317	$C_{21}H_{20}O_{13}$	[40,44]
21	Genistein glucoside (isomer 1)	32.2	431,0985	431,0984	0.3	4.6	269	$C_{21}H_{20}O_{10}$	–
22	Genistein glucoside (isomer 2)	34.3	431,0981	431,0984	0.6	9.9	269	$C_{21}H_{20}O_{10}$	–
23	Rutin	36.4	609,1486	609,1461	4.1	7.3	463	$C_{27}H_{30}O_{16}$	[40,45]
24	Naringenin	37.8	271,062	271,0612	3.1	2.7	non fragmented	$C_{15}H_{12}O_5$	[17,49]
25	Kaempferol glucosylrutinoside	38.6	755,2056	755,204	2.1	12.5	447, 285	$C_{33}H_{40}O_{20}$	[44,45]
26	Kaempferol-glucoside	39.5	447,0937	447,0993	1	9.8	285	$C_{21}H_{20}O_{11}$	[44,45]
27	Myricetin	39.9	317,0308	317,0303	1.6	12.3	non fragmented	$C_{15}H_{10}O_8$	[40,49]
28	Kaempferol rutinoside	40.5	593,151	593,1512	0.3	3.6	447	$C_{27}H_{30}O_{15}$	[45]
29	Morin	43.4	301,0355	301,0354	0.4	4.7	non fragmented	$C_{15}H_{10}O_7$	[50]
30	Theaflavin gallate	44.5	715,1309	715,1305	0.6	16.3	563, 545	$C_{36}H_{28}O_{16}$	[17,40,43]
31	Theaflavin digallate	44.9	867,1387	867,1414	3.1	25.1	715, 563, 545	$C_{43}H_{32}O_{20}$	[17,40,43]
32	Theaflavin	45.3	563,1187	563,1195	0.5	7.3	545	$C_{29}H_{24}O_{12}$	[17,40,43]
33	Quercetin	45.8	301,0362	301,0354	2.6	2.9	non fragmented	$C_{15}H_{10}O_7$	[45,49]
34	Kaempferol-coumaryl-glucoside	46.9	593,1293	593,1301	1.3	17.4	447	$C_{29}H_{24}O_{12}$	[45]
35	Kaempferol	49	285,0418	285,0405	4.7	1.5	non fragmented	$C_{15}H_{10}O_6$	[45,49]

Flavan-3-ol and Its Derivatives

The main flavan-3-ols found were gallate ester derivatives. The deprotonated ions at *m/z* 305 (Peaks 3 and 6) generated the MS^2 fragment ions at *m/z* 261, 219, 179, 169 and 165, which are in keeping with the loss of one CO_2, $C_4H_6O_2$, $C_6H_6O_3$, gallic acid and $C_7H_8O_3$, respectively. The loss of $C_4H_6O_2$ was due to the cleavage of the A ring of flavan-3-ol. The loss of $C_6H_6O_3$ resulted from heterocyclic ring fission (HRF). The loss of $C_7H_8O_3$ occurred through retro-Diels–Alder (RDA) fission. Theses peaks were identified as (epi)gallocatechin isomers [43]. Peak 5 had a $[M-H]^-$ at *m/z* 619. Its MS^2 spectrum showed fragment ions at *m/z* 457 (corresponding to (epi)gallocatechin gallate)) and 305 (corresponding to (epi)gallocatechin)). It was identified as (epi)gallocatechin gallate glucoside.

Peaks 9 and 12, with $[M-H]^-$ at *m/z* 457 ((epi)gallocatechin gallate), produced the MS^2 fragment ions at *m/z* 289 and 169, which corresponded to the deprotonated ion of (epi)catechin and gallic acid, respectively. Peak 10 was identified as (epi)gallocatechin digallate based on the mass spectra data. This compound produced the MS^2 fragment ions at *m/z* 457, 305 and 169, which corresponded to (epi)gallocatechin gallate, (epi)gallocatechin and gallic acid, respectively. Peak 13 had the $[M-H]^-$ at *m/z* 471, with product ions at *m/z* 305 and 183, corresponding to the cleavage of (epi)gallocatechin and methylgalloyl moiety. It was identified as (epi)gallocatechin methyl gallate. The deprotonated ions at *m/z* 441 (Peaks 14 and 15) produced the MS^2 fragment ions at *m/z* 289 and 169, corresponding to the deprotonated ions of catechin (or epicatechin) and gallic acid, respectively. They were identified as (epi)catechin gallate isomers.

Peak 18, which was characterized as (epi)catechin methyl gallate, had a $[M-H]^-$ at *m/z* 455. The product ions were obtained at *m/z* 289 and 183, consistent with the cleavage of (epi)catechin and a methylgalloyl moiety [47]. Peak 19 was identified as epiafzelechin gallate according to previous reports and the interpretation of the mass spectra obtained [48]. Its MS^2 spectrum showed a major fragment ion at *m/z* 169 (gallic acid).

The deprotonated ion at *m/z* 289 produced the MS^2 fragment ions at *m/z* 245, corresponding to the loss of one CO_2. Peaks 7 and 11 were identified as (−)-epicatechin and (+)-catechin based on the retention times and mass fragmentation comparison of $[M-H]^-$ ions with authentic standards. Peak 4, with a $[M-H]^-$ at *m/z* 745, showed MS^2 fragment ions at *m/z* 457 ((epi)gallocatechin gallate)) and 169 (gallic acid). This compound was tentatively identified as (epi)gallocatechin-(epi)gallocatechin gallate. Peaks 8 and 16 were detected at *m/z* 729 and were tentatively assigned as galloyl(epi)catechin-(epi)catechin isomers. This compound produced the MS^2 at *m/z* 577 (loss of a galloyl residue), at *m/z* 441 ((epi)catechin gallate)), at *m/z* 289 (deprotonated (epi)catechin)) and at *m/z* 169 (deprotonated gallic acid).Peaks 30, 31 and 32 were identified as theaflavin and its derivatives (gallate and digallate). These compounds showed the $[M-H]^-$ at *m/z* 715, 867 and 563, which corresponded to theaflavin gallate, theaflavin digallate and theaflavin, respectively. The product ions were obtained at *m/z* 715 (theaflavin gallate), 563 (theaflavin aglycone) and 545 (loss of a 18 amu, which was consistent with the cleavage of one H_2O) [43].

Flavonols

Peaks 20, 23, 25–29 and 33–35 were identified as flavonols and derivatives. Peaks 20 and 27, with $[M-H]^-$ at *m/z* 479 and 317, respectively, were tentatively identified as myricetin glucoside and myricetin, respectively [40]. Peaks 23 (*m/z* 609) and 33 (*m/z* 301) were characterized as rutin and quercetin, respectively. They were confirmed by comparison with the retention times of the standards. Considering the elution order, Peaks 25, 26, 28, 34 and 35, with $[M-H]^-$ at *m/z* 755, 447, 593, 593 and 285, respectively, were identified as kaempferol and its derivatives. Product ion spectra of these peaks showed fragment ions at *m/z* 447 and 285, corresponding to kaempferol glucoside and kaempferol aglycone, respectively. On the basis of the mass spectra and previously published data, these compounds were tentatively identified as kaempferol glucosylrutinoside, kaempferol glucoside, kaempferol rutinoside, kaempferol coumarylglucoside and kaempferol [45]. Peak 29 had a $[M-H]^-$ at *m/z* 301 and was tentatively identified as morin [50].

Flavanones

Two flavanones, with $[M-H]^-$ at *m/z* 287 (peak 17) and 271 (peak 24), were characterized as eriodictyol and naringenin, respectively [17].

Isoflavones

Genistein glucoside isomers (peaks 21 and 22) were found at *m/z* 431. The product ion spectra of these peaks showed a major fragment ion at *m/z* 269, corresponding to genistein aglycone.

Other Compounds

Peak 1 had a $[M-H]^-$ at *m/z* 191 and was identified as quinic acid, for which the most important fragment appeared at *m/z* 127 ($[M-H-CO-H_2O]^-$). Peak 2, with a $[M-H]^-$ at *m/z* 169 and MS^2 fragment ion at *m/z* 125 (decarboxylation of galloyl group), was identified as gallic acid according to the literature [17] and confirmed by comparison with the retention time of the standard.

2.2. Total Phenolic and Flavan-3-ol Contents and In Vitro Antioxidant Activities of Pine Bark and Green Tea Extracts

The antioxidant activity of polyphenols depends on the arrangement of the functional groups around the nuclear structure. Pine bark and green tea extracts are mainly composed of “bricks” of the flavan-3-ols, catechin and epicatechin, linked together into different lengths [13]. These compounds were found to be efficient scavengers of free radicals in a number of *in vitro* systems. The principal naturally occurring catechins in green tea leaves are with galloyl groups [51]. The presence of an *ortho*-dihydroxyl group in the B-ring has been shown to be important for the radical scavenging abilities of tea catechins. The addition of a gallate moiety at the 3 position of the C-ring increases the radical scavenging effectiveness of catechins in a number of systems [52].

Since the methods used to measure total phenolic and flavan-3-ol contents and antioxidant activities are extremely dependent on the reaction conditions and the substrates or products, not all methods yield the same values for activity [53]. Prior to the measurement of the antioxidant activity, the total

phenolic and flavan-3-ol contents of pine bark and green tea extracts were quantified using the Folin–Ciocalteu method and vanillin assays, respectively. The obtained values for each assay are shown in Table 3. On the basis of the dry weight, the total phenolic contents were 847.62 ± 39.74 mg of gallic acid equivalents (GAE) g-1 (pine bark) and 835.23 ± 50.31 mg GAE g-1 (green tea), and total flavan-3-ol contents were 883.33 ± 76.38 mg of (+)-catechin equivalents (CE) g-1 (pine bark) and 906.25 ± 150.26 mg CE g-1 (green tea). According to Ku *et al.*, total polyphenol content in various pine bark varieties ranged from 111 to 862 mg/g [54], and to according Gramza *et al.*, total polyphenol content in tea extracts varied between 245.8–837.6 mg/g [55].

Table 3. Values for different antioxidant measurements performed with pine bark and green tea extracts. Values are expressed as the mean ± SD.

Assays	Pine Bark	Green Tea
Folin–Ciocalteu [a]	847.62 ± 39.74 [54,56]	835.23 ± 50.31 [55]
Vanillin assay [b]	883.33 ± 76.38	906.25 ± 150.26
TEAC [c]	5.72 ± 0.78	9.66 ± 1.27 [57]
FRAP [d]	4.83 ± 0.15	8.4 ± 0.4
ORAC [c]	8.4 ± 0.4 [56]	7.58 ± 0.57 [57]

[a] Expressed in mg gallic acid equivalents g^{-1} extract (dw); [b] expressed in mg (+)-catechin equivalents g^{-1} extract (dw); [c] expressed in mmol Trolox equivalents g^{-1} extract (dw); [d] expressed in mmol $FeSO_4$ equivalents g^{-1} extract (dw).

Different *in vitro* methods were performed to determine the antioxidant activity of pine bark and green tea extracts. TEAC and FRAP are based on a single-electron transfer mechanism (ET). TEAC has been applied to establish the antioxidant properties of components in a large variety of food samples [58], and FRAP is specially indicated for determining the antioxidant capacity of biological samples [59]. The ORAC assay is performed in order to test the capacity of the extracts to quench peroxyl radicals. ORAC determination is based on a hydrogen atom transfer assay and has become one of the most widely accepted methods for measuring the antioxidant capacity of food, botanical and biological samples [58].

Table 3 lists the antioxidant capacities by TEAC and FRAP of both concentrated extracts. According to the single-electron transfer-based methods, TEAC and FRAP assays, the values for the whole pine bark

extract were 5.72 ± 0.78 mmol of Trolox equivalents (TE) g^{-1} and 4.83 ± 0.15 mmol $FeSO_4$ equivalents (FE) g^{-1}, respectively. For the whole green tea extract, the values were 9.66 ± 1.27 mmol TE g^{-1} and 8.4 ± 0.4 mmol of $FeSO_4$ equivalents (FE) g^{-1}, respectively. By the ORAC assay, the values were 8.4 ± 0.4 mmol TE g^{-1} for the pine bark extract and 7.58 ± 0.57 mmol TE g^{-1} for the green tea extract. Seeram *et al.* have determinated the antioxidant activities of green tea dietary supplements by TEAC and ORAC. These values ranged from 1.87 to 15.340 and from 1.66 to 13.690 mmol TE g^{-1}, respectively [57]. The antioxidant activity of different bark extracts was analyzed by Legault *et al.* The ORAC values ranged from 2.4 to 29 mmol TE/g [56].

By comparing all of our assays, both extracts showed high values of antioxidant activities and total phenolic and flavan-3-ol contents. This could be a result of our samples being rich in flavan-3-ol, mainly the oligomeric forms. Other sources, which have been reported to contain oligomeric flavan-3-ols (*i.e.*, cocoa), showed similar antioxidant capacity values [7]. These results showed that, for these two extracts, the green tea extract was a better antioxidant by electron transfer-based mechanisms, and pine bark extract was better by hydrogen atom transfer-based mechanisms. However, as shown in Table 3, total phenolic and total flavan-3-ol contents were similar and could not explain the differences in the antioxidant capacity, demonstrating that these two values only can be used as indicators.

To understand why green tea and pine bark extracts were powerful antioxidants by different mechanisms, an in-depth characterization is needed to identify the phenolic composition of each. According to Tables 1 and 2, the green tea extract was rich in gallic acid and gallate derivatives. In addition to the antitumor [1] and antimicrobial activities [2], gallic acid, as well as gallate derivatives have been described to have notable antioxidant activity by ET-based mechanisms [3–7]. This is, in part, due to the three hydroxyl groups in its phenolic ring [8]. On the other hand, pine bark extract was rich in procyanidins. These compounds have anti-inflammatory [9,10] and anticancer activities [11,12], as well as antioxidant properties, which are commonly determined by HAT-based mechanisms [13–16]. These findings demonstrated that polyphenols (even if they are considered to be universal

antioxidants) act under different mechanisms based on their structure [17–20]. A comparison of these results with previous reports does not yield useful or tenable information due to differences in the nature of the samples and pre-concentration technologies, extraction systems and assay methodologies.

4. CONCLUSIONS

In the present study, HPLC–ESI-QTOF-MS has been confirmed as a powerful analytical technique for separating and detecting phenolic and other polar compounds in concentrated pine bark and green tea extracts. With this method, 37 compounds were tentatively identified in pine bark extract and 35 compounds in green tea extract based on their chromatographic retention, MS data and MS/MS fragmentation pattern. The most representative groups of compounds tentatively identified were flavan-3-ols (oligomeric forms). Of these compounds, (epi)fisetinidol-(epi)catechin isomers and other chalcan-flavan-3ol isomers have been tentatively identified for the first time in pine bark.

These extracts possess significant antioxidant capacity to reduce peroxyl radicals determinated by the ORAC assay. Moreover, both extracts show a strong capacity to donate electrons by FRAP and TEAC assays. Additionally, they both had high phenolic and flavan-3-ol contents.

5. ACKNOWLEDGMENTS

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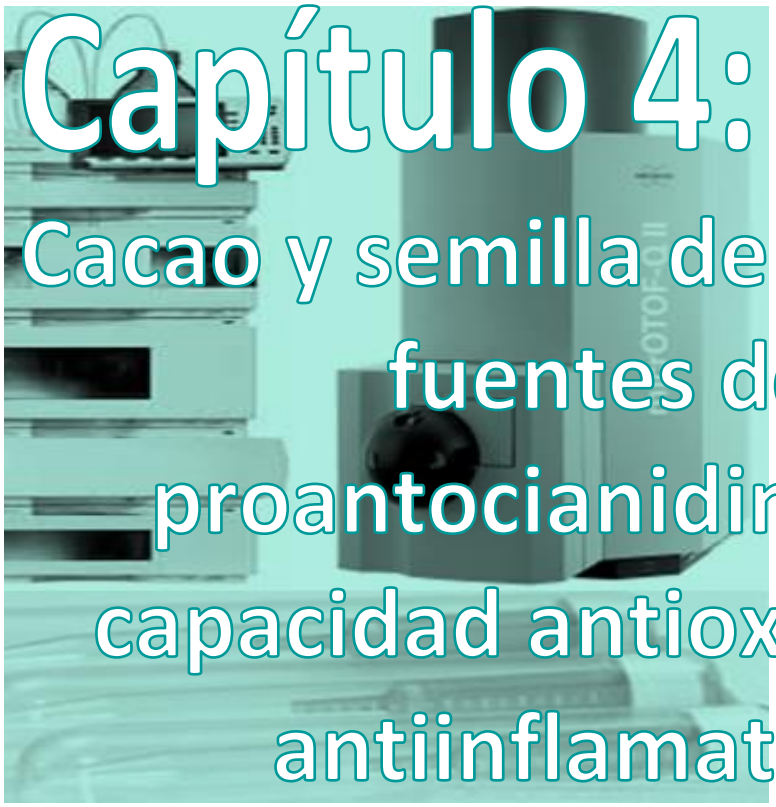

Capítulo 4:

Cacao y semilla de uva como fuentes de proantocianidinas con capacidad antioxidante y antiinflamatoria

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Cocoa and Grape Seed Byproducts as a Source of Antioxidant and Anti-Inflammatory Proanthocyanidins

ABSTRACT

Phenolic compounds, which are secondary plant metabolites, are considered an integral part of the human diet. Physiological properties of dietary polyphenols have come to the attention in recent years. Especially, proanthocyanidins (ranging from dimers to decamers) have demonstrated potential interactions with biological systems, such as antiviral, antibacterial, molluscicidal, enzyme-inhibiting, antioxidant, and radical-scavenging properties. Agroindustry produces a considerable amount of phenolic-rich sources, and the ability of polyphenolic structures to interact with other molecules in living organisms confers their beneficial properties. Cocoa wastes and grape seeds and skin byproducts are a source of several phenolic compounds, particularly mono-, oligo-, and polymeric proanthocyanidins. The aim of this work is to compare the phenolic composition of *Theobroma cacao* and *Vitis vinifera* grape seed extracts by high pressure liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer and equipped with an electrospray ionization interface (HPLC-ESI-QTOF-MS) and its phenolic quantitation in order to evaluate the proanthocyanidin profile. The antioxidant capacity was measured by different methods, including electron transfer and hydrogen atom transfer-based mechanisms, and total phenolic and flavan-3-ol contents were carried out by Folin–Ciocalteu and Vanillin assays. In addition, to assess the anti-inflammatory capacity, the expression of MCP-1 in human umbilical vein endothelial cells was measured.

1. INTRODUCTION

Phenolic compounds are ubiquitous compounds found in most fruits and vegetables and are associated with multiple biological activities, including radioprotective, anti-inflammatory, anti-carcinogenic, antiviral and antibacterial properties attributed mainly to their antioxidant and antiradical activity [1,2]. Increasing the antioxidant compounds intake in the human diet and, for example, enriching food with antioxidant compounds, is considered important. As some synthetic antioxidants may exhibit toxicity and require high manufacturing costs showing lower efficiency than natural antioxidants, there is a need to identify natural and possibly more economic and effective antioxidants with potential to be incorporated into foods [3]. Several studies show the effect of bioactive compounds on gene expression and its impact on metabolic pathways to prevent and/or ameliorate symptoms in some diseases [4] and, therefore, its rational use is an open door in alternate medicine or pharmaceutical industry.

Tannins (comprising hydrolysable and condensed tannins) are one of the major groups of polyphenols which are found in our diets. Proanthocyanidins (belonging condensed tannins) have been identified in several agricultural byproducts, seeds, fruits and vegetables [5] and their biological metabolism and pharmacokinetics have been extensively reviewed [6]. In addition to the free radical scavenging and antioxidant activity [7], proanthocyanidins also exhibit vasodilatory, anti-carcinogenic, anti-allergic, anti-inflammatory, anti-bacterial, cardio-protective, immune-stimulating, anti-viral and estrogenic activities [8], as well as are inhibitors of the enzymes phospholipase A2 [9], cyclooxygenase and lipooxygenase [10]. The anti-inflammatory activity of proanthocyanidins is one of the most widely studied [11–17]. Mechanisms of action include modulation of the arachidonic acid and nuclear factor-κB (NF-κB) pathways, inhibition of eicosanoid generating enzymes, inflammatory mediator secretion and the mitogen-activated protein kinase pathway [18].

Grapes (*Vitis vinifera* L.) are one of the most widely grown fruit crops throughout the world, and their composition and properties have been extensively investigated, with several reports of the

presence of phenolic compounds [19,20]. Grape seeds, amounting to 38%–52% on a dry matter basis [21], are a considerable proportion of the industrial byproduct from the winemaking process. Grape seeds constitute a cheap source of antioxidant compounds due to their incomplete extraction during wine production, providing important economic advantages [20,22]. Cocoa and cocoa products, i.e. cocoa liquor, cocoa powder and chocolates, are worldwide consumed and common ingredients of many food products. The chocolate market has remained stable during the last years [23] and, alternatively, the scientific interest on this potential bioactive source has grown at exponential levels. In grape seeds and cocoa extracts, proanthocyanidins represent the major part of the total polyphenolic extract. These compounds are, in fact, composed of chains of flavan-3-ols units, (+)-catechin and (−)-epicatechin, linked together through C4–C6 and C4–C8 interflavanoid bonds, and various gallate esters [24] (Figure 1).

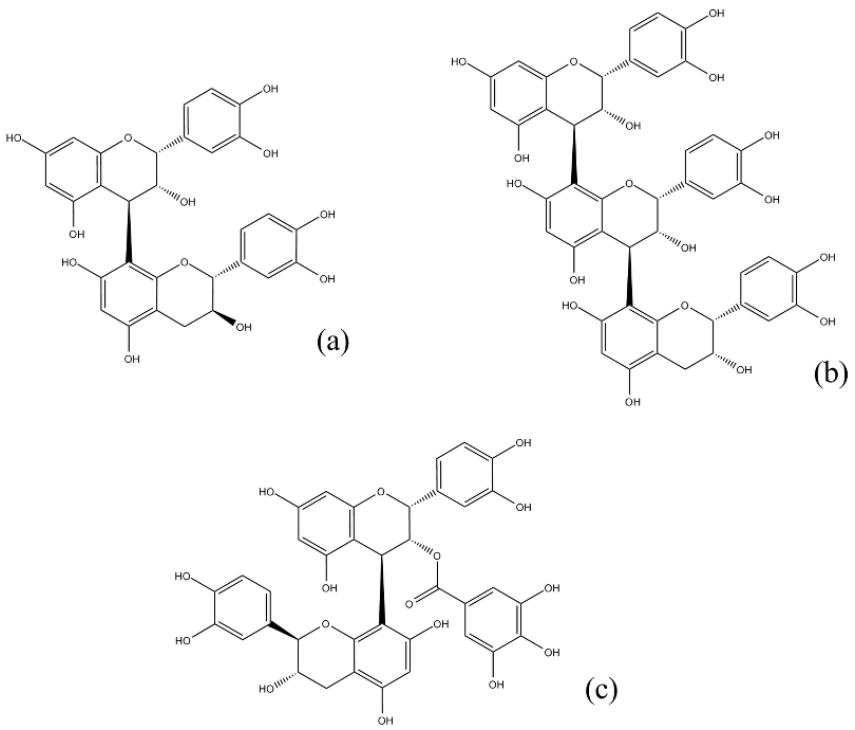

Figure 1. Most common proanthocyanidins in cacao and grape seed extracts: procyanidin dimer (**a**); procyanidin trimer (**b**); and procyanidin dimer gallate (**c**).

Therefore, the main objectives of this work were: (1) to investigate and improve the knowledge of the composition profile at the present time [25], mainly proanthocyanidins, in a grape seed extract using

high pressure liquid chromatography (HPLC) coupled to a quadrupole time-of-flight mass spectrometer (QTOF-MS) and equipped with an electrospray ionization (ESI) interface; (2) to evaluate the total phenolic and flavan-3-ol content by Folin–Ciocalteu and Vanillin assays, respectively; (3) to known how proanthocyanidins operate by determination and comparison of the antioxidant potential in vitro; and (4) to know the anti-inflammatory potential of grape seed and cocoa extracts measuring the expression of MCP-1 in human umbilical vein endothelial cells (HUVECs) [26]. These aims are a first approach in order to find bioactive compounds with biological properties that could be used as preventive or treatment of different pathophysiological disorders.

2. MATERIAL AND METHODS

2.1. Chemicals

All chemicals were of HPLC-MS grade and used as received. Acetic acid and methanol for UHPLC were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Dimethyl sulfoxide (DMSO) was provided from Panreac (Barcelona, Spain). Milli-Q system from Millipore (Bedford, MA, USA) was used to obtain purified water was purified.

The standards, for the calibration curves, procyanidin B2, (+)-catechin, (−)-epicatechin, gallic acid (GA), quercetin and quercetin-3-rutinoside were purchase either from Fluka (Sigma-Aldrich, Steinheim, Germany) or Extrasynthese (Genay Cedex, France).

To measure the antioxidant capacity and total phenolic/flavanol-3-ol content, the following reagents were provided from the indicated suppliers: AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride), ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate)], ferric sulfate, fluorescein, Folin–Ciocalteu reagent, potassium persulfate, TPTZ (2,4,6-tripyridyl-S-triazine), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), vanillin and (+)-catechin from Sigma-Aldrich (St. Louis, MO, USA). From Panreac (Barcelona, Spain), gallic acid, dehydrated sodium phosphate,

trihydrated sodium acetate, sodium acetate, ferric chloride, hydrochloric acid and sodium carbonate was purchased.

2.2. Sample Preparation

V. vinifera seed and *T. cacao* extracts were used in this study (Nutrafur, Murcia, Spain). For polyphenols extraction, 10 mg of extract was dissolved in 1 mL of DMSO, then sonicated for 5 min, vortexed for 1 min, centrifuged for 5 min at 7700×*g* and filtered through a 0.25 mm filter before the HPLC analysis.

2.3. Instrumentation

Analytical characterization of grape seed extract was performed using an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode array detector (DAD). The HPLC system was coupled to a quadrupole time-of-flight mass spectrometer (QTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). Fluorescence (ORAC) and absorbance (Folin–Ciocalteu, Vanillin assay, FRAP and TEAC) measures were carried out on a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) using 96-well polystyrene microplates.

2.4. Chromatographic Conditions

The compounds from grape seeds were separated at room temperature using a Zorbax Eclipse Plus C18 column (1.8 µm, 150 × 4.6 mm). The mobile phases were acetic acid 0.5% (solvent A) and methanol (solvent B). This multi-step linear gradient was applied: 0 min, 0% B; 5 min, 25% B; 15 min, 35% B; 20 min, 39% B; 38 min, 60% B; 40 min, 70% B; 42 min, 80% B; 44 min, 100% B; 46 min, 0% B; 48 min, 0% B. The initial conditions were maintained for 10 min. The injection volume was 10 µL. The flow rate used was set at 0.3 mL/min.

2.5. ESI-QTOF-MS Detection

The HPLC analysis were performed on a QTOF mass spectrometer equipped with an ESI interface. In negative ion mode, the capillary voltage operated at +3.5 kV. The other parameters of the source were set as follows: drying gas temperature, 220 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 2.5 bar. The detection was performed considering a mass range of 50–1200 *m/z*.

Molecular formulae for each analyte were proposed using the measured $[M-H]^-$ ion and processed through the software DataAnalysis 4.0 (Bruker Daltonics), with an accepted accuracy threshold for confirmation of elemental compositions established at 5 ppm [54].

A 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) was used, during the development of the HPLC method, as an external instrument calibration directly connected to the interface, with a sodium acetate cluster solution. The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to the compound identification.

2.6. Total Phenolic and Flavan-3-ol Contents (TPC and TFC)

The TPC was measured by the Folin–Ciocalteu method with some modifications [45,55]. Cocoa and grape seed extracts were dissolved in methanol (different concentrations were tested). Phenol content was calculated based on the calibration curves of GA equivalents and expressed as mg GAE/g of dry matter. Measurements were made in triplicate.

Extract were analyzed for its TFC using a method described by Makkar and Becker (1993) [56], with some modifications [57]. Flavan-3-ol content was calculated based on the calibration curves of (+)-catechin equivalents and expressed as mg CE/g of dry matter. Measurements were made in triplicate.

2.7. Antioxidant Capacity Assays

The reduction of the radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) was performed by TEAC assay using a previously described method [58]. TEAC values were calculated

using Trolox as a standard and reading absorbance at 734 nm in a microplate reader. The FRAP assay was carried out following the method described by Benzie and Strain (1996) [50]. FRAP values were calculated measuring the absorbance at 593 nm in a microplate reader and using $FeSO_4 \cdot 7H_2O$ as standard. To assay the capacity of the extracts to scavenge peroxyl radicals, a validated ORAC method was used [59] with some modifications [58] and measuring the excitation and emission wavelengths at 485 and 520 nm, respectively. A regression equation between the Trolox concentration and the net area of the fluorescence decay curve was used in order to obtain the final ORAC values. In all the antioxidant capacity assays, measurements were made in triplicate.

2.8. Anti-Inflammatory Activity Measurement

Anti-inflammatory experiments with HUVECs followed the procedures approved by our ethics committee, and were made as in Cádiz-Gurrea et al. (2013) [60]. Briefly, after cells reach confluence, they were treated with preconditioning medium containing TNF-α (R&D Systems, MN, USA) (5 ng/mL) for 6 h and then incubated for 48 h with fresh medium containing TNF-α 0.2 ng/mL and different concentrations (10–100 µg/mL) of grape seed and cocoa extracts.

After incubation, the supernatants were collected for ELISA analysis (RNeasy Mini Kit, Qiagen, CA, USA) and high-capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) was used to obtain cDNA using the primers for MCP-1 5′-ATGAAAGTCTCTGCCGCC-3′ and 5′-TTGCTTGTCCAGGTGGTC-3′ and for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) 5′-TCATTGACCTCAACTACATG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′.

3. RESULTS AND DISCUSSION

3.1. Characterization of Grape Seed Extract by HPLC-ESI-QTOF-MS

A comprehensive analytical characterization of phenolic compounds using advanced and powerful techniques is crucial. In this way, suitable methods need to be established for the characterization of bioactive compounds in vegetable matrices. The interpretation was performed

based on both exact mass and tandem mass spectra allowed by the QTOF technologies, which is essential for elemental composition assignment and, thus, for the characterization of small molecules. In the next sections, we do not consider decimals in exact mass and fragments to a better understanding of the manuscript. For exact masses including decimals, refer to Table 1.

A total of 36 compounds distributed in three major categories have been analyzed in grape seeds extract: (1) phenolic acids; (2) flavonoids (flavan-3-ol, procyanidins and others flavonoids); and (3) other compounds. Note that a comprehensive characterization of cocoa extract has already been published by Cádiz-Gurrea et al. (2014) [27]. Figure 2 shows the base peak chromatogram (BPC) of the grape seed extract and the major peaks observed has been assigned in Table 1, identified considering the elution order. All the compounds were characterized by the interpretation of their mass spectra obtained by the QTOF-MS and also taking into account the data provided by the literature, as explained in the following sections.

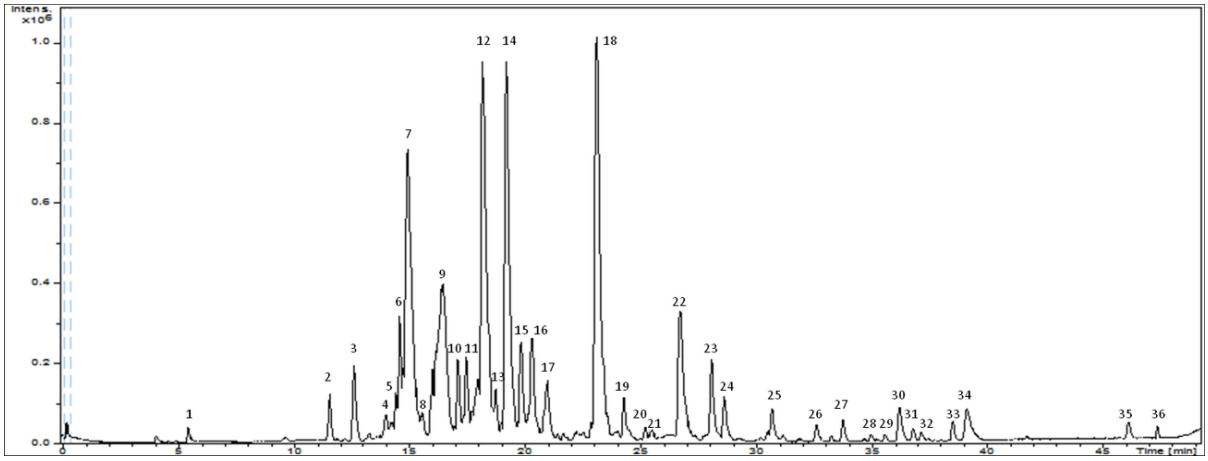

Figure 2. Base peak chromatogram of grape seed extract.

3.1.1. Phenolic Acids

Peak **2**, identified as gallic acid at *m/z* 169 yield a fragment at *m/z* 125 due to the decarboxylation of the galloyl group [20,28] and confirmed by comparison with the retention time of the standard. Peak **34** yield a $[M-H]^-$ at *m/z* 301 and was tentatively characterized as ellagic acid [29].

3.1.2. Flavonoids

Peaks **12**, **18**, **22** and **24** were determined as flavan-3-ol and derivatives. Peak **12** and **18** were characterized as (−)-epicatechin and (+)-catechin, respectively. Both showed a $[M-H]^-$ at *m/z* 289 and a fragment ion at *m/z* 245 corresponding to the loss of CO_2 and they were confirmed by comparison with the retention time of standard. The deprotonated ions (peaks **22** and **24**) at *m/z* 441 produced the MS^2 fragment ions at *m/z* 289, 169 and 125 corresponding to the deprotonated ion of (epi)catechin, gallic acid and decarboxylación of galloyl group, respectively. On the basis of mass spectral data and previously published data [30,31], this compound was identified as (epi)catechin gallate.

Peaks **2**, **4**-**11**, **13**, **14**, **15**, **16**, **17**, **19**, **20**, **21**, **23** and **25** were identified as proanthocyanidins and derivatives.

B-type proanthocyanidins were, qualitatively, the most abundant compounds in this extract [24,32]. Chemical structure of these compounds is based on the presence of (epi)catechin units which are linked by a single bond. Among these, eight procyanidin dimers (peaks **5-7**, **9**, **10**, **16**, **19** and **23**) with $[M-H]^-$ ions at *m/z* 577 and four procyanidin trimers (peaks **2**, **4**, **8** and **15**) with $[M-H]^-$ ions at *m/z* 865 were found. The major fragments were generated at *m/z* 451, after the neutral loss of 126 ($C_6H_6O_3$, phloroglucinol) from the A ring of an (epi)catechin unit, at *m/z* 433 ($[M-H-126-H_2O]^-$), at *m/z* 425 corresponding to the loss of 152 ($C_8H_8O_3$) which come from retro-Diels Alder (RDA) fission of the heterocyclic C ring and, sequentially, at *m/z* 407 ($[M-H-152-H_2O]^-$) and at *m/z* 289, due to the loss of 288 ($C_{15}H_{12}O_6$, (epi)catechin$-H_2$) by cleavages at the interflavanoid bonds [33].

Peaks **11**, **13**, **14**, **20**, **21** and **25** were detected at *m/z* 729 and tentatively assigned as galloyl(epi)catechin-(epi)catechin isomers [28]. These compounds produced the MS^2 at *m/z* 577 (loss of a galloyl residue), at *m/z* 451 ($[M-H-152-126]^-$), at *m/z* 433 ($[M-H-152-126-H_2O]^-$), at *m/z* 425 ($[M-H-152-152]^-$), at *m/z* 289 (deprotonated (epi)catechin) and at *m/z* 169 (deprotonated gallic acid). One peak (**17**) was detected at *m/z* 743 and tentatively identified as a gallate of an A-type dimeric

proanthocyanidin with (epi)catechin and (epi)gallocatechin subunits. This compound showed a MS^2 base peak at *m/z* 591 corresponding to $[M-H-152]^-$ [34].

Peaks **26**, **28**, **30**, **31** and **35** were identified as flavanols and derivatives. Peaks **26** and **31** had a $[M-H]^-$ at *m/z* 463, and peak **28** and **30** at *m/z* 447. Product ion spectra of these peaks showed a major fragment ion at *m/z* 300. This could be due to the formation of the quinone anion (radical anion), which was obtained after homolytic cleavage of the *O*-glycosidic bond yielding the fragment at *m/z* 300.027, and it has been proposed as diagnostic for quercetin glycosides [35]. On the basis of mass spectral data and previously published data, these compounds were tentatively identified as quercetin hexoside (peak **26** and **31**), quercetin rhamnoside (peak **28**) and quercetin glucuronide (peak **30**) [36,37]. Peak **35** (*m/z* 301) was characterized as quercetin and confirmed by comparison with the retention time of the standard.

Peaks **29**, **33** and **36** were identified as flavanones and derivatives. Peak **36** (*m/z* 275) was tentatively identified as phloretin. Peaks **29** and **33**, with $[M-H]^-$ at *m/z* 567 and 435, respectively, showed a MS^2 fragmentation ion at 273 corresponding to phloretin moiety. This compound and its derivatives, mainly its glucoside, phorizin, are abundantly present in apples [38], especially in the peel [39] and seed [40]. Phloretin has been shown to inhibit of protein kinase C, human leukemia cell growth and bladder cancer and rat mammary adenocarcinoma cell growth [41].

3.1.3. Other Compounds

Peak **1**, at *m/z* 341, was tentatively identified as sucrose according to its mass spectra and isotopic distribution as previously reported [42]. Peak **27**, with $[M-H]^-$ ion at *m/z* 523, produced a major fragment at *m/z* 361 (loss of glucose). According to the literature, it was characterized as secoisolariciresinol glucoside [43,44]. Peak **32** (*m/z* 439), which was tentatively identified as amurenisin, has been reported in seeds of *Vitis amurensis* [45].

Table 1. Retention time and mass spectral data of the compounds characterized in grape seed extract by HPLC-ESI-QTOF-MS and MS/MS in negative mode.

Peak	Proposed Compound	RT (min)	$[M-H]^-$ Measured	$[M-H]^-$ Calculated	Error (ppm)	mSigma	Fragmentation Pattern	Molecular Formula
1	Sucrose	5.5	341.108	341.109	2.7	18.0	Not fragmented	$C_{12}H_{22}O_{11}$
2	Procyanidin C (isomer 1)	11.6	865.199	865.198	0.9	42.5	577.114; 289.076	$C_{45}H_{38}O_{18}$
3	Gallic acid	12.6	169.013	169.014	6.7	3.8	125.024	$C_7H_6O_5$
4	Procyanidin C (isomer 2)	14	865.197	865.198	1.4	20.8	577.134; 432.093	$C_{45}H_{38}O_{18}$
5	Procyanidin B (isomer 1)	14.4	577.136	577.135	2.2	46.1	451.124; 289.076	$C_{30}H_{26}O_{12}$
6	Procyanidin B (isomer 2)	14.6	577.136	577.135	1.3	53.1	425.075; 289.074	$C_{30}H_{26}O_{12}$
7	Procyanidin B (isomer 3)	15	577.133	577.135	3.6	38.0	289.076	$C_{30}H_{26}O_{12}$
8	Procyanidin C (isomer 3)	15.6	865.198	865.198	1.0	18.1	577.114; 451.123; 433.072; 289.065	$C_{45}H_{38}O_{18}$
9	Procyanidin B (isomer 4)	16.4	577.136	577.135	1.9	48.5	425.088; 289.074	$C_{30}H_{26}O_{12}$
10	Procyanidin B (isomer 5)	17.1	577.133	577.135	4.0	41.7	425.087; 289.073	$C_{30}H_{26}O12$
11	Galloyl(epi)catechin-(epi)catechin (isomer 1)	17.5	729.146	729.146	0.3	35.7	577.121; 289.074; 169.015	$C_{37}H_{30}O_{16}$
12	(-)-Epicatechin	18.2	289.072	289.072	2.5	24.5	245.083	$C_{15}H_{14}O_6$
13	Galloyl(epi)catechin-(epi)catechin (isomer 2)	18.7	729.148	729.146	2.5	54.4	577.132; 432.094	$C_{37}H_{30}O_{16}$
14	Galloyl(epi)catechin-(epi)catechin (isomer 3)	19.2	729.147	729.146	1.0	50.7	577.131; 432.094; 169.014	$C_{37}H_{30}O_{16}$
15	Procyanidin C (isomer 4)	19.8	865.200	865.198	1.8	22.3	432.094	$C_{45}H_{38}O_{18}$
16	Procyanidin B (isomer 6)	20.3	577.134	577.135	1.8	49.2	432.092; 289.070	$C_{30}H_{26}O_{12}$
17	Galloyl(epi)catechin-(epi)gallocatechin	20.9	743.125	743.125	0.0	28.7	591.170	$C_{37}H_{28}O_{17}$
18	(+)-Catechin	23.1	289.072	289.072	3.4	23.1	245.083	$C_{15}H_{14}O_6$
19	Procyanidin B (isomer 7)	24.3	577.133	577.135	4.4	16.5	407.076; 289.075; 245.044; 125.025	$C_{30}H_{26}O_{12}$
20	Galloyl(epi)catechin-(epi)catechin (isomer 4)	25.2	729.144	729.146	2.1	19.6	577.131; 451.122	$C_{37}H_{30}O_{16}$
21	Galloyl(epi)catechin-(epi)catechin (isomer 5)	25.4	729.144	729.146	3.2	14.1	577.131; 289.072	$C_{37}H_{30}O_{16}$
22	(Epi)catechin gallate (isomer 1)	26.7	441.084	441.083	2.2	25.4	289.072; 169.015; 125.025	$C_{22}H_{18}O_{10}$
23	Procyanidin B (isomer 8)	28	577.134	577.135	1.3	45.4	425.088; 289.073; 125.025	$C_{30}H_{26}O_{12}$
24	(Epi)catechin gallate (isomer 2)	28.6	441.082	441.083	1.5	13.6	289.073; 169.015; 125.025	$C_{22}H_{18}O_{10}$
25	Galloyl(epi)catechin-(epi)catechin (isomer 6)	30.6	729.145	729.146	0.9	35.2	577.117; 407.079; 289.071; 125.023	$C_{37}H_{30}O_{16}$
26	Quercetin hexoside (isomer 1)	32.6	463.086	463.088	3.9	29.5	300.023	$C_{21}H_{20}O_{12}$
27	Secoisolariciresinol glucoside	33.7	523.217	523.219	3.1	5.1	361.180	$C_{26}H_{36}O_{11}$
28	Quercetin rhamnoside	34.9	447.034	447.093	0.3	31.2	300.028	$C_{21}H_{20}O_{11}$
29	Phloretin xyloglucoside	35.5	567.169	567.172	4.9	33.1	273.073	$C_{26}H_{32}O_{14}$
30	Quercetin glucuronide	36.1	477.067	477.067	1.5	7.9	300.028	$C_{21}H_{18}O_{13}$
31	Quercetin hexoside (isomer 2)	36.7	463.071	463.067	1.7	10.5	300.027	$C_{21}H_{20}O_{12}$
32	Amurenisin	37.1	439.066	439.067	3.1	5.3	Not fragmented	$C_{22}H_{16}O_{10}$
33	Phloretin glucoside	38.4	435.129	435.130	0.6	7.2	273.072	$C_{21}H_{24}O_{10}$
34	Ellagic acid	39	301.000	300.999	4.3	25.9	Not fragmented	$C_{14}H_6O_8$
35	Quercetin	46	301.036	301.035	1.5	11.8	Not fragmented	$C_{15}H_{10}O_7$
36	Phloretin	47.2	273.077	273.077	0.1	37.0	Not fragmented	$C_{15}H_{14}O_5$

3.2. Quantification of Grape Seed Extract by HPLC-ESI-QTOF-MS

The sensitivity of the method was studied by defining the limits of detection (LOD) and quantification (LOQ) for individual compounds in standards solutions. The MS detection, based on the extracted ion chromatogram (EIC), was used to measure the peak areas. The EIC for each analyte was chosen regarding the measured $[M-H]^-$ (Table 1). Table 2 summarizes the analytical parameters for the different compounds present in the grape seed extract. In order to quantify the amount of each compound, six calibration curves were prepared with the six standards commercially available: procyanidin B2, (+)-catechin, (−)-epicatechin, gallic acid, quercetin and quercetin-3-rutinoside.

Compounds without a commercial standard available were quantitated using the calibration curve corresponding to the compound with the most similar structure. Oligomeric procyanidins, catechin derivatives, ellagic acid and quercetin derivatives were quantified with procyanidin B2, (+)-catechin, gallic acid and quercetin-3-rutinoside, respectively. Calibration curves were obtained for each standard with a good linearity ($R^2 > 0.99$) by plotting the standard concentration as a function of the peak area obtained from HPLC-ESI-QTOF-MS analyses. The concentration ranges are also stated in Table 2, including the LOD and LOQ, which were calculated according to IUPAC recommendation [46].

The concentration of the extract was set at 2.5 g/L in all cases in order to fix in the considering working ranges. Three replicates of the extract were carried out and the results, expressed in μg/g (m/m, analyte/dry weighted extract), are also summarized in Table 2.

As reported previously [27], cocoa extract shows higher levels of procyanidin oligomers such as tetramers, pentamers and hexamers than grape seed extract. In terms of dimer proanthocyanidins (B-type procyanidins and gallate derivatives), no differences have been observed. However, concerning monomeric flavan-3-ols, such as catechin or epicatechin, grape seed extract presents the highest proportion (Figure 3).

Table 2. Quantification of compounds from grape seed extract, calibration and standard deviation data, where LOD is the limit of detection and LOQ is the limit of quantification. LOD and LOQ values were calculated for the available standards solely. Quantitation values are expressed as µg of analyte per gram of dry extract.

Analyte	LOD (µg/mL)	LOQ (µg/mL)	Calibration Range (µg/mL)	Calibration Equations	R^2	Quantification (µg/g)
Procyanidin C (isomer 1)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	117 ± 2
Gallic acid	0.254	0.848	0.5–12.5	$y = 3.6 \times 10^5 x + 2 \times 10^4$	0.991	2491 ± 118
Procyanidin C (isomer 2)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	79 ± 18
Procyanidin B (isomer 1)	0.096	0.321	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	143 ± 15
Procyanidin B (isomer 2)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	474 ± 41
Procyanidin B (isomer 3)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	2360 ± 296
Procyanidin C (isomer 3)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	89 ± 14
Procyanidin B (isomer 4)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	1623 ± 163
Procyanidin B (isomer 5)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	291 ± 17
Galloyl(epi)catechin-(epi)catechin (isomer 1)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	223 ± 16
(–)-Epicatechin	0.198	0.660	0.25-12.5	$y = 7.7 \times 10^5 x + 3.3 \times 10^4$	0.9991	8900 ± 441
Galloyl(epi)catechin-(epi)catechin (isomer 2)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	129 ± 14
Galloyl(epi)catechin-(epi)catechin (isomer 3)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	1639 ± 156
Procyanidin C (isomer 4)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	287 ± 43
Procyanidin B (isomer 6)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	528 ± 79
Galloyl(epi)catechin-(epi)gallocatechin	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	164 ± 24
(+)-Catechin	0.207	0.688	0.25–12.5	$y = 8.4 \times 10^5 x + 1.6 \times 10^5$	0.993	7747 ± 496
Procyanidin B (isomer 7)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	122 ± 16
Galloyl(epi)catechin-(epi)catechin (isomer 4)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	29 ± 4
Galloyl(epi)catechin-(epi)catechin (isomer 5)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	22 ± 3
(Epi)catechin gallate (isomer 1)	–	–	0.25–12.5	$y = 8.4 \times 10^5 x + 1.6 \times 10^5$	0.993	2744 ± 43
Procyanidin B (isomer 8)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	274 ± 9
(Epi)catechin gallate (isomer 2)	–	–	0.25–12.5	$y = 8.4 \times 10^5 x + 1.6 \times 10^5$	0.993	529 ± 26
Galloyl(epi)catechin-(epi)catechin (isomer 6)	–	–	0.39–6.25	$y = 3 \times 10^6 x - 3.6 \times 10^4$	0.9945	84 ± 2
Quercetin hexoside (isomer 1)	–	–	0.25–6.25	$y = 2 \times 10^6 x - 3.8 \times 10^4$	0.9983	97 ± 2
Quercetin rhamnoside	–	–	0.25–6.25	$y = 2 \times 10^6 x - 3.8 \times 10^4$	0.9983	36 ± 2
Quercetin glucuronide	0.255	0.849	0.25–6.25	$y = 2 \times 10^6 x - 3.8 \times 10^4$	0.9983	124 ± 12
Quercetin hexoside (isomer 2)	–	–	0.25–6.25	$y = 2 \times 10^6 x - 3.8 \times 10^4$	0.9983	62 ± 2
Ellagic acid	–	–	0.5–12.5	$y = 3.6 \times 10^5 x + 2 \times 10^4$	0.991	794 ± 86
Quercetin	0.207	0.690	0.25–10	$y = 2 \times 10^6 x - 2.4 \times 10^5$	0.9992	59 ± 19

3.3. Total Phenolic and Flavan-3-ol Contents and in Vitro Antioxidant Activities Grape Seed Extract

As a previous step to the measurement of the antioxidant activity, the total phenolic and flavan-3-ol contents of the grape seed and cocoa extracts were quantified using the Folin–Ciocalteu method and Vanillin assay, respectively. Since these methods have a weak accuracy, they are widely used as an approximate method to semiquantitate phenolic compounds in plant extracts. The obtained values for each assay are shown in Table 3. On the basis of the dry weight, the total phenolic content in grape seed extract was 964 ± 82 mg GAE g^{-1} and total flavan-3-ol content was 988 ± 124 mg CE g^{-1}. For cocoa extract, the results were 758 ± 82 mg GAE g^{-1} and 724 ± 121 mg CE g^{-1} for Folin–Ciocalteu and Vanillin assays, respectively.

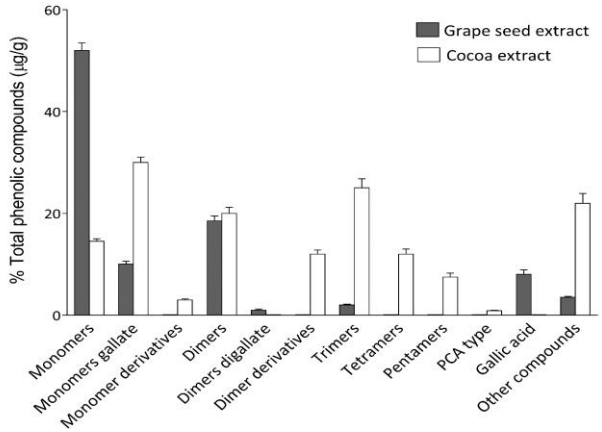

Figure 3. Comparative quantification of main compounds found in cacao and grape seed extracts. Quantitation values are expressed as µg of analyte per gram of dry extract.

Different in vitro methods were carried out in order to determinate the antioxidant activity of cocoa and grape seed extracts. These properties are primarily due to flavonoids, which can perform scavenging action on free radicals, metal chelating properties, reduction of hydroperoxide formation and their effects on cell signaling pathways and gene expression. The presence of the functional group "–OH" in the structure and its position on the ring of the flavonoid molecule determine the antioxidant capacity. Addition of "–OH" groups to the flavonoid nucleus will enhance the antioxidant activity, while substitution by $–OCH_3$ groups diminishes the antioxidant activity. The antioxidant capacity of procyanidins is, in part, governed by the degree of polymerization. Grape seeds, which have a bigger

content than skin and flesh on high-degree of polymerization procyanidins, show, therefore, the highest antioxidant power [47,48].

Trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) are based on single-electron transfer mechanism. TEAC and FRAP are extensively used to establish the antioxidant activity in food [49] and biological samples [50], respectively. The oxygen radical absorbance capacity (ORAC) assay is performed in order to test the capacity of the extracts to quench peroxyl radicals, i.e., the hydrogen atom transfer ability. ORAC determination has become one of the most widely accepted methods to measure the antioxidant capacity of food, botanical, and biological samples [47].

Table 3 lists the antioxidant capacities of cocoa and grape seed extracts by TEAC, ORAC and FRAP methods. Accordingly, in TEAC and FRAP assays (single-electron transfer-based methods), the values for the grape seed extract were 6.1 ± 0.8 mmol of Trolox equivalents (TE) g^{-1} and 6.5 ± 0.5 mmol of Fe^{2+} equivalents (FE) g^{-1}, respectively. Lower values are found for cocoa extract (4.2 ± 0.1 mmol TE g^{-1} and 4.0 ± 0.2 mmol FE g^{-1}, respectively). Concerning ORAC assay, the values were 8.6 ± 0.7 and 7.0 ± 0.5 mmol TE g^{-1} for grape seed and cocoa extracts, respectively.

The comparison of these results with those of previous researchers is untenable due to differences in the nature of the sample and pre-concentration technologies, extraction systems, and assay methodologies.

Table 3. Values for different antioxidant measurements performed

Assays	Grape Seed	*T. cacao*
Folin-Ciocalteu [a]	964.05 ± 82.29	758.33 ± 82.50
Vanillin assay [b]	987.5 ± 123.7	723.6 ± 121.5
TEAC [c]	6.1 ± 0.8	4.19 ± 0.14
FRAP [d]	6.47 ± 0.47	3.95 ± 0.21
ORAC [c]	8.62 ± 0.73	6.99 ± 0.5

a Expressed in mg Gallic acid equivalents/g extract (dw)

b Expressed in mg (+)-Catechin equivalents/g extract (dw)

c Expressed in mmol Trolox equivalents/g extract (dw)

d Expressed in mmol FeSO4 equivalents/g extract (dw)

By comparing all of our assays (which were made in parallel under the same conditions), grape seed extract showed high values of antioxidant activities and total phenolic and flavan-3-ol contents than cocoa extract. This could be a result of the higher content in flavan-3-ol, mainly the oligomeric forms, as well as the higher content in gallic acid in grape seed extract.

3.4. Anti-Inflammatory Activity of Grape Seed and Cocoa Extracts in HUVEC

As expected, the mRNA expression of MCP-1 (a proinflammatory cytokine) is decreased when a concentration more than 50 and 60 mg/mL of cocoa and grape seed extracts, respectively, are used (Figure 4). As observed, cocoa extract has a better response to inflammatory scenarios than grape seed extract. Although antioxidant and anti-inflammatory activities are generally related, we found that, in our case, the anti-inflammatory properties of proanthocyanins are not proportional to the observed antioxidant activity. The fact that polymeric proanthocyanins were found in cocoa extract but not in grape seed extract, could explain this effect, since the anti-inflammatory potential of proanthocyanins with high-degree of polymerization inhibits NF-κB activation and the secretion of eicosanoids and pro-inflammatory cytokines [51]. These results also agree with the activation of IL-4 secretion (an anti-inflammatory cytokine) [52] and the decreased levels on IL-8 (a pro-inflammatory cytokine) [55] by proanthocyanins with a high degree of polymerization.

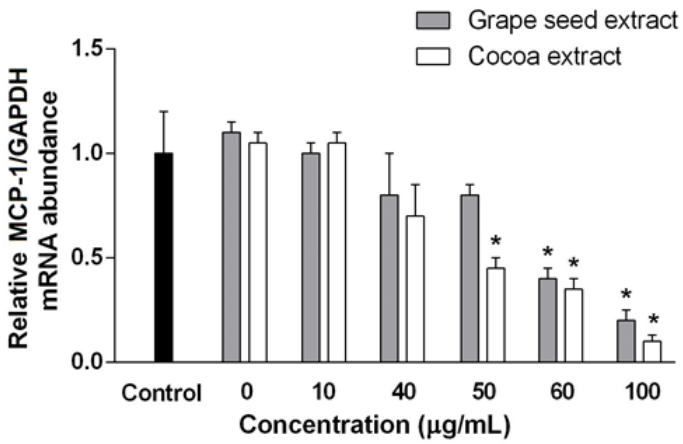

Figure 4. Effect of grape seed and cocoa extracts on production of relative MCP-1 mRNA levels in HUVEC. *mRNA levels of MCP-1 were normalized using mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

4. CONCLUSIONS

In the present work, HPLC-ESI-QTOF-MS has been confirmed to be a powerful analytical technique for separating and detecting phenolic and other polar compounds in a concentrated grape seed extract. With this methodology, 36 compounds were tentatively identified on the basis of their chromatographic retention, MS data, and MS/MS fragmentation pattern, and 30 compounds of them have been quantified. The most representative groups of compounds tentatively identified and quantified were proanthocyanidins (mainly monomers, dimers and galloyl derivatives). Besides these compounds, phloretin and its derivatives have been tentatively identified for the first time in *V. vinifera* seeds. These compounds have been reported to show several activities against diseases, i.e., antitumor effects.

Grape seed and cocoa extracts possess a significant antioxidant capacity to reduce peroxyl radicals by ORAC assay. Moreover, grape seed extract shows a stronger capacity to donate electrons by FRAP and TEAC assays, and a higher phenolic and flavan-3-ol contents. Finally, cocoa extract seems to have a better potential decreasing the expression of MCP-1, and therefore to prevent inflammation than grape seed extract due to its content on proanthocyanidins with high-degree of polymerization.

This work provides a better understanding of industrial byproduct from the winemaking process such as seeds. The importance of knowledge concerning this byproduct composition and activities is increasing due to its cheap source for the extraction of antioxidant compounds.

5. ACKNOWLEDGMENTS

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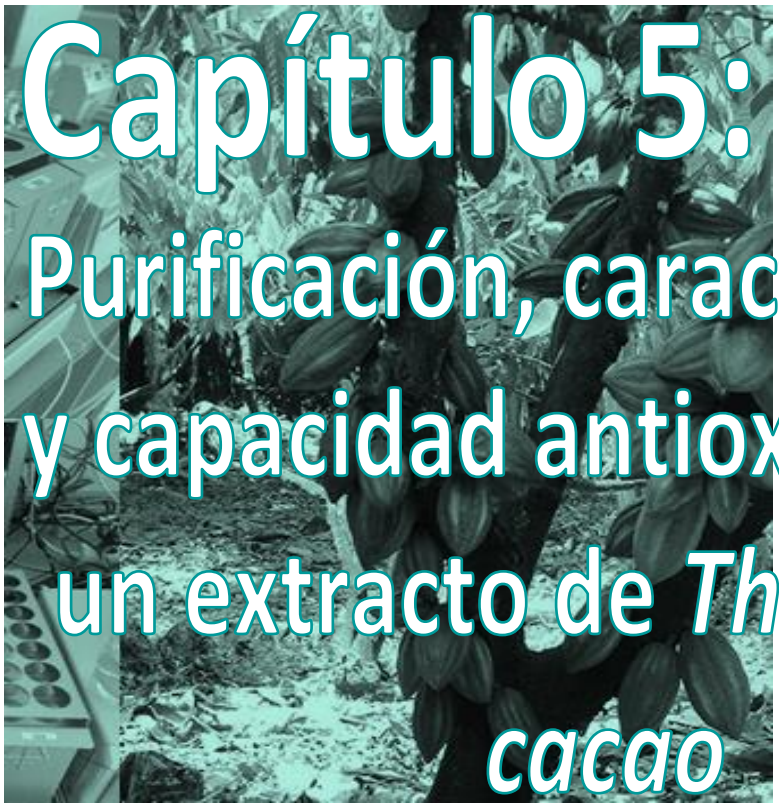
micrOTOF-Q II



Capítulo 5:

Purificación, caracterización y capacidad antioxidante de un extracto de *Theobroma cacao*

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Isolation, comprehensive characterization and antioxidant activities of *Theobroma cacao* extract

ABSTRACT

Cocoa (*Theobroma cacao*) is a major, economically important, international crop and has been associated with several nutritional benefits including high antioxidant capacity. The aim of the present study was to isolate, characterize and quantify phenolic compounds of *T. cacao* extract using HPLC-MSESI-QTOF. A total of 61 compounds were identified and quantified in the *T. cacao* extract and fractions belonging to various structural classes such as flavan-3-ol and derivatives (including procyanidins), flavonols, *N*-phenylpropenoyl-L-amino acids, and other compounds. These compounds were isolated by semi-preparative HPLC. Afterwards, the composition of each fraction was established by the detailed HPLC-DAD and HPLC-MSESI-QTOF method. The relationship between the chemical structure of flavonoids and their radical-scavenging activities was analyzed. In addition, *T. cacao* extract and fractions show antioxidant activity (by TEAC, FRAP and ORAC methods) decreasing the generation of reactive oxygen species.

1. INTRODUCTION

Cocoa, a product derived from the beans of the *Theobroma cacao* tree, consumed by pre-Columbian American civilizations, was introduced to Europe by the Spaniards in the 16th century (Lanaud, Motamayor, & Sounigo, 2003). In the last two decades, the food industry has developed new cocoa-based products, e.g. cocoa liquor, cocoa powder, and chocolate, which are consumed worldwide and used as common ingredients of many food products.The cocoa market has remained stable over the last few years (Ellam & Williamson, 2013), and scientific interest in this potential source of bioactive compounds is growing. Indeed, a large number of studies support the health benefits of cocoa consumption (Ellam & Williamson, 2013; Ramiro-Puig & Castell, 2009; Smith, 2013), being attributed mainly to the flavanol content (Payne, Hurst, Miller, Rank, & Stuart, 2010; Quiñones, Sánchez, Muguerza, Miguel, & Aleixandre, 2011; Ramiro-Puig & Castell, 2009; Smith, 2013). In this respect, the European Food Safety Authority (EFSA) recently issued a positive scientific statement on cocoa flavanols, ascribing beneficial effects primarily to the maintenance of normal endothelium-dependent vasodilation (European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies, 2012). Since chocolate contains additional calories, sugar, and fats (Ellam & Williamson, 2013), raw and highflavanol cocoa powder could receive rapid approval by consumers as well as by legal food authorities defining it as a functional food ingredient.

Cocoa flavanols consist of monomeric (+)-catechin and (−)-epicatechin, and oligomeric flavanols (procyanidins) ranging from dimers to decamers. Concerning the interflavanoid linkage (IFL) nature, B-type procyanidins [C-4 (upper unit)→C-6 or C-8 (lower unit)] are more abundant than A-type procyanidins, which present an additional ether-type bond [C-2 (upper unit)→*O*→C-5 or C-7 (lower unit)], as well as IFL can be either α or β type (Fig. 1). This list of combinations together with the occurrence of glycosylated and methylated derivatives explains the high diversity of this family and the wide range of biological and biochemical activities in plants (He, Pan, Shi, & Duan, 2008). In addition, processing could lead to the formation of the diasteroisomers (+)-epicatechin and (−)-

catechin (Payne et al., 2010). On the other hand, theobromine, a methylxanthine alkaloid, minor amounts of quercetin derivatives and the less known *N*-phenylpropenoyl-L-amino acids have also been reported (Andres-Lacueva et al., 2008; Ortega et al., 2008; Sanbongi et al., 1998; Stark & Hofmann, 2005; Tomas-Barberán et al., 2007), and thus their contribution to the beneficial effects of cocoa should not be ruled out (Ellam & Williamson, 2013; Zeng et al., 2011).

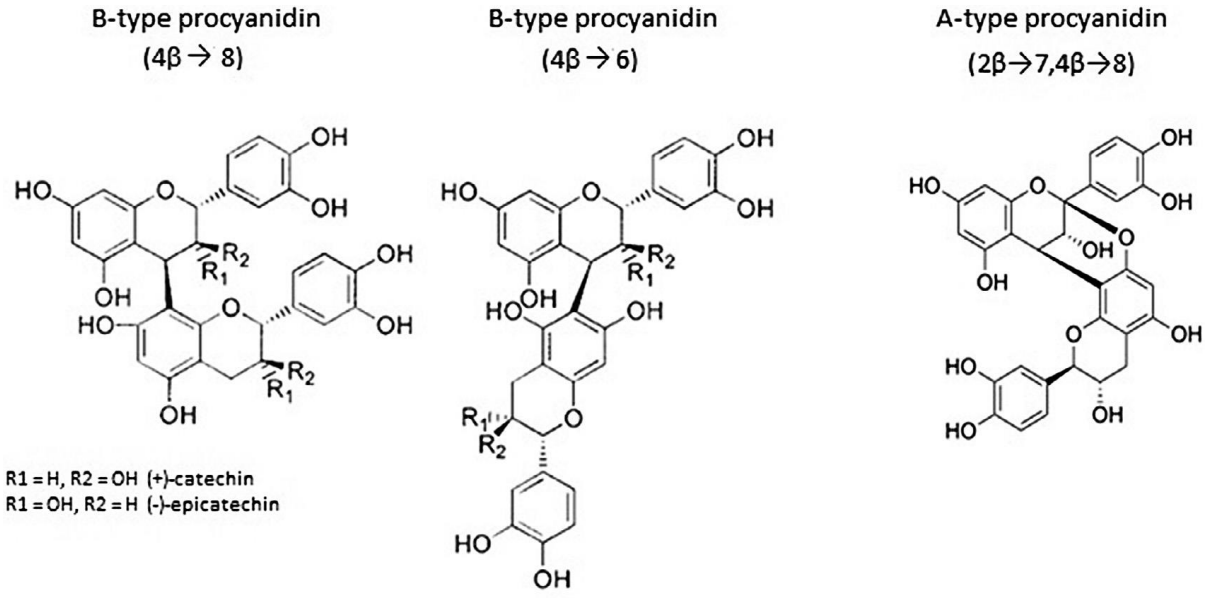


Figure 1. Structures of procyanidin (4β→8) and (4β→6)-dimers (B-type) and (2β→7, 4β→8)-dimer (A-type).

Although the molecular mechanism of these compounds in relation to many diseases could have different cellular targets, the bioactivity of the cocoa polyphenols could be related to different properties, mainly antioxidant activities linked to their chemical structure (Ramiro-Puig & Castell, 2009; Smith, 2013). *In vitro* antioxidant activity of foods and plants is generally studied by ABTS•+ and DPPH• based methods (Wojdylo, Oszmianski, & Czemerys, 2007), but these radicals are not as biologically relevant as peroxyl radicals are. In this case, the oxygen radical absorbance capacity (ORAC) assay could be used. Regarding the key bioactives, the capacity of polyphenols to inhibit free radicals is governed by their chemical structure. For example, the presence of a catechol group in the

B-ring may trap radicals as well as chelating metals (Heim, Tagliaferro, & Bobilya, 2002), and the degree of polymerization of flavanols could also be important (Counet & Collin, 2003; De Gaulejac, Provost, & Vivas, 1999).

Because it is difficult to isolate large amounts of cocoa polyphenols and because there is a lack of commercial standards of procyanidins, almost all *in vitro* and *in vivo* studies make use of whole cocoa matrices. The analytical methodologies applied to purify cocoa bioactives involve laborious pretreatment together with isolation and purification procedures (Hatano et al., 2002; Ortega et al., 2008; Stark & Hofmann, 2005). Among these, isolation by semi-preparative and preparative liquid chromatography (LC) with C18 reversed phase (RP) offers high versatility to separate a wide range of nitrogenous and nonnitrogenous bioactive compounds (Contreras, Carrón, Montero, Ramos, & Recio, 2009; Rzeppa, Von Bargen, Bittner, & Humpf, 2011; Stark & Hofmann, 2005).

The usual technique to analyze polyphenols from cocoa multicomponent extracts or a specific isolated fraction is reversedphase high-pressure liquid chromatography (RP-HPLC) with C8 (Srdjenovic, Djordjevic-Milic, Grujic, Injac, & Lepojevic, 2008), C12 (Pereira-Caro et al., 2013) and C18 (Andres-Lacueva et al., 2008; Calderón, Wright, Hurst, & Van Breemen, 2009; Quiñones et al., 2011; Tomas-Barberán et al., 2007) stationary phase. As solvent system, all of these authors used linear gradients with acidified water (using formic or acetic acid) and acetonitrile or methanol as organic solvent. This separation technique has been coupled to different detectors for the qualitative and quantitative characterization of these compounds, such as ultraviolet and diode-array detection (DAD) (Quiñones et al., 2011; Srdjenovic et al., 2008), fluorescence (Payne et al., 2010; Pereira-Caro et al., 2013) and/or mass spectrometry (MS) (Andres-Lacueva et al., 2008; Ortega et al., 2010; Pereira-Caro et al., 2013; Tomas-Barberán et al., 2007). Other stationary phases have also been reported (Ortega et al., 2010; Payne et al., 2010; Pereira-Caro et al., 2013).

Coupling HPLC with mass spectrometry (MS) further offers a potent analytical alternative to the untargeted characterization of polyphenol composition in plant extracts with high confidence (Abu-

Reidah, Contreras, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2013; Cádiz-Gurrea, Fernández-Arroyo, Joven, & Segura-Carretero, 2013; Iswaldi et al., 2013).

Thus, the objectives of the present study were to: (1) characterize the phenolic composition of a commercial cocoa powder extract by HPLC-MSESI-QTOF, (2) fractionate this extract by semi-preparative HPLC and characterize the obtained fractions, and (3) evaluate the antioxidant capacity of the whole extract and each fraction by three complementary antioxidant activity methods: Trolox equivalent antioxidant capacity (TEAC), ferric-reducing ability power (FRAP), and oxygen radical absorbance capacity (ORAC). This will provide a better understanding of the relationship between antioxidant activity and chemical structure of cocoa polyphenols.

2. MATERIAL AND METHODS

2.1. Chemicals

All chemicals were of HPLC-MS grade and used as received. Acetic acid and methanol for HPLC were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Dimethyl sulfoxide (DMSO) was purchased from Panreac (Barcelona, Spain).Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). The reagents used to measure the antioxidant capacity, AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride), TPTZ (2,4,6-tripyridyl-S-triazine), ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate)], Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein, potassium persulfate, and ferric sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dehydrated sodium phosphate, trihydrated sodium acetate, sodium acetate, ferric chloride, and hydrochloric acid were obtained from Panreac (Barcelona, Spain). The standards, for the calibration curves, procyanidin B2, procyanidin A2, (+)-catechin, (epi)-gallocatechin, quercetin were purchase either from Fluka, Sigma-Aldrich (Steinheim, Germany) or Extrasynthese (Genay Cedex, France).

2.2. Sample preparation

A concentrated *T. cacao* extract was used in this study (Molteoleder, Spain).The polyphenols from whole cocoa matrix were analytically characterized using a solution of cocoa extract of 10 mg/mL. Briefly, 10 mg of cocoa extract were dissolved in 1 mL of DMSO. The sample was sonicated for 5 min, vortexed for 1 min, and then centrifuged for 5 min at 7700 *g* and filtered through a 0.25 mm filter before the HPLC analysis.

For purification of polyphenols from cacao extract, a solution stock of 75 mg/mL was prepared by dissolving the appropriate amount of cacao extract in DMSO. The sample was sonicated for 5 min, vortexed for 1 min and then was centrifuged for 5 min at 7700 *g*.The solution stock was filtered through a 0.25 mm filter before the preparative HPLC analysis.

2.3. Instrumentation

The polyphenols from the *T. cacao* extract were fractionated using a Gilson preparative HPLC system (Gilson Inc., Middleton, WI, USA) equipped with a binary pump (model 331/332), automated liquid handling solutions (model GX-271) and UV-Vis detector (model UV-Vis 156).

T. cacao and isolated fractions were analytically characterized using an Agilent 1200 series rapid-resolution LC system (AgilentTechnologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode-array detector (DAD). The HPLC system was coupled to a quadrupole time-of-flight (QTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface (model G1607A from Agilent Technologies, Palo Alto, CA). Fluorescence (ORAC) and absorbance (FRAP and TEAC) measures were carried out on a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader (Bio-Tek Instruments Inc.,Winooski, VT, USA) by using 96-well polystyrene microplates.

2.4. Fractionation of polyphenols from *T. cacao* extract

The compounds from *T. cacao* were fractionated at room temperature. To separate the target compounds, an Ascentis C18 column (10 µm, 250 × 212 mm) was used. The mobile phases consisted of acetic acid 0.5% (A) and methanol (B). The following multi-step linear gradient was applied: 0 min, 0% B; 10 min, 20% B; 15 min, 25% B; 25 min, 35% B; 35 min, 39% B; 70 min, 60% B; 75 min, 70% B; 78 min, 80% B; 80 min, 100% B; 82 min, 0% B. The initial conditions were held for 15 min. The injection volume was 1 mL.The flow rate used was set at 15 mL/min. The compounds separated were monitored with UV-Vis (220–280 nm). Fraction-collection step consisted of UV-based purification, determining the elution time window for collecting each fraction. Finally, a total of 12 fractions were collected and the solvent was evaporated under vacuum. The residue of each fraction was weighted and dissolved with an appropriate volume of DMSO at concentration level of 100 µg/mL. Finally, all fractions were filtered through a 0.25 µm filter before the HPLC analysis.

2.5. Chromatographic and UV conditions

The compounds from the *T. cacao* and fractions were separated at room temperature using a Zorbax Eclipse Plus C18 column (1.8 µm, 150 × 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA). The mobile phases consisted of acetic acid 0.5% (A) and methanol (B).The following multi-step linear gradient was applied: 0 min, 0% B; 5 min, 25% B; 15 min, 35% B; 20 min, 39% B; 38 min, 60% B; 40 min, 70% B; 42 min, 80% B; 44 min, 100% B; 46 min, 0% B; 48 min, 0% B. The initial conditions were held for 10 min.The injection volume was 10 µL.The flow rate used was set at 0.3 mL/min. The DAD coupled to the HPLC system was set in spectrum range starting at 190 nm and ending at 950 nm.

2.6. MSESI-QTOF detection

The HPLC system was coupled to a QTOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +3.5 kV. The other optimum values of the source

parameters were: drying gas temperature, 220 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 2.5 bar.

The detection was performed considering a mass range of 50–1200 *m/z*. The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonics), which provided a list of possible elemental formulas using Generate Molecular Formula Editor. This uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ringplus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (σ value) for increased confidence in the suggested molecular formula (Bruker Daltonics Technical Note 008, 2004). The widely accepted accuracy threshold for the confirmation of elemental compositions was established at 5 ppm (Bringmann et al., 2005). Even with very high mass accuracy (<3 ppm in most of the cases), many chemically possible formulae were determined depending on the mass regions considered.

Therefore, high mass accuracy alone is not sufficient to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns as a single further constraint removes >95% of the false candidates. This orthogonal filter can reduce several thousand candidates to only a small number of molecular formulas.

During the development of the HPLC method, external instrument calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, with a sodium acetate cluster solution passing through containing 5 mM sodium hydroxide and 0.2% acetic acid in water/isopropanol (1:1, v/v).

The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to the compound identification.

2.7. Antioxidant capacity assays

The TEAC assay, which measures the reduction of the radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by antioxidants, was performed by using a previously described method (Cádiz-Gurrea et al., 2013; Laporta, Pérez-Fons, Mallavia, Caturla, & Micol, 2007) using Trolox as standard and measuring the absorbance at 734 nm. The FRAP assay was carried out following the method described by Benzie and Strain (1996) and Cádiz-Gurrea et al. (2013). FRAP values were calculated using FeSO4·7H2O as a standard and measuring the absorbance at 593 nm. To assay the capacity of the extracts to scavenge peroxyl radicals, a validated ORAC method was used (Ou, Hampsch-Woodill, & Prior, 2001) with the modifications developed by Laporta et al. (2007) and Cádiz-Gurrea et al. (2013). For ORAC assay, Trolox was used as standard and fluorescence at 494/521 nm (excitation and emission wavelengths respectively) was measured. The final ORAC values were calculated by using a lineal regression equation between the Trolox concentration and the net area of the fluorescence decay curve (area under curve, AUC), as previously described in Laporta et al. (2007).

3. RESULTS AND DISCUSSION

3.1. Characterization and quantification of *T. cacao* by HPLC-MSESI-QTOF

The base peak chromatogram of the *T. cocoa* extract obtained by HPLC-MSESI-QTOF is shown in Fig. 2.The compounds characterized are presented in Table 1, numbered according to their elution order. This table includes the retention time, experimental *m/z*, MS/MS fragments, molecular formulas, errors and σ values for all of the compounds detected in the samples analyzed. All the compounds were characterized by the interpretation of their mass spectra determined by the QTOF mass analyzer while taking into account the information provided by the literature and databases.

In order to quantify the amount of phenolic compounds in *T. cacao* extract (Table 1), five calibration curves were prepared with the five standards available: procyanidin B2, procyanidin A2, (+)-catechin, (epi)-gallocatechin and quercetin. In all cases, the linearity was better than 0.99. The other

compounds, for which no commercial standards were available, were tentatively quantified on the basis of the other compounds bearing similar structures. Oligomeric procyanidins, catechin derivatives and quercetin derivatives were quantified with procyanidin B2, (+)-catechin and quercetin, respectively.

In the present study, the compounds were classified intofour groups, according to their family: flavan-3-ol and derivatives (including procyanidins), flavonols, *N*-phenylpropenoyl- L-amino acids, and other compounds.

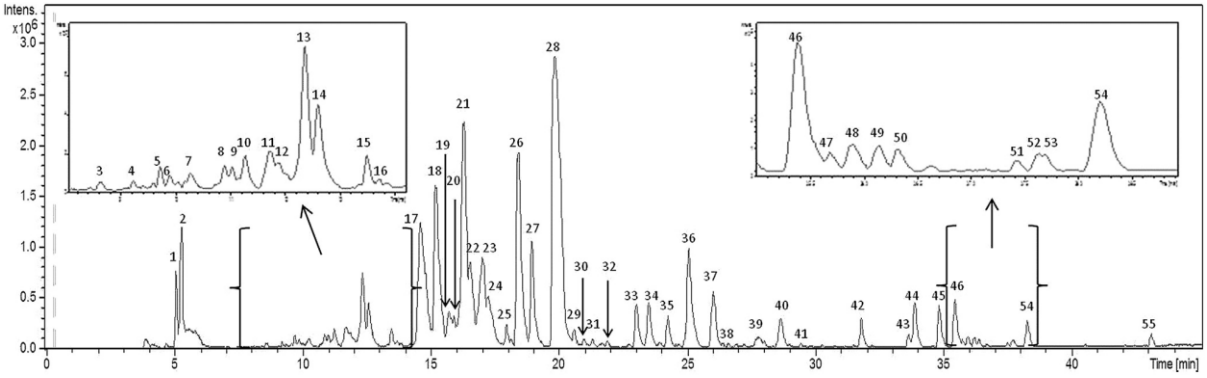


Figure 2. Base peak chromatogram of *Theobroma cacao* extract. Peak numbers correspond to those of Table 1.

3.2. Flavan-3-ols and derivatives

The high-resolution mass-spectrometry method used in this study enabled the characterization of a total of 33 flavan-3-ol derivatives. According to their chemical structures, this group may be divided into monomeric forms, A-type, and B-type oligomeric forms.

3.2.1. Monomeric forms and derivatives

Among these, several compounds were characterized as isomers of (epi)gallocatechin, compounds 25 and 26 at *m/z* 305, and (epi)catechin, peak 28 at *m/z* 289. The MS2 spectra generated by the isomers of (epi)gallocatechin were characterized by the product ion at *m/z* 289, which was assignable to an (epi)catechin moiety generated by the loss of oxygen. In the case of isomers of (epi)catechin,

deprotonated molecules were yielded at *m/z* 245 in MS2 spectra, whereas the difference represented the loss of CO2, as described by Pérez-Magariño, Revilla, González-SanJosé, and Beltrán (1999).

Compounds 15 and 16 showed an [M-H]− at *m/z* 451. They were identified as glycosylated conjugates of (epi)catechin, since the main product ion in MS2 was at *m/z* 289, corresponding to the loss of hexose [(M-H) – 162 amu (C6H10O5]−. In the same manner, compound 31 (*m/z* 593) was tentatively identified as (epi)catechin diglycosylated derivative. Indeed, the major fragment ion at *m/z* 289 revealed two consecutive losses of hexose moieties.

Moreover, compound 47 was tentatively proposed as (epi)catechin derivative (*m/z* 427) because the MS2 spectra had a major fragment ion at *m/z* 289 ((epi)catechin), but the structure could not be completely established.

On the other hand, compound 48 with *m/z* value at 451 had a fragmentation pattern in accordance with Beltrame, Filho, Barros, Cortez, and Cass (2006). Thus, this compound was characterized as the flavalignan (phenylpropanoid-substituted (epi)catechin) cinchonain I.

Monomeric flavan-3-ols have been described in different cocoa sources and products (Ortega et al., 2008; Pereira-Caro et al., 2013; Porter, Ma, & Chan, 1991). Although epicatechin 8-*C*-β-D-galactopyranoside has previously been reported in cocoa liquor (Hatano et al., 2002), the fragmentation pattern of the latter compounds in our MS conditions is typical of flavonoid *O*-glycosides. Thus, this study is the first available to report the occurrence of glycosylated forms of (epi)catechin and cinchonain I in cocoa.

3.2.2. A-type oligomeric forms and derivatives

A-type oligomers are characterized by the existence of a doubly interflavanoid linkage. In this way, compound 49 with *m/z* 575 was tentatively identified as A-type proanthocyanidin dimer (Hatano et al., 2002; Porter et al., 1991). In MS2, the main ions were at *m/z* 289, [(epi)catechin-H]–, and 285, [(epi)catechin-2H2-H]–, both generated by the cleavage at the interflavanoid bonds.

Table 1 – Retention time, mass spectral data and quantification (mean ± SD; n = 3) of the compounds characterized in *T. cacao* extract and fractions by HPLC-MSESI-QTOF and MS/MS in negative mode.

Peak/ compound	Proposed compound	RT (min)	Ion	Measured m/z	Calculated m/z	Error (ppm)	mSigma	Fragmentation pattern	Molecular Formula	Quantification (g analyte/kg extract)	Fractions
1	Sucrose (isomer 1)	5.11	$[M-H]^-$	341.1099	341.1089	2.9	3.5	Non fragmented	$C_{12}H_{22}O_{11}$	nq	F1
2	Sucrose (isomer 2)	5.31	$[M-H]^-$	341.1107	341.1089	5.3	6.9	Non fragmented	$C_{12}H_{22}O_{11}$	nq	F1
3	Unknown	8.62	$[M-H]^-$	265.0936	265.0929	2.6	24.6	187.1429	$C_{10}H_{18}O_8$	nq	F1
4	Tri-O-methylsucrose	9.23	$[M-H]^-$	383.1563	383.1559	1.2	18.7	Non fragmented	$C_{15}H_{28}O_{11}$	nq	F1
5	Unknown	9.71	$[M-H]^-$	442.156	442.1566	1.4	3.6	395.1454	$C_{16}H_{29}NO_{13}$	nq	F1
6	(Epi)catechin tetramer (isomer 1)	9.88	$[M-2H]^{2-}$	576.1258	576.1273	2.6	21.6	407.0789; 289.0737	$C_{60}H_{50}O_{24}$	0.0689 ± 0.0006	nd
7	Procyanidin C (isomer 1)	10.26	$[M-H]^-$	865.1983	865.1985	0.3	19.3	577.1142; 289.0756	$C_{45}H_{38}O_{18}$	0.0334 ± 0.0002	F1
8	Procyanidin B (isomer 1)	10.88	$[M-H]^-$	577.1342	577.1351	1.6	29.7	451.1243; 425.0750; 289.0756	$C_{30}H_{26}O_{12}$	0.1454 ± 0.0068	F1
9	Procyanidin C (isomer 2)	11.02	$[M-H]^-$	865.1981	865.1985	0.5	14.8	577.1337; 432.0929	$C_{45}H_{38}O_{18}$	0.1029 ± 0.0059	F2
10	Procyanidin B (isomer 2)	11.27	$[M-H]^-$	577.1339	577.1351	2.2	32.2	289.0756	$C_{30}H_{26}O_{12}$	0.2323 ± 0.0070	F1
11	(Epi)catechin tetramer (isomer 2)	11.72	$[M-2H]^{2-}$	576.1257	576.1273	2.9	30.9	451.1231; 432.0928; 289.0755	$C_{60}H_{50}O_{24}$	0.2912 ± 0.0075	F2
12	Procyanidin C (isomer 3)	11.87	$[M-H]^-$	865.1959	865.1985	1	19.7	577.1139; 451.1227; 433.0716; 289.0647	$C_{45}H_{38}O_{18}$	0.2344 ± 0.0041	F1
13	Procyanidin C (isomer 4)	12.36	$[M-H]^-$	865.201	865.1985	2.9	22.7	577.1291; 432.0952	$C_{45}H_{38}O_{18}$	1.0450 ± 0.1236	F2
14	(Epi)catechin tetramer (isomer 3)	12.59	$[M-2H]^{2-}$	576.126	576.1273	2.3	51.1	432.0926	$C_{60}H_{50}O_{24}$	0.6758 ± 0.0087	F2, F4
15	(Epi)catechin glucopyranoside (isomer 1)	13.48	$[M-H]^-$	451.1244	451.1246	0.5	20	289.0783	$C_{21}H_{24}O_{11}$	0.7670 ± 0.0086	F2
16	(Epi)catechin glucopyranoside (isomer 2)	13.7	$[M-H]^-$	451.1236	451.1246	2.2	9.3	433.1111; 289.0753	$C_{21}H_{24}O_{11}$	0.0072 ± 0.0008	F2
17	Procyanidin B (isomer 3)	14.62	$[M-H]^-$	577.1367	577.1351	2.8	50.7	289.072	$C_{30}H_{26}O_{12}$	3.3487 ± 0.1534	F2, F3, F4, F5, F6, F7, F8, F9
18	N-caffeoyl-L-aspartate	15.19	$[M-H]^-$	294.0631	294.0619	4.1	5.3	179.0283	$C_{13}H_{13}NO_7$	nq	F1
19	(Epi)catechin tetramer (isomer 4)	15.71	$[M-2H]^{2-}$	576.126	576.1273	2.4	24.6	432.0922; 289.0643	$C_{60}H_{50}O_{24}$	0.4338 ± 0.0060	F5
20	Procyanidin C (isomer 5)	15.91	$[M-H]^-$	865.2002	865.1985	1.9	15.6	577.1335; 432.0953; 289.0751	$C_{45}H_{38}O_{18}$	0.3456 ± 0.0045	F5
21	Procyanidin C (isomer 6)	16.28	$[M-H]^-$	865.2022	865.1985	4.2	46.5	432.0951; 289.0751	$C_{45}H_{38}O_{18}$	4.5791 ± 0.0097	F2, F3, F4, F5
22	(Epi)catechin tetramer (isomer 5)	16.53	$[M-2H]^{2-}$	576.1256	576.1273	2.9	16	432.0921; 289.0751	$C_{60}H_{50}O_{24}$	1.4205 ± 0.1184	F3, F4, F5
23	(Epi)catechin pentamer (isomer 1)	17.01	$[M-2H]^{2-}$	720.1586	720.159	–	–	577.1283	$C_{75}H_{62}O_{30}$	1.9381 ± 0.1144	F4, F5
24	(Epi)catechin hexamer	17.23	$[M-2H]^{2-}$	864.1911	864.1907	–	–	720.1582; 577.1283	$C_{90}H_{74}O_{36}$	1.1402 ± 0.1276	nd
25	(Epi)gallocatechin (isomer 1)	17.95	$[M-H]^-$	305.0707	305.0667	9	28.9	289.075	$C_{15}H_{14}O_7$	3.4995 ± 0.1653	F2, F3
26	(Epi)gallocatechin (isomer 2)	18.42	$[M-H]^-$	305.0717	305.0667	12.8	28.2	289.0749	$C_{15}H_{14}O_7$	5.4829 ± 0.1019	F2, F3
27	L-Aspartic acid. N-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]	18.94	$[M-H]^-$	278.0681	278.067	4	17.5	Non fragmented	$C_{13}H_{13}NO_6$	nq	nd
28	(Epi)Catechin (isomer 2)	19.84	$[M-H]^-$	289.0747	289.0718	10.2	35	245.0824	$C_{15}H_{14}O_6$	4.2031 ± 0.5863	F2, F3, F4, F5, F6, F7, F8, F9

Table 1 – (*continued*)

Peak/ compound	Proposed compound	RT (min)	Ion	Measured m/z	Calculated m/z	Error (ppm)	mSigma	Fragmentation pattern	Molecular Formula	Quantification (g analyte/kg extract)	Fractions
29	L-Aspartic acid. *N*-[3-(4-hydroxy-3-methoxyphenyl)-1-oxo-2-propenyl]	20.58	$[M-H]^-$	308.0787	308.0776	3.6	9.9	Non fragmented	$C_{14}H_{15}NO_7$	nq	nd
30	Procyanidin B (isomer 5)	20.96	$[M-H]^-$	577.1349	577.1351	0.4	14.3	289.0726	$C_{30}H_{26}O_{12}$	0.1166 ± 0.0008	F5, F6
31	Catechin diglucopyranoside	21.87	$[M-H]^-$	593.1507	593.1512	0.8	6.6	451.1053; 289.0715	$C_{27}H_{30}O_{15}$	0.2172 ± 0.0030	F5
32	trans-Clovamide (*N*-[(2E)-3-(3.4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-3-hydroxy-L-tyrosine)	23.01	$[M-H]^-$	358.0944	358.0932	3.3	17.9	178.0501	$C_{18}H_{17}NO_7$	nq	nd
33	(Epi)catechin dimer hexose	23.47	$[M-H]^-$	737.1744	737.1723	2.8	7.9	289.0744	$C_{36}H_{34}O_{17}$	3.1234 ± 0.2651	F5, F6, F7, F8
34	Arabinopyranosyl-(epi)catechin-(epi)catechin (isomer 2)	24.23	$[M-H]^-$	707.164	707.1618	3.1	5.1	289.0743	$C_{35}H_{32}O_{16}$	0.4195 ± 0.0309	F5, F6, F7, F8
35	Procyanidin B (isomer 6)	25.05	$[M-H]^-$	577.1362	577.1351	1.8	26.2	289.0742	$C_{30}H_{26}O_{12}$	1.8376 ± 0.0689	F5, F6, F7, F8, F10
36	Procyanidin C (isomer 7)	25.99	$[M-H]^-$	865.1997	865.1985	1.3	32.1	432.0948	$C_{45}H_{38}O_{18}$	0.9044 ± 0.0531	F7, F8, F9
37	Proanthocyanidin (Type A) (isomer 1)	26.57	$[M-H]^-$	591.1494	591.1508	2.4	9.6	439.1005; 289.0737	$C_{31}H_{28}O_{12}$	0.4142 ± 0.0002	F7
38	(Epi)catechin pentamer (isomer 2)	27.73	$[M-2H]^{2-}$	720.1583	720.159	–	–	575.1197;451.1030	$C_{75}H_{62}O_{30}$	0.2216 ± 0.0198	F7
39	(Epi)catechin tetramer (isomer 6)	28.61	$[M-2H]^{2-}$	576.1257	576.1273	2.8	29.6	437.2017; 245.0926	$C_{60}H_{50}O_{24}$	0.4788 ± 0.0244	F9, F10
40	Quercetin glucuronide	29.4	$[M-H]^-$	477.067	477.0675	1	14.8	431.0979	$C_{21}H_{18}O_{13}$	0.8584 ± 0.0611	nd
41	Deoxyclovamide (*N*-[(2E)-3-(3.4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]-L-tyrosine)	31.73	$[M-H]^-$	326.1045	326.1045	3.4	17.8	282.1137	$C_{18}H_{17}NO_5$	nq	F7, F8, F9
42	Quercetin hexose (isomer 1)	33.57	$[M-H]^-$	463.0879	463.0882	0.7	5	300.0277; 285.0401	$C_{21}H_{20}O_{12}$	0.9891 ± 0.0582	F7, F8, F9, F10
43	Quercetin hexose (isomer 2)	33.84	$[M-H]^-$	463.0882	463.0882	0.1	25	300.0274	$C_{21}H_{20}O_{12}$	1.7070 ± 0.1021	F7, F8, F9
44	Hexenyl xylopyranosyl glucopyranoside (isomer 1)	34.77	$[M-H]^-$	393.177	393.1766	1	22.3	249.1337	$C_{17}H_{30}O_{10}$	nq	F8, F9, F10
45	Quercetin arabinoside	35.38	$[M-H]^-$	433.0788	433.0776	2.6	20.5	300.0276	$C_{20}H_{18}O_{11}$	1.7368 ± 0.1036	F8, F9, F11
46	Hexenyl xylopyranosyl glucopyranoside (isomer 2)	35.9	$[M-H]^-$	393.1769	393.1766	0.8	14.5	249.1357	$C_{17}H_{30}O_{10}$	nq	F9, F10
47	(Epi)catechin derivative	36.15	$[M-H]^-$	427.1611	427.161	0.3	25.6	289.0703	$C_{20}H_{28}O_{10}$	0.3315 ± 0.0007	F10
48	Cinchonain I	36.33	$[M-H]^-$	451.1026	451.1026	1.9	8.4	341.0673; 217.0146	$C_{24}H_{20}O_9$	0.0797 ± 0.0001	F10, F11
49	Procyanidin A (isomer 2)	37.44	$[M-H]^-$	575.1197	575.1195	0.3	18.2	289.0698; 285.0421	$C_{30}H_{24}O_{12}$	0.0330 ± 0.0001	F11
50	Unknown	37.64	$[M-H]^-$	516.2454	516.245	0.8	73.6	Non fragmented	$C_{24}H_{39}NO_{11}$	nq	F9, F10, F11
51	(Epi)catechin methyl dimer (isomer 2)	37.69	$[M-H]^-$	605.1652	605.1664	2	6.6	453.1210; 315.0834; 289.0725	$C_{32}H_{30}O_{12}$	0.2788 ± 0.0035	F12
52	Sweroside	38.19	$[M-H]^-$	357.1227	357.1191	10	25	Non fragmented	$C_{16}H_{22}O_9$	nq	F11, F12
53	Quercetin	43.01	$[M-H]^-$	301.0361	301.0354	2.3	5	Non fragmented	$C_{15}H_{10}O_7$	0.9795 ± 0.0084	F11, F12

nd, compounds not detected in fractions; nq, compounds not quantified.

Compound 37 yielded a deprotonated molecule at *m/z* 591, which was tentatively identified as proanthocyanidin A (Hatano et al., 2002; Natsume et al., 2000). Its MS2 spectra showed fragment ions at *m/z* 439 corresponding to the loss of 152 amu (characteristic fragmentation pathway by retro Diels–Alder reaction, RDA), and at *m/z* 289 corresponding to the (epi)catechin moiety.

Furthermore, glycosylated derivatives were found as compounds 33 (*m/z* 737) and 34 (*m/z* 707). These were tentatively identified as 3T-O-β-D-galactopyranosyl-ent-epicatechin-(2α→7,4α→8)-epicatechin (Calderón et al., 2009; Porter et al., 1991), and arabinopyranosyl-(epi)catechin-(epi)catechin isomer (Hatano et al., 2002; Porter et al., 1991), respectively. Both compounds released (epi)catechin as main fragment.

3.2.3. B-type oligomeric forms and derivatives

B-type procyanidins were the most qualitatively abundant compounds in the extract. The chemical structure of these compounds was based on the presence of (epi)catechin units, which are linked by a single bond. Among these, five dimers (compounds 8, 10, 17, 30, and 35) with [M-H]− ions at *m/z* 577, seven trimers (compounds 7, 9, 12, 13, 20, 21, and 36) with [M-H]− ions at *m/z* 865, six tetramers (compounds 6, 11, 14, 19, 22, and 39) with [M-2H]2– ions at *m/z* 576, two pentamers (compounds 23 and 38) with [M-2H]2– ions at *m/z* 720, and one hexamer (compound 24) with [M-2H]2– ions at *m/z* 864 were characterized in the extract. Although the presence of B-type procyanidins has been reported in cocoa beans and products elsewhere, notably, in our study the number of oligomers up to hexamer is higher than (Cooper et al., 2007; Hatano et al., 2002; Pereira-Caro et al., 2013; Porter et al., 1991; Tomas-Barberán et al., 2007) or comparable to (Ortega et al., 2010) others previously studied. For all of the proposed compounds, the fragmentation pattern was in agreement with the latter studies.The major fragments were generated after the neutral loss of 126 amu ($C_6H_6O_3$, phloroglucinol) from the A ring of an (epi)catechin unit, 152 amu ($C_8H_8O_3$) from RDA fission of the heterocyclic C ring, and sequentially 288 amu ($C_{15}H_{12}O_6$, (epi)catechin − H_2) by cleavages at the interflavanoid bonds (Rockenbach et al., 2012).

In addition, a methylated derivative was also found, compound 51 (*m/z* 605). This compound was tentatively identified as (epi)catechin methyl dimer, corresponding to two (epi)catechin units linked by an ethyl-bridge (Rockenbach et al., 2012). This compound gave fragment ions at *m/z* 453 (formed as RDA reaction product), *m/z* 315 (vinyl-catechin), and *m/z* 289 ((epi)catechin) (Saucier, Guerra, Pianet, Laguerre, & Glories, 1997). These types of compounds were found to be condensed products of (epi)catechin with acetaldehyde and, in fact, acetaldehyde has been described in cocoa beans (Bailey, Mitchell, Bazinet, & Weurman, 1962).

3.3. Flavonols

According to MS and MS/MS data and the HPLC elution profile, five known flavonols were characterized as quercetin at *m/z* 301 (compound 53) and its glucuronide conjugates at *m/z* 477 (compound 40), hexose at *m/z* 463 (compounds 42 and 43) and pentose at *m/z* 433 (compound 45) (Andres-Lacueva et al., 2008; Jalal & Collin, 1977; Ortega et al., 2008; Pereira-Caro et al., 2013; Sanbongi et al., 1998; Tomas-Barberán et al., 2007). In MS2 the major fragments were the even ion at *m/z* 301 and the odd ion at *m/z* 300, which corresponded to quercetin aglycone, according to previous studies (Abu-Reidah et al., 2013; Cádiz-Gurrea et al., 2013). Thus, these major fragments were produced by the respective loss of glucuronyl (C6H8O6, 176 amu), hexosyl, and pentosyl (C5H8O4, 132 amu) groups.

3.4. N-phenylpropenoyl-L-amino acids

Compound 18 had a [M-H]− at *m/z* 294, which MS2 yielded a major fragment at *m/z* 179, [C9H8O4 − H]− (caffeic acid). Thus, it indicated that this compound could be attributed to *N*-caffeoyl-L-aspartic acid (Pereira-Caro et al., 2013; Stark & Hofmann, 2005; Tomas-Barberán et al., 2007).

Compound 27 had a [M-H]− at *m/z* 278. In keeping with the findings of Stark and Hofmann (2005), this compound was tentatively identified as L-aspartic acid, *N*-[3-(4-hydroxyphenyl)- 1-oxo-2-propenyl] (Lechtenberg, Henschel, Liefländer-Wulf, Quandt, & Hensel, 2012). The spectra generated for

compound 29 yielded a [M-H]− at *m/z* 308 and was characterized as L-aspartic acid, N-[3-(4-hydroxy-3-methoxyphenyl)-1-oxo-2-propenyl] (Stark, Justus, & Hofmann, 2006).

On the other hand, compounds 32 and 41 presented deprotonated molecules at *m/z* 358 and 326, respectively, and thus characterized as *N*-coumaroyl-3-hydroxytyrosine (clovamine) and *N*-coumaroyltyrosine (deoxyclovamine), known constituents of cacao beans (Stark & Hofmann, 2005). They yielded major MS/MS fragment at *m/z* 178 [hydroxytyrosine − H2O − H]− and 282 (by the release of CO2), respectively.

3.5. Other compounds

Compounds 1 and 2, at the same *m/z* 341,were tentatively identified as sucrose isomers and compound 4 with *m/z* 383 as Tri-O-methylsucrose. Compounds 44 and 46 had the same deprotonated molecule (at *m/z* 393). These compounds were characterized as hexenylxylopyranosyl glucopyranoside isomers. Both presented a fragment ion at *m/z* 249, corresponding to the loss of 144 amu (C6H8O4, xylosyl plus C). In this sense, the isomer 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside of (*Z*)-3-hexenol has previously been described in tea leaves as aroma precursors (Nishikitani,Wang, Kubota, Kobayashi, & Sugawara, 1999), and the aglycone form in cupuacu (*Theobroma grandiflorum*) (Boulanger & Crouzet, 2000). In the case of compound 52, it was tentatively identified as sweroside (an iridoid glucoside) with *m/z* 357 and previously reported by Li, Li, and Ye (2003). Moreover, there were three compounds (3, 5, and 50) in which it was not possible to elucidate the structure even after the MS/MS.

3.6. Isolation of *T. cacao* by semi-preparative HPLC and characterization of fractions by HPLC-MSESI-QTOF and HPLC-DAD

The compounds in the *T. cocoa* extract were isolated by semipreparative HPLC (Fig. 3). Afterwards, the composition of each fraction was established by the detailed HPLC-MSESI-QTOF and HPLC-DAD method. This complements the characterization of the *T. cocoa* extract. All of these compounds were

correctly separated using the semi-preparative HPLC technique according to their elution order (Table 1).

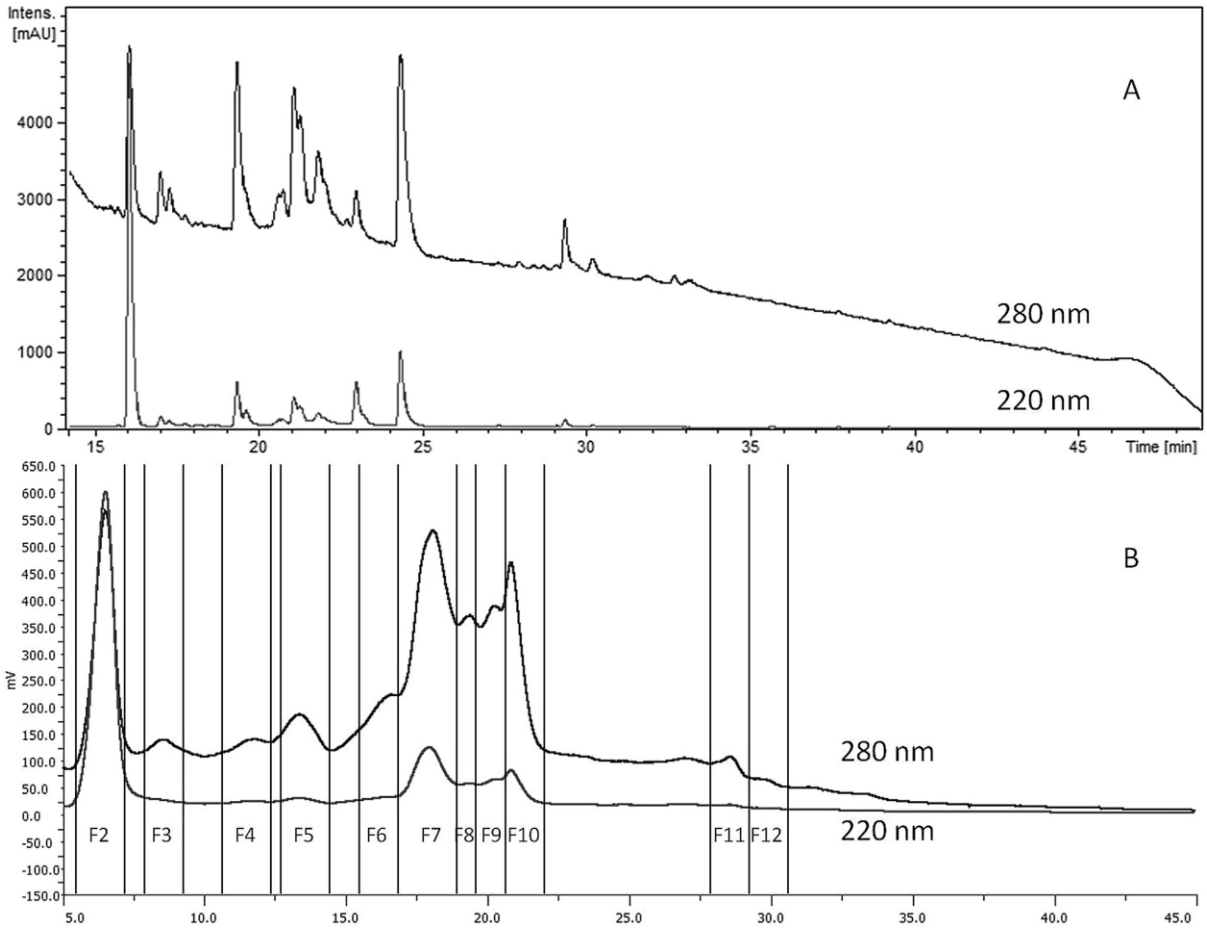


Figure 3. UV-chromatogram of *T. cocoa* extract obtained by (A) analytical HPLC and (B) semi-preparative HPLC indicating the collected fractions.

At least nine new identified compounds were detected only in isolated fractions (Table 2). Most of these compounds were characterized as new isomers of (epi)catechin, procyanidin B, arabinopyranosyl-(epi)catechin-(epi)catechin, procyanidin A, (epi)catechin ethyl dimer, and proanthocyanidin A. Moreover, fraction 1 and 2 yielded a new compound, which had not been detected in the analysis of the complex extract. This compound had a deprotonated molecule at *m/z* 179, and showed two maximum absorptions around 220 and 270 nm (Fig. 4). According to the information provided by QTOF and DAD detector, as well as the literature (Wang, Lu, Miao, Xie, & Yang, 2008), this compound was tentatively characterized as the alkaloid theobromine.

Table 2 – Retention time and mass spectral data of the compounds characterized only in fractions by HPLC-MSESI-QTOF-MS in negative mode.

Proposed compound	RT (min)	Ion	Measured m/z	Calculated m/z	Error (ppm)	mSigma	Molecular Formula	Fractions
Theobromine	15.9	[M-H]-	179.0584	179.0574	5.2	5.6	$C_7H_8N_4O_2$	F1, F2
(Epi)catechin (isomer 1)	19.09	[M-H]-	289.0726	289.0718	2.9	7	$C_{15}H_{14}O_6$	F1
Procyanidin B (isomer 4)	20.31	[M-H]-	577.1302	577.1351	8.7	37.1	$C_{30}H_{26}O_{12}$	F4
Arabinopyranosyl-(epi)catechin-(epi)catechin (isomer 1)	22.34	[M-H]-	707.153	707.1618	12.5	7.4	$C_{35}H_{32}O_{16}$	F5
Procyanidin A (isomer 1)	28.02	[M-H]-	575.1138	575.1195	10	37.5	$C_{30}H_{24}O_{12}$	F5, F7
Procyanidin B (isomer 6)	34.03	[M-H]-	577.1319	577.1351	5.7	17.8	$C_{30}H_{26}O_{12}$	F9
(Epi)catechin ethyl dimer (isomer 1)	39.67	[M-H]-	605.1672	605.1664	1.3	35.3	$C_{32}H_{30}O_{12}$	F11
Proanthocyanidin (Type A) (isomer 2)	40.72	[M-H]-	591.1519	591.1508	1.9	14.9	$C_{31}H_{28}O_{12}$	F11

3.7. Structure–antioxidant activity relationships

Recognition of many health benefits provided by polyphenol compounds has attracted increased scientific interest in determining the antioxidant capacity of various plant-derived products. It is difficult to assess the antioxidant activity of a product on the basis of a single method. A single method can provide basic information about antioxidant properties, but a combination of methods can describe the antioxidant properties of the sample in more detail (Cíž et al., 2010). A complete set of antioxidant assays (TEAC, FRAP and ORAC) was performed in order to fully characterize the antioxidant potential of the whole *T. cacao* extract and each specific fraction (Fn, with "n" being the number of the fraction) (Table 3).

Table 3 – *In vitro* antioxidant capacity by TEAC, FRAP and ORAC methods of *T. cacao* and fractions.

	TEAC[a]	FRAP[b]	ORAC[a]
T. cacao	5.39 ± 0.08	4.8 ± 0.2	7.0 ± 0.5
F1	1.25 ± 0.03	1.81 ± 0.02	0.62 ± 0.02
F2	5.32 ± 0.05	6.7 ± 0.3	9.6 ± 0.3
F3	4.49 ± 0.02	5.39 ± 0.03	4.7 ± 0.1
F4	4.99 ± 0.04	7.4 ± 0.2	5.9 ± 0.4
F5	6.05 ± 0.05	8.23 ± 0.06	5.7 ± 0.2
F6	4.50 ± 0.02	5.72 ± 0.03	3.1 ± 0.2
F7	5.60 ± 0.07	8.4 ± 0.1	5.1 ± 0.2
F8	4.5 ± 0.1	5.7 ± 0.1	3.5 ± 0.2
F9	5.78 ± 0.09	8.00 ± 0.05	3.71 ± 0.06
F10	4.51 ± 0.02	5.54 ± 0.08	3.5 ± 0.2
F11	4.13 ± 0.01	5.23 ± 0.08	3.5 ± 0.2
F12	2.17 ± 0.02	2.51 ± 0.04	1.37 ± 0.02

[a] Expressed in mmol Trolox equivalents/g of extract or fraction (dw).
[b] Expressed in mmol $FeSO_4$ equivalents/g of extract or fraction (dw).

TEAC and FRAP assays are single-electron transfer-based methods. TEAC has been applied to establish the antioxidant properties of components in a large variety of food samples (Huang, Boxin, & Prior, 2005) and it uses Trolox as the standard. FRAP is specially

indicated for determining the antioxidant capacity of biological samples (Benzie & Strain, 1996).The ORAC assay is performed in order to test the capacity of the extracts to quench peroxyl radicals. ORAC determination is a hydrogen atom transfer-based assay and has become one of the most widely accepted methods to measure the antioxidant capacity of food, botanical, and biological samples (Huang et al., 2005).

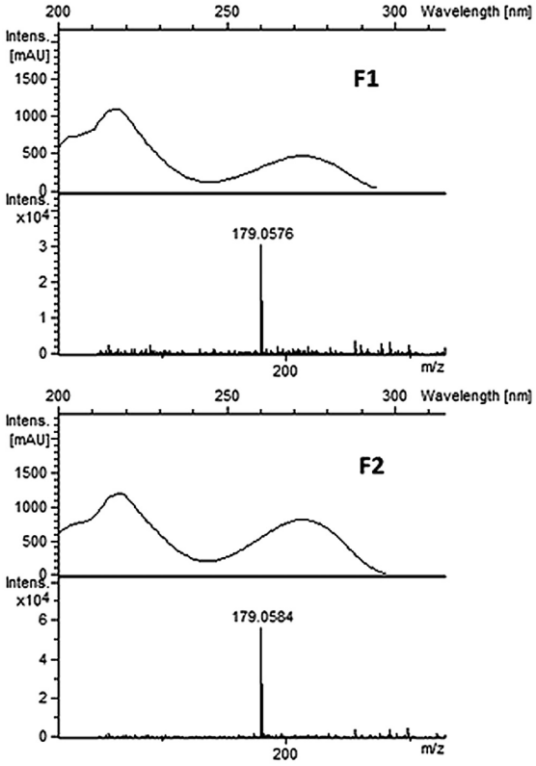


Figure 4. UV-Vis spectra and MS profile of theobromine in fractions 1 and 2.

Regarding single-electron transfer-based methods,TEAC and FRAP assay, the values for the whole *T. cacao* extract were 5.39 ± 0.08 mmol Trolox equivalents per gram (mmol TE/g) of extract and 4.8 ± 0.2 mmol FeSO4 equivalents per gram (mmol FE/g) of extract, respectively.With regard to isolated fractions from whole extract, the TEAC values ranged from 1.25 ± 0.03 to 6.05 ± 0.05 mmol TE/g of fraction and the FRAP value from 1.81 ± 0.02 to 8.4 ± 0.1 mmol FE/g of fraction. In both assays, fractions F5, F7 and F9 showed the highest antioxidant capacity.

Concerning ORAC assay, the value for whole *T. cacao* extract was 7.0 ± 0.5 mmol TE/g of extract, while the values for the isolated fractions ranged from 0.62 ± 0.02 to 9.6 ± 0.3 mmol TE/g of fraction.

However, in this antioxidant assay based on a hydrogen atom transfer-based mechanism, the fractions with highest antioxidant capacities were F2, F4, and F5.

The comparison of these results with those of previous researchers is untenable due to differences in the nature of the sample and pre-concentration technologies, extraction systems, and assay methodologies. In this sense, Gu et al. determined the antioxidant capacity of a whole *T. cacao* extract by ORAC assay.These authors have reported values of 0.008 ± 0.001 mmol TE/g of extract for natural cocoa powder (Gu, House, Wu, Ou, & Prior, 2006). Moreover, Adamson et al. (1999) measured the potential antioxidant activity using the ORAC assay.The ORAC values of the various samples ranged from 0.067 to 0.52 mmol TE/g of extract. By using the ORAC assay, Carrillo, Londoño-Londoño, and Gil (2013) evaluated the antioxidant capacity of the cocoa extracts of 18 cocoa farms, where the results ranged from 0.39 •} 0.01 to 0.64 •} 0.07 mmolTE/g of extract. Nevertheless, the total antioxidant activity assayed by Ortega et al. (2008) showed ORAC values similar to those found in our study.

The antioxidant activity of flavonoids and their metabolites *in vitro* depend upon the arrangement of functional groups about the nuclear structure and substitution pattern of hydroxyl groups. The main requirement for effective radical scavenging is the 3,4-orthodihydroxy configuration in ring B and 4-carbonyl group in ring C. The presence of 3-OH group or 3- and 5-OH groups, giving a catechol-like structure in ring C, is also beneficial for the antioxidant activity of flavonoids. The presence of the C2–C3 double bond configured with a 4-keto arrangement is known to be responsible for electron delocalization from ring B, and it increases the radical-scavenging activity. In the absence of the *o*-dihydroxystructure in ring B, a catechol structure in ring A may compensate for flavonoid antioxidant activity (Heim et al., 2002). The relationship between the chemical structure of flavonoids and their radical-scavenging activities was analyzed as described by Bors, Heller, Michel, and Saran (1990). Due to the relative complexity and diversity of tannins, less is known regarding structure–activity relationships.

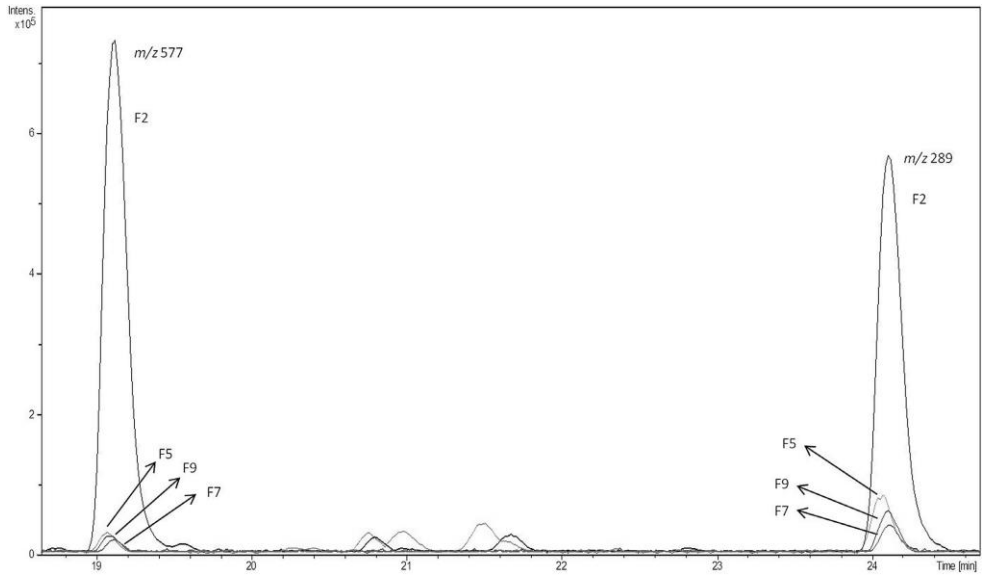
Procyanidins are powerful antioxidant agents since the corresponding oxidized forms acquire additional stabilization due to the extensive electron delocalization induced by the catechol unit on the aromatic B-ring. In addition, the several *o*-dihydroxy phenolic groups in such a high molecular-weight structure give the procyanidins a high capacity to complex metal ions [Fe(III), Cu(II), Al(III)], and proteins (Rice-Evans, Miller, & Paganga, 1996).

Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion, but the activities of dimers and trimers differ little.Tetramers exhibit greater activity against peroxynitrite- and superoxide- than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than do timers and tetramers (Vennat, Bos, Pourrat, & Bastide, 1994). It has been reported that phenolics in whole cocoa extract ranging from monomer to decamers have strong antioxidant capacity (Zhu, Holt, Lazarus, Orozco, & Keen, 2002).

For a better understanding of the contribution of each polyphenol family to the antioxidant capacity of the *T. cacao* extract, the relationship between antioxidant value and polyphenols was established based on the composition of each fraction determined by HPLC-DAD-QTOF-MS. Our study showed that fractions with the most compounds identified as procyanidins had high antioxidant activity (F5, F7 and F9). In the TEAC assay, F5 and F9 showed higher values than F7. This could be explained by the presence of tetramers and pentamers in these fractions. On the contrary, FRAP values were higher in F7 than in F5 and F9. Although TEAC and FRAP values of these three fractions did not show large differences, in the ORAC assay the highest antioxidant values were found in F2. This fraction was characterized by a sharper peak of procyanidin B dimer (at *m/z* 577) than for other fractions (Fig. 5). In addition, another phenolic compound, theobromine, was also identified in F2. Maleyki and Ismail, using the FRAP assay, reported that methylxanthines, such as theobromine, showed low antioxidant capacity (Maleyki & Ismail, 2010). This could explain the fact that this fraction did not have high values in single-electron transfer-based methods (TEAC and FRAP). In addition, the fractions characterized by the presence of quercetin showed lower antioxidant activity (F11-F12) than the ones in which different

procyanidins are detected.A similar effect was noted by Bai, Zhang, and Ren (2013) and Spranger, Sun, Mateus, Freitas, and Ricardo-da-Silva (2008), who reported that the tested methods showed that, on an equimolar basis, polymeric procyanidins appeared the highest antioxidant activities. Moreover, procyanidins presented higher antioxidant activities than did other antioxidants such as quercetin.

Figure 5. Base peak chromatogram of peaks at *m/z* 577 (procyanidin B isomer) and *m/z* 289 ((epi)catechin) in fractions F2, F5, F7 and F9.



Some authors (Djeridane et al., 2006; Katalinic, Milos, Kulisic, & Jukic, 2006; Katsube et al., 2004) have demonstrated a linear correlation between the content of phenolic compounds and their antioxidant capacity. Our results demonstrated that *T. cacao* is rich in phenolic constituents and has good antioxidant activity measured by different methods. This extract, rich in flavonoids and phenolic acids, could be a good source of natural antioxidants. Therefore, qualitative analysis of major individual phenolics in the extract could be helpful in explaining the relationships between total antioxidant capacity and phenolic compounds in *T. cacao*.

4. CONCLUSIONS

A powerful analytical method has been used in the comprehensive characterization of extracts and fractions from *T. cacao*. The combined use of HPLC separation with a small particle size column assisted by mass spectrometric detection with a mass analyzer, QTOF, proved to be a useful tool for

identifying secondary metabolites produced by plants. The method described separated a wide range of phenolic compounds and simultaneously provided the tentative characterization and the quantification of the major compounds of this extract. A total of 61 compounds were characterized and quantified in the *T. cacao* extract and fractions. To the best of our knowledge, this is the first study available to apply this analytical method to the separation of procyanidins in such a complex natural sample. This work also opens new possibilities for the analysis of such complex compounds as procyanidins in other food and natural complex sources. In addition, *T. cacao* extract and fractions exhibited a strong capacity to donate electrons (FRAP and TEAC) and a significant antioxidant capacity to reduce peroxyl radicals by hydrogen atom transfer (ORAC). It could be due to the presence of procyanidins (monomers and oligomers).

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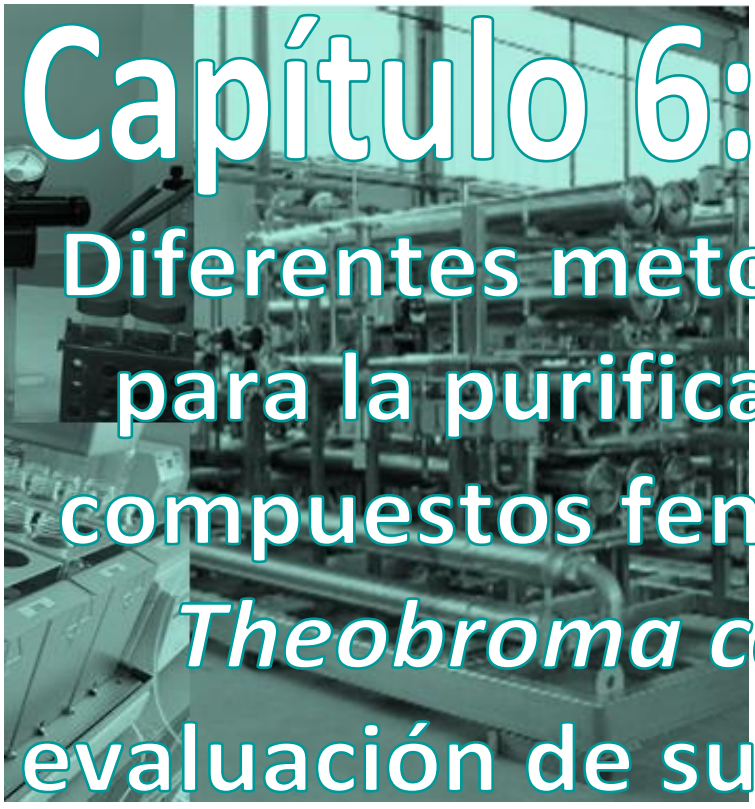

Capítulo 6:

Diferentes metodologías para la purificación de compuestos fenólicos de *Theobroma cacao* y evaluación de su toxicidad

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Different approaches for phenolic compound purification from *Theobroma cacao* and safety evaluation

ABSTRACT

Plants, including most food and feed plants, produce a broad range of bioactive chemical compounds. Among these compounds, polyphenols are classified according to the nature of their carbon skeletons as phenolic acids, flavonoids, stilbenes, or lignans. Beneficial effects as anti-carcinogenic, anti-atherogenic, anti-inflammatory, immune modulating, anti-microbial, vasodilatory and analgesic, are ascribed to these bioactive compounds. Cocoa (*Theobroma cacao*), a major, economically important, international crop, has been related to several nutritional benefits, which have been associated with the phenolic fraction. The main subclass of flavonoids found in cocoa is flavanols, particularly (epi)catechins monomers, and their oligomers, also known as procyanidins which range from dimers to decamers. In this study, these compounds were isolated by different methodologies as Sep-Pak C18 cartridges, semi-preparative HPLC and membrane technologies to obtain different polyphenolic profiles by high-performance liquid chromatography coupled to electrospray time-of-flight mass spectrometry (HPLC-ESI-TOF-MS) and to test their cytotoxicity. Finally, different polyphenolic profiles were collected, where the combination of both semi-preparative HPLC and Sep-Pak C18 cartridges technologies provided the most purified fractions. Filtration with membranes and Sep-Pak gave extracts with different composition depending on the pore size of membranes and the used solvent, respectively. In addition, the results of the cell viability assay indicated a lack of toxicity in all the obtained fractions.

1. INTRODUCTION

Free radicals cause degenerative human diseases through multiple mechanisms. Recently, natural foods and food-derived antioxidants such as phenolic compounds have received growing attention since they are known to act as chemo-preventive agents against oxidative damage.

Polyphenols constitute one of the most large and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently identified (Bravo et al., 1998). Among these compounds, condensed tannins or proanthocyanidins (PA), which are high-molecular-weight polymers, have been recently reported for demonstrating biological activities as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic and cardioprotective (Cádiz-Gurrea et al., 2015). These compounds possess the general structure of polymerized flavan-3-ols, mainly (+)-catechin and (-)-epicatechin, in which the interflavan bonds are most commonly C-4 to C-8 or C-6 (B-type procyanidins), but the unusual double-linked heteropolymers (A-type procyanidins) are also found (Yanagida et al., 2003) (Figure 1). These combinations together with the occurrence of glycosylated and methylated derivatives explain the high diversity of this family and the wide range of biological and biochemical activities in plants (He et al., 2008).

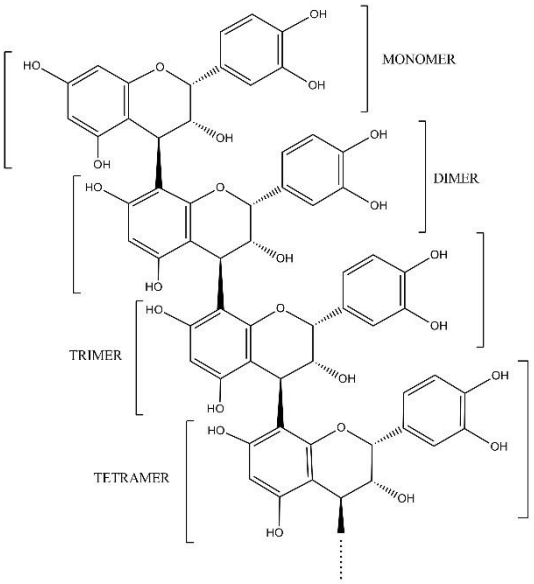

Figure 1. Common structure of procyanidins in *Theoborma cacao* with different degrees of polymerization.

The demonstrated properties of *Theobroma cacao* largely depend on the structures of these compounds, mostly on their degree of polymerization (DP) (Sun et al., 1998). Since last years, it has been suggested that cocoa contains large amounts of polyphenols with several beneficial effects in human health (Caton et al., 2010; Gu et al., 2014; Lee et al., 2003; Sanbongi et al., 1998, 1997; Zeng et al., 2011). PA are important constituent of *Theobroma cacao* (Hammerstone et al., 1999) although the research on putative health effects has been focused on the effects of single procyanidin (PC) fractions demonstrating specific physiological effects for some of the higher oligomeric fraction whereas the lower ones appeared to have no or less activity (Wollgast et al., 2001; Wollgast and Anklam, 2000). Other authors have demonstrated that long-chain PC are by far the best compounds to inhibit lipid oxidation induced by radicals in an aqueous medium (Counet and Collin, 2003), specifically pentameric PCs from *T. cacao* inhibit selectively the growth of human breast cancer cells (Ramljak and Romanczyk, 2005). While the use of PA has become increasingly popular for health promotion and disease prevention as it known, concerns have been raised that high dose may paradoxically induce toxicity (Lao et al., 2015; Shao et al., 2006; Yamakoshi et al., 2002).

Thus, due to their possible beneficial implications in human health, there is a growing scientific and commercial interest to determine not only the PA content in total but also the content of specific oligomers in cocoa products as well as in other food products (Wollgast et al., 2001). The usual technique to analyze polyphenols from cocoa multicomponent extracts or a specific isolated fraction is reversed phase high-pressure liquid chromatography (RP-HPLC) with C18 stationary phase coupled to diode-array detection and mass spectrometry (Andres-Lacueva et al., 2008; Cádiz-Gurrea et al., 2014; Quiñones et al., 2011; Tomas-Barberan et al., 2007).

Because it is difficult to separate large amounts of cocoa polyphenols and because there is a lack of commercial standards of PCs, almost all *in vitro* and *in vivo* studies make use of whole cocoa samples. In addition, the analytical methodologies applied to purify cocoa bioactive compounds involve laborious pre-treatment together with isolation and purification procedures. In this sense, the

evaluation of biological efficacy of individual components is an important step towards drug discovery and development (Jiménez-Sánchez et al., 2017). For these reasons, several studies have developed different strategies in order to separate and fractionate polyphenols and polyphenol-derived compounds into different sub-fractions as a first step for their further identification and characterization (Cádiz-Gurrea et al., 2014; Monagas et al., 2003; Ortega et al., 2008; Sun et al., 1998).

A solid phase extraction (SPE) on C18 Sep-Pak cartridges has been usually applied in beverages such as wine, ciders or beers to isolate from monomers to polymers of PCs (Alonso García et al., 2004; Pinelo et al., 2006; Suárez et al., 1996). The potential of any SPE also depends on the solvents used for elution and the sequence of their application. With different C18 solid-phase supports, the adsorption properties of the cartridges are modified, including physical properties, carbon loading, and pore size (Routray and Orsat, 2013). Moreover, isolation by semi-preparative and preparative liquid chromatography with C18 reversed phase offers high versatility to separate a wide range of nitrogenous and non-nitrogenous bioactive compounds (Cádiz-Gurrea et al., 2014; Jiménez-Sánchez et al., 2017). One alternative to the above-mentioned purification methodologies, which has not been much applied for the analytical purification of PA is the membrane-based micro (MF), ultra (UF) and nano-filtration (NF) techniques. Recently, there has been an increasing interest in the application of membrane technologies for separation, purification and concentration of bioactive compounds from aqueous solutions. Membrane processes have been investigated for high quality concentration of phenolic compounds due to their low operating temperature and minimal energy consumption (Khemakhem et al., 2017).

The aim of this study was to test different methodologies in order to obtain different fractions of phenolic compounds from *T. cacao.* For this purpose, three different methodologies such as a solid phase extraction with Sep-Pak C18 cartridges, a combination of Sep-Pack and semi-preparative HPLC isolation and a sequence of different membrane operations MF, UF and NF, were performed. All obtained fractions were analyzed by HPLC-ESI-TOF-MS in order to discriminate the different

composition of each one and MTT assay was carried out in order to confirm the safety of selected fractions from *T. cacao*.

2. MATERIAL AND METHODS

2.1. Chemicals, solutions and disposables

All chemicals were of HPLC-MS grade and used as received. Acetic acid and methanol for HPLC and semi-preparative were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Diethyl ether and methanol for extraction were purchased from Fisher (Fisher Scientific Co., Fair Lawn, NJ), ethyl acetate from Lab-Scan (Gliwice, Sowinskiego, Poland), ethanol and acetone from AnalaR Normapur (VWR International, Inc., Darmstadt, Germany) and dimethyl sulfoxide (DMSO) was purchased from Panreac (Barcelona, Spain) for analytical assays and from Euromedex for MTT assay. (+)-catechin (≥99%), vanillin (≥98%) and hydrochloric acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

Waters 5 g Sep-Pak C18 Plus cartridges (Mildford, USA) were used as solid-phase extraction minicolumns for purification and concentration.

For cell culture and treatment, Dulbecco's Modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS) 10%, fungizone, 1% penicillin and streptomycin from Gibco (Life technologies, Gaithersburg, MD). 3 (4,5 dimethylthiazol 2 yl)-2,5 diphenyltetrazolium bromide (MTT) was purchase by Sigma-Aldrich (St Louis, MO, USA).

2.2. Sample preparation

A concentrated *T. cacao* extract was used in this study (Monteoleder, Spain). The polyphenols from whole cocoa matrix were analytically characterized using a solution of cocoa extract of 10 mg/mL as described by Cádiz-Gurrea et al. (Cádiz-Gurrea et al., 2014). Briefly, 10 mg of cocoa extract were dissolved in 1 mL of different solvents as ethanol, acetone, methanol, DMSO and acetone:water:acetic

acid (70:28:2) in order to identified the extracted compounds in each condition. The samples were sonicated for 5 min, vortexed for 1 min, and then centrifuged for 5 min at 7700 x *g*. The supernatants and pellets (redissolved in 1 mL of DMSO) were filtered through a 0.25 mm filter before the HPLC analysis and for total flavan-3-ols content assays.

For cartridges purification, a solution stock of 0.1 g/mL was prepared by dissolving the appropriate amount of cacao extract in DMSO. The sample was sonicated for 5 min, vortexed for 1 min, and then centrifuged for 5 min at 7700 x *g* before the cartridges purification.

For semi-preparative HPLC purification, a solution stock of 75 mg/mL was prepared by dissolving the PC fraction of C18 Sep-Pak cartridges in DMSO. The sample was sonicated for 5 min, vortexed for 1 min, and then centrifuged for 5 min at 7700 x *g* before the semipreparative HPLC analysis.

For micro, ultra and nano-filtration purifications, a solution of cacao extract was prepared by dissolving 300 g of cocoa in 50 L of water.

2.3. Instrumentation

The polyphenols from the PC fraction from Sep-Pak cartridges purification were fractionated using a Gilson preparative HPLC system (Gilson Inc., Middleton, WI, USA) equipped with a binary pump (model 331/332), automated liquid handling solutions (model GX-271) and UV-Vis detector (model UV-Vis 156).

In purification by membranes, we used a microfiltration system through 0.2 µm pores size membrane (Microporous membrane TOPER Model, diameter 300mm, CN), a ultrafiltration system of molecular weight cut-off (MWCO) 5 kDa (GE Power & Water, ZeeWeed 1500 Minnetonka, MN) and a nanofiltration system of MWCO 300 Da (GE Osmonics, HL2540TF, Minnetonka, MN).

T. cacao and the isolated fractions were analytically characterized using an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode-array detector (DAD). The HPLC system was coupled to a time-of-flight (TOF)

mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface (model G1607A from Agilent Technologies, Palo Alto, CA).

For Total flavan-3-ols assay, the measures were carried out on a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) by using 96-well polystyrene microplates.

For MTT assay, the results were obtained using a microplate absorbance reader (BioRad, CA, USA).

2.4. Fractionation of polyphenols from *T. cacao* extract

2.4.1. By Sep-Pak C18 cartridges

T. cacao extract prepared as describe above in sample preparation section was fractionated by Waters C18 Sep-Pak cartridges following the reported method by *Sun et al.* (Sun et al., 2006) and *Monagas et al.* (Monagas et al., 2003) with some modifications. A cartridge of C18 Sep-Pak was conditioned with methanol (10 mL) and milliQ water (10 mL). The sample (1.5 mL) was then passed through the cartridge. Phenolic acids were first collected by elution with 10 mL of MilliQ water (PA fraction). After that, the elution of monomeric and oligomeric flavan-3-ols (PC fraction) was carried out with 35 mL of ethyl acetate, followed by the elution of polymeric proanthocyanidins (PP fraction) with 40 mL of methanol. The PC fraction was taken to dryness under vacuum and redissolved in a final concentration of 0.1 g/mL, and finally redeposited (1.5 mL of sample) onto a new cartridge preconditioned as described above. Monomers (MN fraction) were separated from oligomers (OL fraction) by sequential elution with 35 mL of diethyl ether and 40 mL of methanol.

The experiments were performed in triplicate to ensure the repeatability of the fractionation by the cartridges.

The five obtained fractions were evaporated to dryness under vacuum in Speed Vac (Thermo Scientific® SC 250 exp). The residue of each one was weighted and dissolved in DMSO at 100 µg/mL. After that, they were filtered through a 0.25 µm filter to analyze them by HPLC-ESI-TOF-MS.

2.4.2. By combination of Sep-Pak and semi-preparative HPLC

The compounds of PC fraction from C18 cartridges were fractionated according to *Cádiz-Gurrea et al.* (Cádiz-Gurrea et al., 2014). Finally, a total of 11 fractions were collected and the solvent was evaporated under vacuum in Speed Vac. The residue of each fraction was weighted and dissolved with an appropriate volume of DMSO at concentration level of 100 µg/mL. Finally, all fractions were filtered through a 0.25 µm filter before the HPLC analysis.

2.4.3. By membrane separation technology

The fractionation process of *T. cacao* extract was realized through the combination of three membrane operations following the method previously described by *Khemakhem et al.* (Khemakhem et al., 2017) with some modifications. The feed stream was pre-treated in a MF system through 0.2 µm pores size membrane. The MF permeate was submitted to a cross-flow UF system of molecular weight cut-off (MWCO) 5 kDa. Finally, the UF permeate feed a cross-flow NF system of MWCO 300 Da.

Manometers before and after the MF, UF and NF membranes were used to measure the inlet and the outlet pressure so as to control the trans-membrane pressure (ΔP) which was equal to 1 bar for MF and UF. However, the NF was operated at a trans-membrane pressure of 9 bar.

After filtration process, feed solution and permeates of MF, UF and NF systems were evaporated to dryness under vacuum in Speed Vac and redissolved to analyze them by HPLC-ESI-TOF-MS.

2.5. Chromatographic conditions and ESI-TOF-MS detection

The compounds from the *T. cacao* and fractions were separated following the method described by *Cádiz-Gurrea et al.* (Cádiz-Gurrea et al., 2014) with this modifications: the HPLC system was coupled to a TOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +3.5 kV. The other optimum values of the source parameters were: drying gas temperature, 200 °C; drying gas flow, 10 L/min; and nebulizing gas pressure, 2.3 bar. The detection was performed considering a mass range of 50–1200 *m/z*.

The samples were injected in triplicate to ensure the repeatability of the analysis.

2.6. Total flavan-3-ols content assay

Vanillin assay was used to obtain the total flavan-3-ols content for different solvent extractions as described for *Price et al. and Butler et al.* (Butler et al., 1982; Price et al., 1978) with some modifications. For 1 mL of vanillin reactive, 100 µL of samples, standard or blank were used.

(+)-Catechin was selected as standard and the absorbance was measured at 500 nm.

The measurements were performed in triplicate.

2.7. Cell culture and MTT assay

Human Embryonic Kidney cells (HEK) stably expressing the vitronectin receptor (293 VnR) were provided by Dr. R. Baron (Yale University). Cells were incubated in DMEM supplemented with FBS 10%, fungizone, 1% penicillin and streptomycin. Cells were seeded in 96-well plates at a density of 1.400 cells/well in 100 µL medium to assess putative cytotoxic effect of contained compounds in each *T. cacao* fraction. In each plate, four parallel wells were made and the results were collected as the mean of three independent experiments. Cells cultured with complete medium were used as control. After 24 h of seeding, various concentrations (10, 25, 50, 100, 200 and 300 µg/mL) of each fraction were added and the cells were incubated for another 24 h. At the end of culture, medium was substituted by medium without phenol red containing MTT 0.5 mg/mL (Sigma) for 2 h in an incubator. MTT reagent was used to measure the conversion by healthy cells of the soluble MTT dye to formazan crystals. Supernatants were removed and formazan crystals were solubilized in 200 µL of DMSO. Microplate was foiled and gently mixed at room temperature for 10 min. The concentration determined by a purple color change monitored via absorbance measurement at 570 nm. The change in absorbance can be used as an indicator of the HEK293 cell health in the assay. Cell viability was expressed as percentage of the absorbance of treated cells relative to that of the untreated controls.

The measurements were performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Effect of different solvents on the polyphenol content

Extraction of all interested compounds is essential to achieve the best conditions for the following steps in the analytical process. For PAs, many methods have been applied for this purpose since differences in the structure of phenolic compounds also determine their solubility in solvents of different polarity (Boeing et al., 2014; Bosso et al., 2016; Boulekbache-Makhlouf et al., 2013; Złotek et al., 2016). *Kim et al.* tested different processes for extraction of PAs from pine using hexane, ethanol, hot water and combination of some of these solvents. In this way, the ethanol condition provided the highest concentration of total PAs (Kim et al., 2010).

Table 1. Results of total flavan-3-ols assay for different solvent extractions.

Sample	TF (mg Eq. catechin/mg dry extract)	Sample	TF (mg Eq. catechin/mg dry extract)
a	2.87 ± 0.08	-	-
b	2.90 ± 0.07	-	-
c	1.80 ± 0.04	**f**	1.37 ± 0.01
d	2.47 ± 0.05	**g**	0.253 ± 0.006
e	0.146 ± 0.004	**h**	2.601 ± 0.001

Our results indicated that DMSO and acetone:water:acetic acid (70:28:2) (Figure 2a and b) were the best solvents for total dissolution of the extract and provided the largest number of obtained compounds from *T. cacao* extract following by methanol (Figure 2d). As observed in Figure 2e, acetone extract was the least effective solvent for extraction from cocoa. Figure 2 provides the HPLC profiles of different supernatants of each condition (a, b, c, d, e) and their pellets (f, g, h), if available. In addition, values of TF content of each samples were showed in Table 1, where the highest values coincided with the richest HPLC chromatograms. A comprehensive characterization of whole profile of DMSO *T. cacao* extract (Figure 2b) can be found in *Cádiz-Gurrea et al.* (Cádiz-Gurrea et al., 2014).

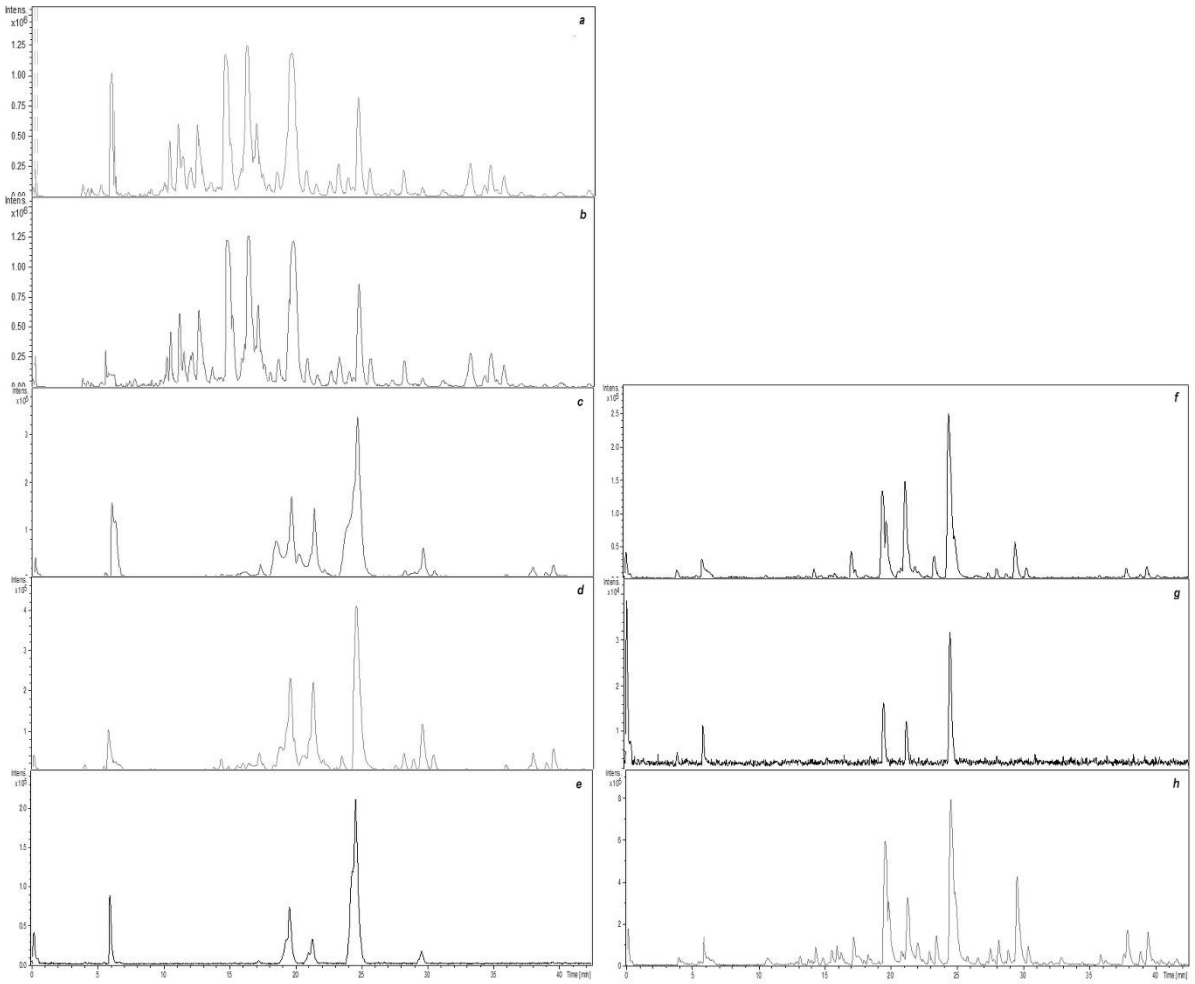

Figure 2. Base peak chromatogram spectrum of each different solvent: supernatants a) acetone:water:acetic acid, b) DMSO, c) ethanol, d) methanol, e) acetone, and DMSO-redissolved pellets of f) ethanol, g) methanol and h) acetone.

For this reason, DMSO was selected as solvent in order to dissolve *T. cacao* extract and every obtained fraction after evaporation.

3.2. Comprehensive characterization of fractions by HPLC-ESI-TOF-MS

3.2.1. Sep-Pak C18 cartridges

In the first separation step of this design, the recovery of phenolic compounds was achieved by passing water in order to obtain the phenolic acids. These acidic polyphenols and other ionizable species such as organic acids did not adsorb to the lipophilic packing material and, thus, they are easily eluted by the aqueous fraction. After that, flavonols, catechins, and anthocyanins were eluted by ethyl acetate.

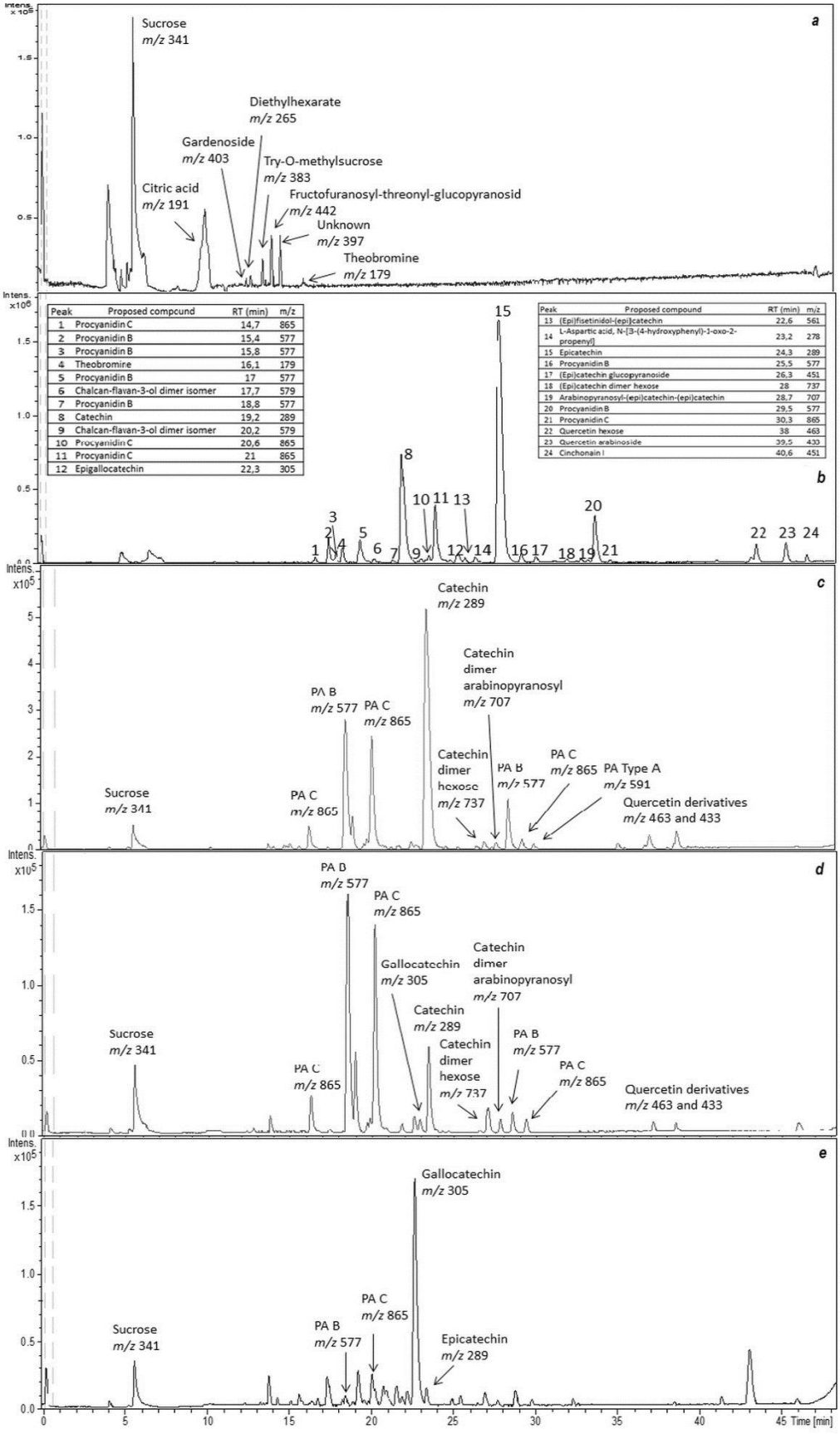
a
Sucrose
m/z 341
Diethylhexarate
m/z 265
Gardenoside
m/z 403
Try-O-methylsucrose
m/z 383
Citric acid
m/z 191
Fructofuranosyl-threonyl-glucopyranosid
m/z 442
Unknown
m/z 397
Theobromine
m/z 179
b
Peak Proposed compcund RT (min) m/z
1 Procyanidin C 14,7 865
2 Procyanidin B 15,4 577
3 Procyanidin B 15,8 577
4 Theobromire 16,1 179
5 Procyanidin B 17 577
6 Chalcan-flavan-3-ol dimer isomer 17,7 579
7 Procyanidin B 18,8 577
8 Catechin 19,2 289
9 Chalcan-flavan-3-ol dimer isomer 20,2 579
10 Procyanidin C 20,6 865
11 Procyanidin C 21 865
12 Epigallocatechin 22,3 305
Peak Proposed compound RT (min) m/z
13 (Epi)fisetinidol-(epi)catechin 22,6 561
14 L-Aspartic acid, N-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl] 23,2 278
15 Epicatechin 24,3 289
16 Procyanidin B 25,5 577
17 (Epi)catechin glucopyranoside 26,3 451
18 (Epi)catechin dimer hexose 28 737
19 Arabinopyranosyl-(epi)catechin-(epi)catechin 28,7 707
20 Procyanidin B 29,5 577
21 Procyanidin C 30,3 865
22 Quercetin hexose 38 463
23 Quercetin arabinoside 39,5 433
24 Cinchonain I 40,6 451
c
Catechin
m/z 289
Catechin
dimer
arabinopyranosyl
m/z 707
PA B
m/z 577
PA C
m/z 865
Catechin
dimer
hexose
m/z 737
PA B
m/z 577
PA C
m/z 865
PA Type A
m/z 591
Sucrose
m/z 341
PA C
m/z 865
Quercetin derivatives
m/z 463 and 433
d
PA B
m/z 577
PA C
m/z 865
Gallocatechin
m/z 305
Catechin
dimer
arabinopyranosyl
m/z 707
Catechin
m/z 289
Catechin
dimer
hexose
m/z 737
PA B
m/z 577
PA C
m/z 865
Sucrose
m/z 341
PA C
m/z 865
Quercetin derivatives
m/z 463 and 433
e
Gallocatechin
m/z 305
PA C
m/z 865
PA B
m/z 577
Epicatechin
m/z 289
Sucrose
m/z 341
Time [min]

Figure 3. Different profiles of Sep-Pak fractions by HPLC-ESI-TOF-MS were; a) PA, b) PC, c) MN, d) OL and e) PL fractions.

Finally, methanol was employed to recover the polymeric fraction as showed Figure 3a, b and e, respectively. Furthermore, after the column had been reconditioned to acidic conditions, the evaporated PC fraction was used to obtain separately the monomers and oligomers with diethyl ether and methanol, respectively (Figure 3b and c).

HPLC-ESI-TOF-MS analysis confirmed that monomers as (+)-catechin were presented with more intensity in PC and MN fractions as expected. On the other hand, (epi)-catechin dimers and derivatives (*m/z* 575, 577, 707 or 737) and trimers were also mainly found in PC and OL fractions (figure 3b and c).

Concerning polymeric fraction (figure 3e), high degree of polymerization compounds were not found and gallocatechin (*m/z* 305) was indeed the most abundant subunit in this condition. Similar results occurred in a study by *Monagas et al.* where described the structural composition of the wine, seed, and skin polymeric PAs from different grapes varieties with the presence of (epi)gallocatechin derivatives (Monagas et al., 2003).

This proposed method permitted separating phenolic compounds from the whole *T. cacao* extract into various different fractions, which were less complex, enabling their use in order to achieve pure fractions of interested compounds.

3.2.2. Combination of Sep-Pak C18 and semi-preparative HPLC

To concentrate and obtain polyphenol rich fractions before analysis, strategies including sequential extraction or liquid-liquid partitioning or SPE based on polarity and acidity have been commonly used. During the investigation of complex matrices, efficient separation and strong methods of identification are required to assess the chemical composition of natural sources. Although as known there are different approaches to the separation and analysis of secondary metabolites from herbal extracts, HPLC is by far the most widely used technique for the separation of complex mixtures of polyphenolic

compounds in plant extracts (Rodrigues et al., 2008). In our study, we have proposed a combination of SPE by Sep-Pak C18 cartridges and semi-preparative HPLC isolation in order to obtain purified fractions of monomeric and oligomeric PAs (Figure 4). Thus, eleven fractions were obtained and the composition of each one was established by the detailed HPLC-ESI-TOF-MS (Figure 5). All of these compounds were correctly separated using the semi-preparative HPLC technique according to *Cádiz-Gurrea et al.* (Cádiz-Gurrea et al., 2014).

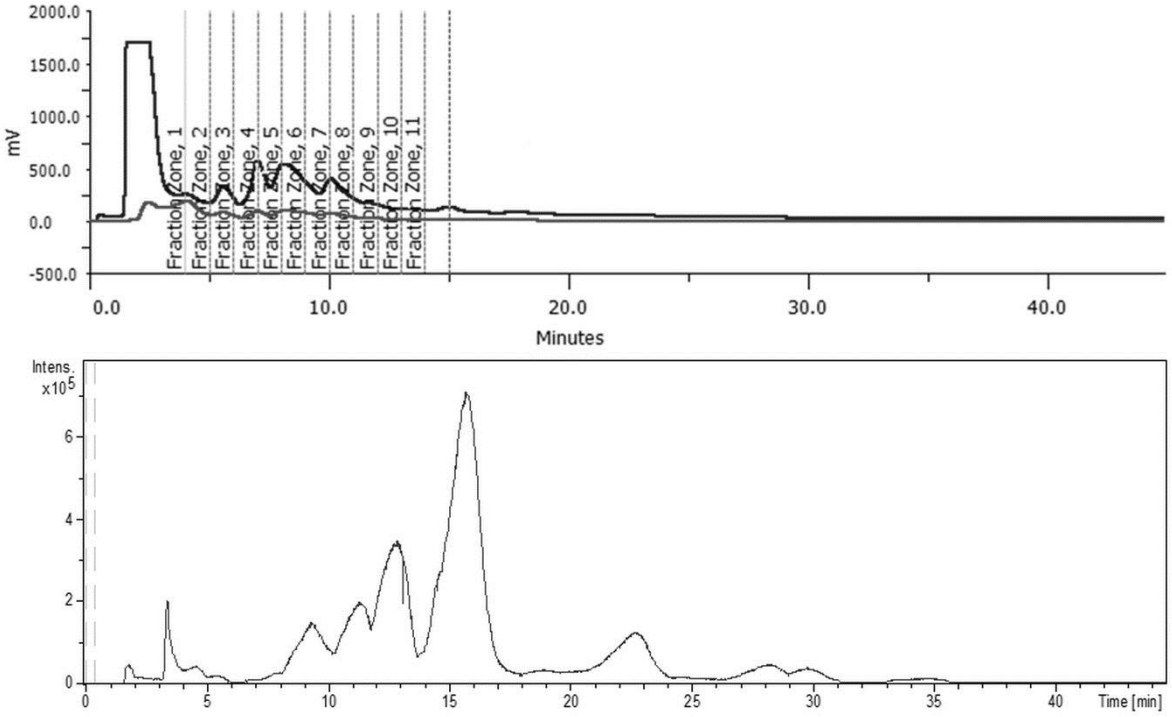

Figure 4. Semi-preparative chromatograms by HPLC-UV with collection and TOF-MS.

Only in F1 we could find catechin (by comparing to the commercial standard) and epicatechin (*m/z* 289). In F2 and F3, there are a B-type dimer or PC and epicatechin, the last one was presented with high intensity in F3. Moreover, a PC trimer appeared in fractions from 4 to 7 (*m/z* 865). Less purified fractions were F7, F8 and F10 with four or more compounds. However, a B-type dimer of PC was detected in F9 being the most purify fraction.

Our results revealed that this approach permits an efficient purification of monomers and oligomers from *T. cacao*. Further studies could use these results in order to clarify the putative candidates for

the attributed biological activities or may evidence synergic effects between some of these compounds.

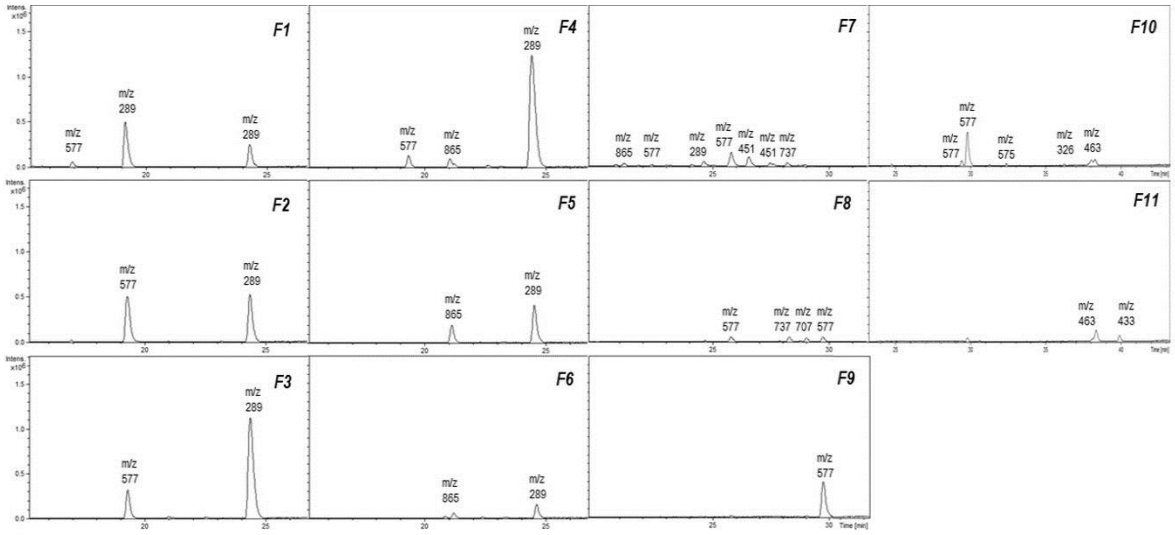

Figure 5. Base peak chromatogram spectrum of eleven semi-preparative HPLC fractions from PC Sep-Pak extract by HPLC-ESI-TOF-MS.

3.2.3. Membrane technologies

Membrane operations, including MF, UF and NF have been proposed to obtain effluent streams from different sources. These methodologies, besides being considered a suitable approach for the selective collecting of phenolic compounds from natural sources or agro-industrial by-products, provide unique separation capability, possibilities to scale-up and low energy consumption (Cassano et al., 2011). Olive mill wastewaters, extracts of grape seeds and grape pomace are the main example of liquid streams derived from industrial activity processed by MF, UF and/or NF for polyphenols recovery, but it also studied the membrane performance in bark extracts (Pinto et al., 2014).

In our work, the membrane process was meant to perform a rich fed stream from water *T. cacao* extract, while the MF, UF and NF processes were meant for the concentration of different compounds from the same extract. Figure 6 shows the different base peak chromatograms from obtained permeates where the compounds appeared according to their elution order.

No much literature is readily available on the performance of filtration membranes for the separation and purification of phenolic compounds in cacao samples. In the light of these considerations, the compounds characterized in each permeate are presented in Table 2. This table includes the retention time, experimental *m/z*, molecular formulas, errors and σ values for all of the compounds found in the samples analyzed. All of this compounds were tentatively identified by the interpretation of their mass spectra determined by the TOF mass analyzer while taking into account the information provided by the literature and databases.

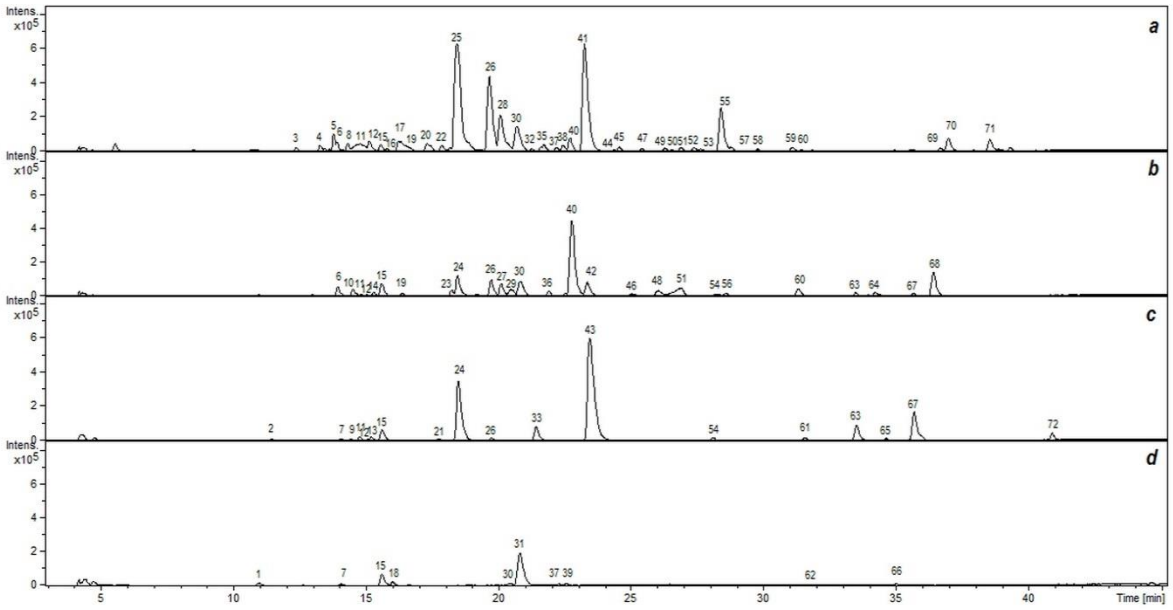

Figure 6. Base peak chromatogram spectrum of fractions by membrane technique for purification: a) feed stream, b) MF permeate, c) UF permeate and d) NF permeate.

In the present study, some sugars and high molecular weight compounds as PC trimers and dimer derivatives at *m/z* 707 were retained in MF membrane. UF membrane showed low rejection capacity towards monomeric and dimeric PC for containing (+)-catechin and (-)-epicatechin, B-type dimer and A-type dimer of PC thus allowed these compounds to be contained in the permeate being the richest and the most concentrated fraction concerning PCs. In addition, low molecular weight compounds as phenolic acids and galloyl derivatives were detected in NF permeated.

Table 2. Compounds characterized in feed stream (FS), micro- (MF), ultra- (UF) and nanofiltration (NF) permeates by HPLC-ESI-TOF-MS in negative mode.

Peak	RT [min]	Calc. m/z	Meas. m/z	Formula [M-H]	Error [ppm]	mSigma	Proposed compound	FS	MF	UF	NF
1	10.9	1.590.657	1.590.663	$C_7H_{11}O_4$	3.9	9.6	Pimelic acid				x
2	11.4	3.690.311	3.690.312	$C_{11}H_{13}O_{14}$	-0.4	33.3	UK1			x	
3	12.3	4.031.246	4.031.234	$C_{17}H_{23}O_{11}$	3.0	3.9	Gardenoside	x			
4	13.2	3.831.559	3.831.558	$C_{15}H_{27}O_{11}$	0.3	3.5	Tri-O-methylsucrose	x			
5	13.7	4.421.566	4.421.568	$C_{16}H_{28}NO_{13}$	-0.4	3.2	Fructofuranosyl-threonyl-glucopyranosid	x			
6	13.9	2.230.612	2.230.609	$C_{11}H_{11}O_5$	1.4	7.4	Sinapic acid	x	x		
7	14.0	1.650.413	1.650.405	$C_5H_9O_6$	-5.2	14.3	Arabonic Acid			x	x
8	14.3	3.971.715	3.971.711	$C_{16}H_{29}O_{11}$	1.2	3.7	Butyl-galactopyranosyl-glucopyranoside	x			
9	14.4	5.771.351	5.771.380	$C_{30}H_{25}O_{12}$	-4.9	6.9	Procyanidin B dimer isomer 1			x	
10	14.5	177.056	1.770.552	$C_{10}H_9O_3$	3.2	5,3	Cinnamic acid, p-methoxy-		x		
11	14.7	5.771.351	5.771.326	$C_{30}H_{25}O_{12}$	4,4	61,6	Procyanidin B dimer isomer 2	x	x	x	
12	15.1	5.771.351	5.771.348	$C_{30}H_{25}O_{12}$	0.7	13.8	Procyanidin B dimer isomer 3	x	x	x	
13	15.2	3.891.089	3.891.109	$C_{16}H_{21}O_{11}$	-5.0	8.6	Glucose pentaacetate			x	
14	15.3	2.050.512	2.050.506	$C_{11}H_9O_4$	-2.9	1.7	Dimethoxycoumarin		x		
15	15.5	1.790.561	1.790.569	$C_6H_{11}O_6$	-4.5	16.7	Hexose	x	x	x	x
16	15.8	4.511.246	4.511.219	$C_{21}H_{23}O_{11}$	5.9	5.0	(Epi)catechin glucopyranoside isomer 1	x			
17	16.2	8.651.985	8.651.966	$C_{45}H_{37}O_{18}$	2.2	10.9	Procyanidin C isomer 1	x			
18	16.0	1.870.037	1.870.049	$C_{10}H_3O_4$	-6.5	38.1	Naphthalenetetrone				x
19	16.3	5.771.351	5.771.333	$C_{30}H_{25}O_{12}$	3.2	18.0	Procyanidin B dimer isomer 4	x	x		
20	17.3	4.511.246	4.511.226	$C_{21}H_{23}O_{11}$	4.4	1.3	(Epi)catechin glucopyranoside isomer 2	x			
21	17.7	3.670.518	3.670.521	$C_{12}H_{15}O_{13}$	-0.8	33.4	Digalacturonate isomer 1			x	
22	17.8	3.690.252	3.690.277	$C_{18}H_9O_9$	-6.9	21.5	Phlorotannin	x			
23	18.2	3.030.568	3.030.569	$C_8H_{15}O_{12}$	0.4	39.3	UK2		x		
24	18.4	2.890.718	2.890.725	$C_{15}H_{13}O_6$	-2.5	8	Catechin		x	x	
25	18.4	5.771.351	5.771.354	$C_{30}H_{25}O_{12}$	-0.5	11.5	Procyanidin B dimer isomer 5	x			
26	19.6	5.771.324	5.771.351	$C_{30}H_{25}O_{12}$	4,7	7	Procyanidin B dimer isomer 6	x	x	x	
27	20.0	2.850.463	2.850.463	$C_8H_{13}O_{11}$	0.0	37.5	UK2		x		
28	20.0	8.651.985	8.651.973	$C_{45}H_{37}O_{18}$	1.4	10.8	Procyanidin C isomer 2	x			
29	20.4	5.771.340	5.771.351	$C_{30}H_{25}O_{12}$	2	43,1	Procyanidin B dimer isomer 7		x		
30	20.6	3.050.667	3.050.692	$C_{15}H_{13}O_7$	-8.2	24.2	(Epi)gallocatechin isomer 1	x	x		x
31	20.8	3.050.661	3.050.667	$C_{15}H_{13}O_7$	2.0	24.2	(Epi)gallocatechin isomer 2				x
32	21.2	5.771.351	5.771.318	$C_{30}H_{25}O_{12}$	5.9	20.3	Procyanidin B dimer isomer 8	x			
33	21.4	3.670.518	3.670.529	$C_{12}H_{15}O_{13}$	-2.8	33.8	Digalacturonate isomer 2			x	
34	21.6	5.611.402	5.611.367	$C_{30}H_{25}O_{11}$	6.3	3.0	Fisetinidol-(epi)catechin	x			
35	21.7	7.071.618	7.071.590	$C_{35}H_{31}O_{16}$	3.9	8.7	Arabinopyranosyl-(epi)catechin-(epi)catechin isomer 1	x			

Table 2. Cont.

36	21.8	1.810.506	1.810.512	$C_9H_9O_4$	-2.9	4.8	Homovallinic acid		x		
37	22.1	2.780.670	2.780.653	$C_{13}H_{12}NO_6$	6.3	8.2	L-Aspartic acid. N-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]	x			x
38	22.4	5.771.258	5.771.281	$C_{19}H_{29}O_{20}$	-4.0	40.5	Procyanidin B dimer isomer 9	x			
39	22.5	5.751.065	5.751.089	$C_{26}H_{23}O_{15}$	-3.9	20.3	di-O-galloylarbutin				x
40	22.7	2.070.663	2.070.648	$C_{11}H_{11}O_4$	7.0	1.4	Dimethoxycinnamic acid	x	x		
41	23.2	2.910.874	2.910.873	$C_{15}H_{15}O_6$	0.3	2.7	Picrotoxinin	x			
42	23.3	5.111.258	5.111.246	$C_{26}H_{23}O_{11}$	-2,4	14,7	(Epi)catechin derivative 1 isomer 1		x		
43	23.4	2.890.718	2.890.731	$C_{15}H_{13}O_6$	-4.5	16.6	Epi-catechin			x	
44	24.3	4.611.301	4.611.278	$C_{19}H_{25}O_{13}$	5.0	18.2	Sibiricose A3	x			
45	24.5	5.771.351	5.771.321	$C_{30}H_{25}O_{12}$	5.3	8.0	Procyanidin B dimer isomer 10	x			
46	25.0	4.951.330	4.951.297	$C_{26}H_{23}O_{10}$	-6.6	5.8	(Epi)catechin derivative 2 isomer 1		x		
47	25.4	5.931.512	5.931.480	$C_{27}H_{29}O_{15}$	5.4	9.9	Catechin diglucopyranoside	x			
48	26.0	4.951.339	4.951.297	$C_{26}H_{23}O_{10}$	-8.6	4.6	(Epi)catechin derivative 2 isomer 2		x		
49	26.2	3.580.932	3.580.927	$C_{18}H_{16}NO_7$	1.3	9.3	trans-Clovamide (N-[(2E)-3-(3.4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-3-hydroxy-L-tyrosine)	x			
50	26.5	5.771.351	5.771.332	$C_{30}H_{25}O_{12}$	3.4	10.5	Procyanidin B dimer isomer 11	x			
51	26.9	7.371.723	7.371.710	$C_{36}H_{33}O_{17}$	1.8	5.9	(Epi)catechin dimer hexose	x	x		
52	27.4	5.751.195	5.751.181	$C_{30}H_{23}O_{12}$	2.4	24.4	Procyanidin A dimer isomer 1	x			
53	27.6	7.071.618	7.071.605	$C_{35}H_{31}O_{16}$	1.8	10.9	Arabinopyranosyl-(epi)catechin-(epi)catechin isomer 2	x			
54	28.1	3.530.303	3.530.304	$C_{18}H_9O_8$	-0.4	13.1	Dicoumarin (dimeresculetin)		x	x	
55	28.4	5.771.351	5.771.343	$C_{30}H_{25}O_{12}$	1.5	13.9	Procyanidin B dimer isomer 12	x			
56	28.6	2.050.515	2.050.506	$C_{11}H_9O_4$	-4.1	7.5	Dimethoxycoumarin		x		
57	29.2	8.651.985	8.651.955	$C_{45}H_{37}O_{18}$	3.5	8.1	Procyanidin C isomer 3	x			
58	29.7	4.151.610	4.151.588	$C_{19}H_{27}O_{10}$	5.2	8.5	β-D-Glucopyranoside, 2-phenylethyl 6-O-β-D-xylopyranosyl-	x			
59	31.0	5.751.195	5.751.172	$C_{30}H_{23}O_{12}$	4.0	49.3	Procyanidin A dimer isomer 2	x			
60	31.4	5.751.195	5.751.174	$C_{30}H_{23}O_{12}$	3.7	83.6	Procyanidin A dimer isomer 3	x	x		
61	31.5	3.030.524	3.030.522	$C_{15}H_{11}O_7$	-3.8	15.2	Taxifolin			x	
62	31.8	1.650.548	1.650.557	$C_9H_9O_3$	5.6	6.1	Paeonol				x
63	33.4	5.751.233	5.751.195	$C_{30}H_{23}O_{12}$	-6.6	5.6	Procyanidin A dimer isomer 4		x	x	
64	34.2	5.111.266	5.111.246	$C_{26}H_{23}O_{11}$	-3.9	8.3	(Epi)catechin derivative 1 isomer 2		x		
65	34.6	5.751.195	5.751.259	$C_{30}H_{23}O_{12}$	-6.0	20.6	Procyanidin A dimer isomer 5			x	
66	34.9	3.261.034	3.260.977	$C_{18}H_{16}NO_5$	17.2	11.0	Deoxyclovamide (N-[(2E)-3-(3.4- Dihydroxyphenyl) -1-oxo-2-propen-1-yl]- L-tyrosine)				x
67	35.6	5.751.232	5.751.195	$C_{30}H_{23}O_{12}$	-6.5	3.5	Procyanidin A dimer isomer 6		x	x	
68	36.4	2.090.829	2.090.819	$C_{11}H_{13}O_4$	-4.4	1.4	Dimethoxyhydrocinnamic acid		x		
69	36.6	4.630.882	4.630.862	$C_{21}H_{19}O_{12}$	4.4	3.9	Quercetin hexose isomer 1	x			
70	36.9	4.630.882	4.630.870	$C_{21}H_{19}O_{12}$	2.6	1.6	Quercetin hexose isomer 2	x			
71	38.5	4.330.776	4.330.766	$C_{20}H_{17}O_{11}$	2.3	4.3	Quercetin 3-O-α-L-arabinopyranoside	x			
72	40.8	5.751.195	5.751.225	$C_{30}H_{23}O_{12}$	-5.1	7.8	Procyanidin A dimer isomer 7			x	

The combination of MF, UF and NF technologies performs the isolation of bioactive compounds from *T. cacao* and provides unique separation capability, possibilities to scale-up and low energy consumption. Moreover, PC-rich fractions make this technology useful in the development of new cosmetic, food or pharmaceutical products with functional properties.

3.3. Cytotoxicity of selected fractions from *T. cacao* extract

There have been many reports where natural products have been used concurrently with conventional medicine and severe reactions have resulted. Several traditional/conventional medicine interactions are not yet well defined and it should be used with caution. Moreover, there is a misconception amongst many people that natural products mean safe (Hübsch et al., 2014).

Since there has not been much information about toxicological studies on PAs, MTT assay of the obtained fractions by the methodologies as describe above was carried out in order to test the cytotoxic effect of different compounds from cacao on HEK293 cell line.

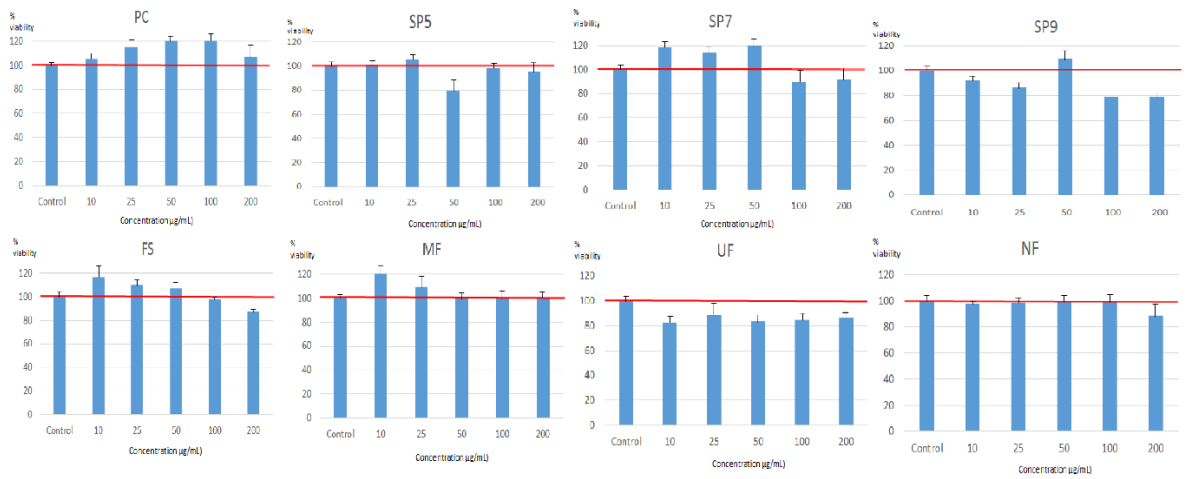

Figure 7. MTT viability cell assay for selected fractions from *Theobroma cacao*.

As observed in Figure 7, almost all fractions were higher than 100% (control), except those obtained with filtration technology by membranes, above all UF fraction which was concentrated in monomers and dimers of PCs. In the same way, collected fractions by combination of Sep-Pak C18 cartridges and semi-preparative HPLC (SP5 and SP9), which were noticed to be the more purified ones, exhibited

cytotoxic results leading to increased cell death at high concentrations. This effect could be explained by purified fractions exert a potential for toxicity at the same concentrations than other less purified since the concentration of the same compound is lower in the mixture and, in addition antagonistic interaction could be happen that also involving a reduction in the efficacy.

4. CONCLUSIONS

This study reports valuable data concerning different methodologies for purification of *T. cacao* compounds and the safety of the obtained fractions in HEK293 cell model. A DMSO cocoa extract was used in order to isolate the bioactive compounds obtaining fractions with different composition profiles, which depend on the used methodology. Regarding Sep-Pak C18 cartridges, this procedure permitted to separate the whole extract in fractions depending on the family or polymerization degree in order to reduce the complexity of the matrix. In addition, the combination of this technique and semi-preparative HPLC provided fractions with one or two compounds that would facilitate knowledge of their individual or synergic bioactivities. On the other hand, the use of membrane technology allowed to obtain a monomeric and dimeric PC-rich fraction in the UF permeate due to the low rejection capacity towards these compounds.

The assessment of the toxicity of polyphenolic fractions through MTT assay indicated a lack of toxicity. In general, the resulting fractions in this study did not deliver toxic values although more purified compounds exhibited cytotoxic values over 80% such as PC-rich fraction (UF) and the most purified ones, SP5 and SP9, but only at higher concentrations.

Overall, this approach should be recommended for several activities ranging from sample purification prior to biological testing to the production of high valued-added compounds such as functional ingredients.

5. ACKNOWLEDGMENTS

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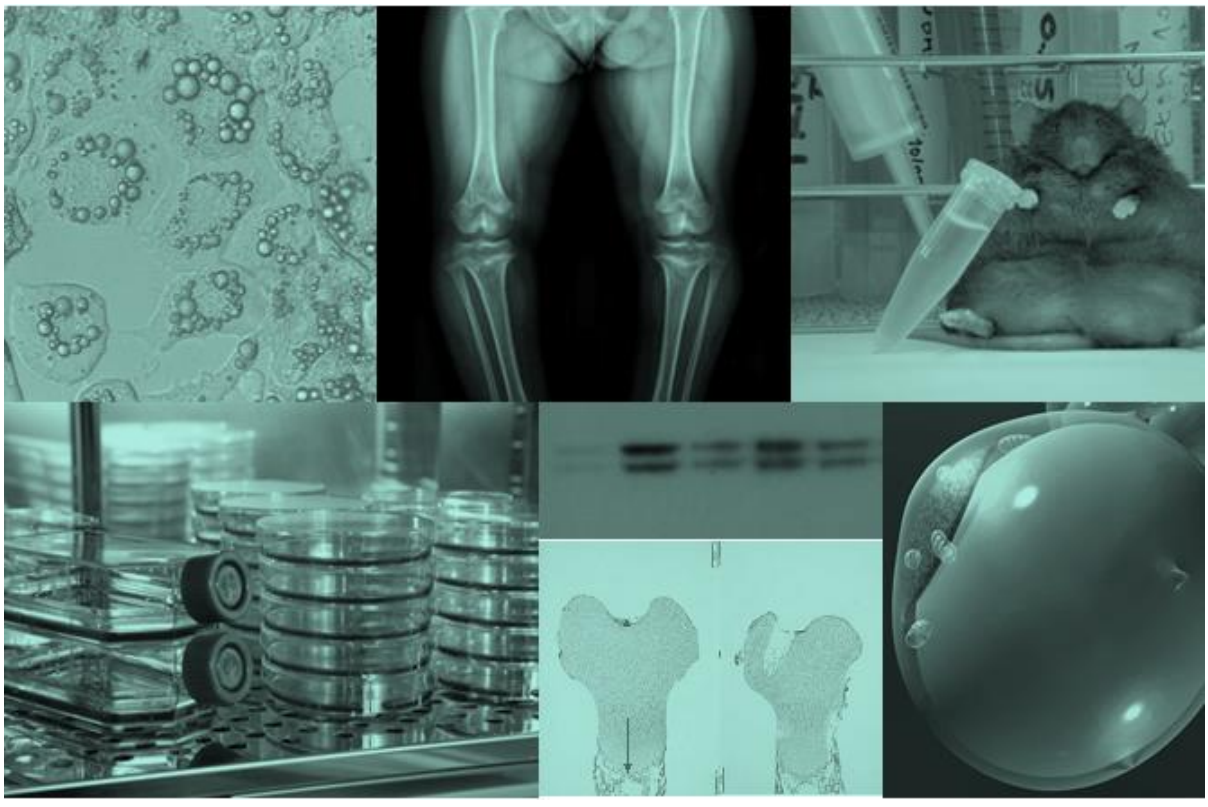
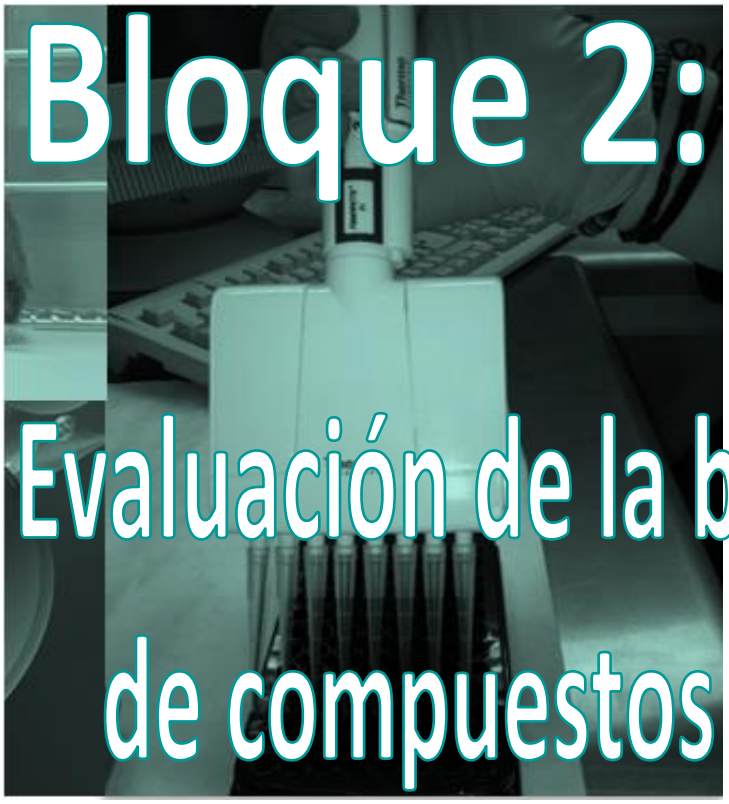
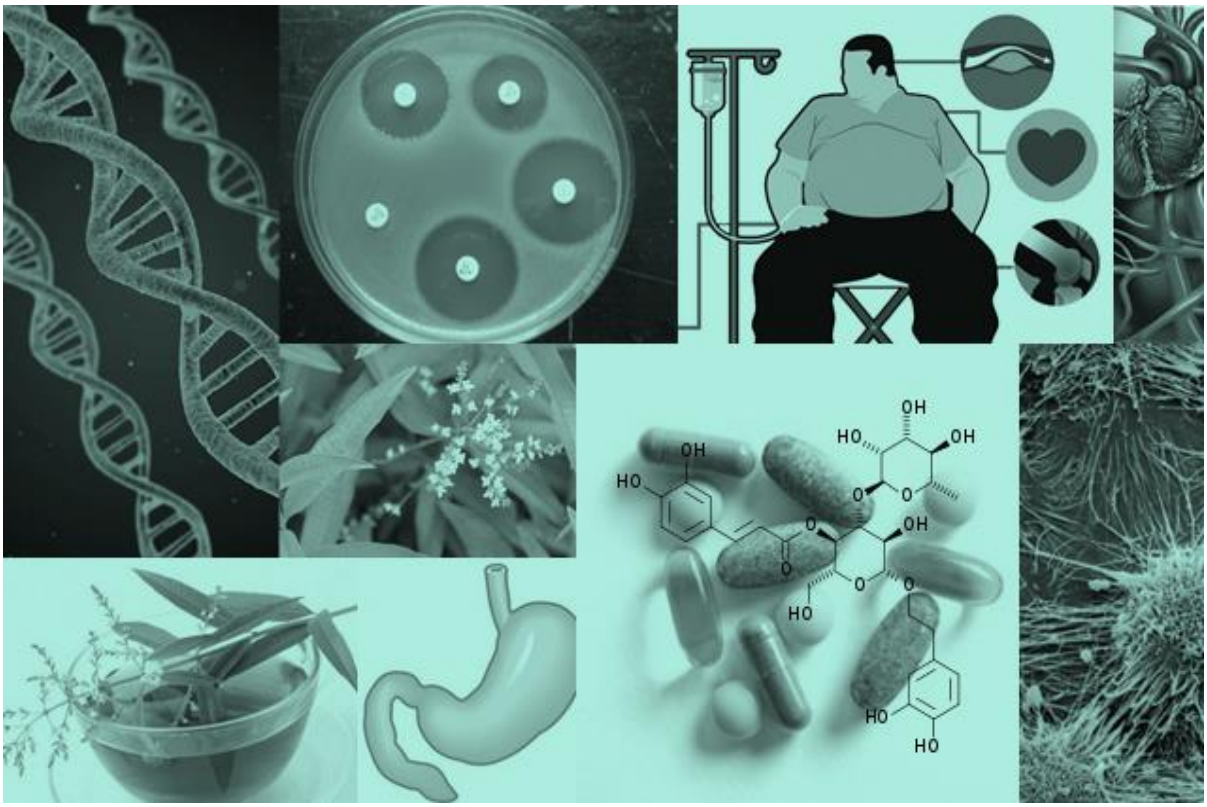




Bloque 2:

Evaluación de la bioactividad de compuestos fenólicos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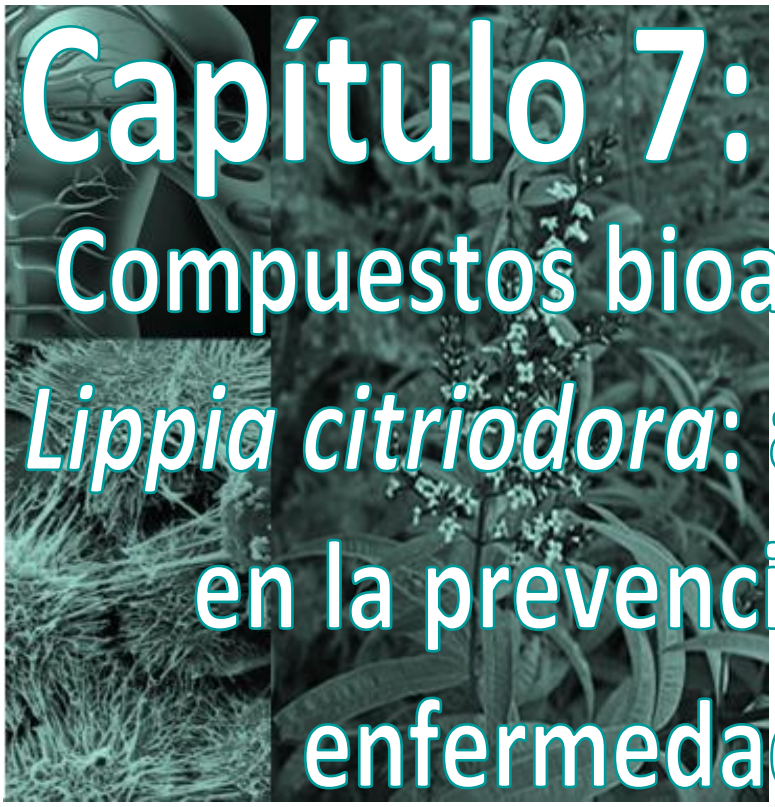


Capítulo 7:
Compuestos bioactivos de *Lippia citriodora*: aplicación en la prevención de enfermedades

In: *Occurrences, Structure, Biosynthesis, and Health Benefits Based on Their Evidences of Medicinal Phytochemicals in Vegetables and Fruits. Volume 7*. 2017, 183-220. ISBN: 978-1-53611-982-4 Nova Science Publishers

Bioactive Compounds from Lippia Citriodora: Application in Diseases Prevention

ABSTRACT

Lippia citriodora (*Lemon verbena*), a shrub indigenous to South America, was introduced into Europe at the end of the 17th century and has been widely used in infusions for its antispasmodic, antipyretic, sedative, and digestive properties. There is a large variety of phytopharmaceutical preparations containing this plant or its extracts used as an antipyretic, antispasmodic and diuretic agents. Furthermore, this plant is used in the food industry to flavour different products.

Lemon verbena infusion is commonly consumed as a treatment for colic, diarrhoea and indigestion, and as a flavoured hot drink. *Lemon verbena* infusion contains significant amounts of polyphenols, including phenylpropanoid glycosides (mainly verbascoside) and flavone diglucuronides such as luteolin 7-diglucuronide, and has a high antioxidant activity. For example, phenylpropanoids and their metabolites are the major compounds responsible for blood-cell protection against oxidative stress after administration of *L. citriodora* in rats.

In recent years, several studies conducted *in vitro* or *in vivo* have produced evidence that polyphenols can modulate intestinal inflammation. For example, verbascoside reduced the severity of intestinal macroscopic and microscopic lesions and decreased pro-inflammatory cytokine levels in various models of murine colitis. Luteolin attenuated dextran sodium sulphate (DSS)-induced colonic injury and inflammation in mice. It has also recently shown that *Lemon verbena* infusion offered beneficial effects against DSS-induced colonic inflammation in rats. *Lippia citriodora* extract has also shown *in vivo* evidences in ameliorating obesity-associated metabolic disturbances and protecting muscle and blood cells against damage caused by strenuous exercise.

The goal of this chapter is to provide an overview of the health benefits associated with bioactive compounds contained in *L. citriodora*.

1. INTRODUCTION

There is growing evidence that natural products, mostly plant-derived polyphenols, are important in the relationship between nutrients and health in human. In recent times, the close relationship between lifestyle, diet and the risk of major human diseases is becoming more evident.

Lippia citriodora is a perennial plant originally from South America, belongs to the family *Verbenaceae*. It is also known as *Aloysia citriodora*, *Aloysia triphylla*, *Lippia triphylla* and popularly as "lemon verbena," "cedrón" or "hierba luisa." Leaves of *L. citriodora* are highly appreciated as species to impart lemon flavor in food or herbal teas. On the other hand, the plant has long been used in traditional medicine in infusions for its calming effects and sedative action as well as for treating asthma, colds, fevers, flatulence, colic, diarrhoea and indigestion [1, 2].

In the last decade, several *in vivo* or *in vitro* studies have been carrying out to reveal scientific evidences that support and throw light to the traditional claims of *L. citriodora*. The potential of *L. citriodora* extract supplementation as a nutraceutical is under consideration with the aim to find phytotherapies, which contribute to treat diseases with less adverse effects and more effective than synthetic drugs.

Polyphenols are the most intensively studied natural products as recognized source of pharmacologic compounds. These compounds are found ubiquitously in a variety of fruits, vegetables, nuts, seeds, flowers, bark, beverages and manufactured foods, as a component of the natural ingredients used. They are primarily synthesized through the pentose phosphate pathway, shikimate and phenylpropanoid pathways (Figure 1) in plants [3]. Phenolics are compounds with one or more aromatic rings with one or more hydroxyl substituents, bearing more than currently known 8,000 phenolic structures, ranging from simple molecules such as phenolic acids to highly polymerized substances [4].

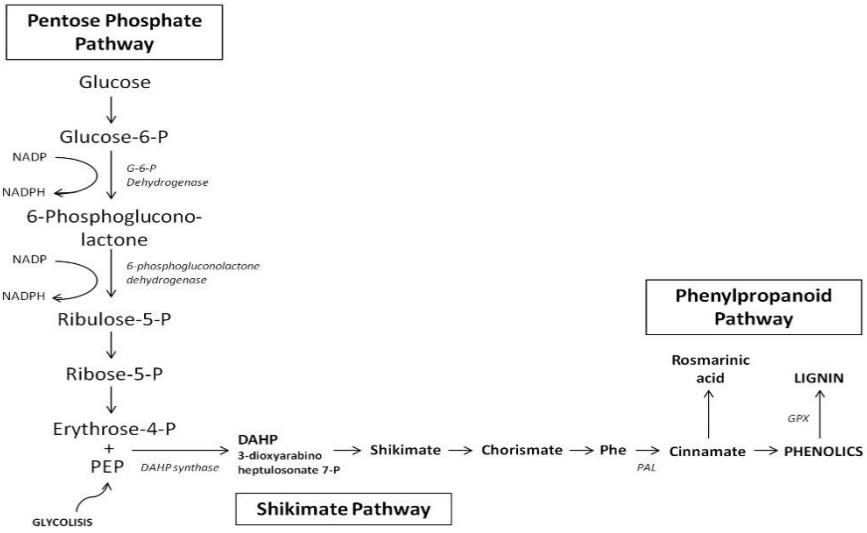


Figure 1. Pentose phosphate, shikimate and phenylpropanoid pathways by *Randhir* et al.

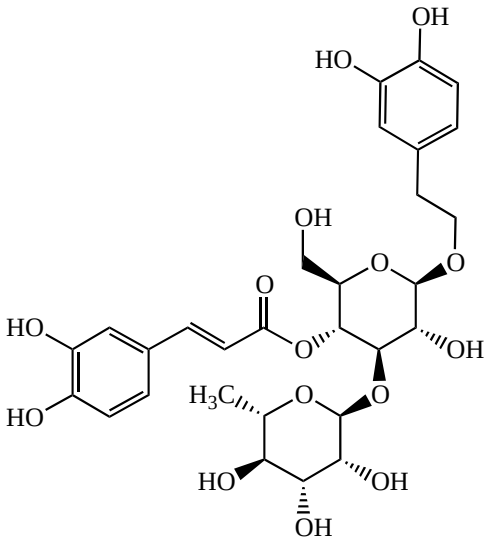
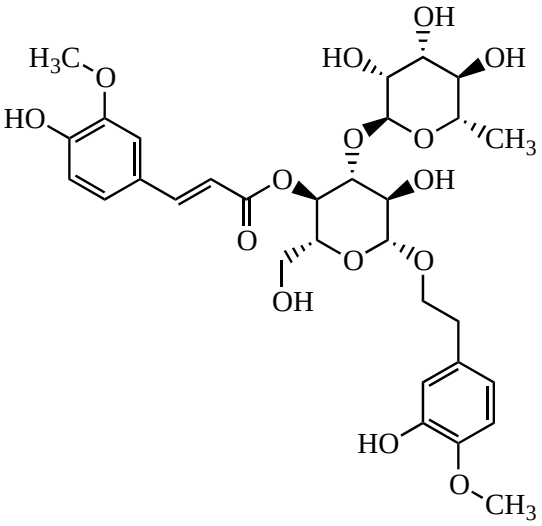
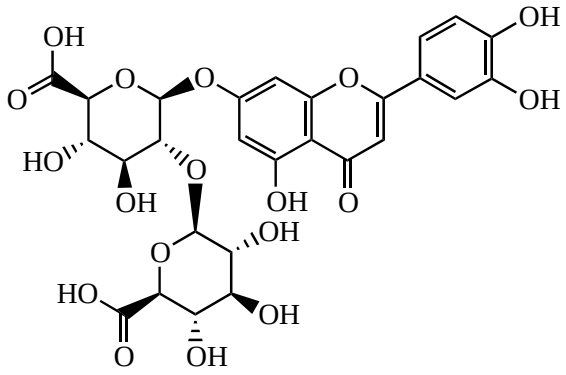


Figure 2. Chemical structure of verbascoside (VB. 1).

The antioxidant, anti-inflammatory and chemopreventive effects of *L. citriodora* seems to rely on their phenolic composition. Flavones, iridoids and phenylpropanoids are the main phenolic families found in *L. citriodora*. Verbascoside (VB. **1**) (Figure 2), a phenylpropanoid, is the most abundant compound quantifying around 10% in extracts [5].



martynoside (**2**)



luteolin 7-diglucuronide (**3**)

Figure 3. Chemical structures of martynoside (2) and luteolin 7-diglucuronide (3).

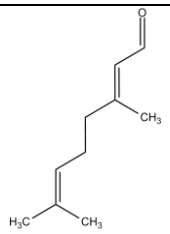
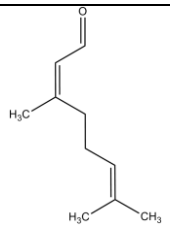
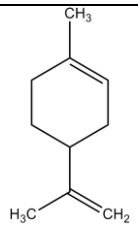
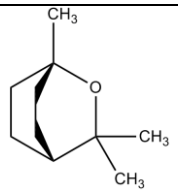
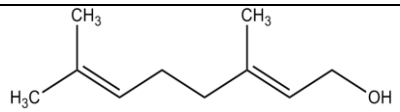
Their demonstrated beneficial effects, occurrence and uses as well as its biotechnological production has been recently reviewed [6, 7]. Consequently, verbascoside (VB. **1**) is regarded as the main responsible for the physicochemical action of *L. citriodora* [8]. However, recent scientific evidences reveal that other phenolic compounds, i.e., martynoside (**2**) or luteolin 7-diglucuronide (**3**) (Figure 3) as well as certain components from essential oil (Table 1) seem should be taken into account due to their contribution to *L. citriodora* properties [9-12]. Hence, the gain of an effective extract has to be relied upon the extraction methods of the secondary metabolites and the analysis and identification of them [13].

Even known polyphenols share the common phenolic feature, due to the structural diversity, these phytochemicals vary significantly in their physicochemical properties. Owing to the chemical complexity and the different process of extraction, separation, identification and analysis of polyphenols remain as challenging as ever, despite the recent advances in new instrumentation. The challenge is multiplied when the complex glycosylation and polymerization patterns. While it is nearly impossible to develop a protocol for all polyphenols, there are some general approaches to these important aspects of polyphenol research [4]. In *L. citriodora*, decoction or infusion are usually used for extraction of phenolic compounds where the extraction solvent is often water, methanol or ethanol in different ratios [1, 9, 14, 15]. Regarding essential oils of *Lippia*, microwave-assisted hydrodistillation [10, 12, 16-19] or improved methods such us supercritical fluid extraction (SFE) [13, 20, 21] are used for obtaining the compounds of these kind of samples. In this way, SFE extraction could significantly represent a valid alternative to traditional ones and an advanced separation technique, which complies with "green" chemistry principles [22]. This "green" method takes advantage of the high solvation power, low viscosity and high diffusion coefficient provided by the supercritical state in order to improve the mass transfer and, consequently, the extraction yield. The most used supercritical fluid is CO_2 (non-toxic, non-flammable and rather inexpensive) that can generate added value products without thermal degradation or massive use of organic solvents, as in organic solvent extraction (OSE) [23-26].

Methods for separation and identification of *L. citriodora* compounds using various chromatographic techniques have been reported by several authors [5, 11, 27, 28]. Reversed-phase high performance liquid chromatography (RP-HPLC) coupled with a diode array detector (DAD) and/or mass spectrometric detector [29-31] is the most widely use analytical tool for quantification of these polyphenols, although when essential oils are studied, these are usually analyzed by gas chromatography (GC) [19, 32].

The aim of this chapter is to provide an overview of the health benefits associated with bioactive compounds contained in *L. citriodora*.

Table 1. Main essential oil components (4-8) of *L. citriodora*

Name	Chemical structure
geranial (4)	
neral (5)	
limonene (6)	
1,8-cineole (7)	
geraniol (8)	

2. ANTIOXIDANT CAPACITY

The antioxidant properties of different lemon verbena extracts, essential oils and pure compounds can be evaluated using various *in vitro* or *in vivo* assays. Free radical intermediates are involved in several metabolic disturbances that are responsible for cell and disease injuries, and are therefore

implicated in many human diseases (cancer, cardiovascular diseases (CVDs), inflammatory processes, cataracts, and even the normal ageing process) [9, 33, 37].

The protective effects of polyphenols in biological systems are ascribed to their capacity to transfer electrons of free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals, and inhibit oxidases [38, 39]. On the other hand, essential oils contain volatile aroma compounds which are complex mixtures of compounds belonging to diverse chemical families (terpenes, alcohols, aldehydes, phenolic compounds, esters, ethers, ketones) [18, 40].

Polyphenols contain at least one aromatic ring with one or more hydroxyl groups in addition to other substituents, and they can be divided into 15 major classes according to their chemical structures. Polyphenols possess ideal structural chemistry for free radical-scavenging activities, and have been shown to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis. There is a hierarchy of polyphenol antioxidant activities that is dependent on structure and defines the relative abilities of these compounds to scavenge free radicals [35, 36]. This relationship between the chemical structure of flavonoids and their radical-scavenging activities was analyzed by *Bors* et al. [41].

As it is known, several factors are responsible for variations in the level of the antioxidant activity of different extracts but one of the most important factors concerns the methodology for evaluating the antioxidant activity. The most commonly used antioxidant methods are 2,20-azino-*bis*(3-ethylbenzthiazoline)-6-sulfonic acid radical ($ABTS^{\cdot+}$) and 1,1-diphenyl-2-picrylhydrasyl radical ($DPPH^{\cdot}$). Both of them are characterized by excellent reproducibility under certain assay conditions, but they also show significant differences in their response to antioxidants [42].

Table 2 show data of antioxidant capacity *in vitro* and *ex vivo* assays of different extracts obtained by different methods.

Table 2. Antioxidant activities of *L. citriodora* extracts

	EXTRACTS		ASSAYS		RESULTS	REFERENCES
In vitro	Dried leaves/Infusion		TEAC		169 mg TE/g DL	[43]
			FRAP		120 mg Fe[II]-E/g DL	
			SRS	Non-enzimatic: PMS/NADH system	IC_{50} 22.8 µg/mL	[9]
				Enzimatic: X/XO system	IC_{50} 3.3 µg/mL	
			HRS deoxyribose	Scavenging ratio	38.3%	
			HAS	Protective effect	67%	
	Dried aerial plants	MeOH under reflux	DPPH of purified compounds		IC_{50} from 8.5 to 44.6 µM	[15]
		Infusion	TRAP		362 µmol TE/g DM	[14]
			ABTS		358 µmol ascorbic acid/g DM	
			DPPH		230 µmol ascorbic acid/g DM	
			TRAP		74 µmol TE/g DM	[44]
			ABTS		186 µmol ascorbic acid/g DM	
			DPPH		135 µmol ascorbic acid/g DM	
			RP		34 µM TE/g DM	
			SRS		37.3%	
			HPRS		6.8%	
		MeOH Soxhlet	DPPH		IC_{50} 30 µg/mL	[45]
	Commercial extract (in water)	25% VB	TEAC		0.81 mM/g extract	[29]
			MDA generation		IC_{50} 6.91 µg/mL	
			ORAC		4075 µmol TE/g extract	
		20% VB	DPPH		IC_{50} 13 µg/mL	[7]
		10% VB	GRed		12 nKat/ng protein	[46]
	Essential oil		DPPH		58% inhibition	[18]
Ex vivo	Dried leaves/Infusion		Human serum	MDA (abs)	12.230 vs 15.660 (*p*-value 0.044)	[47]
				FRA (abs)	1.950 vs 1.670 (*p*-value 0.003)	
	Dried aerial plants/Infusion		Rat brain homogenates	Spontaneous brain CL	IC_{50} 1.8 µg/mL	[14]
				TBARS	IC_{50} 17.1 µg/mL	
			Rat plasma	PC	IC_{50} 29.7 µg/mL	
			Swiss mice brain homogenates	Spontaneous brain CL	IC_{50} 5.4 µg/mL	[44]
				TBARS	IC_{50} 63.5 µg/mL	
			Blood gluthatione level of diabetic rats		34.7 vs 22.3 mg	[48]

	Commercial extract (in water)	25% VB	Rat plasma		MDA	0.22 nmol/mL plasma	[29]
					SOD	3.2 nKat/mL plasma	
		20% VB	Rat blood cells	Lymphocytes	CAT	320 kat/ L blood	[7]
					GPx	400 kat/ L blood	
					GRed	650 kat/ L blood	
				Neutrophils	CAT	590 kat/ L blood	
					GPx	180 kat/ L blood	
					GRed	910 kat/ L blood	
					MOP	170 kat/ L blood	
				Erythrocytes	CAT	15 kat/ L blood	
					GPx	62 kat/ L blood	
					GRed	420 kat/L blood	
		10% VB	Human blood cells	Erythrocytes	CAT	1.2 K/g Hb	[46]
					GPx	31.9 nKat/g Hb	
					GRed	73.1 nKat/g Hb	
					SOD	4.2 pKat/g Hb	
				Lymphocytes	CAT	78.5 K/g Hb	
					GPx	49.5 nKat/g Hb	
					GRed	213 nKat/g Hb	
					SOD	24.1 pKat/g Hb	
					MDA	4.4 µmol/L	
					PC	27.4 µmol/L	
			Human plasma		MDA	5.1 µmol/L	
					PC	788 µmol/L	
Ex vivo	Commercial extract (in water)	10% VB	Human blood cells	Erythrocytes	CAT	2.8 K_5/g Hb	[49]
					GPx	28.0 nKat/g Hb	
					GRed	48.7 nKat/g Hb	
					SOD	26.2 pKat/g Hb	
				Lymphocytes	CAT	32.4 K_5/10^9 cells	
					GPx	68.4 nKat/10^9 cells	
					GRed	1.029 nKat/10^9 cells	
					SOD	41.1 pKat/10^9 cells	
					MDA	27.3 µmol/L	
					PC	17.3 µmol/L	
				Neutrophils	CAT	38.6 K_5/10^9 cells	
					GPx	99.7 nKat/10^9 cells	
					GRed	1.335 nKat/10^9 cells	
					SOD	40.6 pKat/10^9 cells	
					MDA	18.2 µmol/L	
					PC	12.3 µmol/L	
			Human plasma		MDA	174 µmol/L	
					PC	104 µmol/L	
			Human blood cells	Erythrocytes	CAT	5.72 K/10^9 cells	[50]
					GPx	45.7 nKat/10^9 cells	
					GRed	1.37 nKat/10^9 cells	

					SOD	1.28 pKat/10^9 cells	
				Lymphocytes	CAT	16.7 K/10^9 cells	
					GPx	190 nKat/10^9 cells	
					GRed	74.5 nKat/10^9 cells	
					SOD	34.1 pKat/10^9 cells	
					MDA	1.08 µM	
					PC	145%	

3. ANALGESIC AND ANTIHYPERALGESIC PROPERTIES

Neuropathic pain is a widespread disorder induced by autoimmune diseases, drugs or toxin exposure, infections, metabolic insults or trauma. Neuropathic pain is depicted by nerve damage, which causes muscle weakness, altered functionality and sensitivity, and a chronic pain syndrome characterized by allodynia (pain elicited by a non-noxious stimulus) and hyperalgesia (increased pain response to an noxious stimulus), which can persist for a long time after the initial injury is resolved. The molecular mechanisms of neuropathic pain are not fully elucidated, and treatment is still unsatisfactory in most cases [51]. The treatment of neuropathic pain or peripheral neuropathic pain is often hampered by poor activity of the available drugs such as opioids or nonsteroidal anti-inflammatory drugs, and the occurrence of adverse side effects [52]. Consequently, the search for natural alternatives to drugs is of particular interest to develop novel therapeutics more effective against the neuropathic pain. In this sense, attention has been paid on verbascoside (VB. **1**). The analgesic effects of verbascoside (VB. **1**) have been already reported when administered orally and topically but this compound was extracted from plants belonging to *Verbenaea* and *Buddlejaceae* families [53, 54]. Verbascoside (VB. **1**) isolated from *L. citriodora* seemed to revert the mechanical hyperalgesia in two animal models of neuropathic pain: a peripheral mononeuropathy produced either by a chronic constriction injury of the sciatic nerve or by an intra-articular injection of sodium monoiodoacetate [55]. Verbascoside (VB. **1**) administered intraperitoneally at a dose of 100 mg/kg mitigated the mechanical hyperalgesia in both models. The antihyperalgesic effect started 15 min after the verbascoside (VB. **1**) administration

and persisted up to 30-45 min. Verbascoside (VB. **1**) was also effective after oral administration at doses of 300 and 600 mg/kg with a longer lasting effect (60 min). The relatively high effective doses observed could be related to the low bioavailability of this compound possibly due to its limited hydrophobic properties. Furthermore, it is important to take into account that verbascoside (VB. **1**) seems to be unable to increase the pain threshold therefore the antihyperalgesic activity of verbascoside (VB. **1**) is not a consequence of its demonstrated analgesic properties [55].

4. SEDATIVE EFFECTS

Another of the traditional uses of *L. citriodora* infusion has been the treatment of anxiety due to its sedative effects. Recently it was demonstrated that the spontaneous locomotion and the exploratory behavior of mice was reduced as consequence of the administration of *L. citriodora* aqueous extract at doses lower than those orally used in humans [56]. These evidences support the soft sedative properties and benzodiazepine-like effect of the aqueous extract of *L. citridora* on the gamma-aminobutyric acid (GABA. **9**)-A receptors, which seem to be potentiated by diazepam (**10**) and reduced by flumazenil (**11**) (Figure 4). Regarding the sedative components of *L. citriodora*, it still remains undetermined. An apigenin flavone glucoside vitexin (**12**) (Figure 4) seems not to have sedative effects [56]. Other components from the *L. citriodora* essential oil such as limonene (**6**) or citral (**13**) (Figure 4) [1, 57] have been reported as sedative in the other related plant of genus *Lippia* [58]. However, these volatile compounds are not expected to totally remain in the aqueous extract after decoction. On the other hand, luteolin 7-diglucuronide (**3**), which is found in the infusion of *L. citriodora* [57], has also been isolated from *L. alba* and demonstrated to bind the GABA-A receptors [59]. Therefore, the sedative effect of the aqueous extract of *L. citriodora* could be attributable to a combination of several compounds.

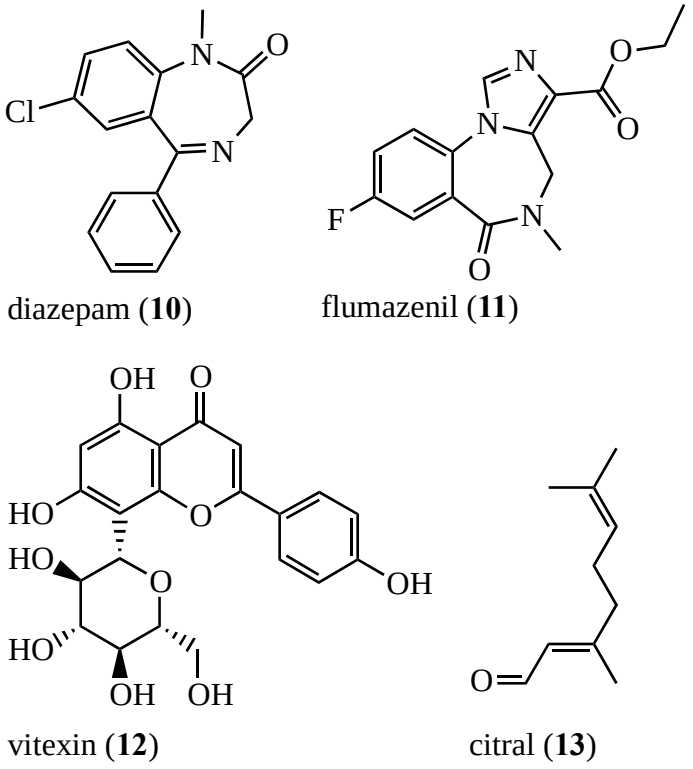


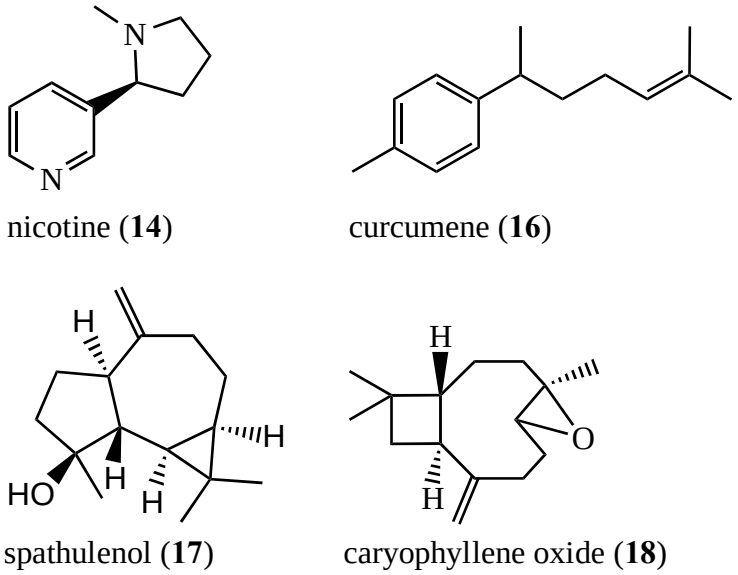
Figure 4. Structures of diazepam (10), flumazenil (11), vitexin (12) and citral (13).

5. CENTRAL NERVOUS SISTEM DISEASES

Multiple sclerosis is a neurodegenerative disease of the central nervous system (CNS) characterized by inflammation, demyelination and axonal damage. The relation between inflammation and neurodegeneration plays a significant role in disease progression. Due to multiple sclerosis heterogeneity and patient response variability, the use of pharmacological therapies has been ineffective in progressive forms of multiple sclerosis and only partially effective in relapsing-remitting phase. Amongst complementary treatments, nutrition has been considered as a decisive factor to ameliorate symptoms and disease progression [60-62]. The *L. citriodora* extract supplementation (200 mg/day) of low-fat diet seemed to be effective on biochemical markers of multiple sclerosis reported in a diet intervention probably due to anti-inflammatory action of verbascoside (VB. **1**) [6]. The assessment of oxidative and inflammatory markers in urine and serum samples showed a decrease in isoprostane-8-isoprostaglandin F2alpha (8-iso-PGF2α) and interleukin-6 (IL-6) levels, whereas C-reactive proteins (CRPs) levels diminished significantly as a consequence of the antioxidant

supplementation [63]. A further study, demonstrated that the effect of *L. citriodora* extract supplementation (600 mg / day) in pro- and anti-inflammatory serum biomarkers depends on the different clinical subtypes of multiple sclerosis (primary progressive, secondary progressive and relapsing-remitting). *L. citriodra* supplementation affects the cytokine profile depending on the clinical subtypes, exhibiting more effectiveness against secondary progressive subtype [64]. Although sample size limits the statistical significance of these studies as well as the interpretation of results, the comparison between placebo and supplemented groups allows the assessment of relevant trends and set the basis for further investigation with the aim to verify the efficacy and the action mechanisms of *L. citriodora* supplements as complementary therapies in multiple sclerosis.

The essential oil of *L. citriodora* derived from the leaves also showed pharmacological properties in neurodegenerative disorders. The essential oil inhibited tritium [^{3}H] nicotine (**14**) (Figure 5) binding in a concentration-dependent manner. Neuronal nicotinic acetylcholine receptors (AChRs) represent novel targets for central nervous system (CNS) therapeutics and may have substantial roles in mediating antinociception and modulating cognitive performance. The iron (II) chelating activity of the *L. citriodora* essential oil *in vitro* is of significance due to the reduction the oxidative damage in many neurodegenerative disorders. The neuroprotective properties of *L. citriodora* were assessed using hydrogen peroxide (**15**)-induced and β-amyloid-induced neurotoxicity with the Cath.-a-differentiated neuroblastome cell lines (CAD cells). Essential oil displayed a neuroprotective profile providing complete and partial protection against H_2O_2 and β-amyloid-induced neurotoxicity, respectively, both relevant to oxidative stress-induced damage in major neurodegenerative disease [19]. The major components of the essential oil were limonene (**6**) (13.6-20.1%), geranial (**4**) (6.3-20.1%), neral (**5**) (3.7-15.1%), 1,8-cineole (**7**) (9.2-9.4%), curcumene (**16**) (Figure 5) (3.5-6.3%), spathulenol (**17**) (Figure 5) (3.1-5.0%) and caryophyllene oxide (**18**) (Figure 5) (2.2-8.4%) [19]. However, to date, a functional group-based fractionating of this oil has not carried out to elucidate the main responsible for the biological activity reported and to confirm its utility *in vivo*.



nicotine (**14**) curcumene (**16**)

spathulenol (**17**) caryophyllene oxide (**18**)

Figure 5. Nicotine (14), curcumene (16), spathulenol (17) and caryophyllene oxide (18).

6. DIGESTIVE DISORDERS

The decoction of the fresh leaves have been frequently used as eupeptic and to treat gastrointestinal disorder such as spasms and indigestion [65, 66]. Scientific evidences support the antispasmodic effect of an aqueous crude extract of *L. citriodora* on isolated rat duodenum in comparison with the effect of papaverine (**19**) (Figure 6), one of the most active relaxant of smooth muscle [67, 68]. The extract, sensitive to methylene blue (MB) (non-selective inhibitor of guanylate cyclase (GC)) [69], appear to reach about 80% of the maximal relaxation of papaverine (**19**). Both mechanisms of action seem to be the same by means of the cyclic guanosine monophosphate (cGMP) increase. The ingestion of aqueous extract directly relaxed the intestinal smooth and non-competitively inhibited the dose-response curve (DRC) of acetylcholine (ACh) 50% inhibitory concentration (IC_{50}) of 1.34 ± 0.49 mg lyophilized/mL). The extract seemed not to be an antagonist of Ca^{2+} influx since it inhibited the DRC of Ca^{2+} on high K^+ media (IC_{50} of 2.64 ± 0.23 mg lyophilized/mL) in a non-competitive way and potentiated the non-competitive inhibition of W-7 (a calmodulin blocker) and papaverine (**19**) on the Ca^{2+} DRC [70]. Other mechanism of the antispasmodic effects was the activation of K^+ channels upon the agonist, which could be a consequence of increasing cGMP, and drives to hyperpolarization and

relaxation. On the other hand, upon a hypoxic-free glucose medium, 2 mg/mL of extract was able to inhibit both phasic and tonic contractions, however lower doses were not enough to relax either the phasic or the tonic hypoxic responses [70].

The antispasmodic action of the two main components found in the extract, vitexin (**12**), whose antispasmodic effect was reported previously [71] and isovitexin (**20**) (Figure 6) were evaluated. Vitexin (**12**) non-competitively inhibited de DRC of Ach on rat duodenums, while no inhibitory effect were observed for isovitexin (**20**). On the other hand, none of the studied flavonoids inhibited the DRC of Ca^{2+} as the extract did, so these flavonoids seem not to be the only responsible for the antispasmodic effect of *L. citriodora*. Indeed, volatile components of essential oil from *L. citriodora* could be related to antispasmodic effects as it was observed for two chemotypes of *L. alba* [72]. Limonene (**6**), neral (**5**), geranial (**4**), and (-)-carvone (**21**) (Figure 6) were the major components of one of chemotype while the other one was rich in linalool (**22**) (Figure 6) content. Both of them exhibited antispasmodic effects being the former chemotype more potent than the other.

Aqueous extract of *L. citriodora* also showed positive effects on healing of experimentally induced gastric ulcers in rats [73, 74]. Indeed, the effectiveness of the plant extract in treatment of gastric mucosal damage was higher than that provided by histamine H2 antagonist ranitidine (**23**) (Figure 6), a drug commonly used to treat and prevent ulcers in the stomach and intestines [73, 74]. The ulceration rate observed by the *L. citriodora* was 40% and the ulcer score was also significantly lower in comparison with those observed by ranitidine treatment. Furthermore, *L. citriodora* exhibited the highest preventive index, 75% [73] and their cytoprotective effect was also confirmed by histological examination that showed protection of mucosa, reduction of mucosal and submucosal hyperemia and edema, and prevention of deep mucosal necrosis [73, 74]. Despite of the unclear mechanisms of ulcer healing effect, it is believed that the anti-inflammatory activity and the strong antioxidant properties of some flavonoids may have caused a part of the observed therapeutic effect of this herb. Antioxidant agents increase collagen fiber proliferation and enhance the quality of gastric ulcer healing [75] and

the cytoprotective and anti-secretory properties of numerous flavonoids have been showed in different experimental models of gastric ulcer [76].

Similarly, the preventive consumption of *L. citriodora* infusion also offered some antioxidative protection against the development of mild-to-moderate colonic inflammation induced by dextran sulfate sodium (DSS) in rats. A daily intake of 5.6 µmol of polyphenols from *L. citriodora* help manage oxidative stress by stimulating antioxidative enzymes such as superoxide dismutase (SOD) and reducing lipid peroxidation (malondialdehyde (MDA)). Meanwhile, other oxidative markers such as glutathione peroxidase (GPx), glutathione reductase (GRed), catalase (CAT) and glutathione (GSH) were not significantly modified [77]. On the other hand, the bioavailability and intestinal absorption of polyphenols derived from *L. citriodora* were examined both healthy and colitic rats by means of *in situ* intestinal perfusion model. Intestinal absorption of verbascoside (VB. **1**) and flavone diglucuronides did not significantly differ between the healthy and colitic rats. Intestinal absorption and urinary excretion of *L. citriodora* flavone diglucuronides were not altered by colonic inflammation, but that urinary excretion of hydroxycinnamic acid derived from verbascoside (VB. **1**) was affected in a colitic situation [28].

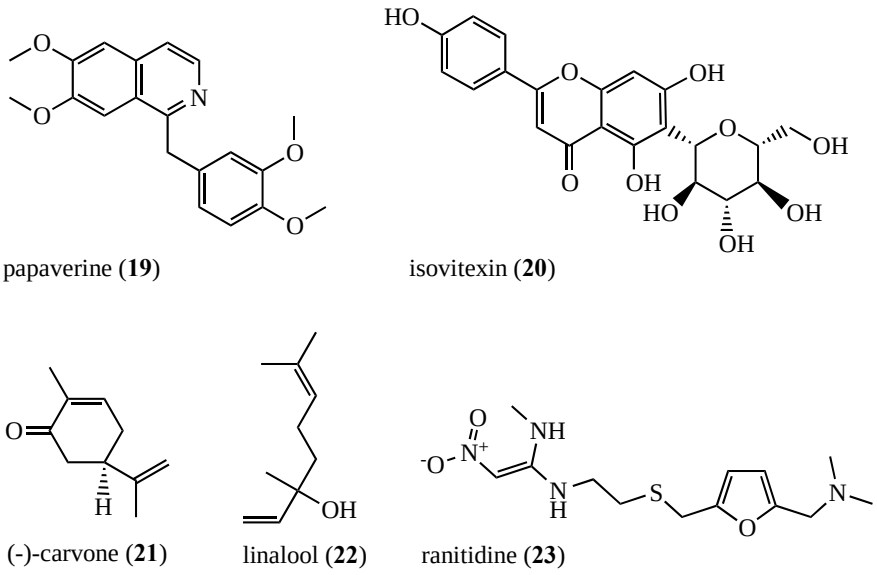


Figure 6. Papaverine (19), isovitexin (20), (-)-carvone (21), linalool (22) and ranitidine (23).

7. METABOLIC DISEASES: DIABETES

The control and management of hyperglycaemia and postprandial hyperglycaemia are a crucial element in the treatment of diabetes. Many researchers have been interested on flavonoids as antidiabetics due to their efficacious role in mitigating the hyperglycaemic effects of diabetes [78, 79]. The α-glucosidase inhibitory properties of several culinary herbs, among them *L. citriodora*, were studied as possible source of functional foods. *L. citriodora* infusion exhibited a weaker inhibitory activity against yeast α-glucosidase than those observed by other herbs. However, it was inactive against rat intestinal α-glucosidases, a more complex system, whose enzymes are more similar to human ones [43]. Using the hyperlipidemic mice model ($LDLr^{-/-}$) treated with high-fat and high-cholesterol diet and *L. citriodora extract*, an increased triglyceride clearance was observed after food ingestion but no changes were noted on postprandial glucose [80]. Therefore, currently there are no strong evidences of the physiological relevance of *L. citriodora* regarding to mitigating postprandial hyperglycaemia in animals or humans.

8. OBESITY

The effects of *L. citriodora* extract and verbascoside (VB. **1**) were also proven in the alleviation obesity-related disturbances in hypertrophic adipocytes. A decrease in triglyceride accumulation, in generation of reactive oxygen species (ROS) and the restoration mitochondrial membrane potential in adipocytes was observed for both substances [80]. The underlying mechanisms seemed to occur *via* ROS-mediated downregulation of nuclear factor kappa B (NF-kappaB) transcription factor (TF) and peroxisome proliferator-activated receptor gamma (PPAR-*γ*)-dependent transcriptional upregulation of adiponectin. A potent activation of AMP-activated protein kinase (AMPK), the mRNA expression upregulation of peroxisomal proliferator-activated receptor alpha (PPAR-□) and the mRNA expression downregulation of fatty acid synthase (FAS) were evidenced. Despite the equivalent verbascoside (VB. **1**) concentrations tested, significant differences were detected between *L. citriodora* extract and verbascoside (VB. **1**), enlightening a higher effect in the presence of *L. citriodora* extract. Therefore,

although verbascoside (VB. **1**) seems to be an important therapeutic aid in the management of obesity and/or associated disturbances, the effect of complete *L. citriodora* extract is greater probably due to other compounds by means of synergistic reactions or complementary effects. Indeed, in animal models that closely resembles the metabolic syndrome, the whole extract prevented the expected weight gain, liver steatosis and hypertrophic adipocytes, concomitantly to alleviation of serum cholesterol, triglycerides and neutral lipids in the liver without altering serum biomarkers of hepatic toxicity was observed by several authors [80, 81].

9. CARDIOVASCULAR DISEASES

Stress-dependent cardiovascular diseases are very frequent, and requires medical treatment on a chronic bases and lifestyle changes, especially food habits. The administration of an aqueous extract for *L. citriodora* induced a transitory hypotension, dose-dependently between 1-30 mg lyophilized per Kg in normotensive rats [56]. This effect seemed not due to cholinergic effect, nitric oxide (NO)-release or α1-adrenergic antagonism. In isolated vas defenders, the aqueous extract showed a non-competitive contractile blockade as it was previously observed in the smooth muscle of intestine [70]. Another contribution to hypotension could be the negative inotropism of the extract observed on isolated rat hearts. All these facts give support to the use of *L. citriodora* as a coadjuvant for treating angor among other cardiovascular diseases (CVDs). Certain flavonoids, due to their antioxidant activity, are potential protectors against cardiovascular diseases. Among the flavonoids present on the aqueous extract from *L. citriodora,* vitexin (**12**) was reported as hypotensive at doses >10 mg/kg in normotensive rats [56]. However, on isolated rat hearths, vitexin (**12**) did not change the inotropism contrary to the effect observed by verbascoside (VB. **1**) which induced a positive inotropism [82, 83]. Furthermore, in a model of ischemia-reperfusion, vitexin (**12**) slightly prevented hearts from the post-ischemic diastolic contracture. Therefore, vitexin (**12**) did not show cardiac anti-ischemic properties.

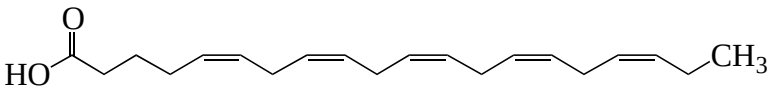
10. BIOCHEMICAL PARAMETERS AFTER EXERCISE

Intense physical activity increases oxygen consumption and the formation of reactive oxygen species (ROS) producing oxidative damage and sings of muscular injury. Therefore, the optimal recovery after physical activity is an essential aspect of sport practice. Several single-blind, parallel group, randomized controlled trials have assessed the effects of commercial *L. citriodora* extract consumption after exercise on several circulating parameters, antioxidant enzyme activities and expression and oxidative stress markers. Results evidenced biochemical changes such as the activation of glutathione reductase (GRed) in erythrocytes and lymphocytes, lower levels of oxidative stress markers, such as malondialdehyde (MDA) and protein carbonyls in plasma, a modulation on circulating lipid profile increased high-density lipoprotein (HDL)-colesterol, a modest decrease in erythrocyte number and acute inflammation and increase in circulating urea after extract intake [46, 49, 50, 84, 85]. Furthermore, a decrease of 20% was observed in circulating myoglobin, suggesting a protective effect of *L. citriodora* in muscle tissue, while the extract did not interfere with the increased glutathione-disulfide reductase gene (GRD) expression due to cell adaptation to oxidative stress [49]. These effects seemed to be attributable to verbascoside (VB. **1**) as the major phenolic compounds found in *L. citriodora* which represented 10% (w/w) of extract content [46]. However, currently, there are not reliable evidences that could prove this hypothesis. In parallel, significantly positive changes in emotional status and quality of sleep seemed to be another consequence of the *L. citriodora* consumption [85].

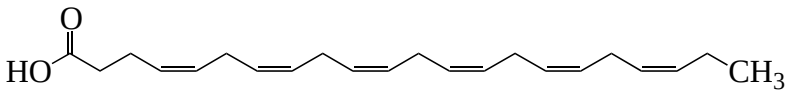
11. JOINT DISEASES

Joint diseases such as osteoarthritis and rheumatoid arthritis seem to be related to inflammation and oxidative stress. Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) are responsible for damage on cartilage, synovial fluid structures and mitochondria [86, 87]. Consequently, plants, common sources of antioxidants, might help minimize the muscular and joint injuries related to oxidative stress and inflammation. In this sense, a nutritional supplement based on

an *L. citriodora* combined with omega-3-fatty acids demonstrated the improvement of general condition of joint status (pain, stiffness and function) in subjects with joint discomforts in a randomized, double-blinded, placebo-controlled study [88]. The supplement contained 370 mg of fish-oil powder eicosapentaenoic acid (EPA. **24**)/docosahexaenoic acid (DHA. **25**) (10/8) (Figure 7) and 230 mg of the standardized *L. citriodora* extract (14% of verbascoside (VB. **1**) w/w) per capsule. The daily dosages of verbascoside (VB. **1**) administrated to patients were 193 mg during the first 5 weeks and 97 mg the last three weeks. After 9 weeks, volunteers revealed a significant improvement of joint status and pain relief. However, these effects cannot to be attributable exclusively to *L. citriodora* extract.



eicosapentaenoic acid (EPA. **24**)



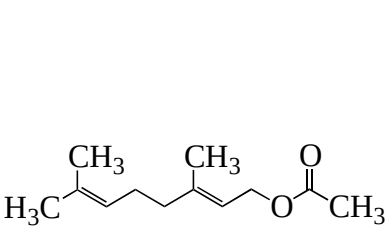
docosahexaenoic acid (DHA. **25**)

Figure 7. Eicosapentaenoic acid (EPA. 24) and docosahexaenoic acid (DHA. 25).

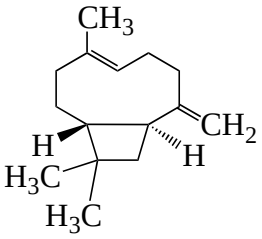
12. ANTIMICROBIAL ACTIVITY

The antibiotic era during the 20th century has substantially reduced the threat of infectious diseases. Nevertheless, over the years, there has been a decrease in microbial susceptibility to existing antimicrobial agents responsible for critical point drug resistance in hospitals and in communities [89]. Nowadays, natural products of higher plants may give a new source of antimicrobial agents [32]. Many compounds in lemon verbena are known to exert antimicrobial activity, above all compounds contained in essential oils. Differences in the chemical composition of them related to variety, agronomic practice and processing are also likely to influence antimicrobial properties, since these factors contribute to both the profile and relative concentrations of active ingredients [12]. The

components commonly found in *L. citriodora* essential oils are: neral (**5**), geranial (**4**), limonene (**6**), geranyl acetate (**26**) (Figure 8), betacaryophyllene (**27**) (Figure 8), curcumene (**16**), spathulenol (**17**), *etc* [16, 19]. The antimicrobial activity of *L. citriodora* has been mainly associated to the terpene fraction of essential oils, nevertheless aqueous or methanolic extracts enriched in phenylpropanoids have exhibited significant antimicrobial activity, especially against Gram-positive bacteria [90], likely due to verbascoside (VB. **1**) [91], which may be useful for food preservation. Table 3 shows the antimicrobial activities of both essential oils and leaves extracts against different strains of microorganism by the most common assays such as disk diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).



geranyl acetate (**26**)



betacaryophyllene (**27**)

Figure 8. Geranyl acetate (26) and betacaryophyllene (27).

Table 3. Antimicrobial activities of *L. citriodora* extracts

EXTRACTS	STRAINS		ASSAYS	RESULTS	REFERENCES
Essential oil of aerial parts/Clevenger	Dengue virus	DENV-1	Virucidal activity	IC_{50} 1.9 µg/mL	[10]
		DENV-2		IC_{50} 2.9 µg/mL	
		DENV-3		IC_{50} 2.6 µg/mL	
		DENV-4		IC_{50} 33.7 µg/mL	
	Escherichia coli O157 ATCC 1659		Disc-difussion	22 mm	[18]
			MIC	0.5% v/v	
			MBC	>1% v/v	
	Staphylococcus aureus ATCC 13565		Disc-difussion	17 mm	
			MIC	1% v/v	
			MBC	>1% v/v	
	Klebsiella ozaenae	URO2	Disc-difussion	9 mm	[16]
			MIC	20 µg/mL	
		URO4	Disc-difussion	10.75 mm	
			MIC	10 µg/mL	
		BLEE URO7	Disc-difussion	9 mm	
			MIC	50 µg/mL	
		MultiR URO11	Disc-difussion	8 mm	
			MIC	20 µg/mL	
		SV1	Disc-difussion	9.75 mm	
			MIC	20 µg/mL	

		Enterobacter aerogenes	URO6	Disc-difussion	8.75 mm	
				MIC	30 µg/mL	
EXTRACTS		**STRAINS**		**ASSAYS**	**RESULTS**	**REFERENCES**
		Proteus mirabilis	URO9	Disc-difussion	7 mm	
				MIC	10 µg/mL	
		Escherichia coli	BLEE URO1	Disc-difussion	9.75 mm	
				MIC	20 µg/mL	
			URO5	Disc-difussion	11 mm	
				MIC	20 µg/mL	
			URO8	Disc-difussion	9.50 mm	
				MIC	20 µg/mL	
			SV5	Disc-difussion	12.75 mm	
				MIC	20 µg/mL	
			SV6	Disc-difussion	11 mm	
				MIC	40 µg/mL	
Essential oil of aerial parts/Clevenger		*Escherichia coli*	SV8	Disc-difussion	10 mm	[16]
				MIC	20 µg/mL	
			SV10	Disc-difussion	13.50 mm	
				MIC	30 µg/mL	
			SV11	Disc-difussion	9 mm	
				MIC	10 µg/mL	
		Staphylococcus aureus	SV4	Disc-difussion	30 mm	
				MIC	20 µg/mL	
		Enterococcus faecalis	SV2	Disc-difussion	14.5 mm	
				MIC	20 µg/mL	
			SV3	Disc-difussion	23 mm	
				MIC	20 µg/mL	
			SV7	Disc-difussion	21.75 mm	
				MIC	20 µg/mL	
			SV9	Disc-difussion	25 mm	
				MIC	20 µg/mL	
		Staphylococcus aureus		Disc-difussion	20 mm	[12]
				MIC	28.1 mg/mL	
		Staphylococcus epidermidis		Disc-difussion	22 mm	
				MIC	28.1 mg/mL	
		Micrococcus luteus		Disc-difussion	33 mm	
				MIC	7 mg/mL	
		Enterococcus faecalis		Disc-difussion	13.5 mm	
				MIC	56.25 mg/mL	
		Bacillus cereus		Disc-difussion	38 mm	
				MIC	7 mg/mL	
		Escherichia coli		Disc-difussion	8 mm	
				MIC	900 mg/mL	
		Klebsiella pneumoniae		Disc-difussion	10 mm	
				MIC	900 mg/mL	
		Proteus mirabilis		Disc-difussion	10 mm	
				MIC	450 mg/mL	
Essential oil of aerial parts/Clevenger		*Candida albicans*		Disc-difussion	39 mm	[12]
				MIC	28.1 mg/mL	
		Rhodotorula sp.		Disc-difussion	34 mm	
				MIC	7 mg/mL	
		Hansenula sp.		Disc-difussion	16 mm	
				MIC	7 mg/mL	
		Candida krusei		GM-MIC	99.21 µg/mL	[92]
		Aspergillus fumigatus			62.5 µg/mL	
Leaves extract/Soxhlet	MeOH	*Staphylococcus aureus*		Disc-difussion	17 mm	[45]
		Bacillus subtilis			13 mm	
		Micrococcus flavus			17 mm	
		MultiR *Staphylococcus epidermidis*			20 mm	
		MultiR *Staphylococcus haemolyticus*			20 mm	

	EtOH	*Staphylococcus aureus* ATCC 6538P	Disc-difussion	14 mm	[93]
			MIC	10 mg/mL	
			MBC	16 mg/mL	
		Klebsiella pneumoniae CCM 2318	Disc-difussion	6 mm	
			MIC	30 mg/mL	
			MBC	50 mg/mL	
		Proteus vulgaris ATCC 6998	Disc-difussion	9 mm	
			MIC	22 mg/mL	
			MBC	26 mg/mL	
		Candida albicans	Disc-difussion	22 mm	
			MIC	6 mg/mL	
			MBC	10 mg/mL	
Dried leaves/Pressurized CO_2		*Aeromonas sp.*	MBC	3125 µg/mL	[94]
Acetone extract		*Escherichia coli*	Disc-difussion	24 mm	[95]
		Klebsiella pneumoniae		25 mm	
		Proteus vulgaris		23.9 mm	
		Staphylococcus aureus		32 mm	
		Bacillus subtilis		31.8 mm	
Ethanolic extract		*Listeria monocytogenes*	Disc-difussion	14 mm	[32]
			MIC	11.75 mg/mL	
Commercial extract (in water)		*Escherichia coli*	MIC	12.8 mg/mL	[90]
		Salmonella enterica		6.4 mg/mL	
		Enterobacter aerogenes		12.8 mg/mL	
		Bacillus cereus		0.8 mg/mL	
		Staphylococcus aureus		0.2 mg/mL	

13. CANCER RISK: ANTIANGIOGENIC PROPERTIES

Angiogenesis, the process of new endothelial blood vessel development, is a necessary component of different physiological process such as normal tissue repair, embryonic development as well as pathological diseases as tumor growth. *L. citriodora* showed selective antiangiogenic activity against the endothelial cell proliferation, demonstrating a direct inhibitory activity against the key step in tumor angiogenesis [96].

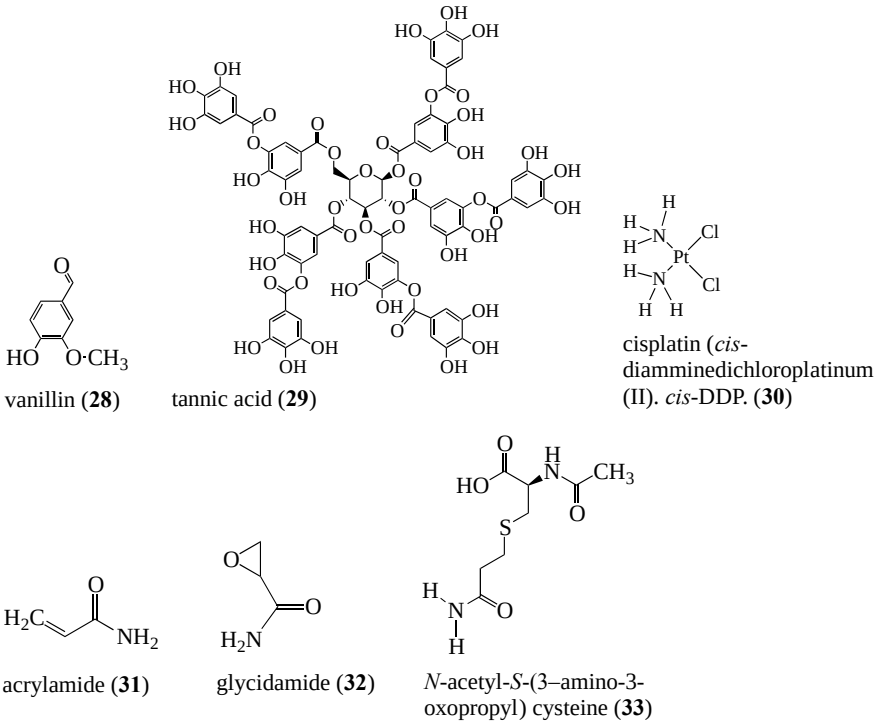


Figure 9. Vanillin (28) and tannic acid (29), cisplatin (cis-diamminedichloroplatinum (II). cis-DDP. (30), acrylamide (31), glycidamide (32) and N-acetyl-S-(3–amino-3-oxopropyl) cysteine (33).

14. PROTECTIVE ACTIVITY OVER DNA-DAMAGE

In recent years, interest over the use of bioactive compounds to prevent anti-tumor agent-induced has considerable increased. Epidemiological evidence suggests that a diet high in fruit and radicals are likely to protect against cancer, cardiovascular disease (CVD) and maybe of other degenerative or age-related diseases [97]. There are a considerable number of reports of antimutagenic effects of polyphenols, dependent upon structure-activity relationships [14, 98-104]. In many cases, whether a compound is antimutagenic or not depends not only upon the exact chemical nature of the molecule, but also upon the locus studied and whether the polyphenol is present before, during or after exposure to the relevant mutagen. Many of the chemicals described as antimutagens may also act as co-mutagens, such as for example vanillin (**28**) and tannic acid (**29**) (Figure 9) [105]. There are a number of different mechanisms which have been implicated in antimutagenic effects of polyphenols. *Araújo* et al. have described that polyphenols are promising chemopreventive agents for colorectal

cancer management because they restore normal cell growth by modulating proliferation, apoptosis, angiogenesis, metastasis, and inflammation and by targeting multiple molecular and biochemical pathways implicated in tumor development [106]. In case of *L. citriodora*, Zamorano-Ponce et al. have studied the protective activity of cedron infusion over genetic damage induced by cisplatin (*cis*-diamminedichloroplatinum (II). *cis*-DDP. **30**) (Figure 9) and the acrylamide (**31**) (Figure 9) [107, 108]. The inorganic compound cisplatin (*cis*-diamminedichloroplatinum (II). *cis*-DDP. **30**) is an effective anti-neoplastic agent in treatment of solid malignant tumors, but it also induces a high reduction of antioxidant plasma levels and generation of free radical in normal cells [109]. Zamorano-Ponce et al.'s results suggest that cedron (*Aloysia triphylla*) infusion may have therapeutic power to inhibit the cisplatin (*cis*-diamminedichloroplatinum (II). *cis*-DDP. **30**)'s side effect, preventing cisplatin (*cis*-diamminedichloroplatinum (II). *cis*-DDP. **30**)-induced genetic damage, through its action as a free radical scavenger since cisplatin (*cis*-diamminedichloroplatinum (II). *cis*-DDP (**30**) is a free radical generator. In addition, these results represent the first evidence that cedron (*Aloysia triphylla*) infusion has an effective anti-genotoxic activity in an *in vivo* mammalian system using comet assay [107]. In the same way, cedron (*Aloysia triphylla*) leaf infusion has an effective anti-genotoxic activity against acrylamide-induce DNA damage. Zamorano-Ponce et al. provide two possible pathways to explain why pretreatment with cedron (*Aloysia triphylla*) infusion exerts the preventive action against DNA damage induced by acrylamide (**31**) [108]: 1) using cytochrome p450 metabolic enzyme to transform acrylamide (**31**) into an easier excreting glycidamide (**32**) (Figure 9) and 2) involving the conjugation of acrylamide (**31**) to glutathione (GSH) to form *N*-acetyl-*S*-(3–amino-3-oxopropyl) cysteine (**33**) (Figure 9) as the final product.

15. CONCLUSION

An overview of the applications of *L. citriodora* in disease prevention has been offered. Reported data provide a basis for reviving the traditional therapeutic uses of *L. citriodora* based on modern scientific knowledge supported by safety issues. This could attract the attention of scientists and researchers

looking for new drugs based on *L. citriodora* product which is supposed to be a potential alternative to chemical drugs. However, the study of the mechanism of action might require a complex approach due to the multitargeted character of most polyphenols.

The potential pharmacological applications of *L. citriodora* have been explored through *in vitro* or in animal studies. However, these effects need to be verified in further human studies which should be performed on the basis of metabolomics approaches and pharmacokinetic studies with the aim to detect key metabolites from *L. citriodora* and unravel the potential health effects in humans.

Among the chemical components of *L. citriodora*, verbascoside (VB. **1**) is the major component detected. Although some authors reasoned that the effects of the extract could be simplified by the use of verbascoside (VB. **1**) as a promising functional element, evidences revealed that other minor components might contribute to the beneficial properties of *L. citriodora*. This fact would explain the higher efficacy of the *L. citriodora* extract in comparison with the isolated verbascoside (VB. **1**). Therefore, the comprehensive study of the chemical composition of *L. citriodora* leaves using high resolution mass spectrometry techinques becomes necessary, taking into account that a variety of geographical, ecological and physiology factors can lead to qualitative and quantitative differences in its composition.

Therefore, based on the evidences reviewed in this chapter, *L. citriodora* deserves further attention for additional preclinical and clinical studies in order to develop herbal ingredients or formulations that may act as adjuvants to current medications, studies which will necessarily encompass pharmacokinetic and metabolomic approaches.

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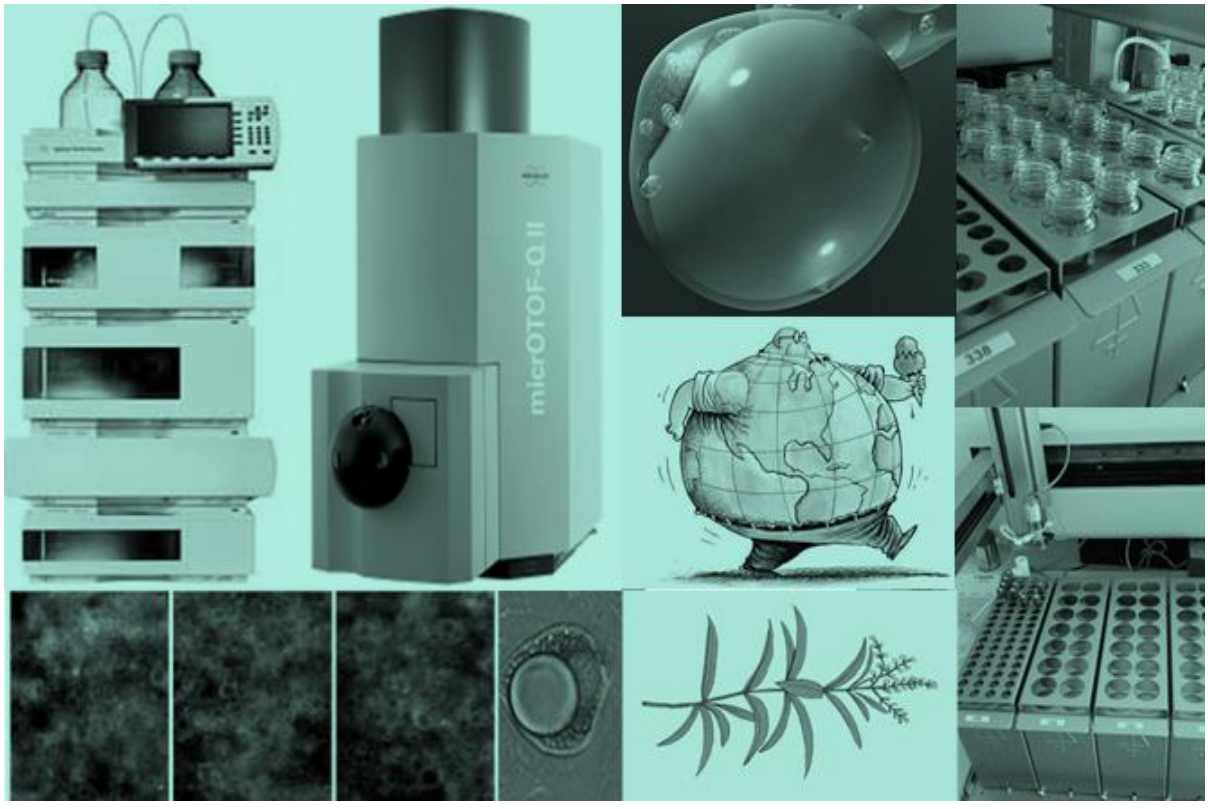
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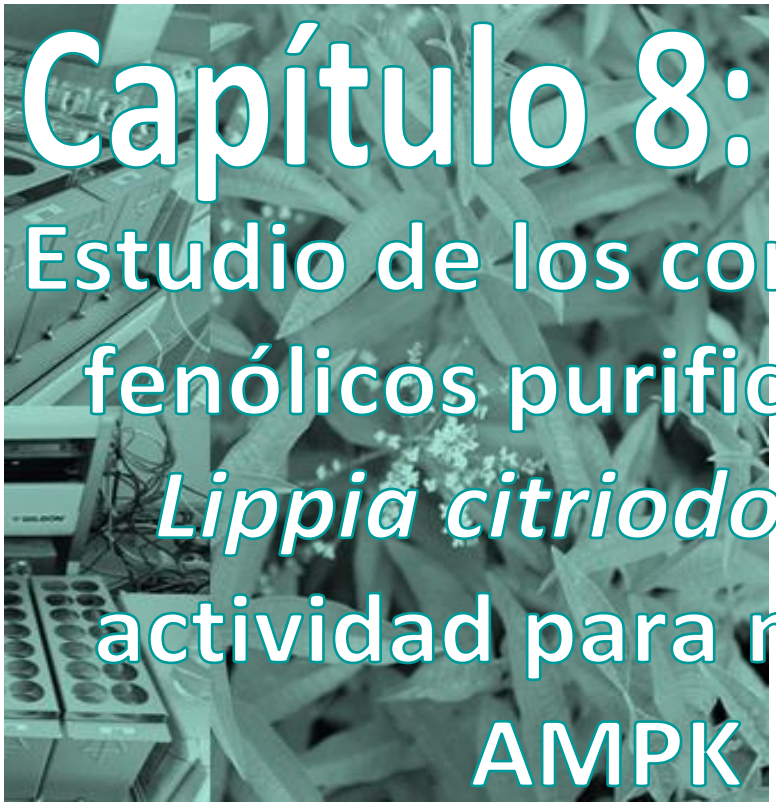

Capítulo 8:

Estudio de los compuestos fenólicos purificados de *Lippia citriodora* con actividad para modular AMPK

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Bioassay-guided purification of *Lippia citriodora* polyphenols with AMPK modulatory activity

ABSTRACT

Recently, the relationship between nutrients and health is becoming. Natural dietary products as polyphenols are being considered by their potential for the management of several diseases. We aimed to investigate the responsibility of *Lippia citriodora* compounds to modulate AMP-activated protein kinase activity (AMPK) on a hypertrophic adipocyte model. HPLC semi-preparative purification method and reverse phase high performance liquid chromatography coupled to time-of-flight mass detection with electrospray ionization (RP-HPLC-ESI-TOF/MS) were used to obtain de compounds from *L. citriodora* extract. AMPK activity was measured on the hypertrophic 3T3-L1 adipocyte model by immunofluorescence microscopy. Four compounds of 29 total compounds have been tentatively identified in *L. citriodora* for first time by HPLC-ESI-TOF-MS. Phenylpropanoids (verbascoside), iridoids (gardoside) and flavonoids (luteolin-7-diglucoronide) were the best candidates to account for activating AMPK capacity. The combination of specific polyphenols from *L. citriodora,* which showed strong activating AMPK capacity, could be an alternative in the management of obesity-associated diseases.

1. INTRODUCTION

Aromatic-medicinal plants, which have been used in folk medicine for centuries to treat several disorders all over the world, are natural resources presenting an important economic potential in health and wellness. This added value is directly related to the extract composition. Plants have unbounded ability to synthesize aromatic substances such as polyphenols, mainly flavonoids and phenolic acids, which exhibit antioxidant capacity (Zamorano-Ponce, Fernández, Vargas, Rivera, & Carballo, 2004). Reactive oxygen species (ROS) have been related with the mediation of several pathological processes, including cancer, obesity, cardiovascular and inflammatory diseases. Phenolic compounds can help to limit the oxidative damage caused by ROS either by acting directly on ROS or by stimulating endogenous defence systems (Tsao, 2010). It is known that many infusions from natural sources have plenty of these bioactive compounds, and consequently it is reasonable to determine whether plants have the capacity to prevent disorders related with oxidative stress (Lasagni Vitar, Reides, Ferreira, & Llesuy, 2014).

In this context, the Verbenaceae family is composed of approximately 2000 species of wide geographical distribution and it is highlighted by including species mainly used in popular medicine. Among the most important, *Lippia citriodora* also called lemon verbena grows spontaneously in South America and is cultivated in northern Africa and southern Europe. The leaves of this species are traditionally utilized to treat many digestive disorders as anti-inflamatory, analgesic, antipyretic, tonic and stimulating (Oliva et al., 2010) and contain a large number of polar compounds such as phenylpropanoids, flavonoids, phenolic acids, and iridoid glycosides, verbascoside being the most abundant (Quirantes-Piné, Funes, Micol, Segura-Carretero, & Fernández-Gutiérrez, 2009).

AMPK has been revealed to be involved in the regulation of carbohydrate and lipid metabolism and, meanwhile, AMPK activation stimulates ATP production by increasing fatty acid oxidation, muscle glucose transport, mitochondrial biogenesis and caloric intake (Bijland, Mancini, & Salt, 2013; Carling, Woods, Thornton, & Sanders, 2012). For example, it is reported that olive-tree leaves extract

decreases intracellular lipid accumulation through AMPK-dependent mechanisms in a hypertrophic and insulin resistant adipocyte model (Jiménez-Sánchez et al., 2017).

Previously, our studies revealed that polyphenols from *Lippia citriodora* extract decreased NF-κB and increased adiponectin protein levels. Further, the anti-inflammatory action of adiponectin was accompanied by the down-regulation of selected inflammatory genes and a significant activation of AMPK in hypertrophic adipocytes. This is important because the ability of polyphenols to act on both, adiponectin and AMPK, may represent important regulators of glucose and lipid metabolism, modulators of inflammation, oxidative stress and insulin resistance and consequently a therapeutic opportunity in the management of obesity (Herranz-López et al., 2015).

Not all secondary metabolites or natural products can be fully synthesized due to their very complex structures that are too difficult and expensive on industrial scale. Hence, there is an urgent need to search for alternative remedies as naturally occurring biologically active secondary metabolites from plant origin. At present, there are innumerable number of such bioactive compounds isolated from crude extracts and their chemical structure were elucidated (Bajpai, Majumder, & Park, 2016). Although natural extract are known to contain high concentrations of polyphenols, it is not clear which of these polyphenols are the actual contributing components to the known biological activities (Si et al., 2006). For these reasons, a preferred approach would be the bioassay-guided fractionation and purification technique that could directly link the activity of *Lippia citriodora* to its components. In this way, isolation by semi-preparative and preparative liquid chromatography (LC) with C18 reversed phase (RP) offers high versatility to separate a wide range of nitrogenous and non-nitrogenous bioactive compounds (Cádiz-Gurrea et al., 2014; Jiménez-Sánchez et al., 2017).

Thus, the aims of this study were to: (1) identified the composition of a commercial *Lippia citriodora* extract by HPLC-ESI-TOF-MS, (2) fractionate this extract by semi-preparative HPLC and characterize the obtained fractions, and (3) evaluate the capacity to modulate AMPK activity using the well-established 3T3-L1 adipocyte model. This will provide a better understanding of the relationship

between AMPK modulation activity and chemical structure of isolation polyphenols from *Lippia citriodora* in order to be included as a supplement or potential ingredient in functional foods.

2. MATERIAL AND METHODS

2.1. Materials

For the identification and semi-preparative isolation all chemicals were of HPLC-MS and used as received. Acetic acid and methanol for HPLC were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

3T3-L1 mouse embryo fibroblasts were purchased from the American Type Culture Collection (Manassas, VA, USA). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, penicillin-streptomycin, calf serum, fetal bovine serum (FBS) (both being HyClone), paraformaldehyde solution, and Triton X-100 were obtained from Sigma-Aldrich (Madrid, Spain). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (ThermoFisher Scientific, Waltham, MA, USA). Cellulose acetate filters, 0.2 µm, were obtained from Advantec MFS, and Dulbecco's phosphate buffered saline (PBS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The Hoechst nucleus specific fluorescence probe for toxicity evaluation, were obtained from Molecular Probes, Invitrogen (Carlsbad, CA, USA).

2.2. Sample preparation

A commercial extract (PLX) of *Lippia citriodora* 10% verbascoside (Monteloeder, Spain) was used in this study. Analytical characterization of polyphenols was carried out using a solution of this extract of 2 mg/mL. Briefly, 10 mg of extract were dissolved in 1 mL of water. The sample was vortexed for 1 min and then filtered through a 0.25 mm filter before the HPLC analysis.

Concerning purification of polyphenols from lemon verbena extract, a solution stock of 70 mg/mL was prepared by dissolving the appropriate amount in water. The sample was vortexed for 1 min and then filtered through a 0.25 mm filter before the HPLC analysis.

2.3. Instrumentation

Fractionation of polyphenols from *L. citriodora* extract was achieved using a Gilson preparative HPLC system (Gilson, Middleton, USA) equipped with a binary pump (model 331/332), automated liquid handling solutions (model GX-271) and UV-Vis detector (model UV-Vis 156).

LC analyses of *L. citriodora* extract and fractions were made with an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode array detector (DAD). The HPLC system was coupled to a TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI interface. Separation was carried out with a Zorbax Eclipse Plus C18 (1.8 µm, 150×4.6 mm).

2.4. Fractionation of polyphenols from L. citriodora extract

The compounds from *L. citriodora* extract were fractionated at room temperature. An Ascentis C18 column (10 µm, 250×212 mm) was used for the separation of the target compounds. The mobile phases consisted of acetic acid 0.5% (A) and methanol (B). The following multi-step linear gradient was applied: 0 min, 0% B; 5 min, 15% B; 12 min, 25% B; 15 min, 35% B; 20 min, 39% B; 38 min, 60% B; 40 min, 70% B; 42 min, 80% B; 44 min, 100% B; 47 min, 0% B. The initial conditions were held for 15 min. The injection volume was 1mL. The flow rate used was set at 15 mL/min. The compounds separated were monitored with UV-Vis (220-280nm) and TOF mass spectrometer. Fraction collection step consisted of UV-based purification, determining the elution time window for collecting each fraction. Finally, a total of 32 fractions were collected and the solvent was evaporated under vacuum. The residue of each fraction was weighted and dissolved with an appropriate volume of water at concentration level of 100 µg/mL. Finally, all fraction were filtered through a 0.25 µm filter before the HPLC analysis.

2.5. Chromatographic, UV and spectrophotometric conditions

The compounds from *L. citriodora* extract and fractions were separated at room temperature. The mobile phases consisted of acetic acid 0.5% (A) and methanol (B). The following multi-step linear gradient was applied: 0 min, 0% B; 5 min, 15% B; 12 min, 25% B; 15 min, 35% B; 20 min, 39% B; 38 min, 60% B; 40 min, 70% B; 42 min, 80% B; 44 min, 100% B; 47 min, 0% B. The initial conditions were held for 10 min. The injection volume in the HPLC and HPLC systems was 10 µL. The flow rate used was set at 0.3 mL/min. The DAD coupled to the HPLC system was set in spectrum range starting at 190 nm and ending at 950 nm.

2.6. ESI-TOF-MS detection

The HPLC system was coupled to a TOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +4.5 kV. The other optimum values of the source parameters were: drying gas temperature, 210 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 2.3 bar. The detection was performed considering a mass range of 50–1200 *m/z*.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonics), which provided a list of possible elemental formulas using Generate Molecular Formula Editor. This uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (σ value) for increased confidence in the suggested molecular formula (Bruker Daltonics Technical Note 008, 2004). The widely accepted accuracy threshold for confirmation of elemental compositions was established at 5 ppm (Bringmann et al., 2005). Even with very high mass accuracy (<3 ppm in most of the cases), many chemically possible formulae were determined depending on the mass regions considered.

Therefore, high mass accuracy alone is not sufficient to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns as a single further constraint removes >95% of the false candidates. This orthogonal filter can reduce several thousand candidates to only a small number of molecular formulas.

During the development of the HPLC method, external instrument calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, with a sodium formate cluster solution passing through containing 5 mM sodium hydroxide and 0.2% acetic acid in water:isopropanol 1:1 v/v.

The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to the compound identification.

2.7. Cell culture and treatment

The 3T3-L1 preadipocyte cell line was cultured and differentiated into adipocytes as described elsewhere (Green & Kehinde, 1975; Jiménez-Sánchez et al., 2017). In order to obtain hypertrophied adipocytes, the adipocytes were incubated with a high glucose medium (4.5 mg/mL) containing insulin for 17-18 days. (Herranz-López et al., 2012). Under these conditions, adipocytes accumulate a high level of cytoplasmic lipids, and develop insulin resistance and a high oxidative state (Herranz-López et al., 2012, 2015). On day 17-18, hypertrophied adipocytes were treated with the respective LC fractions at 200 and 400 µg/mL in the culture medium for 48 h.

2.8. Quantification of the AMPK and pAMPK levels

An immunofluorescence assay was carried out as describe *Jimenez-Sánchez et al.* (2017) (Jiménez-Sánchez et al., 2017) for the detection and quantification of AMPK and phospho-AMPK (pAMPK). After 48 h of treatment with the fractions, adipocytes were fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.25% Triton X-100 for 5 min. After blocking in 4% goat serum at room temperature for 1 h, the cells were co-incubated overnight at 4°C with anti-AMPK alpha 1 + AMPK alpha 2 mouse

antibody (Abcam, Cambridge, UK) and anti-phospho-AMPKα (Thr172) rabbit antibody (Cell Signaling Technology, Danvers, MA, USA). Cells were washed 3 times with PBS and co-incubated at room temperature for 6 hours with each corresponding secondary antibody, goat anti-rabbit IgG-CF594 and goat anti-mouse IgG-FITC, all from Sigma-Aldrich (St. Louis, MO, USA). In addition, cells were incubated with the Hoechst nucleic acid dye to dismiss the possible cytotoxic effects of the fractions at the working concentrations. Then, cells were washed 3 times with PBS and read with a Cytation 3 cell imaging multi-mode microplate reader (Biotek Instruments, Winooski, VT, USA). AMPK activation was determined as the ratio between pAMPK and total AMPK levels by measuring the fluorescence at 490/ 520 nm for total AMPK and 590/620 nm for pAMPK. To ensure that the fractions were not cytotoxic, the cell count was performed by taking microphotographs of the Hoechst-stained cells at 4x, using the DAPI imaging filter cube.

2.9. Statistical analysis

Values are represented as the mean ± standard deviation (S.D.) of the mean. The values were subjected to statistical analysis (one-way ANOVA, and Tukey's test for multiple comparisons/ non-parametric approaches). The differences were considered to be statistically significant at $p < 0.05$. All analyses were performed using Graph Pad Prism 6 (GraphPad Software, Inc. La Jolla, CA, USA). *$p <$ 0.05, **$p < 0.01$ and ***$p < 0.001$ on the bars indicate statistically significant differences compared to the control, unless otherwise stated. All cellular measurements were performed in fivefold, unless otherwise specified.

3. RESULTS AND DISCUSSION

3.1. Characterization of the L. citriodora extract by RP-HPLC-ESI-TOF-MS

The base peak chromatogram of the lemon verbena extract obtained by RP-HPLC-ESI-TOF-MS is shown in Figure 1. A total of 29 compounds, which were distributed in different categories as phenylpropanoids, iridoids and flavonols among others, were analyzed in the present study. The

compounds characterized are presented in Table 1, numbered according to their elution order. This table includes the retention time, experimental *m/z*, molecular formulas, errors and σ values for all of the compounds detected in the samples analyzed. All the compounds were tentatively characterized by the interpretation of their mass spectra determined by the TOF mass analyzer while taking into account the information provided by the literature and databases.

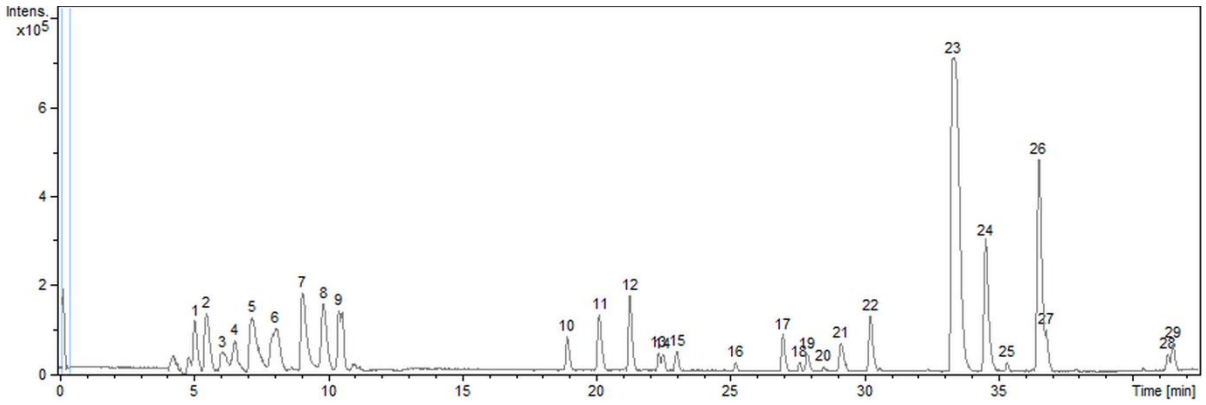

Figure 1. Base peak chromatogram of *L. citriodora extract*. Peak numbers correspond to those of Table 1.

As expected, phenylpropanoids are the major class of compounds found in this extract, being verbascoside (Peak 23) the most abundant one which is in agreement with the data previously reported (Quirantes-Piné et al., 2013) followed by isoverbascoside (Peak 26). In this category, eight compounds (peaks 12-14, 18, 23, 26, 27, 29) were detected and previously identified in *L. citriodora,* (Wang et al., 2015) amongst them a new isomer (peak 14) from caffeoyl-rhamnopyranosyl-, -glycopyranoside, also known as cistanoside (peak 13), was characterized for first time in this specie.

Regarding iridoids, five compounds (peaks 10, 11, 15-17) were detected in lemon verbena extract. One of them (peak 16) with [M-H]- at *m/z* 405 was assigned for first time in *L. citriodora* to shanziside methyl ester previously reported in *Lippia alba* (Barbosa, Lima, Braz-Filho, & Silveira, 2006).

Table 1. Retention time and mass spectral data of the compounds characterized in *L. citriodora* extract by RP-HPLC–ESI-TOF-MS in negative mode.

Peak	RT (min)	[M-H]- measured	[M-H]- calculated	Error (ppm)	mSigma	Molecular Formula	Proposed compound
Organic acid							
1	5,1	195,0512	195,051	0,7	6,3	C 6 H 12 O 7	Gluconic acid
Sugars							
2	5,5	341,1091	341,1089	0,5	13,5	C 12 H 22 O 11	Disaccharide I
3	6,1	503,1603	503,1618	2,9	4,8	C 18 H 32 O 16	Raffinose
4	6,6	341,1072	341,1089	5	2,7	C 12 H 22 O 11	Disaccharide II
5	7,2	665,2123	665,2146	3,4	4	C 24 H 42 O 21	Tetrasaccharide I
6	8,1	665,2123	665,2146	3,4	4	C 24 H 42 O 21	Tetrasaccharide II
7	9	827,2651	827,2674	2,8	4,1	C 30 H 52 O 26	Pentasaccharide I
8	9,9	827,2657	827,2674	2,1	2,6	C 30 H 52 O 26	Pentasaccharide II
9	10,4	989,318	989,3202	2,3	3,8	C 36 H 62 O 31	Maltohexaose
Iridoids							
10	18,9	391,1227	391,1246	4,8	3,5	C 16 H 24 O 11	Shanziside
11	20,1	373,1131	373,1140	2,5	5	C 16 H 22 O 10	Gardoside
15	23	375,1278	375,1297	5	3,7	C 16 H 24 O 10	Loganic acid
16	25,1	405,1384	405,1402	4,4	2,3	C 17 H 26 O 11	Shanziside methyl ester
17	26,9	389,1075	389,1089	3,7	5,4	C 16 H 21 O 11	Theveside
Phenylpropanoids							
12	21,2	461,1674	461,1664	3,7	1,2	C 20 H 30 O 12	Verbasoside
13	22,3	487,1440	487,1457	-2,0	5,8	C 21 H 28 O 13	Caffeoyl-rhamnopyranosyl-glycopyranoside I
14	22,5	487,1441	487,1457	3,4	8,1	C 21 H 28 O 13	Caffeoyl-rhamnopyranosyl-glycopyranoside II
18	27,5	639,1896	639,1931	5,4	15,8	C 29 H 36 O 16	β-hydroxy-(iso)verbascoside
23	33,2	623,1956	623,1981	4,1	6,5	C 29 H 36 O 15	Verbascoside
26	36,4	623,193	623,1981	8,2	2,9	C 29 H 36 O 15	Isoverbascoside
27	36,7	637,208	637,2138	9,2	22,8	C 30 H 38 O 15	Eukovoside

29	41,4	651,2246	651,2294	7,4	6,2	C 31 H 40 O 15	Martinoside
Flavonoids							
22	30,2	637,1017	637,1046	4,6	5,5	C 27 H 26 O 18	Luteolin-7-diglucuronide
24	34,4	651,1147	651,1203	8,7	5	C 28 H 28 O 18	Chrysoeriol-7-diglucoronide
25	35,3	621,1762	621,1766	0,7	18,1	C 36 H 30 O 10	Diooflavone
28	41,2	635,1198	635,1254	8,8	9	C 28 H 28 O 17	Acacetin-7-diglucuronide
Other compounds							
19	27,8	387,1652	387,1661	2,3	16,6	C 18 H 28 O 9	Tuberonic acid glucoside I
20	28,4	445,2073	445,2079	1,4	23,3	C 21 H 34 O 10	Sacranoside A
21	29,1	387,2006	387,2024	4,8	5,4	C 18 H 28 O 9	Tuberonic acid glucoside II

As flavonoids, the diglucuronidic derivatives of luteolin, chrysoeriol and acacetin were detected. Moreover, a new biflavonoid was tentatively identified for first time in *L. citriodora*. Peak 25 with *m/z* at 621 was characterized as diooflavone (Moawad & Amir, 2016), an amenthoflavone methyl derivative (Figure 2).

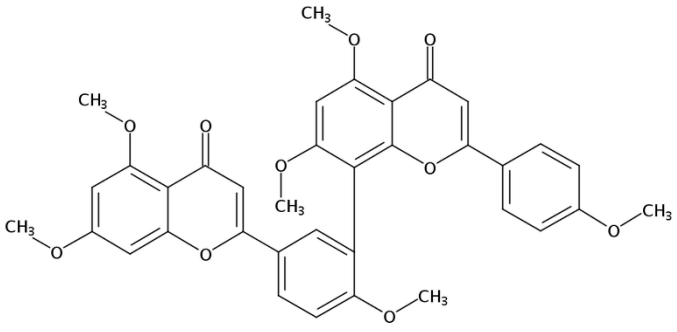

Figure 2. Chemical structure of diooflavone.

Other compounds belongs to monoterpenes group were identified in this extract, two isomers of tuberonic acid glucoside (peaks 19 and 21) and sacranoside A (peak 20). The latter has been tentatively

identified in *L. citriodora* for first time although it was detected in methanolic extracts from medicinal and aromatic species as *Origanum majorana* (Taamalli et al., 2015).

3.2. Isolation of *L. citriodora* extract by semi-preparative HPLC and characterization of fractions by RP-HPLC-ESI-TOF-MS

To delimit the bioactivity of compounds contained in lemon verbena extract as AMPK modulators, a semi-preparative HPLC methodology was developed in order to obtain isolated compounds according to UV and MS information (Figure 3 a and b) and to their elution order.

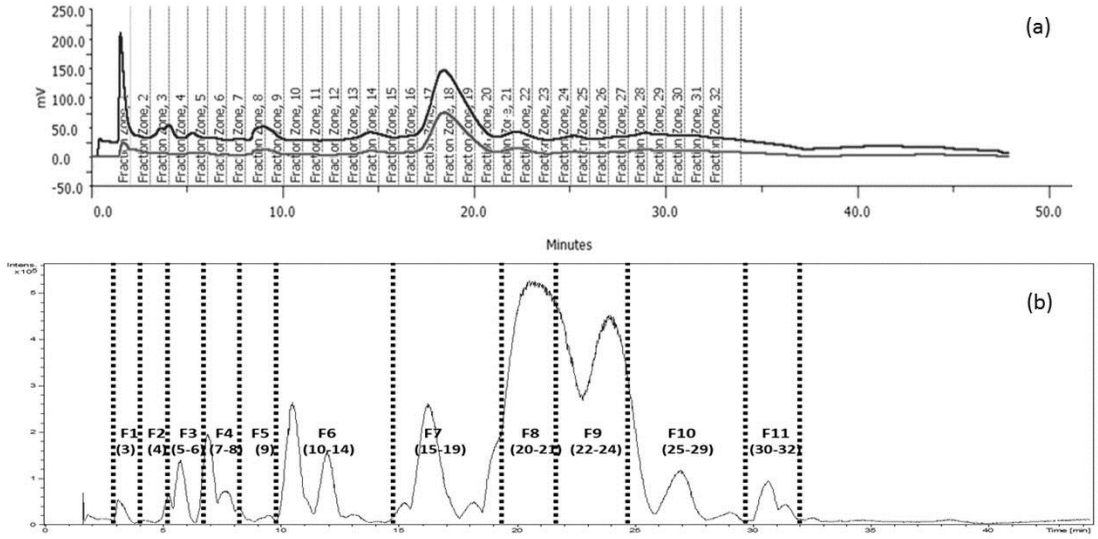

Figure 3. UV-chromatogram obtained by semi-preparative HPLC-DAD (a) and BPC spectrum by semi-preparative HPLC-ESI-TOF-MS (b) of *L. citriodora* extract indicating the collected fractions.

Table 2. Contained compounds in each collected and selected fractions of *L. citriodora* extract according to Table 1.

Selected fractions for AMPK modulation	Collected fractions by semi-preparative HPLC	Compounds (Peaks)
F1	3	10, 11*, 12
F2	4	12*, 13, 14, 15
F3	5-6	13, 14, 15*
F4	7-8	16, 17*, 18, 19
F5	9	19
F6	10-14	20, 21, 22*
F7	15-19	23
F8	20-21	23*, 24, 25
F9	22-24	26
F10	25-29	27*, 28
F11	30-32	29

***Peak with major contribution in each fraction.**

Afterwards, a total of 32 fractions were collected and the composition of each fraction was established by the detailed HPLC-MSESI-TOF method in order to select the most appropriate to be analysed for AMPK modulation (Table 2).

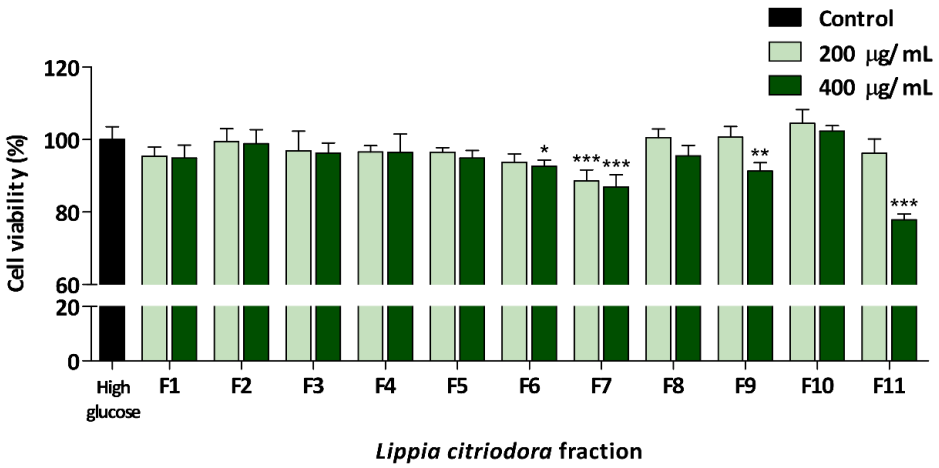

Figure 4. Cell viability of hypertrophied adipocytes treated with *L. citriodora* fractions. Cell viability was determined by counting the Hoechst stained-nuclei. Values were normalized with respect to the high glucose control. **p*<0.05, ***p*<0.01, ****p*<0.001 indicate significant differences compared to the control.

3.3. AMPK modulation of *L. citriodora* fractions in hypertrophied 3T3-L1 adipocytes

A total of 11 fractions of lemon verbena were analysed in a cell model of hypertrophied adipocytes to determine which polyphenols are able to modulate the activation of AMPK by phosphorylation. The cell count from microphotographs of the Hoechst dye (Figure 4) revealed a cell toxicity around 10-20% for the fractions F6, F9 and F11 at 400 µg/mL and for F7 at 200 and 400 µg/mL after incubating 48 hours. Most of the fractions showed a significant AMPK activation (F1, F3, F5, F6, F7, F8, F9, F10 and F11) (Figure 5). F1 which contains compounds 10 (shanziside), 11 (gardoside) and 12 (verbasoside) and F5 composed of compound 19 (tuberonic acid glucoside I) activated AMPK at 400 µg/mL. F8 containing compounds 23 (verbascoside), 24 (chrysoeriol-7-diglucoronide) and 25 (diooflavone) and F9 which contains compound 26 (isoverbascoside) showed a significant activation of AMPK at 200 µg/mL. F3 which is composed of compounds 13 (caffeoyl-rhamnopyranosyl-glycopyranoside I), 14

(caffeoyl-rhamnopyranosyl-glycopyranoside II) and 15 (loganic acid), F6 which contains compounds 20 (sacranoside A), 21 (tuberonic acid glucoside II) and 22 (luteolin-7-diglucuronide), F7 composed of compound 23 (verbascoside), F10 containing compounds 27 (eukovoside) and 28 (acacetin-7-diglucuronide) and F11 consisting of compound 29 (martinoside) activated AMPK at both concentrations tested.

Obesity is no longer considered to be only a cosmetic problem but that, in many cases, are associated with an increased risk for the development of metabolic disorders. Plant-derived polyphenols such as catechins, anthocyanines, phenylpropanoids, resveratrol or curcumine have been studied to modulate physiological and molecular pathways that are related with energy metabolism, adiposity, and obesity (Herranz-López et al., 2012; Jiménez-Sánchez et al., 2017; Meydani & Hasan, 2010; Rayalam, Della-Fera, & Baile, 2008). In this sense, lemon verbena extract and verbascoside have demonstrated a beneficial effect in high glucose-induced insulin-resistant hypertrophic adipocytes and in a murine model of hyperlipidemia (Herranz-López et al., 2015). For this reason, the fractionation of the *L. citriodora* extract by semi-preparative HPLC has allowed to delimit the putative candidate compounds responsible for the activation of AMPK.

In this work, fractions which exhibited a potent AMPK activation through phosphorylation at both 200 and 400 µg/mL are mostly composed of phenylpropanoids. It should be highlighted the different behaviour of compounds as verbascoside. F7, where this compound was detected alone in a high purified fraction, showed the best result at 400 µg/mL, while this is not the case for F8. This fraction, which also presented as main compound verbascoside, exhibited lower values than F7. The different between these two may be attributed to the fact that F8 included two flavonoids which could attain a synergic effect reducing the AMPK activation. In this scenario, F2 (non-effect in AMPK activation capacity) and F3 (effect at both concentrations) shared almost the same composition apart from the fact that F2 presented as mayor component verbasoside (phenylpropanoid) along with compounds 13, 14 and 15. These three last were detected in F3. On the other hand, our results noticed that pure

isoverbascoside (F9) showed a different effect than the pure verbascoside (F7). This might be explained by the cytotoxic effect of fraction F9 or because of these isomers may act as direct modulators of AMPK at different binding sites but the molecular mechanism by which these lemon verbena compounds exert their AMPK activation needs to be elucidated.

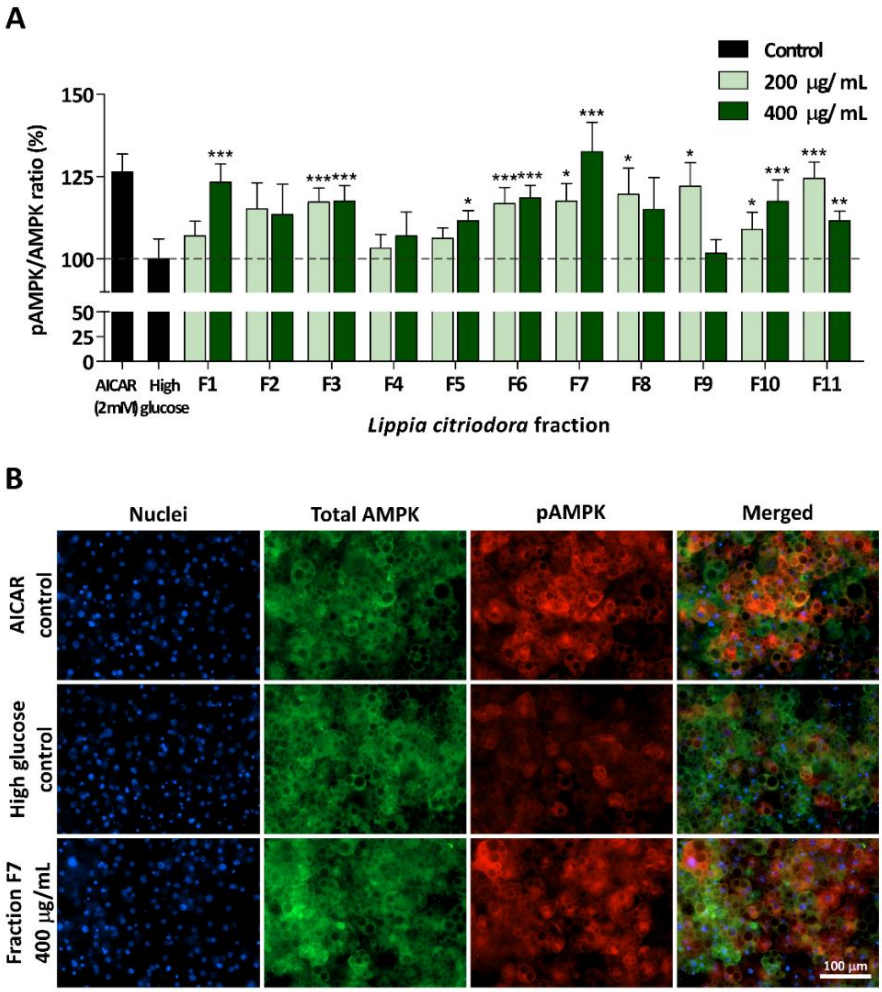

Figure 5. Measurement of the activation rate of AMPK (%) as the ratio pAMPK/AMPK of the selected *L. citriodora* fractions (A). Inmunofluorescence microscopy of hypertrophic adipocytes treated with 400 µg/mL of the F7 and incubated in high glucose medium. Values were normalized with respect to the high glucose control. With comparative aims, the positive control 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) has also been included. *, **, and *** indicate significant differences with respect to the control incubated in high glucose medium ($p<0.05$, $p<0.01$, and $p<0.001$, respectively). Representative microphotographs taken with a fluorescence microscope at 4x: control cells incubated in high glucose medium (B).

4. CONCLUSIONS

A total of 29 compounds were tentatively identified in the present study by HPLC-ESI-TOF-MS being phenylpropanoids the major class of compounds found in the extract. For first time in this specie, a new isomer from caffeoyl-rhamnopyranosyl glycopyranoside (peak 14), shanziside methyl ester (peak 16), sacranoside A (peak 20) and diooflavone (peak 25) were detected.

Since *Lippia citriodora* extract has exhibited higher capacity to active AMPK than pure verbascoside in insulin-resistant hypertrophic adipocyte model (Herranz-López et al., 2015). In an attempt to identify those compounds contributing to such capacity, the bioassay-guided fractionation of *Lippia citriodora* extract has been achieved by HPLC semi-preparative. Fraction containing pure verbascoside (F7) showed the highest activating capacity. Other fractions enriched in a combination of iridoids and phenylpropanoids (F1, F3) or fractions containing terpenes, phenylpropanoids and flavones (F6, F10) also showed a significant activating capacity. Fractions containing a combination of phenylpropanoids and flavones (F8, F9 and F11) activated AMPK but showed a cytotoxic effect when the concentration was increased, probably due to the presence of some flavones. Based on the results, we hypothesized that the combination of specific phenylpropanoids, iridoids and flavones may be the responsible for the strong activating capacity observed in the whole extract. Further research using combinations of pure compounds may be required to delimit the compounds responsible for such activity and to find for a potential synergistic effect.

The importance of knowledge concerning this product composition and activities is interesting due to its potential source for the obtainment of bioactive compounds in order to be used as a functional ingredients in food industry.

5. ACKNOWLEDGMENTS

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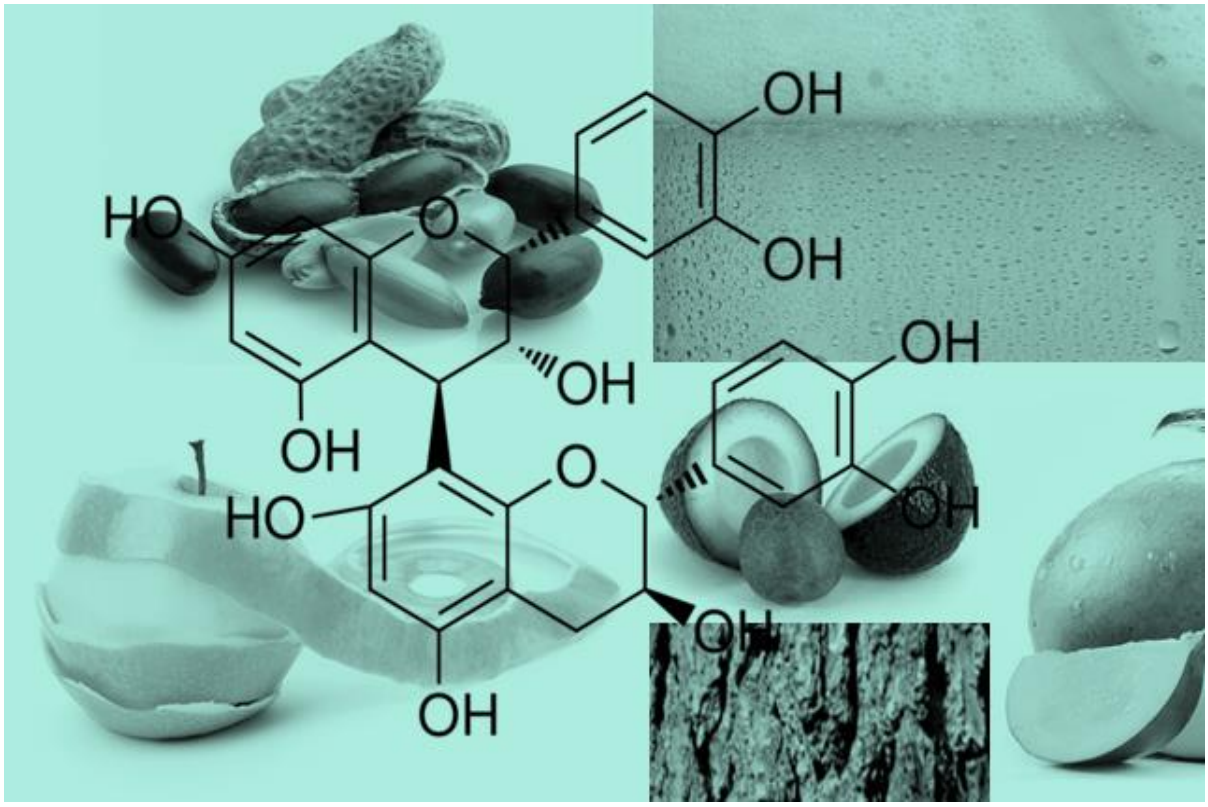
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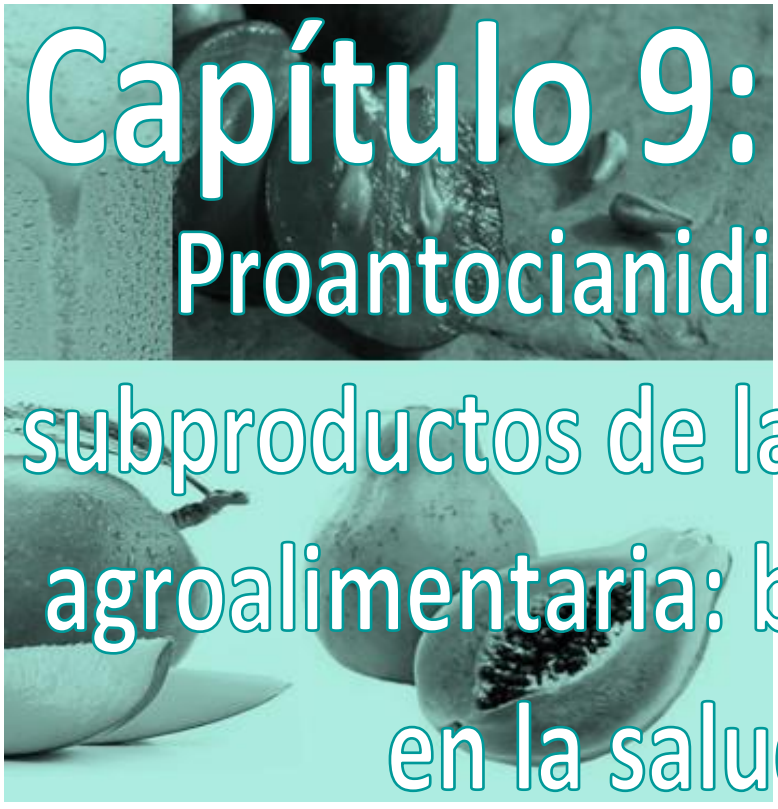


Capítulo 9:
Proantocianidinas en subproductos de la industria agroalimentaria: beneficios en la salud

In: *Occurrences, Structure, Biosynthesis, and Health Benefits Based on Their Evidences of Medicinal Phytochemicals in Vegetables and Fruits. Volume 3*. 2015, 63-114. ISBN: 978-1-63482-710-2 Nova Science Publishers

Proanthocyanidins in Agro-Industrial By-Products: Health Benefits

ABSTRACT

Phenolic compounds, ubiquitous in plants are an essential part of the human diet and are of considerable interest due to their physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects.

The food and agricultural product processing industries generate substantial quantities of phenolic-rich by-products. The peels of several fruit, i.e. apples have also been reported to contain higher amounts of phenolics than the edible fleshy parts. Grape seeds and skin, by-products of grape juice and wine production are also sources of several phenolic compounds, particularly mono-, oligo-, and polymeric proanthocyanidins (PAs). Several authors have shown that the total phenolic content of fruit seeds, i.e., mango, longan, avocado, and jackfruit were higher than that of the edible flesh and as such these seeds could be a valuable source of antioxidant phenolics. The phenolic profile of mango seeds was assumed to be mainly gallic and ellagic acids, gallotannins and condensed tannin-related polyphenols. PAs found in peanut skins can provide potential benefits by acting as antioxidants, a natural anti-microbial property in laboratory media, and vasodilators.

This class of polyphenols consists of a mixture of oligomers and polymers composed of monomeric (+)-catechin and (-)-epicatechin ranging from dimers to decamers. Regarding the interflavanoid linkage (IFL) nature, B-type procyanidins (PCs) [C-4→C-6 or C-8] are more abundant than A-type PCs, which present an additional ether type bond [C-2→O→C-5 or C-7], as well as IFL, which can be either α or β type. The PAs consisting of (epi)catechin are known as PCs. This list of combinations, together with the occurrence of derivatives such as O-methylation, and C- and O-glycosylation, explain the high diversity of this family and the wide range of biological and biochemical activities in plants. Reports of several in vitro assays demonstrate potentially significant interactions with biological systems, such as anti-viral, bacterial, molluscicidal, enzyme-inhibiting, antioxidant, and radical-scavenging properties. Their tendency to interfere with biological systems is, at least in part, due to a characteristic ability to form complexes with macromolecules, combined with a polyphenolic nature. As well as the free radical scavenging and antioxidant activity, PAs also exhibit vasodilatory, anti-carcinogenic, anti-allergic, anti-inflammatory, anti-bacterial and cardioprotective properties.

1. INTRODUCTION

Largely distributed across the plant kingdom and abundant in our diet, plant polyphenols are today among the most talked-about concerning the classes of phytochemicals. These compounds are naturally occurring secondary metabolites found in a variety of fruits, vegetables, nuts, seeds, flowers, bark, beverages and manufactured foods, as a component of the natural ingredients used. They are primarily synthesized through the pentose phosphate pathway, shikimate and phenyl-propanoid pathways (Figure 1) in plants [1]. Phenolics are compounds with one or more aromatic rings with one or more hydroxyl substituents, bearing more than currently known 8,000 phenolic structures, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins [2] (Table 1).

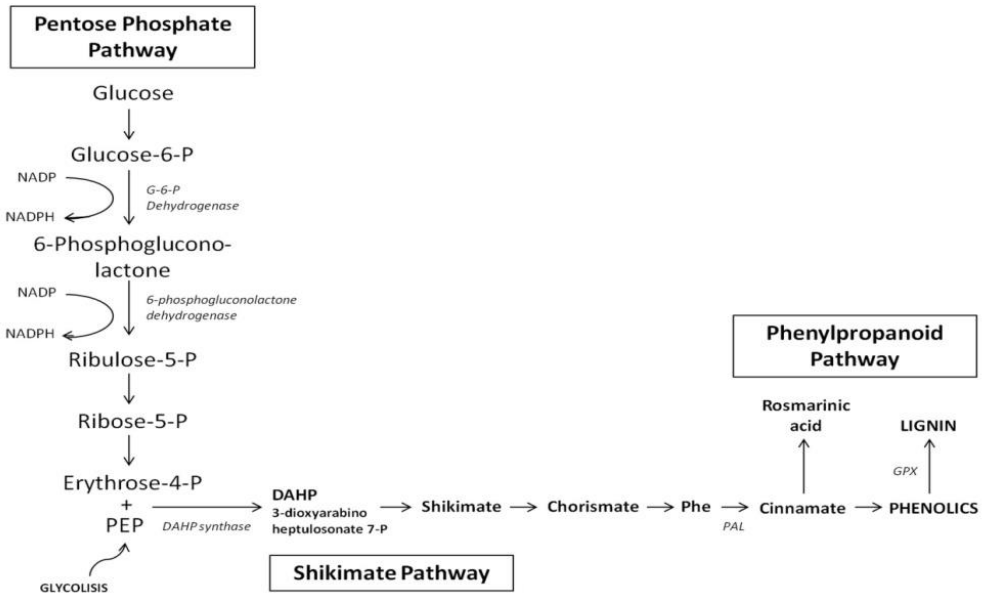
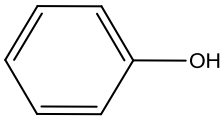
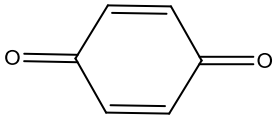
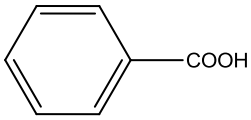
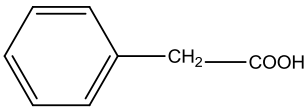
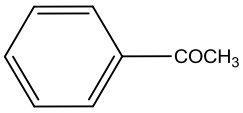
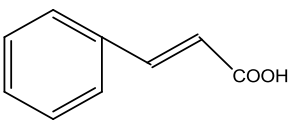
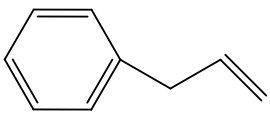


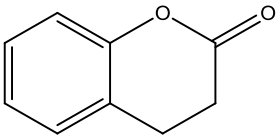
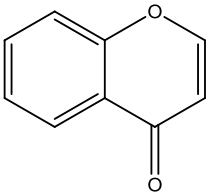
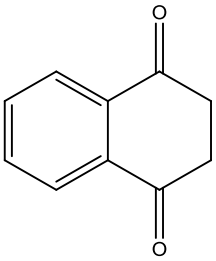
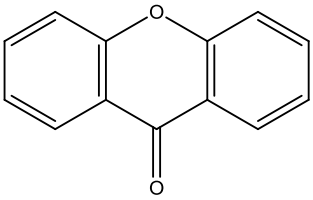
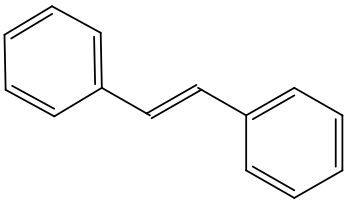
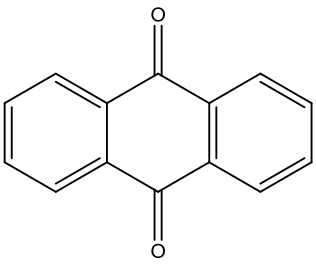
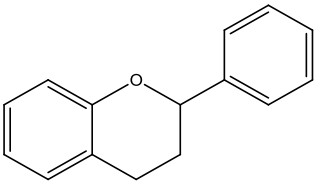
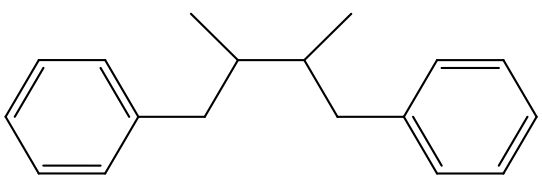
Figure 1. Pentose phosphate pathway, shikimate and phenyl-propanoid pathways.

Tannins are one of the major groups of polyphenols found in our diets and are usually subdivided into two groups: hydrolysable tannins and condensed tannins. The term "tannin" has been employed classically to designate the substances of vegetable origin capable of transforming fresh hide into leather. Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also known as

ellagitannins. PAs, forming the second group of tannins, are far more common in our diet. They are polymers made of elementary flavan-3-ol units. The great variety in the structure of these compounds is due to the many possibilities in forming oxidative linkage [3].

Table 1. Structures of the main classes of polyphenols

Backbone	Family	Basic structure
C_6	simple phenols	
	benzoquinones	
C_6-C_1	phenolic acids	
C_6-C_2	phenylacetic acids	
	acetophenones	
C_6-C_3	hydroxycinnamic acids	
	phenylpropenes	

	coumarins	
	chromones	
C_6-C_4	naphthoquinones	
C_6-C_1-C_6	xhantones	
C_6-C_2-C_6	stilbenes	
	anthraquinones	
C_6-C_3-C_6	flavonoids	
$(C_6$-$C_3)_2$	lignans (neolignans)	

$(C_6\text{-}C_3)_n$	lignins	complex polymers of aromatic alcohols
$(C_6\text{-}C_1)_n$	hydrolyzable tannins	complex polymers of phenolic acids and simple glycosides

Flavonoids comprise the largest group of plant phenolics, with more than 4,000 structures identified [4]. Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C_6–C_3–C_6 configuration and are classified according to substitutions (Figure 2).

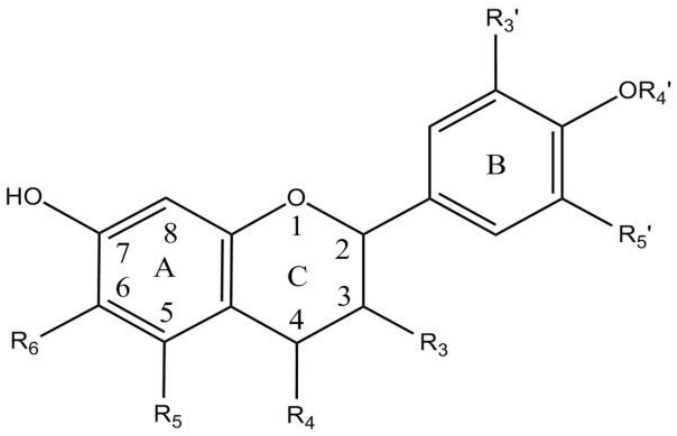


Figure 2. General structure and numbering pattern for common food flavonoids.

Variations in substitution patterns to ring C result in the major flavonoid classes, *i.e.*, flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins (Table 2) [5], of which flavones and flavonols are the most widely occurring and structurally diverse flavonoids. Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups and in the conjugation between the A- and B- rings. These substitutions give rise to the different compounds within each class of flavonoids [6].

Table 2. Flavonoid subclass, chemical characteristics and examples of common food flavonoids

Flavonoid subclass	C-ring unsaturation	C-ring functional group	Examples
flavanols	none	3-hydroxy	(+)-catechin (-)-epicatechin (+)-gallocatechin
		3-*O*-gallate	(-)-epicatechin-3-gallate
flavanones	none	4-oxo	eriodyctiol naringenin
flavones	2-3 double bond	4-oxo	apigenin luteolin
isoflavones	2-3 double bond	4-oxo	genistein
flavonols	2-3 double bond	3-hydroxy, 4-oxo	quercetin kaempferol myricetin
anthocyanidins	1-2, 3-4 double bonds	3-hydroxy	delphinidin petunidin

Proanthocyanidins (PAs) are oligomers or polymers of flavan-3-ols and these units are linked mainly through C4→C8 bond, but the C4→C6 linkage also exists. These linkages are both called B-type linkages. An additional ether bond between C2→C7 resulting in a double linkage of the flavan-3-ol units is called an A-type linkage (Figure 3). The PAs that exclusively consist of (epi)catechin units are designated procyanidins (PCs), the major type of PAs in plants. The most common PAs in food are PCs with a 3',4'-dihydroxy substitution on the B-ring. The less common PAs, with a 3',4',5'-trihydroxy substitution, containing epiafzelechin or epigallocatechin subunits, are called propelargonidin or prodelphinidin, respectively (Table 3). The flavan-3-ol subunits may carry acyl or glycosyl substituents, being the most common gallic acid to form 3-*O*-gallates, which is found as an ester with the hydroxyl

in the C3 position as in tea or wine. Several glycosylated PA oligomers have been identified with the sugar linked to the C3 or the C5 position as the most common glycosylation [3, 7–9].

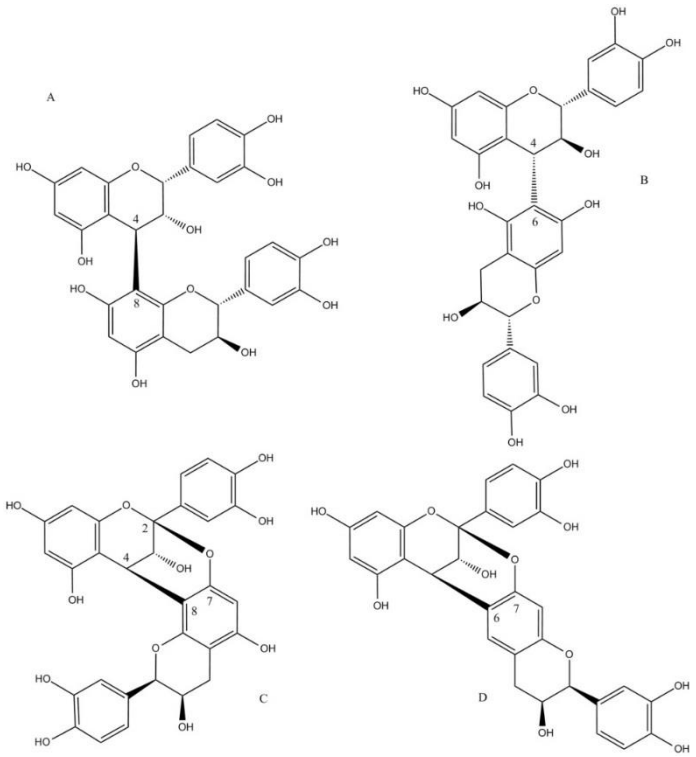
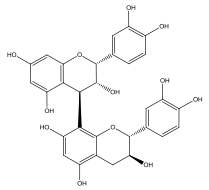
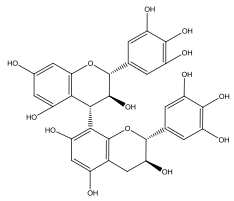
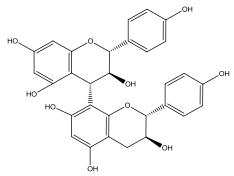


Figure 3. Structures of proanthocyanidins type B C4→C8 (A), C4→C6 (B) and Type A C2→C7, C4→C8 (C), C2→C7, C4→C6 (D).

All analytical methods for quantifying these biologically active compounds present in different sources involve extraction, separation and analysis. The extraction efficiency greatly depends on the time of extraction and the solvents used [10]. For the extraction of PAs, several solvent systems, such as absolute methanol, ethanol, acidified methanol, acetone, water and their combinations have been used. For example, 1% HCl in methanol was used for the extraction of PAs from sorghum and dry beans, whereas acetone–water (70:30, v/v) was found to serve as the best solvent system for the extraction of PAs from rapeseed, beach peas and blueberries. On the other hand, acetone–water (60:40, v/v) was used for the extraction of PAs from cider apple and grape skins. PAs of cloves and allspice may be extracted with boiling water [11]. Dimethyl sulphoxide was used to obtain PAs from *Theobroma cacao* extracts [12].

Table 3. Common proanthocyanidin subgroups

PAs subgroup	Monomeric unit	C3'	C5'	Examples
procyanidin	epicatechin	OH	H	
prodelphinidin	epigallocatechin	OH	OH	
propelargonidin	epiafzelechin	H	H	

The diversity in phenolic profiles places high demands on the analytical methods used for their separation, identification and quantification, necessitating adjustment of methods for specific purposes. Separation and determination of PAs are very difficult because of the variety of isomers and oligomers, which exhibit different degrees of polymerization. Therefore, the kind of chromatography selected depends on the PAs in the matrix to be analyzed [13]. Bioactive compounds have been analysed using different techniques, such as gas-chromatography coupled with mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). HPLC and CE allow for efficient separation of flavonoids in different plant extracts. Several aspects of an HPLC method that can be adapted to improve the separation of target compounds include the stationary phase (column), mobile phase composition, gradient program and column temperature. The use of different detectors may also be advisable for quantification of compounds from multiple phenolic groups. A diode array detector (DAD) allows detection at a range of wavelengths in the UV and visible spectra simultaneously, allowing classification of compounds from their UV-Vis spectral

characteristics and quantification of different phenolic groups at appropriate wavelengths. Fluorescence detection (FLD), on the other hand, can be used to obtain higher sensitivity for compounds with fluorescent properties than UV-Vis absorption, *i.e.*, flavan-3-ols [14]. Mass spectrometric (MS) detection is used to identify compounds based on their molecular weights and fragmentation patterns and is a very effective and highly sensitive method for characterizing PCs from complex matrices [14,15]. In recent years, electrospray ionization (ESI) has been shown to be suitable for the analysis of polar compounds in aqueous solutions without any previous sample derivatization [16]. ESI permits the identification of the molecular weight of PC with different degrees of polymerization. The negative ion ESI mass spectra show the presence of a series of non-galloylated and galloylated oligomeric PCs up to a trigalloylated octamer [17]. In addition, tandem mass spectrometry (MS/MS) can give even more information about the structural details of the different molecules. These techniques, due to their high accuracy and higher sensitivity, have been used for the conclusive identification of PCs in a broad range of materials, such as fruits, cereals, nuts and spices [18,19]. Reversed-phase high performance liquid chromatography (RP-HPLC) coupled with DAD and/or MS are usually employed for analysis of these compounds [12,20–22]. Quadrupole time-of-flight mass spectrometry (QTOF–MS) combines high sensitivity and mass accuracy for both precursor and product ions, providing the elemental composition of the parent and fragment ions. This feature helps to identify compounds thoroughly and to differentiate between isobaric compounds. The potential of HPLC–ESI–QTOF–MS for qualitative purposes has been highlighted in several studies [12,22,23].

Polyphenols have attracted increasing attention as potential agents for preventing and treating many oxidative stress-related diseases. Reactive oxygen species (ROS) from both endogenous and exogenous sources may be involved in the etiology of diverse chronic and degenerative diseases, such as coronary artery disease, stroke, rheumatoid arthritis, cancer, diabetes mellitus and ageing [24]. ROS are capable of oxidizing cellular proteins, nucleic acids and lipids [25]. The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activity and this is

referred to as structure–activity relationships (SAR). This relationship between the chemical structure of flavonoids and their radical-scavenging activities was analyzed by *Bors* et al. [26].

The structural arrangements imparting the greatest antioxidant activity as determined from these studies are:

- The *ortho* 3',4'-dihydroxy moiety in the B ring (*i.e.* in catechin, luteolin and quercetin).
- The *meta* 5,7-dihydroxy arrangements in the A ring (*i.e.* in kaempferol, apigenin and chrysin).
- The 2,3-double bond in combination with both the 4-keto group and the 3-hydroxyl group in the C ring, for electron delocalization (*i.e.* in quercetin), as long as the *o*-dihydroxy structure in the B ring is also present. However, alterations and substitution of contributing hydroxyl groups by glycosylation decreases the antioxidant activity.

For metal chelation, the two points of attachment of transition metal ions to the flavonoid molecule are the *o*-diphenolic groups in the 3',4'-dihydroxy positions in the B ring, and the ketol structures 4-keto, 3-hydroxy or 4-keto and 5-hydroxy in the C ring of the flavonols. Glycosylation in all these hydroxyl positions influences the antioxidant activity of the flavonoids [27–30].

The study of the catechins is important for understanding the antioxidant properties of sources rich in PAs. Catechins (including epicatechins) with three hydroxyl groups in the B ring are the gallocatechins and those esterified to gallic acid at the 3-OH group in the C ring are the catechin gallates. There is no electron delocalization between the A and B rings due to the saturation of the heterocyclic ring; the antioxidant activity responds broadly to the tenet that the structures with the most hydroxyl groups exert the greatest antioxidant activity, with the catechin isomers more than twice as effective as vitamins E and C.

The catechin-gallate esters reflect the contribution from gallic acid. An ester linkage *via* the 3-OH group to gallic acid and an incorporation of an additional 5'-OH group in the B ring can enhance its antioxidant potential. Moreover, the insertion of a third adjacent hydroxyl group in the B ring such as epigallocatechin also enhances the antioxidant activity [28].

Table 4. Antioxidant activity of epicatechins and proanthocyanidins using different methods by *Muselík* et al. [31]

Compound	% inhibition of tyrosine nitration	TEAC[1)]	FRAP[2)]	IC_{50} value (mM)[3)]
catechin	45.1 ± 1.6	2.85 ± 0.12	1.08 ± 0.03	3.1 ± 0.2
epicatechin	44.1 ± 3.7	2.93 ± 0.02	1.10 ± 0.02	3.2 ± 0.2
Gallocatechin	44.3 ± 2.9	3.31 ± 0.09	1.74 ± 0.04	15.9 ± 3.0
epicatechin-3-*O*-gallate	66.0 ± 1.2	5.31 ± 0.38	3.10 ± 0.12	15.7 ± 2.1
dimer B1 (Ec 4-8 cat)	72.3 ± 3.8	6.29 ± 0.09	3.15 ± 0.03	4.6 ± 1.0
dimer B2 (Ec 4-8 Ec)	79.1 ± 2.3	8.36 ± 0.48	3.05 ± 0.03	3.6 ± 1.1
dimer B3 (cat 4-8 cat)	59.1 ± 5.0	5.59 ± 0.10	2.39 ± 0.02	4.8 ± 1.5
dimer B4 (cat 4-8 Ec)	63.9 ± 2.5	6.03 ± 0.25	2.75 ± 0.01	5.3 ± 1.9
dimer B7 (Ec 4-6 cat)	59.2 ± 1.2	4.37 ± 0.04	1.84 ± 0.03	5.1 ± 0.6
trimer (Ec-Ec-cat)	90.6 ± 0.9	8.60 ± 0.56	4.35 ± 0.03	6.7 ± 3.1

1) TEAC: trolox equivalent antioxidant capacity; 2) FRAP: ferric reducing antioxidant power; 3) IC_{50} value: 50% inhibitory concentration.

Polymeric flavonoids present a complex extension of structure-activity relationship (SAR) that is poorly understood. The great variety in the structure of these compounds is due to the many possibilities in forming oxidative linkage. PC dimers and trimers are more effective than monomeric flavonoids against superoxide anion, whereas the activities of dimers and trimers differ only very slightly. Tetramers exhibit greater activity against peroxynitrite- and superoxide-mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimmers and tetramers. It appears that increasing the degree of polymerization only enhances the effectiveness of PCs against a variety of radical species up to a point. Extensive conjugation between 3-OH and B-ring catechol groups, together with abundant linkages, endow a polymer with significant radical scavenging properties by increasing the stability of its radical [29]. Moreover, the linkage between positions 4 and 8 in PCs significantly increase antioxidant efficiency in

aqueous phase assays with respect to 4 and 6 linkage [31]. Table 4 shows the remarkable antioxidant activities of (epi)catechins and some PAs in different systems.

Table 5. Different crops, their common by-products and main type of proanthocyanidins

Crop	Main by-products	Main group of PAs
grapes	pomace, seeds, stalks, peels	Type B - PC monomers and dimers [35]
apples	pomace, peels, seeds, core, stems	Type B - PC dimers [36]
beer (barley, hop)	straw	Prodelphinidin dimers and trimers [37,38]
peanut	hull, skin, kernel	Type A - PC dimers [39]
mango	skin, seeds, leaves	Type A - PC dimers and galloyl dimers [40]
avocado	skin, seeds, pulp	Type B - PC monomers to octamers [41]
papaya	skin, seeds	Type B - PC monomers and dimers [42]
pine	bark	Type B - PC dimers to heptamers [43]

Agricultural by-products contain a variety of biologically active species, which mostly go to waste with most plant-derived materials being particularly rich in antioxidant compounds such as polyphenols. The processing of fruits, vegetables, oilseeds and forest resources result in high amounts of waste materials, *i.e.* peels, seeds, stones, roots, barks, oilseed meals (Table 5). A disposal of these materials usually presents a problem that is further aggravated by legal restrictions. Plant waste is prone to microbial spoilage, thus limiting further exploitation. On the other hand, costs of drying, storage, and shipment of by-products are additional economic limitations to waste utilization with the result that agro-industrial waste is often utilized as feed or fertilizer. Thus new aspects concerning the use of this waste as by-products for further exploitation on the production of food additives or supplements with high nutritional value have gained increasing interest because these are high-value products and their recovery may be economically attractive [27, 32, 33]. PAs have been identified in several agricultural by-products such as skin, peels, pomace, stones, bark and seeds of grape, apple, beer, peanut, mango, avocado and pine and their biological, pharmacological and medicinal properties have been extensively reviewed. In addition to the free radical scavenging and antioxidant activity, PAs also exhibit vasodilatory, anti-carcinogenic, anti-allergic, anti-inflammatory, anti-bacterial, cardioprotective, immune-stimulating, anti-viral and estrogenic activities, as well as being

inhibitors of the enzymes phospholipase A2, cyclooxygenase (COX) and lipooxygenase [34]. The goal of this chapter is to provide an overview of the health benefits associated with agro-industrial by-products, which are rich in PAs and the primary focus is on the effects that have been clinically demonstrated in humans, although results of animal, *ex vivo* and *in vitro* studies are also discussed for different health areas.

2. GRAPES

Grape (*Vitis vinifera*) is one of the world's largest fruit crops, with more than 60 million tons produced annually. Grape is also one of the most commonly consumed fruits in the world both as fresh fruit (table grape) and processed fruit (wine, grape juice, molasses, and raisins) [44]. About 80% of the total crop is used in wine-making and pomace represents approximately 20% of the weight of grapes processed [32, 45, 46]. An estimated 13% (by weight) of the grapes processed by the wine industry ends up as by-product after pressing. Skins, peels, seeds and stems of grapes are produced in large quantities by the wine-making industry and these by-products have become valuable raw materials for extraction of polyphenols [47, 48]. Grapes and their by-products therefore, such as wine, grape juice, jams and raisins constitute an increasing economic significance.

The economical feasibility of the extraction process involves the search for optimal extraction conditions, in order to maximize process efficiency. Different novel extraction methods including subcritical water, polymeric adsorber resins and pressurized liquid extraction have been reported to enhance secondary metabolite extraction from grape by-products. The application of advanced technologies such as ultrasonics, high hydrostatic pressure and pulsed electric fields has demonstrated an extraordinary potential and selectivity for extraction. The combination of effective extraction technologies and low-cost raw materials represent an environmental and economical alternative to conventional extraction methods where large amounts of organic solvents and long extraction times are required. The use of these novel processing technologies will reduce food processing waste and

facilitate the production of natural valuable products which will guarantee food sustainability and meet consumer demand [49, 50].

Grape by-products contain a high amount of secondary metabolites including phenolic acids, flavanols and anthocyanins [51, 52] which are reported to possess anti-bacterial, anti-viral, antioxidant, anti-inflammatory, anti-carcinogenic properties and can prevent cardiovascular diseases [2, 53–56]. The greatest concentration of polyphenolics can be found in skin and seed with 20-30% and 60-70% of the total phenolics, respectively. For this reason, it is extremely important to recover the discarded by-products to extract their polyphenols for the health benefits associated with these compounds.

2.1. Oral Health

Dental caries is a multi-factorial infectious disease that depends on diet and nutrition, microbial infection and host response. In addition to dental caries, gingivitis and periodontal disease also affect most of the adult population with the prevalence of severe disease increasing with age. Periodontal disease is a group of chronic inflammatory diseases caused by specific anaerobic Gram-negative bacteria that activate immuno-inflammatory mechanisms within the local periodontal tissues, leading to the destruction of collagen and bone supporting the teeth [57, 58]. In recent years, much attention has been focused on research related to the identification of food components with disease-preventing and health-promoting benefits. Numerous naturally occurring components in foods and vegetables have been shown to promote health and reduce risks for many common diseases and despite these advances, the general public seems less aware of foods that promote oral health. It is thought that plant-derived anti-microbial compounds may serve as alternatives to the commonly used chemicals for dental plaque and oral disease control [57,59–63]. The following summarizes some of the studies related to grape products and their potential oral health benefits.

Root caries is especially prevalent among the elderly population due to gingival recession and the exposure of susceptible root surface. *Xie* et al. studied the effect of grape seed extract (GSE) on the

remineralization and demineralization of the collagen-rich root tissue of human teeth [64]. The GSE used consisted mainly of 97.8% PAs and in order to evaluate the effect of this extract on the remineralization of artificial root caries, an *in vitro* pH-cycling model was used. Results obtained from this study showed that treatment with GSE may positively affect the remineralization process through two distinct mechanisms. First, GSE may contribute to mineral deposition on the superficial layer of the lesion. Thus, it is likely that after treatment with GSE, the latter present in the lesion may combine with Ca^{2+} from the remineralizing solution, thereby enhancing remineralization. Secondly, GSE may interact with the organic portion of the root dentin through PA-collagen interaction, thereby stabilizing the exposed collagen matrix. Moreover, GSE treated dentin demonstrated increased microhardness value and wider precipitation band. The potential remineralizing effect of GSE may also be attributed to the changes in the organic matrix, specifically by the presence of newly induced collagen crosslinks. In summary, GSE may be a potential adjunct or alternative to fluoride in the treatment of root caries during minimally invasive therapy.

Several strains of oral streptococci are capable of initiating the formation of dental plaque, which plays an important role in the development of caries and periodontal disease in humans. *In vitro* recent studies have reported several anti-bacterial constituents of raisins with inhibitory effects on the growth of oral pathogens (*Streptococcus mutans* and *S. sanguis*). Grape extracts with different qualitative and quantitative phenolic content showed activity against glucosyltransferases [65,66] and protected the oral cavity from the cariogenic action of *S. mutans* [67]. In addition, grape products also exerted *in vitro* anti-bacterial activity against several oral streptococci, such as *S. pyogenes,* by inducing post-contact effects against *S. mutans* [68], and against potential respiratory pathogens [69].

In oral cancer, *in vitro* studies have shown that the anti-proliferative activity of GSE has been assayed using oral squamous cell carcinoma (SCC) cell lines (CAL-27 and SCC-25). Apoptosis is induced through the up-regulation of caspase-2 and -8 pre-mRNA, and the observed anti-tumour activity is attributable to extract polyphenol content, which comprises PAs, flavonoids and stilbenes [70, 71].

p53 gene has been reported to be responsible for the suppression of malignant transformation and block tumorigenesis. Grape seed PCs (GSP) treatment of oral squamous carcinoma cell lines exerted an effective and dose-dependent inhibitory effect on cell growth with slight differences. These findings indicated that GSP may have cytotoxic effects on these cells and play an important role as novel chemopreventive on therapeutic agent for oral squamous cell carcinoma [72].

2.2. Cancer

Cancer and chemoprevention have provided a major challenge for health professionals around the world. A large number of investigations have demonstrated a broad spectrum of pharmacological and therapeutic benefits of grape seed PAs against various stages of neoplastic processes and carcinogenesis including detoxification of carcinogenic metabolites [73–76]. A recent review of GSP by *Bagchi* et al*.* has highlighted the beneficial and pharmacological effects of this by-product extract in cancer chemoprevention and concluded that GSP is a superior scavenger when compared to vitamins C, E and β-carotene, and prevents hepatic and brain lipid peroxidation and DNA damage in animals. Moreover, GSP protects against structurally diverse drug- and chemical-induced multi-organ toxicity and induces selective cytotoxicity toward human breast, lung, gastric and pancreatic cancer cells while maintaining growth and viability of normal cells. A broad spectrum of studies further demonstrated that GSP prevents against dimethylnitrosamine (*N*-nitrosodimethylamine, DMN)-induced hepatic carcinogenesis by selective preventive and cell death patterns, by modulating gene expression profiles and protecting genomic integrity [77]. *Kaur* et al.'s work focuses on recent advancements largely in GSE and to a lesser extent on other grape by-products regarding different kinds of cancer [76]. In skin cancer, *Zhao* et al*.* found that a polyphenolic fraction isolated from grape seeds afforded significant protection against tumor promotion in the mouse skin tumorigenesis model and that this effect of GPS may largely be due to the exceptionally high antioxidant activity of PCs present therein. Moreover, they showed that PC B5-gallate was the most potent antioxidant compared with other polyphenols isolated from the extract by HPLC [78]. *Bomser* et al*.* [79,80] also

reported anti-tumor-promoting activity of GSP in a CD-1 mouse model. This data suggested that grape polyphenolic fraction is effective as an inhibitor of mouse epidermal ornithine decarboxylase activity when applied before the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and that the magnitude of inhibition is independent of TPA-induced epidermal ornithine decarboxylase protein content. In an ultraviolet B (UVB) radiation-induced mouse skin carcinogenesis model, dietary feeding of GSP was effective in preventing photocarcinogenesis at both initiation and promotion stages and malignant transformation of skin papillomas to carcinomas. Studies provide conclusive evidence that dietary GSPs have the potential to attenuate ultraviolet B (UVB)-induced oxidative stress and to inhibit the activation of the cellular signaling cascades involving the MAPK and NF-κB pathways that are associated with high risk of photocarcinogenesis [81–84].

Colorectal cancer is a major cause of morbidity and mortality throughout the world. It accounts for over 9% of all cancer incidences, is the third most common cancer worldwide and the fourth most common cause of death [85]. Studies have shown that GSE inhibits cell growth and induces cell cycle arrest and apoptosis in human colorectal cancer cells and modulates cell cycle regulators with a strong effect on Cip1/p21 up-regulation [86,87]. The anti-cancer effects of whole black grape (seeds included) extract are also reported in the cancerous colon tissues of humans via inhibition in DNA turnover enzymes [88]. The anti-cancer effects of PAs from grape seeds against colon cancer Caco2 cells have also been demonstrated through inhibition of the survival pathway and induction of apoptosis by *Engelbrecht* et al. [89] who concluded that GSP treatment inactivates protein kinase B (PKB) to decrease Bcl-2-associated death promoter (BAD), cAMP response element-binding protein (CREB) and forkhead transcription factor Foxo1 (FKHR) phosphorylation. This, in turn, leads to increased apoptosis as indicated by increased caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage. The anti-proliferative effect of GSP on colon cancer cells is associated with decreased pi3 kinase and phosphatidylinositol-3 kinase (PI3-K) activation and points towards the PI3-K/PKB signalling pathway as a potential therapeutic target. The effects of GSP should be further characterized in cancer cells and in normal cells for the purpose of development as an agent for the management of colon cancer.

Nomoto et al. reported that a low dose of PA effectively induced apoptosis in one colon cancer cell line and large intestine treated with COL2A1(AOM). It also markedly reduced the number of aberrant crypt foci (ACF) in the rat colon. These results suggested that PA can be applied as a chemopreventive agent against colorectal cancer [90]. For the first time *Dinicola* et al. demonstrated that GSE enhances a significant apoptosis-inducing factor (AIF) release in colon cancer cells together with an increase in caspase activity. These results suggested that GSE-induced apoptosis in Caco-2 cells can be considered a biphasic process, obtained through both caspase-dependent and caspase-independent pathways [91]. As per the statistics provided by the American Cancer Society in 2008, prostate cancer (PCA) remains the most commonly diagnosed cancer in men. In this way, findings by *Agarwal* et al. showed that GSE exerts anti-proliferative and anti-angiogenic effects and interferes with insulin-like growth factor 1 (IGF-1) signalling in (?) a cell line made from human prostate cancer cells DU145 xenografts, thereby exerting an overall growth inhibitory effect against DU145 xenografts in nude mice [92,93]. In addition, investigations by *Singh* et al. provided *in vivo* evidence for anti-proliferative, apoptotic and anti-angiogenic effects of GSE, and their correlation with the inhibition of advanced human prostate tumor xenograft growth in athymic nude mice. Up-regulation of insulin-like growth factor binding protein 3 (IGFBP-3) by GSE could be one of the possible *in vivo* mechanisms leading to inhibition of PCA cell growth and survival. GSE-induced decrease in vascular endothelial growth factor (VEGF) secretion from PCA cells might also play a role in the inhibition of tumor angiogenesis. Based on these findings, further studies are needed to explore the preventive/therapeutic efficacy of GSE with mechanistic details in other PCA pre-clinical models for its possible implication in humans [94]. Regarding breast cancer, which is the second leading cause of cancer-related deaths after lung cancer in women, a study conducted by *Mantena* et al. [95] reported that the metastatic potential of 4T1 breast cancer cells was inhibited by GSP. The anti-angiogenic effects of GSE were also observed by *Lu* et al. They showed that GSE inhibits VEGF expression in human cancer cells and presented one possible mechanism underlying this action. Their data indicated that the effect of GSE can be mediated through inhibition of Akt activation and higher intellectual function (HIF)-1α protein synthesis. This finding

added new insight into the potential mechanisms of the anti-cancer activity of GSE and offers a new molecular mechanism underlying the anti-angiogenic action of GSE. These results would help in the design of future strategies of developing GSE as a chemopreventive agent for a potential clinical therapy in combination with current anti-cancer drugs [96]. *Bhise* et al*.* also discussed the functional characteristics of grape by-product and their health benefits against cancer [97]. Animal studies and cell models suggest that the polyphenolics contained in grape seed act as anti-carcinogens by influencing molecular events at the initiation, promotion, and progression stages of cancer [98]. Flavonoids can act as anti-carcinogens by scavenging free radicals, regulation of signal transduction pathways of cell growth and proliferation, suppression of oncogenes and tumor formation, induction of apoptosis, governance of enzyme activity related to detoxification, oxidation, and reduction, stimulation of the immune system and DNA repair, and the regulation of hormone metabolism [99].

2.3. Cardiovascular Diseases

Epidemiological evidence indicates that consumption of polyphenolics results in a reduction of cardiovascular disease risk factors and decreased mortality [100]. The protective effect of polyphenols is in part due to their ability to retard the development and progression of early atherosclerotic lesions to advanced atherosclerotic plaques. Antioxidant flavonoids found in red wine for example can reduce the oxidation of low density lipoprotein (LDL), which is a key and early event in the atherogenic process, platelet aggregation, and nitric oxide (NO) dependent dilation [101]. There is evidence to support that oxidative stress resulting from increased production of free radicals associated with decreased amount of antioxidants in the myocardium plays a crucial role in cardiovascular diseases [102–106]. Several authors discuss the beneficial effects of grape polyphenols in their reviews [97, 107–109]. LDLs that transport lipids throughout our bodies are targets for oxidation. Studies of PAs from grape seeds suggest that they are effective at protecting against LDL oxidation *via* free radical scavenging activities [110]. Grape products, especially grape seeds and skins, are potent accelerators of nitric oxide synthase (NOS) activity, quenchers of *in vitro* oxidation of LDL, effective platelet

inhibitors and appear to reduce the development of atherosclerosis through this pathway. Consumption of grape and grape extracts and/or grape products such as red wine may be beneficial in preventing the development of chronic degenerative diseases such as cardiovascular disease [97, 111]. Improved endothelial function is a mechanism by which cardiovascular events might be prevented by grape products. *In vitro* studies have shown that flavonoid components of grape juice promote arterial endothelium-dependent vasodilation *via* the nitric oxide-guanosine 3',5' cyclic monophosphate pathway [112]. PCs have recently been shown to possess endothelium dependent relaxing (EDR) activity in blood vessels. Furthermore, increased levels of cyclic GMP, the vascular smooth muscle cell messenger through which NO acts accompanied EDR activity. It has recently been shown that vasodilating compounds tend to be of the PA type. Activity of isolated PCs tended to increase with degree of polymerization, epicatechin content, and with galloylation [113]. Interestingly, *Clifton* reported a significant difference in flow-mediated dilatation (FMD) as measured by ultrasound in subjects who had above average cardiovascular risk factors, which included high cholesterol, smoking, or high blood pressure (BP). Therefore, sufficient grape seed antioxidant polyphenols from grape seed extract were absorbed to influence FMD. It is conceivable that "bioavailability" of the polyphenols may have been a determinant in the failure of grape seed extract to reduce BP despite improvement in FMD in this study [114]. *Soares De Moura* et al. demonstrated that oral administration of grape skin extract significantly reduced systolic, mean, and diastolic arterial pressure in a hypertensive rat model and this extract was shown to concentration-dependently inhibit lipid peroxidation and induce endothelium-dependent vasodilation in norepinephrine-induced contracted mesenteric vessels *ex vivo* [115]. Several studies in humans report enhanced vasodilation following regular supplementation with grape extract. *Lekakis* et al. reported increases in flow-mediated dilation of the brachial artery in adults with coronary heart disease following acute ingestion of red grape polyphenol extract [116]. According to *Chaves* et al. acute administration of a standardized grape product, such as a freeze-dried preparation of red, green, and blue-black California seeded and seedless grapes, significantly increased endothelial shear stress-induced vasorelaxation responses in

healthy normal subjects, and this effect was further enhanced during chronic administration. The single consumption of a standardized high fat meal caused a significant reduction in endothelial function, and with a modest intake the standardized grape product completely abrogated this meal-induced vascular dysfunction. The mechanism involved was independent of circulating triglyceride levels. This data suggested that standardized grape product may have beneficial effects on endothelial health and function in subjects with no evidence of cardiovascular disease, and may also have substantial protective effects against the vascular toxicities associated with a typical Western high-fat diet [117]. In another study investigating the potential cardioprotective effects of grape seed PAs, the extent of ischemia/reperfusion-induced cardiac arrhythmias was measured [101]. After 3 weeks on the grape seed PA–supplemented diets, the incidence of reperfusion-induced arrhythmias was 25% in the supplemented group compared with 92% in the control group. The importance of inflammation for all stages of atherosclerosis is increasingly recognized and there is data suggesting that grape polyphenols have anti-inflammatory effects. In humans, treatment with lyophilized grape powder was associated with a reduction in tissue necrosis factor-α which may be due to the decrease in lipid peroxidation [118].

2.4. Neurodegenerative Diseases

Neurodegenerative disorders such as Parkinson's and Alzheimer's diseases represent an increasing problem in our aging societies. Results from such investigations suggest that grape seed extract might be effective against developing Alzheimer's disease and potentially other neurodegenerative disorders [54, 119–122]. *Thomas* et al. utilizing Alzheimer's disease and wild-type mouse models in a study involving two dietary treatments with grape seed extract, observed reductions in DNA damage in the Alzheimer's disease model mice after they consumed free grape seed extract. The results of this study are suggestive of a potential protective effect of a polyphenol enriched diet in terms of reducing DNA damage events, such as micronuclei and telomere length in different somatic tissue types in this transgenic mouse model, with no adverse effects on cell

proliferation rates or cell death markers [123]. In addition, decreases in amyloid β-protein concentrations in brain and serum and reductions in amyloid β-plaque deposition and inflammation in the brains of Alzheimer's mouse models have been reported by *Wang* et al. following supplementation of grape seed extract [124]. Similarly hopeful results have also been observed *in vitro*, with decreases in amyloid β-protein formation being noted subsequent incubation in solutions of grape seed extract [125]. *Pasinetti* et al. reported that *in vivo* preclinical studies support the efficacy of grape seed polyphenolic extract (GSPE) to mitigate amyloid β-protein (Aβ)- or mutant tau [tau protein. a highly soluble microtubule-associated protein (MAP)]-mediated neuropathologic phenotypes. Thus, GSPE might benefit Alzheimer's disease by simultaneously interfering with the two hallmark neuropathologies of the disease. These new observations of the effects of GSPE treatment on Alzheimer's disease's pathological features, taken together with the demonstrated bioavailability as well as safety and tolerability, strongly support the continued development of GSPE for Alzheimer's disease prevention and therapy [54].

2.5. Diabetes

Diabetes mellitus is the most common metabolic disorder, being ranked as the fourth most common cause of mortality. Several studies have been conducted that suggest grapes or constituents of grapes may have some protective effects against the metabolic disturbances observed in type 2 diabetics [126–129]. *Kar* et al. have demonstrated that GSE in Type 2 diabetic subjects has favourable effects upon inflammation (hsCRP), glycaemia (fructosamine) and a sole marker of oxidative stress [glutathione (GSH)], while also decreasing total cholesterol levels. These changes may have important clinical implications [130]. Research in animal models is limited, but supplementation with grape seed PAs and grape powder appears to benefit blood glucose control [120]. PCs extracted from grape seed showed an anti-hyperglycemic agent with insulinomimetic properties in streptozotocin-induced diabetic rats. These PCs mimic and/or influence insulin effects by directly acting on specific components of the insulin-signaling transduction pathway. This insulin-like role of PCs must be added

to their very well described effect of improving altered oxidative states [131]. In another study, *El-Alfy* et al. observed a decline in hyperglycemia and an increase in serum insulin levels in Wistar rats fed daily with a pretreatment orally administered from grape seed PAs. These compounds decreased lipid peroxidation and increased pancreatic glutathione levels. This data indicated that the grape seed PAs protected β-cell function and suggested a protective effect against the generation of damaging reactive oxygen species [132]. *Hogan* et al. [133] were the first to report that the grape pomace extract exerted significant anti-postprandial hyperglycemic effect, suggesting that it could be a valuable food derived bioresource that is rich in antioxidants and anti-hyperglycemic compounds. These dual bioactive attributes derived from the grape pomace could play a complementary and alternative role in managing the poorly regulated blood glucose levels and oxidative stress associated with type 2 diabetes. *Zunino* et al. noted a reduction in the incidence of type 1 diabetes in non-obese, diabetic, young mice after replacing 1% of their total energy intake with freeze-dried grape powder for 6 months. By 7 months of age, 77% of the mice consuming the control diet and only 33% of those receiving the grape powder supplement had developed diabetes [120,134].

2.6. Immune Function

Grape products have recently been implicated in protecting and strengthening immune function in animals. Very few studies have been published in human beings or intact animals to study immunity after consuming grapes and grape products [135]. The review by *Magrone* et al. illustrates the effects of polyphenols on immune cells from human healthy peripheral blood. Data shows that grape polyphenols are able to stimulate both innate and adaptive immune responses. In particular, the release of cytokines such as interleukin (IL)-12, interferon (IFN)-γ, and IL-10 as well as immunoglobulins may be important for host protection in different immune related disorders. Some molecular cytoplasmatic pathways elicited by polyphenols able to regulate certain immune responses are also discussed. In particular, it seems that p38, a molecule belonging to the mitogen-activated protein kinase (MAPK) family, is involved in the release of interferon-gamma (IFN-γ) and, therefore, in

NO production [136]. *Zhang* et al. observed that an intraperitoneal administration of doxorubicin (DOX) along with grape seed extract possessed anti-tumor effects and enhances the anti-tumor activity of DOX in tumor-bearing mice. The mechanism might be partially related to immunopotentiating activities through the enhancements of lymphocyte proliferation, natural killer cell (NK cell) cytotoxicity, the $CD4^+$ antigen ($CD4^+$)/$CD8^+$ antigen ($CD8^+$) ratio, and IL-2 and IFN-gamma productions. The protective effect on the immune system was not observed when it was administered without the PA grape seed extract [137].

3. APPLES

Apples (*Malus* sp.) are a rich source of nutrient as well as non-nutrient components and contain high levels of polyphenols and other phytochemicals. Much of the protective effect of fruits and vegetables has been attributed to phytochemicals. Main structural classes of apple constituents include hydroxycinnamic acids, dihydrochalcones, flavonols, catechins and oligomeric PCs, which have long been recognised as the major contributors to antioxidant activity of apples [138,139] and derivatives [140], whose capacity depends on their polymerisation degree and substituents [141].

Apples are a very significant source of flavonoids in people's diet in the US and Europe. Since fruits and vegetables are high in antioxidants, a diet high in these foods should help prevent oxidative stress and may therefore help prevent chronic disease and slow aging. However, apple by-products, consisting of pomace, peel, seeds, core, stems and exhausted soft tissue have long been recognized as a valuable material for nutritional, pharmacological or cosmetic purposes, as it is rich in dietary fibre and polyphenols [142–147]. The compounds most commonly found in apple peels consist of PCs, catechin, epicatechin, chlorogenic acid, phloridzin, and quercetin conjugates. There is some catechin, PC, epicatechin, and phloridzin in apple flesh, but these compounds are found in much lower concentrations than in the peels [36,148].

Asturias (northern Spain) is one of the largest producers of cider of the world (AICV, 2000). More than 20,000 tons of apple pomace are produced per year and are primarily used as cattle feed,

although it still remains as a waste which causes economic and environmental problems [149]. Apple pomace represents about 20–35% of the original fruits. Apple peels are waste products of apple sauce and canned apple manufacturing. Washington State dominates U.S. organic (and conventional) apple production. Washington State apple producers managed approximately 13,000 acres under certified organic production systems in 2008 and Washington State produced 5.4 billion pounds of apples in 2009 [150]. *Wolfe* et al. estimated that 16 million pounds of peels were generated [151]. Several lines of evidence have suggested that apples and apple products possess a wide range of activities which may contribute to health benefits against cardiovascular disease, asthma and pulmonary dysfunction, diabetes, obesity, cancer; and various authors have summarized the knowledge on apples and apple by-products and their relationship to human health [148, 152, 153].

3.1. Cancer

Several studies have specifically linked apple consumption with a reduced risk of cancer. Recent trials have added data that suggests a protective effect of apple PCs on risk of cancer.

Colon cancer has been the focus of many investigations because it is one of the most common cancers in Western countries. Polyphenol-rich apple extracts have been reported to possess bioactive properties, suppressing human colon cancer cell growth *in vitro* [154–157]. In addition, polyphenol-rich apple extracts and selected apple polyphenols were found to reduce oxidative cell damage in human colon carcinoma cell lines *in vitro* [158]. Expression of the protein-tyrosine phosphatase DEP-1, a candidate tumor suppressor for colon carcinogenesis, is elevated upon treatment of colon epithelial cells with apple polyphenols. The first report by *Balavenkatraman* et al. showed that the effects of these protective dietary nutrients are attributed to the up-regulation of a tumor suppressor [159]. This data showed that apple polyphenols modulate signaling cascades, which are crucial for the regulation of cell growth. According to *Kern* et al., apple polyphenols were found to inhibit protein kinase C (PKC) activity in a cell-free system [160]. *McCann* et al. have also shown that a crude mixture of apple phenolics can affect three biomarkers of colon cancer risk *in vitro*. In terms of the

concentration required, the effects of the extract appeared to be more potent in the bioassays that modelled the later stages of carcinogenesis namely transepithelial electrical resistance (TER, an assay for tumour promotion) and matrigel invasion (tumour metastasis). Protection against DNA damage may also reduce the acquisition of mutations leading a tumourigenic phenotype or delay the rate of aggressive mutations. Increased barrier function can potentially prevent abnormal cellular interactions with the surrounding extracellular environment. Inhibition of invasive restricts the process of metastasis and abnormal extracellular interactions. Therefore, apple consumption may serve to protect against the risk of colon cancer by protecting colonic cells against DNA damage and abnormal extracellular behaviour [161]. A study using rats injected with the chemical carcinogen azoxymethane (AOM) found protective effects and extract of apple PCs provided to the animals. There was a significant reduction in preneoplastic lesions in the animals exposed to the apple phytochemical [155]. An epidemiological study by *Liu* et al. suggested that whole apple extracts possess potent activity to suppress 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in a rat model and that this suppression is at least partly attributed to both the inhibition of cell proliferation and induction of apoptosis. Animal feeding and *in vitro* studies have reported the potential health benefits of the apple in breast cancer reduction. The inhibition of cell proliferation and induction of apoptosis in mammary cancer may be regulated through the down-regulation of cyclin D1 and B-cell leukemia/lymphoma 2 protein (Bcl-2) expression as well as the up-regulation of Bcl-2-associated X protein (Bax) expression [162]. Very few of the individual fruits and vegetables examined had a significant effect on lung cancer risk in women, however apples were one of the individual fruits associated with a decreased risk in lung cancer [163].

3.2. Cardiovascular Diseases

A reduced risk of cardiovascular disease has been associated with apple consumption. It is estimated that over 80 million American adults have one or more types of cardiovascular disease, such as hypertension, coronary artery disease, myocardial infarction, angina, heart failure and stroke [164].

The Women's Health Study surveyed nearly 40,000 women with a 6.9-year follow-up, and examined the association between flavonoids and cardiovascular disease [165]. Women ingesting the highest amounts of flavonoids had a 35% reduction in risk of cardiovascular events.

Byun et al*.* demonstrated that apple PCs play crucial roles in promoting hyperpolarization *via* multiple K^+ channel activations as well as increased NO release *via* promotion of NO/cGMP pathway responsible for anti-angiogenic effect by a reduction of cell proliferation in rat aorta endothelial cells (RAECs). Apple PCs induced bimodal actions would be caused by the increasing influx of Ca^{2+} into RAECs. However, further studies are needed to clarify which upstream signaling mediator(s) is involved in apple PCs actions using apple PCs or its individuals [166]. Elevated lipids and aberrations in lipid metabolism are well-established risk factors for many types of cardiovascular disease. *Pearson* et al*.* examined the effects of Red Delicious apples (whole apples, peels alone, and flesh alone) on human LDL oxidation *in vitro*. LDL oxidation was measured using headspace analysis of hexanal produced from copper-induced lipid oxidation *in vitro*. The dose of the whole apple, apple peel and apple flesh were standardized for gallic acid equivalents. Whole apples inhibited LDL oxidation by 34%.

Apple peels inhibited LDL oxidation by 34%, while the flesh alone showed significantly less inhibition (21%) [167]. A moderate supplementation of lyophilized apple (15% of the diet) exerts a slight cholesterol-lowering effect in plasma and liver and elicits noticeable modifications of lipoprotein cholesterol distribution [depressed triglyceride rich lipoprotein cholesterol (TGRLP-C) and increased high-density lipoprotein cholesterol (HDL-C)] with an anti-atheromatous significance.

Some of the apple's protective effect against cardiovascular disease may come from its potential cholesterol-lowering ability. The conclusions obtained by *Aprikian* et al*.* were that the apple diet increased the faecal excretion of neutral sterols and the apparent cholesterol absorption was markedly reduced. Adaptation to the apple diet induced a greater malondialdehyde (MDA) excretion in urine, together with an increase of the ferric reducing-antioxidant power (FRAP) value in plasma, which suggests effective antioxidant properties in the body [168].

3.3. Diabetes and Weight Loss

The incidence of diabetes, chiefly type 2 diabetes, has increased dramatically and is the subject of intensive study around the world. In 2010, an estimated 19.7 million Americans had been diagnosed with diabetes mellitus, representing 8.3% of the adult population [164]. New data has suggested a possible link between apple PAs consumption and a reduced risk of diabetes. In a large ongoing trial by the Women's Health Study, apples were identified as the only flavonoid-rich food that might be protective. The authors of this study also searched for an association between total flavonol and flavone intake and a limited number of subtypes of these flavonoids (5 total) and reduced risk [169].

Current guidelines recommend daily consumption of fruit- and vegetables that are good sources of dietary fibers and low in energy density to promote healthy weight maintenance or weight loss. Based on this premise, a study was conducted by *Conceição de Oliveira* et al. on 49 overweight women with high blood cholesterol levels to determine the effect of fruit intake on blood lipids and body weight [170]. The women were randomized to 1 of 3 diet groups including a daily intake of apple, pear or oat cookies.

All 3 groups were matched for the additional dietary fiber provided by each of the treatments. The addition of apples as part of an average daily caloric intake resulted in a significant weight loss. The authors proposed that the weight loss was due in part to the significant decrease in energy density of the diet due to the addition of apples compared to the oat cookies in spite of the comparable fiber content of the two.

3.4. Other Diseases

Herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) (family Herpesviridae) are responsible for a broad spectrum of clinical symptoms in humans, varying from mild skin vesicular lesions to severe manifestations such as encephalitis, conjunctivitis, eczema, pneumonia and hepatitis [171]. There is limited information available on the anti-viral activity of apple pomace. In this work, *Suárez* et al.

presented the phenolic profiles and antioxidant abilities of extracts from apple pomace, and preliminary data on their inhibitory activity against HSV-1 and HSV-2 *in vitro* propagation [172].

Growing evidence demonstrates the gastroprotective effects of apple polyphenols on chemically induced injuries. *Pastene* et al. demonstrated that flavonoids present in apple peel extract possess an anti-*Helicobacter pylori* activity. Additionally, this effect is linked to an inhibition of extracellular reactive oxygen species (ROS) production induced by non opsonized *H. pylori*. The results suggest that apple peel extract exerts a protective effect, inhibiting the mechanism by which *H. pylori* and neutrophils collaborate to cause gastric mucosa damage [173].

Saito et al. demonstrated that the polyphenol fraction from unripened apples inhibited enzymatic activities of cholera toxin in dose dependent manner in mice. It also transpired that the two fractions with highly polymerized catechin compounds inhibited cholera toxin catalyzed ADP-ribosylation by 95% and 98%, respectively [174].

The prevalence of pulmonary disorders, particularly asthma, has been increasing over the past several decades worldwide [175]. Apple consumption has been inversely linked with asthma and has also been positively associated with general pulmonary health, including bronchitis and emphysema, as well as a general benefit to ventilatory function in healthy individuals as highlighted by *Boyer* et al. [148].

4. BEER

Beer is a very popular alcoholic beverage consumed in large amounts in most countries and contains various classes of polyphenolic compounds, from simple phenols to complex oligomeric PAs. Polyphenols that occur in beer in relatively high concentrations are: the phenol tyrosol, the benzoic acid derivative *p*-hydroxybenzoic acid, the cinnamic acids, (+)-catechin and (-)-epicatechin, PA dimers (PC B3 and prodelphinidin B3) and the flavanone isoxanthohumol [37, 176, 177]. Different profiles of *in vitro* biological activities have been described for these compounds, which in combination exert a

synergistic effect. However, to extrapolate these results and evaluate the *in vivo* physiological effects of beer consumption, it is necessary to study their bioavailability in the body [74]. About 80% of phenolic compounds present in beer are derived from barley malt and the remaining come from hops. Hops (*Humulus lupulus L.*) is the raw material of beer which serves as an important source of phenolic compounds. About 20-30% of beer polyphenols originate from hops, whereas 70-80% are malt-derived. Only 15% of the hops constituents end up in the beer, whereas 85% will become spent hops material [38, 178]. *McCarthy* et al. have demonstrated in a recent study that phenolic extracts from brewers' spent grain (BSG) which is a low-value by-product of the brewing industry, are produced in large quantities annually. This BSG has antioxidant potential by protecting against oxidant-induced DNA damage and also possibly by iron chelation. This opens up new possibilities for use of this brewery by-product [179]. *Fărcas* et al. evaluated the high polyphenol content and antioxidant activity of BSG in order to highlight their potential for the extraction of natural antioxidants and their efficiency for incorporation in functional foods [180]. The abundant polyphenolic compounds in beer and beer ingredients have been shown to possess a variety of beneficial effects on health, such as anti-bacterial, anti-oxidative, anti-inflammatory, anti-angiogenic, anti-osteoporotic and anti-carcinogenic activities [181].

There are several lines of evidence suggesting beer as an interesting starting material for chemopreventive agent development. Recently, several reports have demonstrated that feeding beer or freeze dried beer inhibited chemically induced mammary and colonic carcinogenesis in rats [182, 183]. *Gerhäuser* et al. have reviewed scientific evidence accumulated over the past 10 years which points to the cancer preventive potential of selected hop-derived beer constituents including prenylated flavonoids and hop bitter acids. Moreover, selected hop compounds were also tested for estrogenic and anti-estrogenic properties [184] and 48 beer constituents have been tested for cancer preventive potential in a battery of nine test systems designed to detect antioxidant and radical-scavenging activity, potential to inhibit the metabolic activation of xenobiotics by cytochrome P450 1A (Cyp1A) and to enhance phase 2 detoxification, anti-inflammatory and estrogenic/anti-estrogenic

effects. Overall, catechins and PAs were identified as the most potent radical scavengers against 2,2-diphenyl-1-pycrylhydrazyl (DPPH), superoxide anion and peroxyl radicals. On the other hand, catechins were also identified as cyclooxygenase (COX)-1 inhibitors [177]. The four procyanidin (PC) dimers (B1-B4), a PC trimer, and the mixture of oligomers in hop investigated by *Stevens* et al. are potent inhibitors of neuronal nitric oxide synthase (nNOS) activity and linsidomine (SIN1)-induced oxidation of low density lipoprotein (LDL), suggesting a potential for dietary PCs in preventing diseases associated with reactive nitrogen or oxygen species [185]. *Zanoli & Zavatti* describes the morphological, phytochemical and ethnopharmacological aspects of hop and summarizes the most interesting findings obtained in the preclinical and clinical research related to the plant. They report the data from their experiments as well as those obtained from other researchers, focusing on the variability of the results. Other traditional applications of hops as a stomachic, anti-bacterial and anti-fungal remedy have been supported by *in vivo* and/or *in vitro* investigations. In recent years some bioactive compounds present in hops have received considerable attention for their biological effects: in particular, xanthohumol has been shown to exert cancer chemopreventive activity *in vitro* experiments [186].

5. PEANUT

Peanut is an important crop, which is grown worldwide and although it originates from South America, the peanut has spread beyond the Mediterranean to China, Africa, India, Japan and the United States of America. The substantial amounts of by-products are generated in the process of peanut harvest and peanut oil extraction, which are potential pollutants. A large portion of peanut meals, skins, hulls, and vines is regarded as agriculture waste. Many researchers are currently focusing on the investigation of producing edible oil and kernel but few studies are paying any attention to peanut by-products. Over 0.74 million metric tons of peanut skins are produced annually worldwide as a by-product of the peanut processing industry [187]. The high concentration of polyphenols present in peanut skins supports the utilization of agricultural by-products as a source of natural

antioxidants [188]. Seventeen percent of peanut skin weight are PAs, consisting of low and high molecular weight oligomers [189]. *Lou* et al. made a comprehensive analysis of the water-soluble phenolic extract from peanut skins, resulting in six A-type proanthocyanidins (PAs), including procyanidins (PCs) A1 and A2, and three newly found epicatechin oligomers [39]. In 2004, they isolated five oligomeric proanthocyanidins (PAs), B2, B3 and B4 from the water-soluble fraction and two new polyphenols, epicatechin-(2β-*O*-7,4β-6)-[epicatechin-(4β-8)]-catechin and epicatechin-(2β-*O*-7,4β-8)-[epicatechin-(4β-8)]-catechin-(4α-8)-epicatechin, based on their spectral data [190,191].

In recent years, new scientific research has focused on the effects of nutritional composition from peanut by-products on human performance. *Bansode* et al. hypothesised that polyphenols present in peanut skin extract would exert the same health promoting and hypolipidemic effects as observed by the previous studies related to the polyphenols from various food sources. Their findings suggest that peanut skin-derived polyphenols provide resistance to Western diet-induced hyperlipidemia in rats. This may have a broader implication in humans for its potential use in the prevention of obesity and obesity-related disorder. This will impart a significant therapeutic potential for using peanut skin as a value-added ingredient in peanut-based product and in other food products as a source of bioactive phenolic compounds [192]. *Tatsuno* et al. isolated 11 PAs oligomers, monomer to tetramer, from an aqueous extract of peanut skin. They showed that these compounds suppressed not only production of inflammatory cytokines but also melanogenesis in cultured human melanoma cell co-stimulated with phorbol 12-myristate 13-acetate (PMA). Moreover, anti-melanogenesis activity of four type A PAs have been reported in human malignant melanoma cell line (HMV-II) co-stimulated with PMA. Three compounds of this peanut skin extract resulted in suppressed melanogenesis in murine B16F1 melanoma, whose activities were similar to those in the case of HMV-II. Therefore, the suppressive activity of PCs on melanogenesis might be more effective in type A PC rather than type B. These results suggest that type A PAs may be useful for preventing the inflammation and melanin synthesis that occur during skin damage, or as a result of dermatological diseases [193]. Its main constituent procyanidin A1 (PA) was also investigated by *Takano* et al. whose findings suggest that peanut skin

extract may have the potential to suppress allergic diseases by inhibiting immunoglobulin synthesis and regulating systemic T helper cytokine productions. It was also revealed that procyanidin A1 (PA) isolated might be one of the anti-allergic constituent of this extract [194]

6. TROPICAL FRUITS

World production and trade of fresh tropical fruit is expected to expand over the next decade. Developing countries account for about 98 percent of total production, while developed countries account for 80 percent of world import trade. Major tropical fruits account for approximately 75 percent of global fresh tropical fruit production. Mango is the dominant tropical fruit variety produced worldwide, followed by pineapples, papaya and avocado [195]. Their attractive sensory properties and nutritional and therapeutic value have significantly increased the production, trade and consumption of these fruits [196, 197].

Of the potential uses that can be considered for tropical fruits by-product, one of the most important is as food additives [198], which are a valuable dietary source of many phytochemical compounds. Several studies have reported polyphenolic compounds including various flavonoids, xanthones, phenolic acids and gallotannins [197, 199, 200] playing a preventive role in degenerative diseases, such as cancer, cardiovascular and neurodegenerative diseases [32, 201, 202].

6.1. Mango

Mango (*Mangifera indica* L., Anacardiaceae) is one of the most popular tropical fruits in the world with annual production of over 40 million metric tonnes in 2012 (FAOSTAT). It is grown in more than 100 countries in both tropical and subtropical regions, especially India. Mango is generally consumed as a dessert fruit, but there has been an increase in consumption of mango products such as juice, nectar, powder, canned mango slices in syrup, and chutneys. The residue resulting from its processing primarily consists of peel and seeds, amounting to 35-60% of the total fruit weight [197, 203]. Recent studies have reported that mango by-products have a great deal of antioxidant activity attributed to

their high bioactive compound content such as carotenoids, tocopherols, sterols and phenolic compounds [40,204–206] including PAs, mainly gallate derivatives [102, 207]. Mango extracts from leaves, fruit, seed kernel, fruit pulp, roots, and stem bark have been used for medicinal purposes in many countries. They have also been found effective against several diseases such as cancer, diabetes, asthma, infertility, lupus, prostatitis, prostatic hyperplasia, gastric disorders, arthralgias and mouth sores, among others [208–210].

As well as its antioxidant activities [206, 211, 212], several biological properties of mango by-products have been studied in recent years. Gastroenteritis is a clinicopathological term that refers to inflammation of the intestines causing diarrhea as a result of increased motility in the colon. Diarrhea is caused by a variety of enteric pathogenic bacteria such as enterotoxigenic *Escherichia coli* (enteropathogenic and enteroinvasive), *Salmonella sp*., *Shigella flexneri*, *Vibrio cholerae*, *Campylobacter jejuni* etc. Viruses, protozoans and helminthes can also cause diarrhoea in humans [213]. Flavonoids and tannins present in the seed kernels could be implicated as having anti-diarrheal activity and inhibit the intestinal motility. Reduction of intestinal motility may be due to the presence of these compounds in the seed kernel extract since animals treated with aqueous and alcoholic extracts from the seed kernel by *Rajan* et al*.* showed reduced intestinal motility and faecal score in Swiss albino mice [214]. Anti-microbial activities of different seed kernel extracts were investigated by *Mirghani* et al*.* using the agar well diffusion method. From observation and measurement of diameter of inhibition zones, it could be demonstrated that the mango extracts had significant potential anti-bacterial activities capable of inhibiting the growth of pathogenic bacteria [215]. Moreover, *Hannan* et al*.* also studied the anti-bacterial activity of leaf extracts from mango against *Salmonella typhi*. They demonstrated that the zone of inhibition size was directly proportional to the increase in concentration of extract [216]. Gallotannin extracted from mango kernels using semipreparative high performance liquid chromatography (HPLC) showed anti-bacterial activity against pathogenic bacteria [217]. Regarding seed extracts, anti-bacterial activity against four strains of both Gram-positive and Gram-negative bacteria has been reported by *Khammuang* et al*.*, where

the most sensitive pathogenic strain inhibited was *Pseudomona aeruginosa* [218]. These results indicate that there is a possibility of sourcing alternative antibiotic substances in these by-products for the development of newer anti-bacterial agents.

Mango has been reported to have hypoglycemic effects in animal and human studies [219–221]. Therefore, the study of *Daud* et al. which tested the hypothesis that mango extracts (peel and flesh) contain bioactive molecules capable of modulating endothelial cell migration, was an essential step in the formation of new blood vessels or angiogenesis [222], together with demonstrated bioactivities such as peroxisome proliferator-activated receptor (PPAR) activation and inhibition of cancer cell proliferation [223]. On the other hand, *Lucas* et al. examined the effects of a low-dose dietary supplementation of mango extract, in comparison with the hypolipidemic drug, fenofibrate, and the hypoglycaemic drug, rosiglitazone, in reducing adiposity and alterations in glucose metabolism and lipid profile in mice which were fed a high-fat diet [224]. Anti-obesity effects of mango seed kernel extract was also investigated by *Kobayashi* et al. in 3T3-L1 adipocytes and in a high fat diet (HFD)-induced obesity rat model. This extract caused a significant decrease in the activity of glycerol 2-phosphate dehydrogenase (GPDH) in 3T3-L1 adipocytes without eliciting cell cytotoxicity and inhibited cellular lipid accumulation through down-regulation of transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein □ (C/EBPα) using *in vitro* assays. In an animal model, rats fed an HFD containing the extract gained less weight and their visceral fat mass tended to be lower than rats fed an HFD alone. Furthermore, histological examination of rat livers from an HFD only showed steatohepatitis. The others showed no histopathological changes in liver tissue [225]. All these results demonstrate the diverse bioactivities present within mango products.

6.2. Avocado

Avocado (*Persea americana*) is an oleaginous fruit native to tropical America and is an important commercial tropical fruit. Avocados are rich in unsaturated fatty acids, fibre, vitamins B and E, and

other nutrients. Industrial processing of avocados generates a large amount of peels and seeds as waste. Exploiting the phytochemical content of this waste may lead to new products and add value to the avocado industry [41, 226]. A few studies have focused on their phytochemical compositions. The characterization of phenolic compounds has revealed the presence of flavonoids in leaves, pulp, skin and seeds [202, 227]. Avocado seeds are claimed to be rich in a complex mixture of polyphenolic compounds, ranging from (+)-catechin and (-)-epicatechin to highly polymeric PAs [18, 228]. The Californian Avocado Commission, has also driven efforts to publicize the fruit as a health promoter, including joint publications with other associations and more recently, some press releases. For this reason, the avocado is gaining worldwide recognition as a healthy food and consequently, as a product of significant economic value. The non-edible parts of the fruit (skin and seed) have also been studied in order to assess their potential use as a cheap source of bioactive compounds for the food, pharmaceutical and dermocosmetic industries [227].

There is little information available about the total phenolic content and antioxidant and anti-microbial capacities of pulp or residues from avocado fruit (peel, pulp or seed) [229]. *Fulgoni* et al*.* were the first to explore associations between avocado consumption, diet quality, nutrient and energy intakes, and metabolic disease risk factors [230]. Anti-microbial activity of avocado seed extracts against *Staphylococcus aureus, Staphylococcus pyogenes, Corynebacterium ulcerans* and *Corynebacterium albicans* was carried out by *Idris* et al*.* and the different solvent extracts exhibited potential activity against clinical microbial isolates that are associated with various infectious diseases, may provide scientific justification for the ethnomedicinal uses of fruit by-products [231]. *Nagaraj* et al*.* have also reported its potential as an antioxidant and anti-bacterial agent for different fractions of seed extract of avocado. The results showed that these fractions could also be used as phytochemical analysis in order to obtain highly efficient concentrated compounds that could be protected from oxidative stress and prevent deterioration of food ingredients and to protect from diseases in humans [232]. Avocado seeds have been traditionally used to treat mycoses and parasitic infections. Anti-fungal potential was evaluated by *Leite* et al*.* using the microdilution method against *Candida* spp.,

Cryptococcus neoformans and *Malassezia pachydermatis.* They concluded that the extracts obtained from avocado seeds and their constituents represent possible candidates for use as alternative dengue control agents and also act as anti-fungal agents against pathogenic yeasts [233].

To justify the use of avocado seed extract in the treatment of hypertension and to determine the effect of such usage on cholesterol levels, *Anaka* et al*.* indicated that the aqueous seed extract possesses blood pressure lowering properties in normotensive Sprague-Dawley rats. The effect of the extract may be involved in the blood pressure. Acute administration of the extract to naïve rats resulted in blood pressure reduction. The mechanism involved in the lowering effect of the extract appears to be cardio-dependent as the heart rate was significantly decreased following pretreatment with the extract or its acute administration to naïve rats [234]. This reduction is the main mechanism by which β-adrenoceptor blockers such as propranolol reduce blood pressure in hypertensive animals although these drugs do not reduce blood pressure in normotensive humans [235].

Imafidon et al*.* also evaluated the effects of aqueous seed extract on blood pressure, plasma and tissue lipids of albino rats. They found that this extract reduced blood pressure in rats. However, the use of avocado seed in the treatment of hypertension should be dose dependent since high concentrations of this herbal drug could produce an increase in cholesterol levels by alterations in the metabolism of some hormones. For this reason, analysis and phytochemical screening of the avocado seed could be important in order to determinate and isolate the chemical components responsible for its pharmacological and biochemical actions [236]. Moreover, studies on the hypolipidemic effects of the avocado seed have been reported where different extracts showed low toxicity and could significantly reduce the cholesterol and low-density lipoprotein cholesterol (LDL-C) levels in hypercholesterolemic mice [237] and triglycerides, very low density lipoprotein cholesterol (VLDL-C) and high-density lipoprotein cholesterol (HDL-C) in hypercholesterolemic rats [238, 239].

6.3. Papaya

Papaya (*Carica papaya* L.) is an important fruit crop throughout tropical and sub-tropical countries with a total global production of about 12.5 million metric tonnes worldwide in 2012. India and Brazil are the major producers with annual production of 5.2 and 1.5 million metric tons, respectively (FAOSTAT 2012).

Papaya fruit is highly appreciated worldwide for its flavor, nutritional qualities and digestive properties and is grown mostly for fresh consumption and papain production; however, it can be processed into jelly, jam, candy, and pickles and its seeds are usually discarded. Papaya is harvested almost all year round and during fruit harvest by-products are produced from removal of plants at the end of their productive cycle (approximately 2 years continuous production) or diseased plants, as well as in the form of unripe fruit or fruit that does not meet quality control specifications (2-5% of total production) [240].

Papaya is known for its plethora of folkloric uses and pharmacological activities, such as antioxidant, anti-fungical, anti-proliferative, haematopological, among others [241–244]. It is used in Nigeria in treating malarial fever, diabetes mellitus, and bacterial infection such as a dewormer and as an ecbolic agent [245]. In this way, the acute anti-inflammatory of *Carica papaya* extract (CPE) was evaluated using the carrageenin-induced oedema model by *Anaga* et al. The extract showed moderate and typical biphasic anti-inflammatory activity on the carrageenin model. The findings suggest the inhibition of serotonin and histamine synthesis as observed in formalin-induced nociception. Therefore, the antinociceptive and anti-inflammatory activities of CPE may be due to the presence of alkaloids, flavonoids and other polyphenols [246]. Gastro protective effects of aqueous *Carica papaya* seed extract on an ethanol induced gastric ulcer were also investigated in male rats. *Abisola* et al. reported that this extract has significantly protected the gastric mucosa against the (?) ethanol challenge as shown by significant reduction in gastric juice volume, gastric acidity, ulcer index and percentage ulcer inhibition as compared to the control group suggesting its potent gastro-protective

effect on ethanol induced gastric ulcer in rats [247]. As for papaya leaves, *Otsuki* et al. examined the potential role of *Carica papaya* as anti-cancer therapy and analyzed the anti-tumor activity of CPE against various cancer cell lines, as well as its potential immunomodulatory effects. They observed significant growth inhibitory activity of the extract on tumor cell lines. In peripheral blood mononuclear cells (PBMC), the production of IL-2 and IL-4 was reduced following the addition of CPE, whereas that of IL-12p40, IL-12p70, IFN-γ and TNF-α was enhanced without growth inhibition. In addition, cytotoxicity of activated PBMC against K562 cells was enhanced by the addition of CPE. Since *Carica papaya* leaf extract can mediate a T helper 1 (Th1) type cell shift in human immune system, these results suggest that the CPE may potentially provide the means for the treatment and prevention of selected human diseases such as cancer, various allergic disorders, and may also serve as immunoadjuvant for vaccine therapy [248].

7. PINE BARK

Pinus is the largest extant genus of conifers in the Pinaceae family with more than 100 species. Economically, pines are an important source of wood, paper, resins, charcoal, food (particularly seeds), and ornamentals [249]. The bark removed from logs is mostly used as boiler fuel, but a huge surplus is still discarded as waste residue. In fact, pine bark is an important biomass resource, amounting to about 10%-15% of the total tree weight. From a commercial point of view, simultaneous utilization of wood and waste bark is required, but, in particular, Pinus species with its thick and polyphenol-rich bark is of significant interest due to the high cost of the extraction process [250].

In terms of chemical composition, bark differs from wood by its high content of water and/or organic solvent soluble extractives, ashes and polyphenolics. The main polyphenolic structures found in pine bark are catechin/epicatechin, epigallocatechin and epicatechin gallate. Pine is one of the plants with the highest content of PCs (dimers, trimers, oligomers and polymers) which have diverse biomedical properties applications [251, 252].

Pine bark extract (PBE) from European coastal pine has been used by native Indians in Quebec. They introduced pine bark tea which proved highly effective in preventing scurvy, a disease caused by vitamin C deficiency. Fascinated by this information, Professor Jack Masquelier who was working on bioflavonoids suspected that they might be used in the treatment of scurvy. Later he determined that pine bark extract was rich in bioflavonoids including organic acids [253].

PAs in pine bark have been recognized as a significantly effective material for activities such as antioxidant activity, anti-microbial effect, anti-inflammatory property, and anti-allergy activity [253–255]. In fact, pycnogenol (PYC), a standardized extract from the bark of the French maritime pine (*Pinus pinaster*), showed good results in the treatment of chronic venous insufficiency and cardiovascular diseases. In addition, oral intake of this extract was reported to be effective against oxidative stress in several cell systems and to protect against UV-radiation-induced erythema [256–258]. PYC consists of a concentrate of polyphenols, mainly PCs, pharmacologically active biopolymers composed from units of catechin and epicatechin.

7.1. Antioxidant and Anti-Microbial Activities

Several studies have addressed the antioxidant and anti-microbial capacity of pine bark extracts (PBE) from different varieties [259–261]. Ultrasonic assisted extraction has been performed under various solvent conditions on the bark of seven boreal forest conifers used by Native Americans including: *Pinus strobus, Pinus resinosa, Pinus banksiana, Picea mariana, Picea glauca*, in order to evaluate the total phenolic content, as well as oxygen radical absorption capacity assay with fluorescein as the fluorescent probe ($ORAC_{FL}$) potency and cellular antioxidant activity (IC_{50}) and to compare with the standardized water extract PYC. Interestingly, total phenolic content of these bark extracts was similar to PYC but their antioxidant activity was higher [262]. *Wood* et al. reported that various PBEs are more effective superoxide radical-scavengers than the antioxidant vitamin C and Trolox [251]. *Jerez* et al. compared the anti-radical activity of PCs from the bark of two kinds of pine, *P. pinaster* and *Pinus radiata*. In this study, they observed that *P. radiata* was a richer source of PCs

than *P. pinaster*. The PC composition of the fraction, which mainly contained catechin monomers and oligomers, soluble in ethyl acetate and water, presented significant differences: the mean degree of polymerisation is higher for radiata (2.9 vs. 2.3) and this source is richer in catechin moieties. Moreover, PCs from radiata appear to be more hydrophobic than those from pinaster [263].

Resistance of the *Helicobacter pylori* strain to antibiotics, alternative therapy for the treatment of this infection was tried in the form of PBE [264]. The results showed a significant, yet limited inhibition of growth and adherence of *H. pylori* to gastric cells by PYC. Comparative anti-microbial effect against *Staphylococcus aureus* was analyzed in cooked meat by *Hames Kocabas* et al. [265]. The experimental assay showed a significant effect of *Pinus pinea* against *S. aureus* in terms of inhibiting the progress of bacterial growth.

As regards anti-viral activities, *Matsumori* et al. analyzed the effect of PBE on viral myocarditis in mice [266]. The area of myocardial infiltration and necrosis was significantly smaller in the hearts of the treated mice. The results suggested that PYC exerts its beneficial effects on viral myocarditis by decreasing virus replication and by suppressing expression of pro-inflammatory cytokines, genes related to cardiac remodeling, and mast cell-related genes in the hearts of mice.

7.2. Cardiovascular Diseases

Different studies have shown an inverse association with supplementary pine bark extract (PBE) intake and coronary artery disease (CAD) risk and mortality [112, 253, 267]. Atherosclerosis is a major vascular disorder responsible for most cardiovascular ill health. High blood pressure is responsible for 40,000 deaths annually in the United States, while being the most modifiable risk factor for stroke. For this reason, *Hosseini* et al. tested the possible protective effect of oral pycnogenol (PYC), administered for eight weeks to non-smoking, mildly hypertensive patients. A significant decrease in the systolic blood pressure and serum thromboxane B2 levels were observed during PYC supplementation [268]. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis. *Nishioka* et al. determined PYC's effects on endothelial function by measuring the

responses of forearm blood flow (FBF) to the endothelium-dependent vasodilator acetylcholine (ACh) and to the endothelium-independent vasodilator sodium nitroprusside (SNP) before and after PYC administration. Their findings were that PBE induced forearm blood flow response to acetylcholine (ACh) was completely eliminated by the administration of the NO synthase inhibitor; NG-monomethyl-L-arginine supporting the fact that PBE played its role in endothelium-dependent vasodilation by increasing NO production [269]. In this way, *Devaraj* et al*.* studied the effect of PYC supplementation on measures of oxidative stress and the lipid profile in humans. In addition to its anti effects measured by the oxygen radical absorbance capacity (ORAC) assay, PYC significantly reduced low-density lipoprotein cholesterol (LDL-C) levels and increased high-density lipoprotein cholesterol (HDL-C) levels in plasma and thus exerted beneficial effects on the lipid profile [270].

Recently we found that a single dose of PYC prevented smoke-induced platelet aggregation. Therefore, smokers were used by *Araghi-Nikam* et al*.* to determine the long-term effects on platelet aggregation of PYC. They investigated whether changes in platelet aggregation in smokers could be produced by long-term PYC supplementation. This supplementation reduced smoke-induced platelet aggregation to the level of non smokers [271]. These observations suggest that PYC supplementation reduces risk factors for cardiovascular diseases.

7.3. Diabetes

Epidemiological studies have demonstrated that type 2 diabetes increases the risk of cardiovascular morbidity and mortality. *Zibadi* et al*.* demonstrated that PYC supplementation helps to maintain control of blood pressure with a lower dose of angiotensin-converting enzyme inhibitors in individuals with type 2 diabetes, so that PYC could improve diabetes control, lowered cardiovascular disease (CVD) risk factors, and reduced anti-hypertensive medicine use *vs.* controls [272]. A double-blind, placebo-controlled, randomized, multi-center study was performed with diabetes type 2 patients to investigate anti-diabetic effects of the French maritime pine bark extract (PBE), PYC by *Liu* et al*.* Blood glucose and hemoglobin alpha 1 (HbA_1) levels of the PYC group exhibited greater decreases

than the control group despite the pronounced placebo effect [273]. Diabetic retinopathy is the result of weakened enzymatic anti defenses by uncontrolled diabetes mellitus. *Kamuren* et al. reported that when diet was combined with *Pinus maritima* treatment, both retinal glutathione (GSH) peroxidase and glutathione (GSH) reductase activities increased, suggesting that a low-carbohydrate diet plus *Pinus maritima* may be an effective anti and anti-hyperglycemic therapy, reducing the risk of diabetic retinopathy and cataract formation [274].

7.4. Cancer

In light of the research in progress on the benefits of various phytochemicals in foods, it appears feasible that the chemical compounds from herbs could also be helpful in the prevention or treatment of cancer. *In vitro* and mice studies have suggested that pine bark extract (PBE) may be useful for preventing disorders associated with oxidative damage and can increase natural killer cell cytotoxicity [275–278], but human trials are needed [279].

Kinases are known to trigger the major signal transduction systems in the course of a wide spectrum of cell responses and are involved in many of the cellular processes affected by pine bark extract (PBE), such as anti-tumoral, anti-inflammatory and anti-proliferative activities. In order to further investigate the mechanism of action of this extract, the *in vitro* effect of PBE on the activity of highly purified phosphorylase kinase (PhK), protein kinase A (PKA) and protein kinase C (PKC) were tested by *Nardini* et al. Their findings showed that PBE displayed the ability to strongly inhibit *in vitro* the activity of the three kinases considered. The strong inhibition of PhK, a key regulatory enzyme involved in the metabolism of glycogen and PKC, may contribute to the understanding of some of the effects reported for this extract, such as the anti-proliferative, anti-tumoral and anti-inflammatory properties. The inhibition follows a non-competitive mechanism and, due to the absence of regulatory subunits, it may only be ascribed to a direct interaction of polyphenols with the catalytic subunit. PKA participates in many of the cellular processes affected by PCs from PBE. PKA has been suggested as a potential molecular target for therapeutical intervention in cancer [280].

Skin cancer is one of the most common types of cancer, with a tremendous impact on health and morbidity. The three main types of skin carcinomas are basal cell carcinomas (BCCs), squamous cell cancers (SCCs) and cutaneous melanomas (CMs). UV radiations and chemical agents can initiate damage to biomolecules either by direct photochemical reactions or/and *via* oxidative mechanisms generated by reactive oxygen species (ROS) [281]. Chemoprevention represents a relatively new and promising strategy, which can slow down, reverse or completely halt the process of carcinogenesis by the use of natural or synthetic antioxidants. Owing to the chemical structure of its components, the pine bark extract (PBE) presents remarkable antioxidant activity [256]. The PBE anti-cancer activity was examined on non-melanoma skin carcinomas in hairless mice without melanin and with melanin by *Kyriazi* et al*.* The tumors were induced by ultraviolet radiation (UVR) and combination of UVR and 7,12-dimethylbenz[*a*]anthracene (DMBA), for simulating the UVR carcinogenic action and the genotoxic effects of UVR with chemical agents combination. The results showed that there is strong evidence to suggest that PBE exhibits potent non-melanoma skin cancers preventive and anti-cancer activity. This chemopreventive action was demonstrated in the late appearance of first skin papillomas/tumors, the reduced number of skin tumors per animal, the reduced percentage of animals bearing skin tumors and the prolongation of animal viability [282]. In keeping with the chemopreventive effect of PBE, ovarian cell lines and polymorph nuclear neutrophils (PMN) were treated with talc and with PBE followed by talc. Soft agar assay was used to measure cell viability, ROS generation and neoplastic transformation. *In vitro* data suggests that talc may contribute to ovarian carcinogenesis in humans by way of inducing aberrant ROS generation and that pycnogenol (PYC) reduces talc-induced neoplastic transformation of ovarian cells. Taken together, PYC may prove to be a chemopreventative agent against ovarian carcinogenesis [283].

8. CONCLUSION

Since billions of metric tons of biomass are generated every year by the agricultural industry worldwide including liquid, solid and gaseous residues, they may be considered one of the most

abundant, cheap and renewable resources on earth. Many of these residues can cause environmental problems when they are not managed properly. In this way, different strategies of residue utilization from the agriculture industry have been developed [200] as a source for high value-added products

Phenolic compounds with antioxidant activity have been identified in several agricultural by-products [27]. These compounds can be subdivided into the following classes: phenolic acids, flavanones, flavones and flavonols, anthocyanidins, flavan-3-ols and proanthocyanidins (PAs). PAs comprise condensed polyphenols or condensed tannins. Apart from lignin, they represent the most abundant class of natural phenolic compounds. There is a variety of different classes of PAs depending on the substitution pattern of the monomeric flavan-3-ol units [13].

Taken together, the above studies clearly demonstrate that PAs from different kinds of agro-industrial by-products have significant potential to scavenge free radicals both *in vitro* and *in vivo* models. Furthermore, a number of pharmacological effects have also been reported, *e.g.*, anti-viral, anti-microbial, antioxidative, anti-tumor promoting properties, as well as cardiotonic and anti-arteriosclerotic activities. The results suggest that these by-products may potentially provide the means for the treatment and prevention of human diseases.

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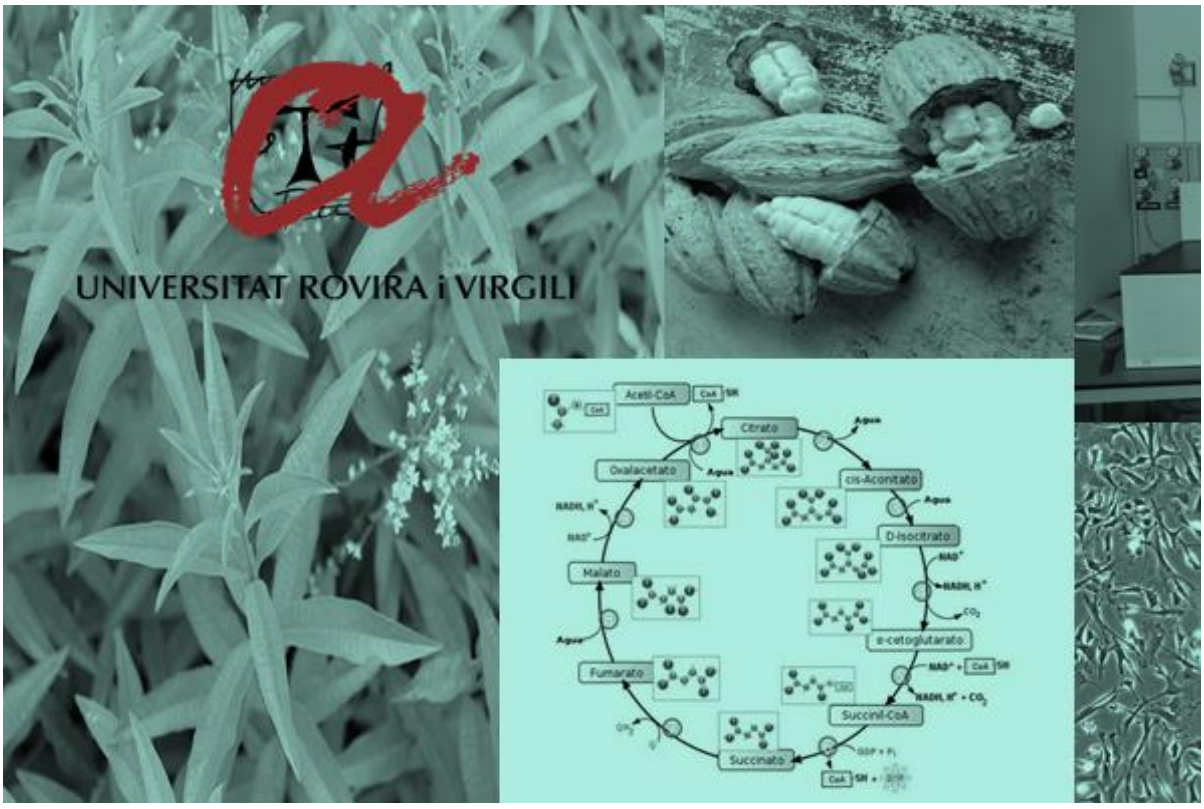
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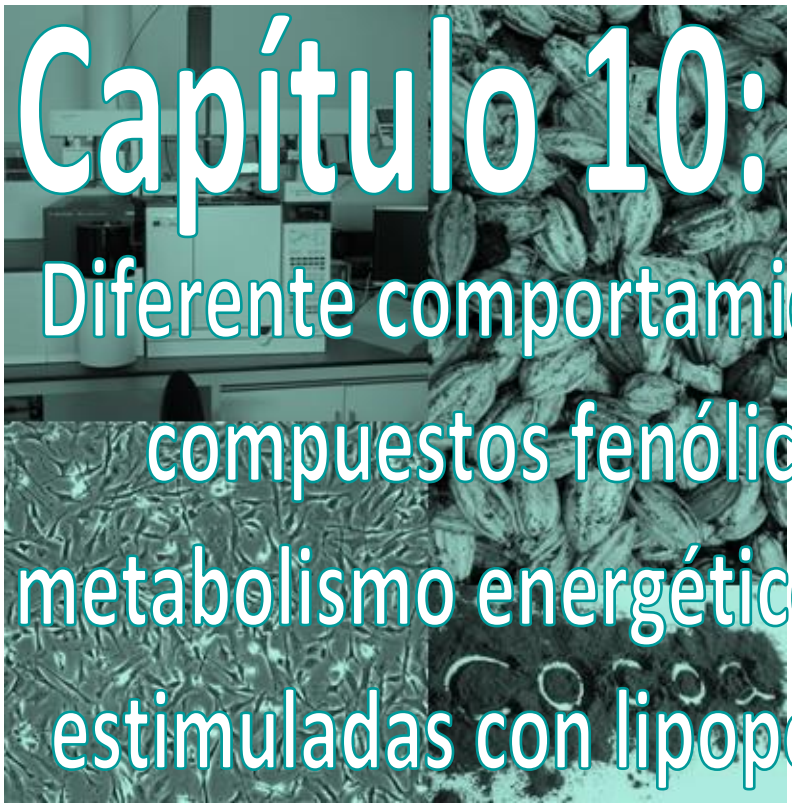

Capítulo 10:

Diferente comportamiento de los compuestos fenólicos en el metabolismo energético de células estimuladas con lipopolisacárido

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Different behavior of polyphenols in energy metabolism of lipopolysaccharide-stimulated cells

ABSTRACT

Energy metabolism is one of the main sources of reactive oxygen species leading to oxidation and inflammation in pathophysiological processes. Lipopolysaccharide (LPS)-activated mouse embryonic fibroblast (MEF) cell lines from knock-out mice for paraoxonase-1 and from transgenic mice overexpressing monocyte chemoattractant protein-1 were obtained as model of pro-oxidant and pro-inflammatory scenarios. *Theobroma cacao* and *Lippia citriodora* (worldwide consumed and common ingredient of many food products) were tested in these cell models to assess the action of polyphenols in the energy management. Our metabolomics experiments show a different behavior of polyphenols: *T. cacao* extract partially reverts the effect of LPS in a pro-oxidant scenario through the antioxidant properties of theobromine, flavonols and procyanidins, while *L. citriodora* seems to act mainly in a pro-inflammatory cell model through the action of verbascoside decreasing the production of pro-inflammatory cytokines and MCP-1. Nevertheless, the action of polyphenols cannot be attributed only to a mechanism of action but the sum of different modulations in biological pathways. The capacity of both plant extracts to decrease α-ketoglutarate levels merits special attention due to the implications in future medicine. The action of polyphenols modulating oxidative stress, cytokine production and epigenetic changes make an interesting source of bioactive compounds for nutraceutical or functional food purposes.

1. INTRODUCTION

Oxidative stress and inflammation are the basis of the most diseases and their mechanisms are inextricably linked. An increased oxidative stress starts a cascade of pro-inflammatory cytokines that triggers inflammation (Camps et al., 2014). Dysfunctions in mitochondria, as the main organelle producing reactive oxygen species (ROS) (Handy & Loscalzo, 2012), lead to non-communicable diseases (obesity, diabetes, atherosclerosis, cancer, etc.) characterized by oxidation, inflammation and metabolic disorders (Camps & García-Heredia, 2014).

Polyphenols are secondary metabolites naturally synthetized in plants and have demonstrated several biological activities, including antioxidant and anti-inflammatory properties (A. N. Li et al., 2014). *Theobroma cacao* (cocoa) is worldwide consumed and common ingredient of many food products. Cocoa is a valuable source of antioxidant compounds with health benefits mainly attributed to the flavanol content (Andujar, Recio, Giner, & Rios, 2012; M. L. Cadiz-Gurrea et al., 2017) and the proanthocyanidins with high-degree of polymerization (Belscak, Komes, Horzic, Ganic, & Karlovic, 2009; M.L. Cadiz-Gurrea et al., 2014; Martin & Ramos, 2016; Schinella et al., 2010; Yasmeen, Fukagawa, & Wang, 2017). In the past decades, the bioactive constituents of cocoa have been targeted in multiple research studies confirming its health benefits and, for these reasons, the market has developed new cocoa-based products such as cocoa liquor, cocoa powder, chocolate and other derivatives and has remained stable over the last few years (Ellam & Williamson, 2013).

Lippia citriodora (lemon verbena) is a perennial plant originally from South America which was introduced into Europe at the end of the 17th century and has been widely used in infusions for its antispasmodic, antipyretic, sedative, and digestive properties. Furthermore, this plant is used in the food industry to flavor different products. Lemon verbena infusion contains significant amounts of polyphenols, including phenylpropanoid glycosides (mainly verbascoside) and flavone diglucuronides such as luteolin 7-diglucuronide, with high anti-inflammatory and antioxidant properties (Funes et al., 2009; Quirantes-Piné, Funes, Micol, Segura-Carretero, & Fernández-Gutiérrez, 2009). In the last

decade, the potential of *L. citriodora* extract supplementation as a nutraceutical is under consideration with the aim to find phytotherapies, which contribute to ameliorate diseases with less adverse effects than synthetic drugs (Cádiz-Gurrea et al., 2017).

In this work, antioxidant and anti-inflammatory properties of food polyphenols found in cocoa and lemon verbena were tested on two mouse embryonic fibroblast (MEF) cell lines: 1) knock-out mice for paraoxonaxe-1, an antioxidant endogenous enzyme, as model of oxidation (PON-1 KO cells) and 2) transgenic mice overexpressing the monocyte chemoattractant protein 1 (MCP-1), a proinflammatory cytokine, as model of inflammation (tgMCP-1 cells). To analyze the impact of polyphenols in the energy management in these conditions, a targeted metabolomics approach was performed on gas chromatography coupled to a quadrupole time-of-flight mass spectrometer and an electron impact source.

2. MATERIAL AND METHODS

2.1. Chemicals.

Methanol, dimethylsulphoxide (DMSO), methoxyamine hydrochloride, pyridine and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-methyl-N-trimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane (TMS) and RIPA lysis and extraction buffer were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure type-1 water was obtained from a Milli Q water system (Merck Millipore, Darmstadt, Germany). DMEM medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), L-glutamine and penicillin/streptomycin were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Cell culture.

Cells (5 replicates) were grown to 80% confluence in 6-well plates using DMEM medium containing 10% FBS, 5 mM L-glutamine and penicillin/streptomycin. After depletion of FBS (0.1%), *L. citriodora* (standardized with 10% of verbascoside) and *T. cacao* extracts (Monteloeder, Elche, Spain) at a final

concentration of 100 µg/mL in wells, dissolved in DMSO, were tested for 48 hours in PON-1 KO and tgMCP-1 cells. Complete polyphenol characterization of both extracts is detailed in Supplementary Information Figures S1 and S2, Tables S1 and S2. Prior to the assay, cells were incubated 24 hours with LPS from *E. coli* (100 ng/mL) to stimulate the pro-oxidant and pro-inflammatory states. Controls (with or without LPS and with or without plant extracts) were also made. Then, cells were scrapped in PBS and centrifuged at 2500 rpm 5 minutes. Supernatants were removed and pellets were stored at -80 °C until use.

The total amount of protein (in mg) was used to normalize the metabolite concentrations. For this purpose, cells (3 replicates) where scraped using 200 µL of RIPA lysis and extraction buffer, and protein concentration was calculated using the absorbance at 280 nm analyzed in a NanoDrop 2000 system (Thermo Scientific, Waltham, MA, USA).

2.3. Metabolite extraction.

Metabolites were extracted from cells following the protocol performed by (Riera-Borrull et al., 2016). Briefly, 200 µL of methanol:water (8:2) was added to cell pellets and lysed with three cycles of freezing and thawing using liquid N_2. After precipitating proteins, samples were centrifuged at 14000 rpm for 10 min, supernatants were collected, dried under N_2 and derivatized using methoxyamine in pyridine (40 mg/mL) and TMS.

2.4. Instrumentation, chromatographic conditions and mass spectrometry detection.

A 7890A gas chromatograph coupled with an electron impact source to a 7200 quadrupole time-of-flight mass spectrometer equipped with a 7693 autosampler module and a J&W Scientific HP-5MS column (30 m x 0.25 mm, 0.25 µm) (Agilent Technologies, Santa Clara, USA) were used. Optimized parameters for chromatographic separation and QTOF detection are explained in *Riera-Borrull et al., 2016*. Metabolites were quantitated using standard calibration curves and values normalized using the total amount of protein.

2.5. Data analysis

Raw data were processed and compounds were detected and quantified using Qualitative and Quantitative Analysis B.07.00 software (Agilent Technologies), respectively. Statistical analysis was performed using the Kruskal-Wallis non-parametric test with SPSS 23.0 software (IBM Corporation, Armonk, NY, USA). Sparse partial least square discriminant analysis (sPLS-DA) were made using Metaboanalyst 3.0 (www.metaboanalyst.ca) (Xia, Sinelnikov, Han, & Wishart, 2015).

3. RESULTS AND DISCUSSION

Understanding oxidation and inflammation as the two fronts of a same coin is crucial to assess the mechanisms involved in appearance, progression and resolution (or chronicity) of these pathophysiological conditions and how they are interconnected to metabolism (Griffiths, Gao, & Pararasa, 2017). Inflammation and oxidation are related to several "metabolic diseases", including obesity, atherosclerosis and cancer (Camps, García-Heredia, Hernández-Aguilera, & Joven, 2016), and mitochondrial impairments play a key role in these pathophysiological processes (Hernández-Aguilera et al., 2013).

Dietary polyphenols are one of the most valuable sources of these compounds, and, for this reason, numerous efforts have been realized to demonstrate how polyphenols can help to prevent or ameliorate these pathologies characterized by oxidation and inflammation (Hussain et al., 2016; Joven et al., 2014; Santini, Tenore, & Novellino, 2017).

To test the antioxidant and anti-inflammatory properties of food polyphenols, MEF PON-1 KO and tgMCP-1 cell lines previously incubated with LPS were used as oxidant and inflammatory scenarios, respectively. WT cells also were used as control cells. Although the concentration of plan extracts (100 µg/mL) used in this study is in a supra-physiological dose, these results demonstrate the effect of dietary polyphenols contained in cocoa and lemon verbena to act as antioxidant and/or anti-inflammatory compounds.

Our results showed changes in the concentrations of metabolites in both PON-1 KO and tgMCP-1 cells in comparison with WT cells. These changes include intermediates of glycolysis, tricarboxylic acid cycle (TCA), amino acids catabolism, glutamine catabolism and pentose phosphate pathway (Supplementary Information, Table S3). LPS-activated WT MEF cells experience, specially, a decrease in amino acid concentrations (Supporting information, Table S3) compared to the control WT cells, mostly branched-chain amino acids (BCAAs) (valine, leucine, isoleucine), serine, alanine and glutamate.

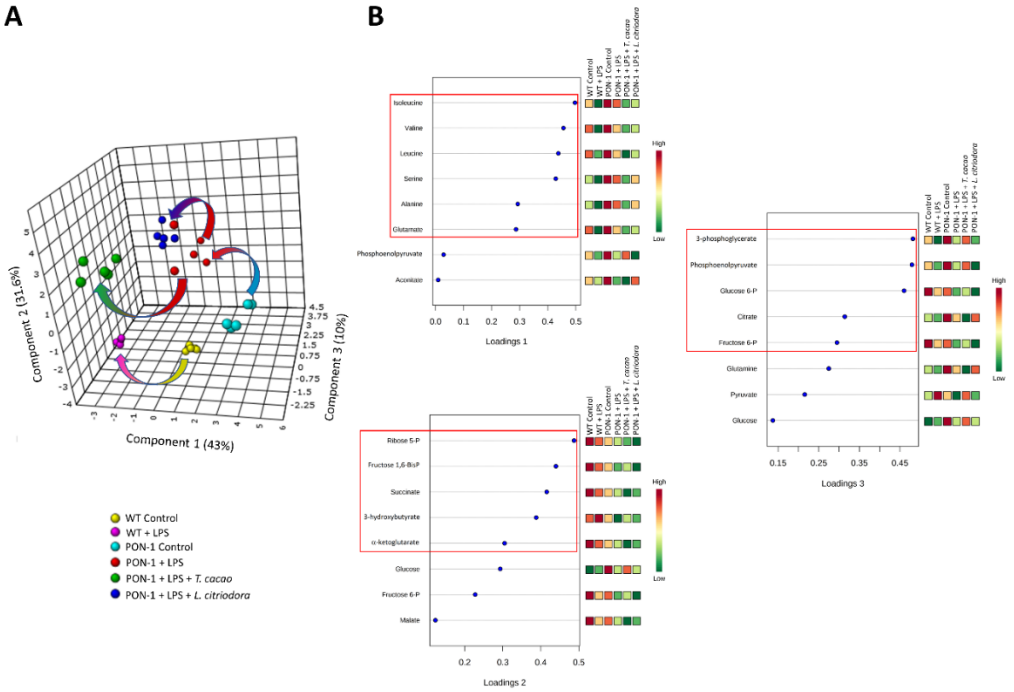

Figure 1. Effect of the treatment with cocoa and lemon verbena extracts in LPS-activated PON-1 KO MEF cells. A) Sparse partial least square discriminant analysis (sPLS-DA). B) Importance in loading metabolites in each sPLS-DA component.

In LPS-activated PON-1 KO cells, as a pro-oxidant scenario, a shift in the sPLS-DA in the 3 components (Figure 1A) was also observed as result of a decrease in the concentrations of, mainly, amino acids and glycolysis intermediates (Figure 1B). *T. cacao* extract had an effect on energy metabolism in LPS-activated MEF PON-1 KO cells (Figure 1A), partially recovering the glycolysis intermediates and amino acids to similar values to these found in MEF WT (Figure 1B). Although, this effect is not so obvious when cells are incubated with *L. citriodora*. In this case, significant shifts in the component of the sPLS-

DA (Figure 1A) were not detected, but only a partial recovery in the concentration of some amino acids (alanine, aspartate, glutamate and serine) and intermediates of TCA (aconitate, fumarate and malate) (Supporting information, Table S3).

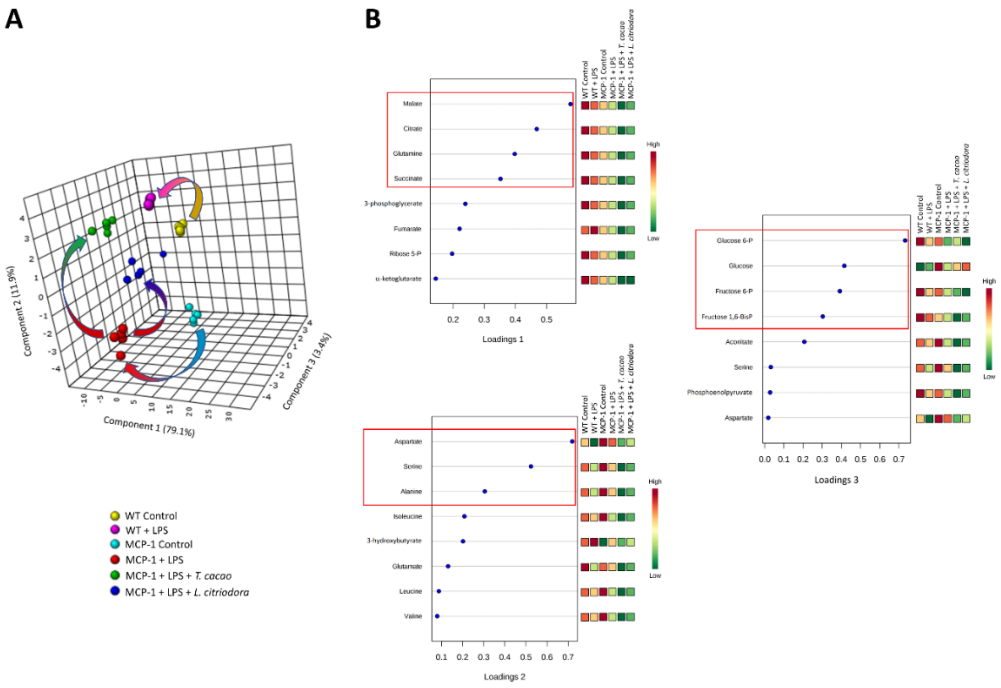

Figure 2. Effect of the treatment with cocoa and lemon verbena extract in LPS-activated tgMCP-1 MEF cells. A) Sparse partial least square discriminant analysis (sPLS-DA). B) Importance in loading metabolites in each sPLS-DA component.

In a pro-inflammatory scenario, LPS-activated tgMCP-1 cells as well as PON-1 KO cells experienced a decrease in all of the intermediates in the energy production. Incubation with *T. cacao* extract did not recover cells from the effects of LPS when regarding the concentrations of the analyzed metabolites (Supplementary Information Table S3), even though in the sPLS-DA we can observe this group in a position near the WT cells. Contrarily, *L. citriodora* had a slight action protecting tgMCP-1 MEF cells from LPS (Figure 2A).

Although *T. cacao* has previously demonstrated anti-inflammatory activity (Selmi, Mao, Keen, Schmitz, & Eric Gershwin, 2006), our results are highly related with its antioxidant properties. The antioxidant capacity of cocoa extract can be attributed to its main compounds theobromine (a methylxanthine), flavonols (specially catechin and derivatives) and procyanidins, among other compounds that are present in a minor proportion. These compounds have been strongly reported for their antioxidant capacity (Hu, Kim, & Baik, 2016; S. Li et al., 2015; Schinella et al., 2010). Moreover, high quantities of theobromine and catechin derivatives, and, in a minor quantity, procyanidins, were found in human plasma after ingestion of these compounds at a dietary dose (Baggott et al., 2013; Holt et al., 2002; Lee et al., 2002). These data, together with the ability of catechin and derivatives to interact with, and cross, the cell membrane (Sirk, Brown, Sum, & Friedman, 2008), result in an antioxidant activity of cocoa polyphenols not only at plasma level but also intracellularly.

L. citriodora showed the opposite effect, acting as anti-inflammatory more than as antioxidant agent. The phenylethanoid glycoside verbascoside, also called acteoside, is the main compound of this extract (10% w/w) and anti-inflammatory properties have been already conferred on it (Alipieva, Korkina, Orhan, & Georgiev, 2014). Its mechanism of action is through inhibiting the production of cytokines such as TNF-α, IL-1β and IL-6, among others. Verbascoside also contributes to the inhibition of the nuclear factor kappa B (NF-κB) (Jing, Chunhua, & Shumin, 2015). Because MCP-1 expression depends, partially, on the action of NF-κB, the inhibition of this nuclear factor may decrease the production of MCP-1 and, thus, the inflammatory process.

Although the anti-inflammatory properties of polyphenols can be attributed to an interaction with cytoplasmic and nuclear pathways rather than their direct antioxidant properties (Pastore et al., 2012), the action of a complex mixture of polyphenols, such as cocoa or lemon verbena extracts, where synergic effects occur, cannot be assigned only to one mechanism of action. As reported previously, energy generation impairment and inflammatory conditions are closely related to disease. A correlation between pathophysiological processes that present oxidative stress and/or

inflammation produce mitochondrial imbalance that affects the energy management. The properties of polyphenols as exogenous antioxidant/anti-inflammatory molecules modulates the energy expenditure by the action on the AMP-activated protein kinase (AMPK), the cytokine production or acting as epigenetic regulators (Charles et al., 2013; Joven et al., 2013; Most, Goossens, Jocken, & Blaak, 2014). Thus, the action of polyphenols improving the endogenous antioxidant systems, modulating several biological pathways related to inflammation, cancer and other pathophysiological processes make these molecules an attractive source of bioactive compounds for pharmaceutical, nutraceutical or functional food formulations.

Special attention merits α-ketoglutarate (α-KG). This key and pivotal intermediate of the TCA cycle is formed, mainly, from isocitrate or glutamate. α-KG is involved in multiple diseases such as non-alcoholic fatty liver disease (Rodríguez-Gallego et al., 2015) or cancer (Dang, Yen, & Attar, 2016). It is also related to the inhibition of histone and DNA demethylases (Lempradl, Pospisilik, & Penninger, 2015). Interestingly, both plant extracts decrease strongly the levels of α-KG. The mechanism of action of polyphenols decreasing α-KG is still unclear and deserves more efforts due to the implications in future treatments for pathophysiological processes with an hypermethylated phenotype.

4. CONCLUSIONS

In the present study, we highlight the different behavior of polyphenols as antioxidant and anti-inflammatory molecules. *T. cacao* extract is responsible to partially return the impairment in the energy management during a pro-oxidant status in PON-1 KO cells probably through the antioxidant properties of theobromine, flavan-3-ol monomers and procyanidins. On the contrary, *L. citriodora* extract possess a partial effect on the energy metabolism in a pro-inflammatory scenario probably due to the action of verbascoside which may inhibit NF-κB and, thus, decrease the expression of MCP-1. However, action of polyphenols, especially in complex mixtures, cannot be attributed to only one mechanism of action but to the sum of different pathways and mechanisms that can be modulated.

More investigations are needed to assess the implications and impact of polyphenols to decrease the levels of α-KG.

5. ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

Figure S1. Base peak chromatogram of *Lippia citriodora* extract. Peak numbers correspond to those included in Table S1.

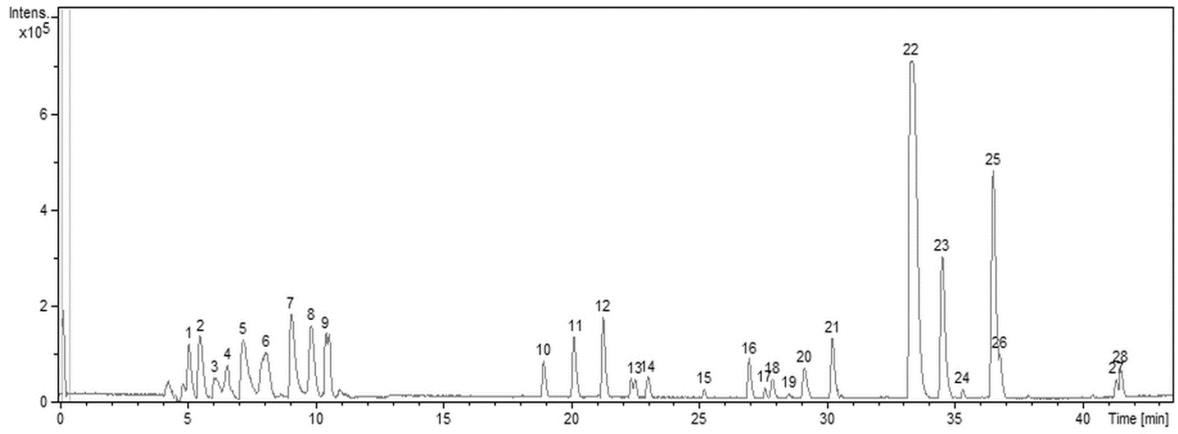

Table S1. Characterization of *Lippia citriodora* extract by HPLC-ESI-TOF-MS.

Peak	Proposed compound	RT (min)	Measured [M-H]-	Error (ppm)	Molecular Formula
1	Gluconic acid	5,1	195,0512	0,7	$C_6H_{12}O_7$
2	Sucrose	5,5	341,1091	0,5	$C_{12}H_{22}O_{11}$
3	Raffinose	6,1	503,1603	2,9	$C_{18}H_{32}O_{16}$
4	Sucrose (I)	6,6	341,1072	5	$C_{12}H_{22}O_{11}$
5	Stachyose	7,2	665,2123	3,4	$C_{24}H_{42}O_{21}$
6	Stachyose (I)	8,1	665,2123	3,4	$C_{24}H_{42}O_{21}$
7	Maltopentose	9	827,2651	2,8	$C_{30}H_{52}O_{26}$
8	Maltopentose (I)	9,9	827,2657	2,1	$C_{30}H_{52}O_{26}$
9	Maltohexaose	10,4	989,318	2,3	$C_{36}H_{62}O_{31}$
10	Shanziside	18,9	391,1227	4,8	$C_{16}H_{24}O_{11}$
11	Gardoside	20,1	373,1131	2,5	$C_{16}H_{22}O_{10}$
12	Verbasoside	21,2	461,1674	3,7	$C_{20}H_{30}O_{12}$
13	Cistanoside F	22,3	487,1429	5,7	$C_{21}H_{28}O_{13}$
14	Loganic acid	23	375,1278	5	$C_{16}H_{24}O_{10}$
15	Caryoptoside	25,1	405,1384	4,4	$C_{17}H_{26}O_{11}$
16	Theveside	26,9	389,1075	3,7	$C_{16}H_{21}O_{11}$

17	β-hydroxy-(iso)verbascoside	27,5	639,1896	5,4	$C_{29}H_{36}O_{16}$
18	Tuberonic acid glucoside	27,8	387,1652	2,3	$C_{18}H_{28}O_9$
19	Dendromoniliside D	28,4	445,2073	1,4	$C_{21}H_{34}O_{10}$
20	Tuberonic acid glucoside	29,1	387,2006	4,8	$C_{18}H_{28}O_9$
21	Luteolin-7-diglucuronide	30,2	637,1017	4,6	$C_{27}H_{26}O_{18}$
22	Verbascoside	33,2	623,1956	4,1	$C_{29}H_{36}O_{15}$
23	Chrysoeriol-7-diglucoronide	34,4	651,1147	8,7	$C_{28}H_{28}O_{18}$
24	Dioonflavone	35,3	621,1762	0,7	$C_{36}H_{30}O_{10}$
25	Isoverbascoside	36,4	623,193	8,2	$C_{29}H_{36}O_{15}$
26	Eukovoside	36,7	637,208	9,2	$C_{30}H_{38}O_{15}$
27	Acacetin-7-diglucuronide	41,2	635,1198	8,8	$C_{28}H_{28}O_{17}$
28	Martinoside	41,4	651,2246	7,4	$C_{31}H_{40}O_{15}$

Figure S2. Base peak chromatogram of *Theobroma cacao* extract. Peak numbers correspond to those included in Table S2.

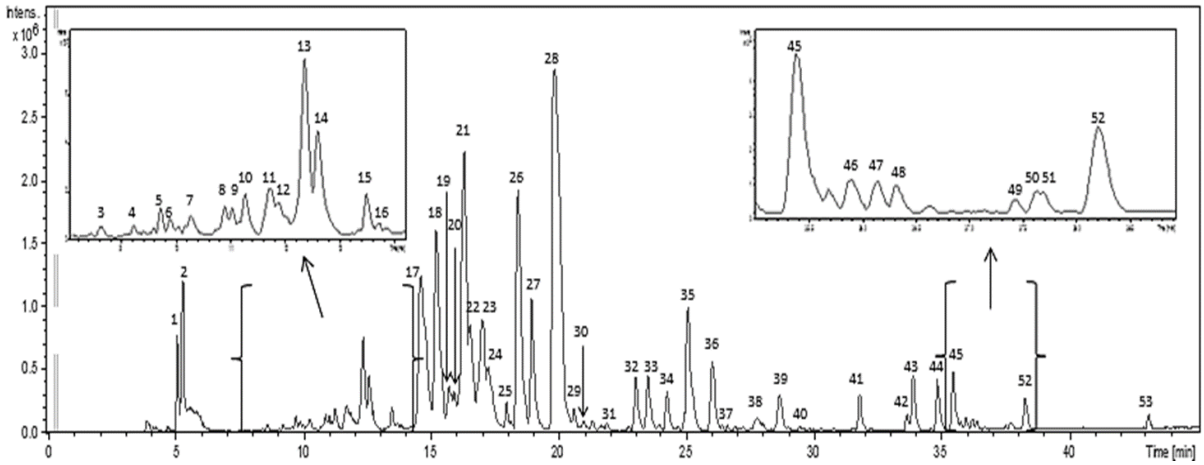

Table S2. Characterization of *Theobroma cacao* extract by HPLC-ESI-TOF-MS. The quantitation of the main polyphenols found in cocoa extract is already published (Cádiz-Gurrea et al., 2017).

Peak	Proposed compound	RT (min)	Measured [M-H]-	Error (ppm)	Molecular Formula
1	Sucrose (isomer 1)	5,11	341,1099	2,9	$C_{12}H_{22}O_{11}$
2	Sucrose (isomer 2)	5,31	341,1107	5,3	$C_{12}H_{22}O_{11}$
3	unknown	8,62	265,0936	2,6	$C_{10}H_{18}O_8$
4	Tri-O-methylsucrose	9,23	383,1563	1,2	$C_{15}H_{28}O_{11}$
5	Unknown	9,71	442,156	1,4	$C_{16}H_{29}NO_{13}$
6	(Epi)catechin tetramer (isomer 1)	9,88	576,1258	2,6	$C_{60}H_{50}O_{24}$
7	Procyanidin C (isomer 1)	10,26	865,1983	0,3	$C_{45}H_{38}O_{18}$
8	Procyanidin B (isomer 1)	10,88	577,1342	1,6	$C_{30}H_{26}O_{12}$
9	Procyanidin C (isomer 2)	11,02	865,1981	0,5	$C_{45}H_{38}O_{18}$
10	Procyanidin B (isomer 2)	11,27	577,1339	2,2	$C_{30}H_{26}O_{12}$
11	(Epi)catechin tetramer (isomer 2)	11,72	576,1257	2,9	$C_{60}H_{50}O_{24}$
12	Procyanidin C (isomer 3)	11,87	865,1959	1	$C_{45}H_{38}O_{18}$
13	Procyanidin C (isomer 4)	12,36	865,201	2,9	$C_{45}H_{38}O_{18}$
14	(Epi)catechin tetramer (isomer 3)	12,59	576,126	2,3	$C_{60}H_{50}O_{24}$
15	(Epi)catechin glucopyranoside (isomer 1)	13,48	451,1244	0,5	$C_{21}H_{24}O_{11}$
16	(Epi)catechin glucopyranoside (isomer 2)	13,7	451,1236	2,2	$C_{21}H_{24}O_{11}$
17	Procyanidin B (isomer 3)	14,62	577,1367	2,8	$C_{30}H_{26}O_{12}$
18	*N*-caffeoyl-L-aspartate	15,19	294,0631	4,1	$C_{13}H_{13}NO_7$
19	(Epi)catechin tetramer (isomer 4)	15,71	576,126	2,4	$C_{60}H_{50}O_{24}$
20	Procyanidin C (isomer 5)	15,91	865,2002	1,9	$C_{45}H_{38}O_{18}$
21	Procyanidin C (isomer 6)	16,28	865,2022	4,2	$C_{45}H_{38}O_{18}$
22	(Epi)catechin tetramer (isomer 5)	16,53	576,1256	2,9	$C_{60}H_{50}O_{24}$
23	(Epi)catechin pentamer (isomer 1)	17,01	720,1586	-	$C_{75}H_{62}O_{30}$
24	(Epi)catechin hexamer	17,23	864,1911	-	$C_{90}H_{74}O_{36}$
25	(Epi)gallocatechin (isomer 1)	17,95	305,0707	9	$C_{15}H_{14}O_7$
26	(Epi)gallocatechin (isomer 2)	18,42	305,0717	12,8	$C_{15}H_{14}O_7$
27	L-Aspartic acid, *N*-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]	18,94	278,0681	4	$C_{13}H_{13}NO_6$
28	(Epi)Catechin (isomer 2)	19,84	289,0747	10,2	$C_{15}H_{14}O_6$
29	L-Aspartic acid, *N*-[3-(4-hydroxy-3-methoxyphenyl)-1-oxo-2-propenyl]	20,58	308,0787	3,6	$C_{14}H_{15}NO_7$
30	Procyanidin B (isomer 5)	20,96	577,1349	0,4	$C_{30}H_{26}O_{12}$
31	Catechin diglucopyranoside	21,87	593,1507	0,8	$C_{27}H_{30}O_{15}$
32	trans-Clovamide (*N*-[(2E)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]-3-hydroxy-L-tyrosine)	23,01	358,0944	3,3	$C_{18}H_{17}NO_7$
33	(Epi)catechin dimer hexose	23,47	737,1744	2,8	$C_{36}H_{34}O_{17}$

34	Arabinopyranosyl-(epi)catechin-(epi)catechin (isomer 2)	24,23	707,164	3,1	$C_{35}H_{32}O_{16}$
35	Procyanidin B (isomer 6)	25,05	577,1362	1,8	$C_{30}H_{26}O_{12}$
36	Procyanidin C (isomer 7)	25,99	865,1997	1,3	$C_{45}H_{38}O_{18}$
37	Proanthocyanidin (Type A) (isomer 1)	26,57	591,1494	2,4	$C_{31}H_{28}O_{12}$
38	(Epi)catechin pentamer (isomer 2)	27,73	720,1583	-	$C_{75}H_{62}O_{30}$
39	(Epi)catechin tetramer (isomer 6)	28,61	576,1257	2,8	$C_{60}H_{50}O_{24}$
40	Quercetin glucuronide	29,4	477,067	1	$C_{21}H_{18}O_{13}$
41	Deoxyclovamide (*N*-[(2E)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]-L-tyrosine)	31,73	326,1045	3,4	$C_{18}H_{17}NO_5$
42	Quercetin hexose (isomer 1)	33,57	463,0879	0,7	$C_{21}H_{20}O_{12}$
43	Quercetin hexose (isomer 2)	33,84	463,0882	0,1	$C_{21}H_{20}O_{12}$
44	Hexenyl xylopyranosyl glucopyranoside (isomer 1)	34,77	393,177	1	$C_{17}H_{30}O_{10}$
45	Quercetin arabinoside	35,38	433,0788	2,6	$C_{20}H_{18}O_{11}$
46	Hexenyl xylopyranosyl glucopyranoside (isomer 2)	35,9	393,1769	0,8	$C_{17}H_{30}O_{10}$
47	(Epi)catechin derivative	36,15	427,1611	0,3	$C_{20}H_{28}O_{10}$
48	Cinchonain I	36,33	451,1026	1,9	$C_{24}H_{20}O_9$
49	Procyanidin A (isomer 2)	37,44	575,1197	0,3	$C_{30}H_{24}O_{12}$
50	Unknown	37,64	516,2454	0,8	$C_{24}H_{39}NO_{11}$
51	(Epi)catechin methyl dimer (isomer 2)	37,69	605,1652	2	$C_{32}H_{30}O_{12}$
52	Sweroside	38,19	357,1227	10	$C_{16}H_{22}O_9$
53	Quercetin	43,01	301,0361	2,3	$C_{15}H_{10}O_7$

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Table S3. Concentration values for metabolites involved in energy generation in wild-type cells (WT), PON-1 double knock-out cells (PON-1 KO) and transgenic cells overexpressing MCP-1 (tgMCP-1), incubated with or without lipopolysaccharide (LPS) and with or without plant extracts. Data are expressed as Median (Interquartile range).

	WT Control	WT + LPS	PON-1 KO Control	PON-1 KO + LPS	PON-1 KO + LPS + T. cacao	PON-1 KO + LPS + L. citriodora	tgMCP-1 Control	tgMCP-1 + LPS	tgMCP-1 + LPS + T. cacao	tgMCP-1 + LPS + L. citriodora
Pyruvate	2.24 (0.47)	3.33 (0.19)	2.80 (1.10)	3.53 (0.96)	3.00 (0.51)	2.10 (0.91)	1.84 (0.25)	1.27 (0.10)	0.29 (0.04)	0.55 (0.04)
Lactate	50.08 (3.54)	60.47 (8.32)	240.60 (34.81)	161.57 (7.28)	93.30 (10.97)	87.41 (6.34)	31.25 (3.40)	16.55 (5.59)	3.81 (0.92)	14.25 (1.39)
Alanine	17.18 (3.99)	5.75 (1.06)	40.29 (4.50)	34.64 (6.57)	13.27 (3.55)	18.44 (4.87)	20.57 (4.25)	11.62 (3.37)	1.54 (0.45)	4.24 (0.56)
3-hydroxybutyrate	1.01 (0.33)	0.98 (0.20)	0.61 (0.04)	0.45 (0.04)	0.45 (0.05)	0.39 (0.17)	0.42 (0.05)	0.55 (0.05)	0.49 (0.12)	0.50 (0.06)
Valine	2.92 (0.68)	0.99 (0.07)	5.25 (1.64)	2.46 (0.58)	1.24 (0.35)	1.55 (0.69)	4.02 (1.07)	0.69 (0.16)	0.09 (0.01)	0.29 (0.08)
Leucine	1.19 (0.28)	0.36 (0.05)	2.74 (1.15)	0.88 (0.10)	0.31 (0.12)	0.57 (0.13)	2.23 (0.63)	0.21 (0.06)	0.02 (0.01)	0.09 (0.03)
Isoleucine	0.99 (0.10)	0.33 (0.04)	3.45 (1.17)	1.04 (0.10)	0.40 (0.14)	0.78 (0.12)	2.53 (0.67)	0.29 (0.09)	0.02 (0.01)	0.11 (0.03)
Succinate	18.98 (2.24)	15.60 (1.05)	10.11 (0.92)	9.38 (0.89)	5.51 (1.58)	7.21 (0.73)	6.46 (1.17)	6.63 (1.75)	3.50 (0.39)	5.22 (1.03)
Fumarate	1.14 (0.12)	2.07 (0.23)	4.05 (0.42)	3.03 (0.14)	0.94 (0.38)	1.72 (0.56)	0.60 (0.19)	0.42 (0.06)	0.06 (0.01)	0.19 (0.02)
Serine	0.51 (0.04)	0.09 (0.01)	17.91 (6.35)	1.27 (0.24)	0.31 (0.08)	0.65 (0.18)	6.13 (3.49)	0.22 (0.04)	0.01 (0.00)	0.06 (0.02)
Malate	16.94 (3.41)	12.50 (2.07)	16.99 (2.47)	13.40 (1.95)	4.42 (1.33)	7.87 (2.12)	3.03 (0.64)	1.76 (0.16)	0.23 (0.05)	0.90 (0.06)
Aspartate	16.66 (4.60)	2.26 (0.20)	26.68 (4.05)	21.70 (3.18)	19.08 (3.35)	24.07 (2.45)	31.68 (2.62)	18.33 (4.17)	2.39 (0.82)	10.81 (2.06)
a-ketoglutarate	1.11 (0.19)	1.02 (0.27)	0.87 (0.25)	0.31 (0.05)	0.08 (0.04)	0.22 (0.05)	0.19 (0.03)	0.09 (0.03)	ULOD	ULOD
Phosphoenolpyruvate	0.09 (0.01)	0.06 (0.00)	0.64 (0.17)	0.11 (0.02)	0.12 (0.05)	0.05 (0.01)	0.06 (0.02)	0.01 (0.00)	0.003 (0.001)	0.01 (0.00)
Glutamate	170.72 (20.85)	54.27 (5.99)	185.61 (18.56)	130.21 (8.97)	74.50 (10.39)	100.45 (14.44)	151.66 (8.95)	82.29 (6.63)	5.85 (2.02)	35.57 (2.63)
Aconitate	11.80 (1.14)	8.50 (1.77)	8.85 (2.16)	5.62 (1.04)	2.56 (0.28)	11.26 (2.16)	43.69 (7.18)	1.82 (1.06)	0.14 (0.06)	0.69 (0.05)
3-phosphoglycerate	0.64 (0.07)	0.38 (0.07)	2.23 (0.35)	0.39 (0.10)	0.94 (0.14)	0.47 (0.12)	0.20 (0.08)	0.08 (0.02)	0.03 (0.02)	0.07 (0.02)
Citrate	2.20 (0.42)	1.42 (0.29)	2.83 (0.77)	2.98 (0.69)	1.02 (0.29)	2.67 (0.19)	0.71 (0.15)	0.59 (0.16)	0.19 (0.06)	0.31 (0.04)
Glutamine	1.85 (0.13)	1.26 (0.11)	3.26 (0.96)	3.49 (0.84)	1.11 (0.17)	3.11 (0.15)	0.77 (0.15)	0.65 (0.16)	0.22 (0.06)	0.33 (0.05)
Glucose	0.94 (0.15)	1.17 (0.08)	16.80 (5.79)	6.58 (0.78)	14.38 (2.85)	6.58 (1.18)	12.63 (3.78)	2.45 (1.75)	2.32 (0.88)	2.92 (0.66)
Ribose 5-P	0.63 (0.08)	0.45 (0.06)	0.25 (0.05)	0.17 (0.02)	0.10 (0.02)	0.09 (0.02)	0.09 (0.02)	0.04 (0.01)	0.02 (0.00)	0.03 (0.01)
Fructose 6-P	0.40 (0.06)	0.18 (0.05)	0.32 (0.11)	0.07 (0.01)	0.10 (0.01)	0.05 (0.01)	0.19 (0.04)	0.04 (0.00)	0.02 (0.00)	0.02 (0.00)
Glucose 6-P	0.47 (0.06)	0.17 (0.02)	0.50 (0.19)	0.10 (0.02)	0.16 (0.03)	0.07 (0.01)	0.29 (0.03)	0.04 (0.01)	0.04 (0.01)	0.04 (0.01)
Fructose 1,6-BisP	1.22 (0.15)	0.95 (0.10)	0.52 (0.15)	0.21 (0.04)	0.24 (0.08)	0.17 (0.04)	0.36 (0.05)	0.10 (0.06)	0.07 (0.02)	0.08 (0.02)


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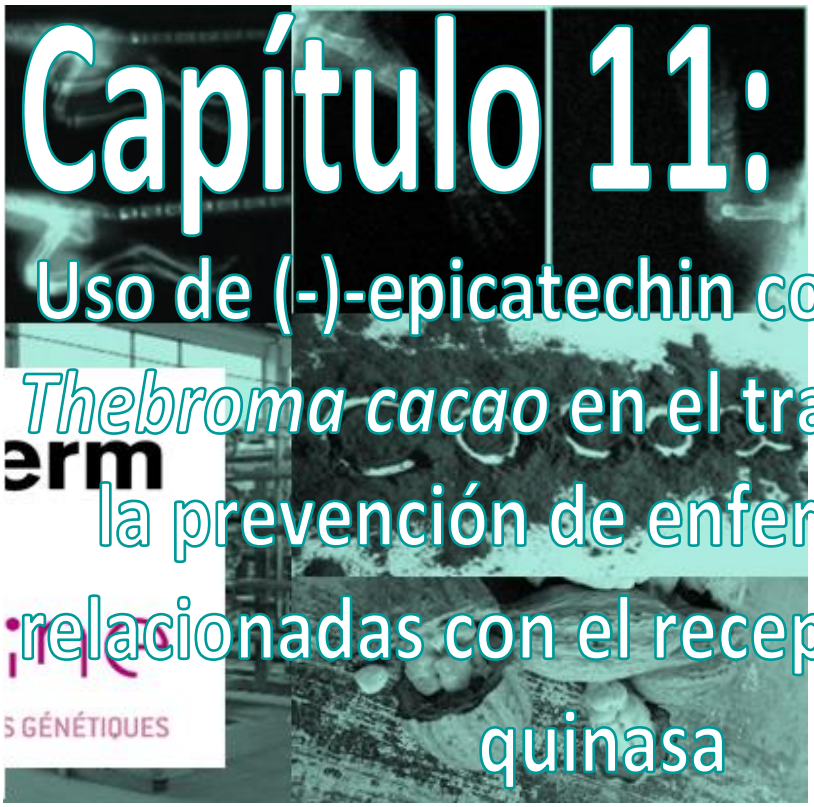


Capítulo 11:

Uso de (-)-epicatechin contenida en *Thebroma cacao* en el tratamiento o la prevención de enfermedades relacionadas con el receptor tirosina quinasa

PATENTE solicitada EP18305053.3

Methods and pharmaceutical compositions for the treatment of treatment of FGFR3-related chondrodysplasias

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of treatment of FGFR3-related chondrodysplasias.

BACKGROUND OF THE INVENTION:

"Rare diseases" are diseases with a particularly low prevalence. In this manner, the 10 European Union considers diseases to be rare when they affect not more than 5 per 10,000 persons (Rodwell and Ayme, 2014a). As example, achondroplasia presents an estimated prevalence of 2.6 per 100,000 persons in EU (Orphanet, 2014). In this sense, rare diseases were defined for first time in EU Regulation (EC) N°141/2000 (*Regulation (EC) No 141/2000 of the European Parliament and of the Council of 16 December 1999 on orphan medicinal products*). In general, patients with rare diseases are particularly isolated and psychologically, socially, economically and culturally vulnerable, with a lack of specific health policies for these diseases and the scarcity of expertise; translate into delayed diagnosis and difficult access to care, inadequate or even harmful treatments and loss of confidence in the health care system (Orphanet, n.d.; Rodwell and Ayme, 2014b).

Receptor tyrosine kinases (RTKs) are cell surface receptors contributing to cell signalling, but some mutations on these receptors are associated to specific rare diseases. RTKs play pivotal roles in development, tissue repair and normal cellular homeostasis as well as mediate cellular responses to a broad array of extracellular signals involved in the regulation of cell proliferation, migration, differentiation and survival signalling. This RTKs type plays an essential role in the regulation of homeostasis of the cartilage e.g. chondrocyte differentiation, proliferation and apoptosis, and is required for normal skeleton development as well as regulation of both osteogenesis and postnatal

bone mineralization by osteoblasts (UniProt, n.d.). Achondroplasia, a nonlethal form of chondrodysplasia, is the most common type of dwarfism. It is mostly due to de novo mutation and has an autosomal dominant inheritance (Rousseau et al., 1994; Di Rocco et al., 2014). The mutation, which produce an increase of FGFR3 function, affects many tissues, most strikingly the cartilaginous growth plate and bone in the growing skeleton, leading to a variety of manifestations and complications. As other RTKs, the binding of the ligand, that is fibroblast growth factors (FGFs), leads to dimerization and transautophosphorylation, resulting in the stimulation of its tyrosine kinase activity (Huete et al., 2011). At molecular levels, four main signaling pathways for FGFR3 may be implicated: signal transducer and activator of transcription 1 (STAT1), mitogen activated protein kinase (MAPK), phospholipase C γ (PLCγ), and phosphatidylinositol phosphate-3-kinaseserine/threonine kinase (PI3K-AKT; protein kinase B) and others. (Horton et al.,5 2007, Ornitz DM & Legeai-Mallet L 2017).

Cocoa (*Theobroma cacao*) is a major, economically important, international crop, which has been related to several nutritional benefits including high antioxidant capacity. These healthy properties have been associated with the phenolic fraction (Andújar et al., 2012; Cádiz-Gurrea et al., 2015). The main subclass of flavonoids found in cocoa is 10 flavanols, particularly (epi)catechins monomers, and their oligomers, also known as procyanidins which range from dimers to decamers (Cádiz-Gurrea et al., 2014). This extract has been reported to show an inhibitory effect of both FGFR3 activated with FGF2 ligand and the phosphorylation cascade related to FGFR3 signalling (Legeai-Mallet and Segura-

Carretero, 2015 and WO2016046375).

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of treatment of FGFR3-related chondrodysplasias. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to a method of treating a FGFR3-related chondrodysplasia in a patient in need thereof consisting in administering to the subject a therapeutically effective amount of a substantially pure (-)-epicatechin.

The present invention also relates to a pharmaceutical composition comprising a therapeutically effective amount of a substantially pure (-)-epicatechin for use in a method of treating a FGFR3-related chondrodysplasia in a patient in need thereof.

As used herein, the term “FGFR3-related chondrodysplasia” is intended to mean a skeletal disease that is caused by an abnormal increased activation of FGFR3, in particular by expression of a constitutively active mutant of the FGFR3 receptor, in particular a constitutively active mutant of the FGFR3 receptor as described after. As used herein, the terms “FGFR3”, “FGFR3 tyrosine kinase receptor” and “FGFR3 receptor” are used interchangeably throughout the specification and refer to all of the naturally-occurring isoforms of FGFR3. As used herein, the expressions "constitutively active FGFR3 receptor variant", "constitutively active mutant of the FGFR3" or "mutant FGFR3 displaying a constitutive activity" are used interchangeably and refer to a mutant of said receptor exhibiting a biological activity (*i.e.* triggering downstream signaling), and/or exhibiting a biological activity which is higher than the biological activity of the corresponding wild-type receptor in the presence of FGF ligand. A constitutively active FGFR3 variant according to the invention is in particular chosen from the group consisting of (residues are numbered according to their position in the precursor of fibroblast growth factor 5 receptor 3 isoform 1 - 806 amino acids long -): a mutant wherein the serine residue at position 84 is substituted with lysine (named herein below S84L); a mutant wherein the arginine residue at position 248 is substituted with cysteine (named herein below R200C); a mutant wherein the arginine residue at position 248 is substituted with cysteine (named herein below R248C); a mutant wherein the serine residue at position 249 is substituted with cysteine (named herein below S249C); a mutant wherein the proline residue at position 250 is substituted with arginine (named

herein below P250R); a mutant wherein the asparagine residue at position 262 is substituted with histidine (named herein below N262H); a mutant wherein the glycine residue at position 268 is substituted with cysteine (named herein below G268C); a mutant wherein the tyrosine residue at position 278 is substituted with cysteine (named herein below Y278C); a mutant wherein the serine residue at position 279 is substituted with cysteine (named herein below S279C); a mutant wherein the glycine residue at position 370 is substituted with cysteine (named herein below G370C); a mutant wherein the serine residue at position 371 is substituted with cysteine (named herein below S371C); a mutant wherein the tyrosine residue at position 373 is substituted with cysteine (named herein below Y373C); a mutant wherein the glycine residue at position 380 is substituted with arginine (named herein below G380R); a mutant wherein the valine residue at position 381 is substituted with glutamate (named herein below V381E); a mutant wherein the alanine residue at position 391 is substituted with glutamate (named herein below A391E); a mutant wherein the asparagine residue at position 540 is substituted with Lysine (named herein below N540K); a mutant wherein the termination codon is eliminated due to base substitutions, in particular the mutant wherein the termination codon is mutated in an arginine, cysteine, glycine, serine or tryptophane codon (named herein below X807R, X807C, X807G, X807S and X807W, respectively); a mutant wherein the lysine residue at position 650 is substituted with another residue, in particular with methionine, glutamate, asparagine or glutamine (named herein below K650M, K650E, K650N and K650Q). Typically, a constitutively active FGFR3 variant according to the invention is K650M, K650E or Y373C mutant.

In some embodiments, the FGFR3-related skeletal diseases are FGFR3-related chondrodysplasias and FGFR3-related craniosynostosis. In some embodiments, the FGFR3-related skeletal osteochondrodysplasias correspond to an inherited or to a sporadic disease. As used herein, the term “FGFR3-related skeletal dysplasias” includes but is not limited to thanatophoric dysplasia type I, thanatophoric dysplasia type II, hypochondroplasia, achondroplasia and SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans). In some embodiments, the FGFR3-related skeletal osteochondrodysplasia is caused by expression in the subject of a constitutively

active FGFR3 receptor variant such as defined above. In some embodiments, the FGFR3-related chondrodysplasia is an achondroplasia caused by expression of the G380R constitutively active mutant of the FGFR3 receptor. In some embodiments, the FGFR3-related chondrodysplasia is a hypochondroplasia caused by expression of the N540K, K650N, K650Q, S84L, R200C, N262H, G268C, Y278C, S279C, V381E, constitutively active mutant of the FGFR3 receptor. In some embodiments, the FGFR3-related chondrodysplasia is a thanatophoric dysplasia type I caused by expression of a constitutively active mutant of the FGFR3 receptor chosen from the group consisting of R248C, S248C, G370C, S371C; Y373C, X807R, X807C, X807G, X807S, X807W and K650M FGFR3 receptors. In some embodiments, the FGFR3-related chondrodysplasia is a thanatophoric dysplasia type II caused by expression of the K650E constitutively active mutant of the FGFR3 receptor. In some embodiments, the FGFR3-related chondrodysplasia is a severe achondroplasia with developmental delay and acanthosis nigricans caused by expression of the K650M constitutively active mutant of the FGFR3 receptor. In some embodiments, the FGFR3-related craniosynostosis corresponds to an inherited or to a sporadic disease. In some embodiments, the FGFR3-related craniosynostosis is Muenke syndrome caused by expression of the P250R constitutively active mutant of the FGFR3 receptor or Crouzon syndrome with acanthosis nigricans caused by expression of the A391E constitutively active mutant of the FGFR3 receptor.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative, improving the patient’s condition or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing

used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at regular intervals, e.g., daily, weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

As used herein the term “(-)-epicatechin” has its general meaning in the art and refers to (2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol. The methods of producing or extracting (-)-epicatechin are well known to the skilled person.

As used herein, the term “substantially pure" refers to the total absence, or near total absence, of impurities, such as related-substance impurities. For example, when a (-)-epicatechin composition is said to be substantially pure, there are either no detectable related substance impurities, or if a single related-substance impurity is detected, it is present in an amount no greater than 0.1 % by weight, or if multiple related- substance impurities are detected, they are present in aggregate in an amount no greater than 0.6% by weight.

Accordingly, the patient is administered with a pharmaceutical composition comprising the substantially pure (-)-epicatechin as active principle and at least one pharmaceutically acceptable

excipient. As used herein the term “active principle” or “active ingredient” are used interchangeably. The active principle is used to alleviate, treat or prevent a medical condition or disease. By the term “pharmaceutically acceptable excipient” herein, it is understood a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredient(s) and which is not excessively toxic to the host at the concentration at which it is administered. Said excipients are selected, depending on the pharmaceutical form and the desired method of administration, from the usual excipients known by a person skilled in the art. In some embodiments, the pharmaceutical composition of the present invention does not comprise a second active principle. Accordingly, in some embodiments, the pharmaceutical composition of the present invention is not a *Theobroma cacao* extract as described in WO 2016046375. In some embodiments the pharmaceutical composition does not comprises an amount of flavonols, sweroside, and hexenyl 5 xylopyranosyl glucopyranoside. In some embodiments, the pharmaceutical composition of the present invention does not comprise an amount of procyanidin, catechin, cinchonain and derivatives thereof. In some embodiments, the pharmaceutical composition does not comprises an amount of 3,4-N-phenylpropenoyl-Laminoacid or derivative thereof chosen from /V-caffeoyl-L-aspartate, L-Aspartic acid, A/-[3- (4-hydroxyphenyl)-1 - oxo-2-propenyl], L-Aspartic acid, A/-[3-(4-hydroxy-3-methoxyphenyl)- 1 -oxo-2- propenyl], trans-clovamide (A/-[(2E)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1 -yl]- 3-hydroxy-L-tyrosine), deoxyclovamide (A/-[(2E)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1 -yl]-L-tyrosine) and derivatives thereof. In some embodiments, the pharmaceutical composition of the present invention does not comprise a compound selected from the group consisting of quercetin, quercetin glucuronide, quercetin hexose, quercetin arabinoside and isomers thereof and/or said at least one procyanidin, catechin, cinchonain and derivative thereof chosen from proanthocyanidin A, (epi)catechin, (epi)catechin dimer hexose, arabinopyranosyl-(epi)catechin-(epi)catechin, (epi)gallocatechin, (epi)catechin glucopyranoside, catechin diglucopyranoside, cinchonain I, (epi)catechin tetramer, (epi)catechin pentamer, (epi)catechin hexamer, (epi)catechin methyl dimer, (epi)catechin ethyl dimer, procyanidin A, procyanidin B, procyanidin C and isomers

thereof. In some embodiments, the pharmaceutical composition does not comprises an amount of procyanidin B; (epi)catechin tetramer; (epi)catechin pentamer; hexenyl xylopyranosyl glucopyranoside; (epi)catechin dimer hexose; arabinopyranosyl- (epi)catechin-(epi)catechin; - procyanidin C; - proanthocyanidin A; (epi)catechin ethyl dimer; quercetin; quercetin hexose; - cinchonain I; procyanidin A; and sweroside.

By a "therapeutically effective amount" of the substantially pure (-)-epicatechin as above described is meant a sufficient amount to provide a therapeutic effect. It will be understood, however, that the total daily usage of the substantially pure (-)-epicatechin will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0,5 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight 10 per day.

The invention will be further illustrated by the following figures and examples.

However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE:

MATERIAL AND METHODS

1. Chemicals and reagents.

All chemicals were of HPLC-MS grade and used as received. Acetic acid and methanol for HPLC and semi-preparative were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Diethyl ether and methanol for extraction were purchased from Fisher (Fisher Scientific Co., Fair Lawn, NJ), ethyl acetate from Lab-Scan (Gliwice, Sowinskiego, Poland), ethanol and acetone from AnalaR Normapur (VWR International, Inc., Darmstadt, Germany) and dimethyl sulfoxide (DMSO) was purchased from Panreac (Barcelona, Spain) for analytical assays. (-)-epicatechin was purchased from Sigma-Aldrich (St Louis, MO, USA). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). Deuterated solvents such as dimethylsulfoxide were purchased from Eurisotop (France). For NMR analysis, the sample was dissolved in dimethylsulfoxide-d6 and transferred in an oven-dried 5 mm NMR tube for spectral analysis.

Waters 5 g Sep-Pak C18 Plus cartridges (Mildford, USA) were used as solid-phase extraction minicolumns for purification and concentration.

2. Sample preparation.

A concentrated *T. cacao* extract was used in this study (Molteoleder, Spain). The polyphenols from whole cocoa matrix were analytically characterized using a solution of cocoa extract of 10 mg/mL in DMSO.

For cartridges purification, a solution stock of 0.1 g/mL was prepared by dissolving the appropriate amount of cacao extract in DMSO. The sample was sonicated for 5 min, vortexed for 1 min, and then centrifuged for 5 min at 7700 g before the cartridges purification.

For semi-preparative HPLC purification, a solution stock of 75 mg/mL was prepared by dissolving the PC fraction of C18 Sep-Pak cartridges in DMSO. The sample was sonicated for 5 min, vortexed for 1 min, and then centrifuged for 5 min at 7700 g before the semi-preparative HPLC analysis.

Each obtained sample was filtered through a 0.25 mm filter before the preparative HPLC analysis.

3. Instrumentation.

The polyphenols from the PC fraction from Sep-Pak cartridges purification were fractionated using a Gilson preparative HPLC system (Gilson Inc., Middleton, WI, USA) equipped with a binary pump (model 331/332), automated liquid handling solutions (model GX-271) and UV-Vis detector (model UV-Vis 156).

T. cacao and isolated fractions were analytically characterized using an Agilent 1200 series rapid-resolution LC system (AgilentTechnologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode-array detector (DAD). The HPLC system was coupled to a time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface (model G1607A from Agilent Technologies, Palo Alto, CA).

4. Fractionation of polyphenols for obtaining of (-)-epicatechin from T. cacao extract.

4.1. By Sep-Pak C18 cartridges.

T. cacao extract prepared as describe above in sample preparation section was fractionated by Waters C18 Sep-Pak cartridges following the method describe by *Sun et al.* (Sun et al., 2006) and *Monagas et al.* (Monagas et al., 2003) with some modifications. Five different fractions were obtained: phenolic acids (PA fraction), monomeric and oligomeric flavan-3-ols (PC fraction), polymeric proanthocyanidins (PP fraction), monomers (MN fraction) and oligomers (OL fraction).

The experiments were performed in triplicate to ensure the repeatability of the fractionation by the cartridges.

The five obtained fractions were evaporated to dryness under vacuum in Speed Vac. The residue of each one was weighted and dissolved in DMSO at 100 µg/mL. After that, they were filtered through a 0.25 µm filter to analyze them by HPLC-ESI-TOF-MS.

4.2. By combination of Sep-Pak and semi-preparative HPLC.

The compounds from PC fraction from C18 cartridges were fractionated according to Cádiz-Gurrea et al. (Cádiz-Gurrea et al., 2014). Finally, a total of 11 fractions were collected and the solvent was evaporated under vacuum. The residue of each fraction was weighted and dissolved with an appropriate volume of DMSO at concentration level of 100 µg/mL. Finally, all fractions were filtered through a 0.25 µm filter before the HPLC analysis.

5. Chromatographic conditions and ESI-TOF-MS detection.

The compounds from the T. cacao and fractions were separated following the method described by *Cádiz-Gurrea et al.* (Cádiz-Gurrea et al., 2014) with this modifications: the HPLC system was coupled to a TOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +3.5 kV. The other optimum values of the source parameters were: drying gas temperature, 200 °C; drying gas flow, 10 L/min; and nebulizing gas pressure, 2.3 bar. The detection was performed considering a mass range of 50–1200 *m/z*.

The samples were injected in triplicate to ensure the repeatability of the analysis.

6. NMR conditions.

NMR spectra was recorded at 293 ± 0.1 K on a Bruker Avance III 600 spectrometer operating at a proton frequency of 600.13 MHz using a 5 mm QCI quadruple resonance pulse field gradient cryoprobe. The multiplicities observed are labeled as s = singlet; d = doublet; dd = doublet of doublets; t = triplet; m = multiplet; and bs = broad singlet. The sample was measured, without rotation and using

8 dummy scans prior to 128 scans. Acquisition parameters have been set as follows: size of fid = 64K, spectral width = 20.5 ppm, acquisition time = 2.73 s, relaxation delay = 10 s, receiver gain = 20.2, FID resolution = 0.25 Hz. A pre-saturation pulse sequence (Bruker 1D noesygppr1d) was used to suppress the residual H_2O signal via irradiation of the H_2O frequency during the recycle and mixing time delays. The resulting spectrum was automatically phased, baseline-corrected, and calibrated to the TSP signal at 0.0 ppm. The t1 time was set to 4 us and the mixing time (d8) to 10 ms. The spectrometer transmitter was locked to DMSO-d6 frequency. Acquisition and processing of spectra were carried out with TOPSPIN software (version 3.1). 1H–1H total correlation spectroscopy (TOCSY), 1H-13C heteronuclear single quantum coherence (HSQC), 1H-13C heteronuclear multiple bonds coherence (HMBC) spectra were recorded using standard Bruker sequences. The TOCSY spectrum was obtained applying a relaxation delay of 2.0 s, spectral width in both dimensions of 7194.25 Hz and a receiver gain of 64.0. TOCSY spectrum was processed using sine-bell window function (SSB = 2.0). The HSQC spectrum was acquired using a relaxation delay of 1.0 s, spectral width of 7211.54 Hz in F2 and 24900.71 Hz in F1. Quadratic sine window function (SSB = 2.0) was used for the HSQC spectrum. The HMBC spectrum was recorded with the same parameters used in the HSQC spectra except for 37729.71 Hz of spectral width in F1. The coupling constant for HSQC experiment was fixed to 145 Hz whereas HMBC experiment was obtained using fixed coupling constants of 145 and 8 Hz (long range).

7. Evaluation of the efficacy of F5 with human chondrocyte lines expressing FGFR3 gain-of-function mutations

The F5 from cocoa extract was evaluated in vitro using chondrocyte lines expressing human FGFR3 gain-of-function mutations. We have studied two human chondrocyte lines, the first one expresses the heterozygous achondroplasia (ACH) mutation (Gly380Arg) and the other one the heterozygous thanatophoric dysplasia (TD) mutation (Tyr373Cys) (Benoist-Lasselin C. et al. FEBS Lett. 2007).

The cells were depleted during 24 hours, we tested various concentrations (50, 100, 200 µg/mL) of F5 from cocoa. The cocoa fraction was added in the medium then the cells were stimulated with a ligand

of the receptor FGF2 (Fibroblast Growth Factor). The efficacy of the cocoa fraction on the activation of FGFR3 was evaluated by western blotting (n= 3). We evaluated the level of phosphorylation of Erk1/2, two proteins of the canonical Mapkinase pathway.

The levels of phosphorylated Erk1/2 and Erk1/2 were measured using the Li-Cor technology and Imager.

8. Evaluation of the efficacy of Fraction 5 from cacao extract and (-)-epicatechin on *ex vivo* femur cultures isolated from Fgfr3 $^{Y367C/+}$ mice

Heterozygous Fgfr3$^{Y367C/+}$ mice ubiquitously expressing the Y367C mutation and exhibiting a severe dwarfism were used (Pannier et al., Biochim Biophys Acta, 1792: 140-147, 2009). Several sets of *ex vivo* experiments were performed. Femur embryos at day E16.5 from Fgfr3$^{+/+}$ (n=41) and Fgfr3$^{Y367C/+}$ (n=30) mice were used and incubated for 5 days in DMEM medium with antibiotics and 0.2% BSA (Sigma) supplemented with cocoa F5 or (-)-epicatechin (Sigma-0394-05-90) or DMSO (as control) at a concentration of 10 and 30 µg/mL. Right femur was cultured in supplemented medium and compared with the left one cultured in control medium. To establish the effect of the inhibitors, the right femur was cultured in supplemented medium and compared with the left one cultured in control medium. The bone length was measured at the beginning (before treatment) and at the end of time course. The genotype of Fgfr3$^{+/+}$ and Fgfr3$^{Y367C/+}$ mice were determined by PCR of tail DNA as previously described (Pannier et al., Biochim Biophys Acta, 1792: 140-147, 2009). All experimental procedures and protocols were approved by the Animal Care and Use Committee.

9. Evaluation of the efficacy of Fraction 5 from cacao extract and (-)-epicatechin on growth plate cartilage

In order to appreciate the impact of the treatment on the growth plate cartilage, we performed histological and immunohistological studies. We selected specific markers of the cartilage, SOX9 and

Collagen type X, we tested the expression of the protein FGFR3 and we also evaluated the expression of the phosphorylated Erk1/2 proteins of the Mapkinase signalling and activated by FGFR3.

Limb explants were fixed after culture in 4% paraformaldehyde at 4°C and embedded in paraffin. Serial 5□m sections were stained with Hematoxylin-Eosin using standard protocols for histological analysis or were subjected to immunohistochemical staining.

For immunohistochemistry, sections were stained with antibodies specific to FGFR3 (1:100 dilution; Sigma), anti-Collagen type X (1:50 dilution; Quartett anti-SOX9 (1:1000 dilution; Abcam), anti-phosphorylated Erk1/2 (1:100 dilution; Cell signaling) using the Dako Envision system kit. Images were captured with an Olympus PD70-IX2-UCB microscope.

RESULTS

1. Comprehensive characterization of fractions by HPLC-ESI-TOF-MS.

The Figure 1 showed the different profiles (base peak chromatograms and characterized compounds), which were obtained by Sep-Pak C18 cartridges. This proposed method permitted separating phenolic compounds from the whole *T. cacao* extract into various different fractions, which were less complex, enabling their use in order to achieve pure fractions of interested compounds. In addition, eleven fractions were obtained by combination of solid phase extraction by Sep-Pak C18 cartridges and semi-preparative HPLC isolation in order to obtain purified fractions of monomeric and oligomeric PAs (Figure 2). The composition of each one was established by the detailed HPLC-ESI-TOF-MS (Figure 3). Only in F1 we could find (+)-catechin (by comparing to the commercial standard) and (-)-epicatechin (*m/z* 289). In F2 and F3, there are a B-type dimer or PC and (-)-epicatechin, the last one was presented with high intensity in F3. Moreover, a PC trimer appeared in fractions from 4 to 7 (*m/z* 865). Less purified fractions were F7, F8 and F10 with four or more compounds. However, a B-type dimer of PC was detected in F9 being the most purify fraction.

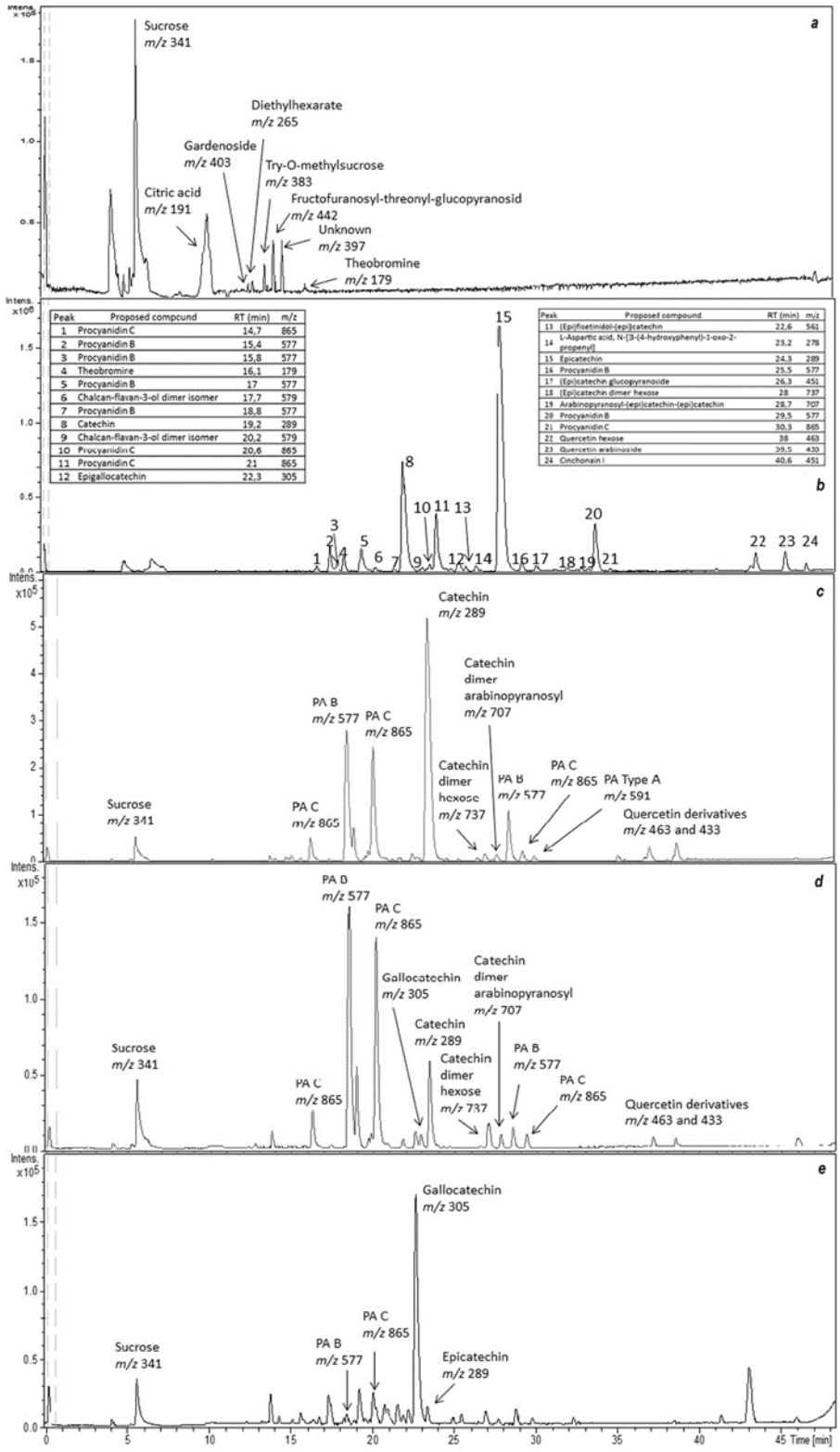


Figure 1. Different profiles of Sep-Pak fractions by HPLC-ESI-TOF-MS were; a) PA, b) PC, c) MN, d) OL and e) PL fractions.

After preliminary biological tests of the obtained fractions, F5 was selected as a candidate for the biological experiments in achondroplasia model.

2. NMR identification of compounds from selected F5.

Figure 4 showed the ^{1}H spectra in DMSO-d_6 for F5 where the most abundant compound corresponded to (-)-epicatechin (Figure 5).

Table 1 contains the ^{1}H and ^{13}C NMR data obtained in this work for (-)-epicatechin in F5 from cacao sample. The identification of similar substances as (+)-catechin and (-)-epicatechin was carried out on the basis of the spectroscopic data of aliphatic protons H-3(C) and H-2(C). High values of the coupling constant $^{3}J_{2,3}$ (8-10 Hz) indicate the presence of (+)catechin, whereas $^{3}J_{2,3}$ values round 2 Hz or broad singlet demonstrated the presence of (-)-epicatechin in our cacao fraction.

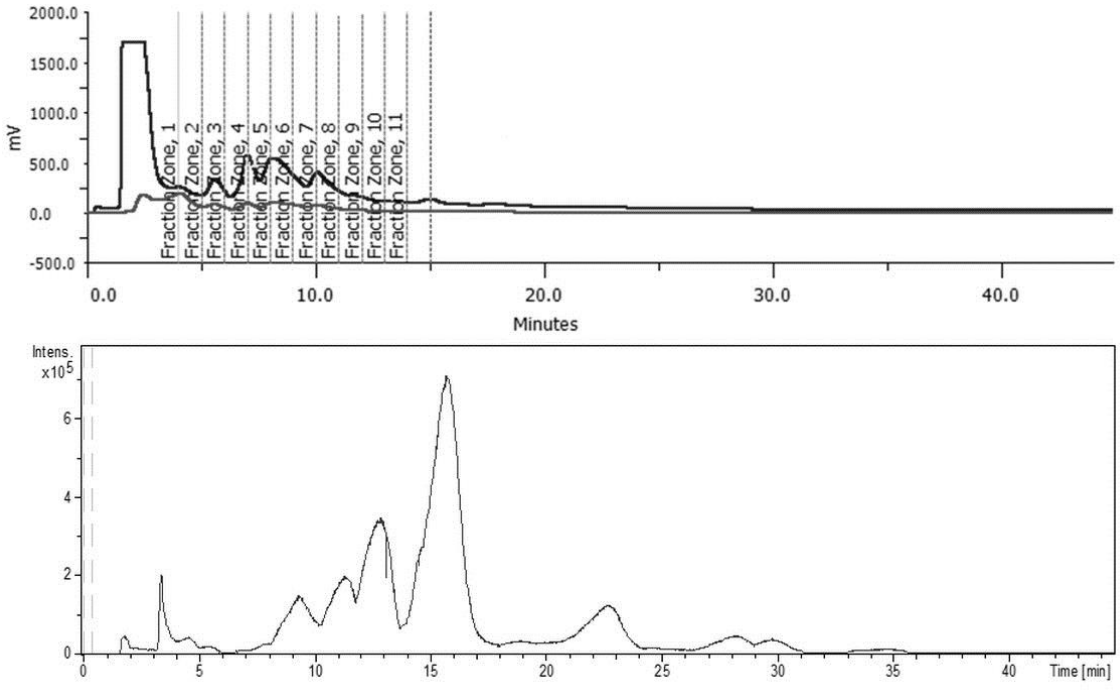


Figure 2. Semi-preparative chromatograms by HPLC-UV with collection and TOF-MS.

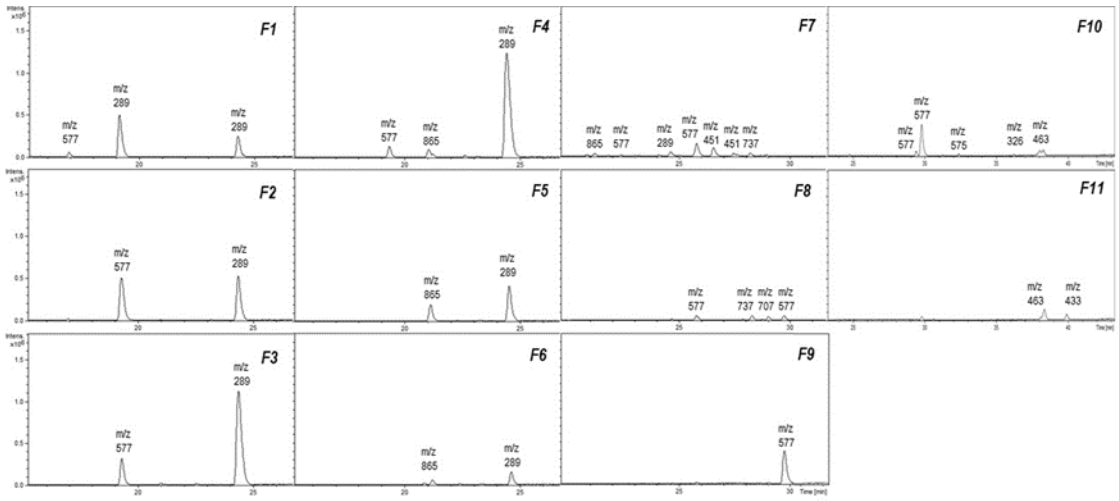


Figure 3. BPC of eleven obtained fractions from PC Sep-Pak fraction by HPLC-ESI-TOF-MS.

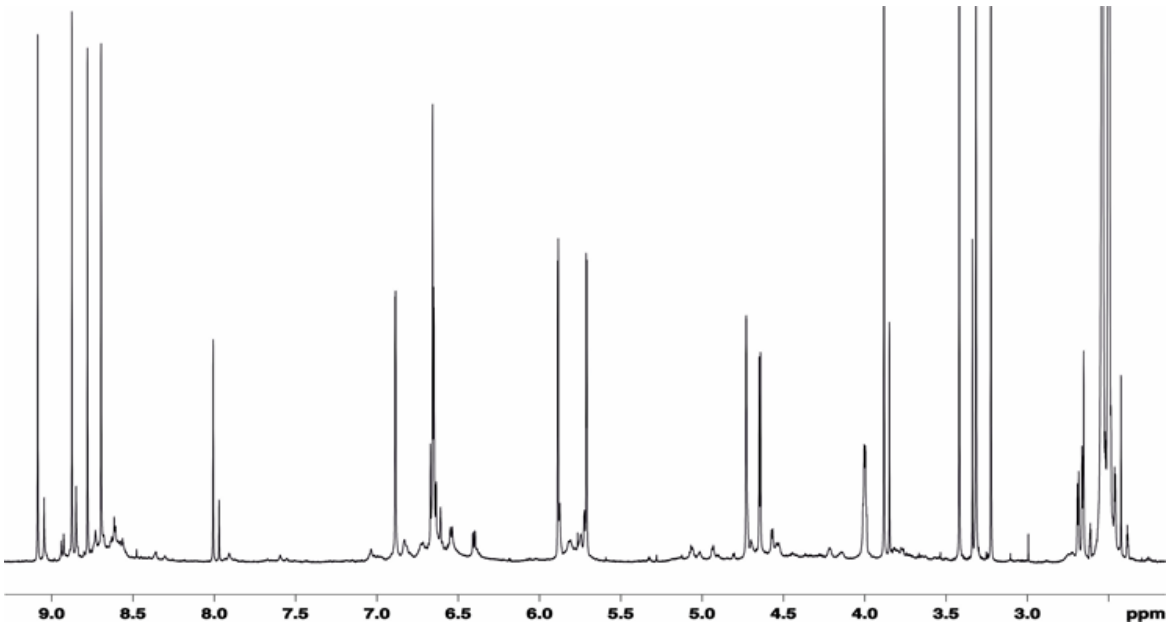


Figure 4. NMR ^{1}H spectra in DMSO-d_6 of F5 from cacao extract.

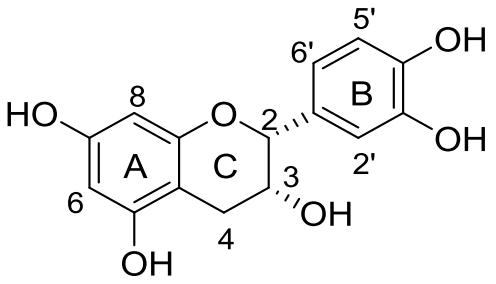


Figure 5. Chemical structure of (-)-epicatechin.

Table 1. ^{1}H and ^{13}C NMR chemical shift data for (-)-epicatechin from F5 in d_6-DMSO.

Ring	Position	^{13}C	^{1}H		
		δ(ppm)	δ(ppm)	multiplicity	Coupling constant data (Hz)
A	6	94.54	5.71	d	2.3
A	8	95.52	5.89	d	2.3
B	5'	115.21	6.66	d	8.0
B	6'	118.41	6.65	dd	1.6; 8.0
B	2'	115.36	6.89	d	1.6
C	2	78.52	4.73	s (br)	-
C	3	65.37	4.00	m	-
C	4	28.67	2.67	dd	4.6; 16.5
C	4	28.67	2.47	dd	3.5; 16.5

d = doublet, dd = double doublet; s (br) = broad singlet; m = multiplet

3. Evaluation of the efficacy of F5 on the Mapkinase pathways using human chondrocyte lines expressing FGFR3 gain-of-function mutations

We evaluated the level of phosphorylation of the protein Erk1/2, two specific proteins of the Mapkinase pathway, by *in vitro* assays using human TD chondrocyte lines. Considering the data obtained (Figure 6), we decided to treat the TD and ACH lines with 10 µg/ml of F5.

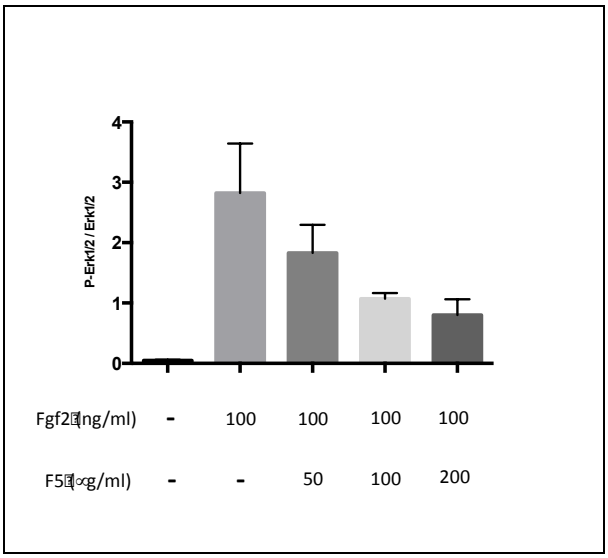
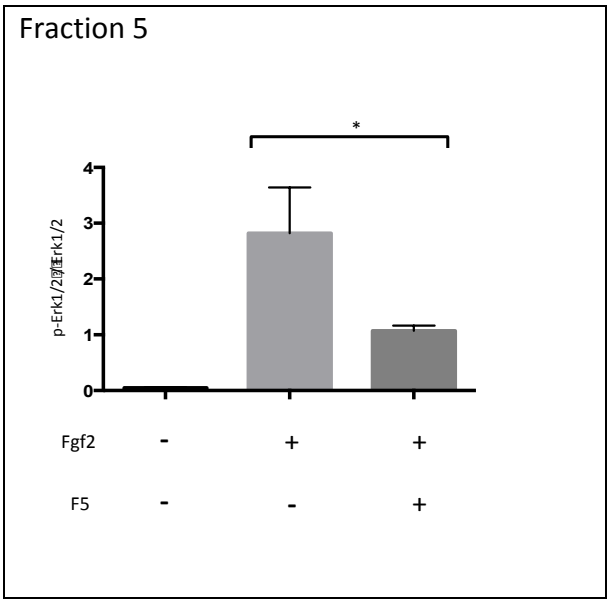


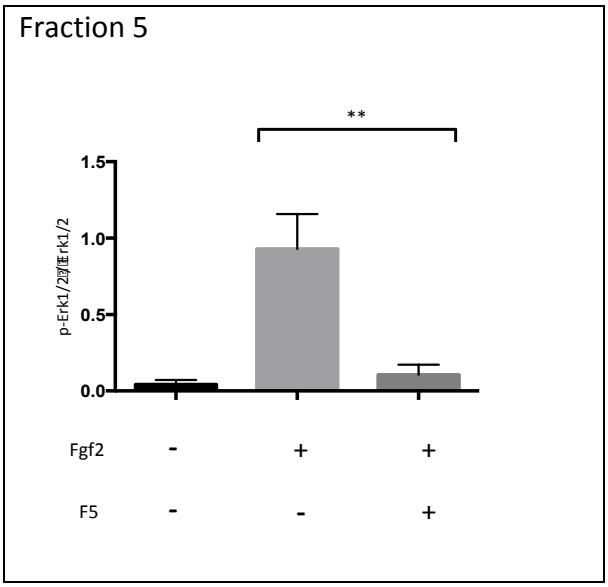
Figure 6. Dose related inhibition of Erk1/2 phosphorylation in TD chondrocyte lines.

The treatment of the TD chondrocyte lines with F5, showed a strong reduction of the level of Erk1/2 phosphorylation (Figure 7). We tested also F5 with ACH chondrocyte lines. A significant reduction of Erk1/2 phosphorylation was observed for this fraction (Figure 8).



Fraction 5: p* 0.0212

Figure 7. Representative data of treatments of TD human chondrocyte lines using FGF2 (100 ng/ml).



Fraction 5: p** 0.0038

Figure 8. Representative data of treatments of ACH human chondrocyte lines using FGF2 (100 ng/ml).

4. Evaluation of the efficacy of F5 from cacao extract and (-)-epicatechin on ex vivo femur cultures isolated from Fgfr3 $^{Y367C/+}$ mice mimicking achondroplasia

Regarding the data obtained *in vitro* and the data of the NMR analysis, we tested F5 and (-)-epicatechin in femur cultures.

F5 has been evaluated for a concentration of 10 µg/ml. F5 significantly increased the length of the *Fgfr3*$^{Y367C/+}$ femurs comparing to *Fgfr3*$^{+/+}$ femurs (Figure 9). The percentage of femur growth was more important in *Fgfr3*$^{Y367C/+}$ mice than in the *Fgfr3*$^{+/+}$ mice. F5 did not impair the growth of the *Fgfr3*$^{+/+}$ femurs (Figure 10).

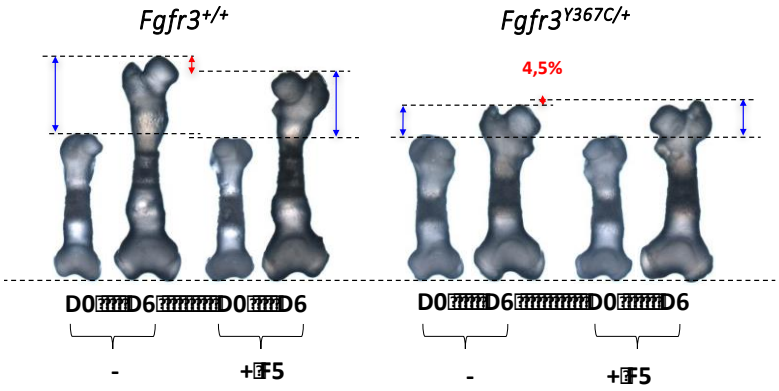


Figure 9. Representative picture of femurs treated with fraction 5 (10 µg/ml).

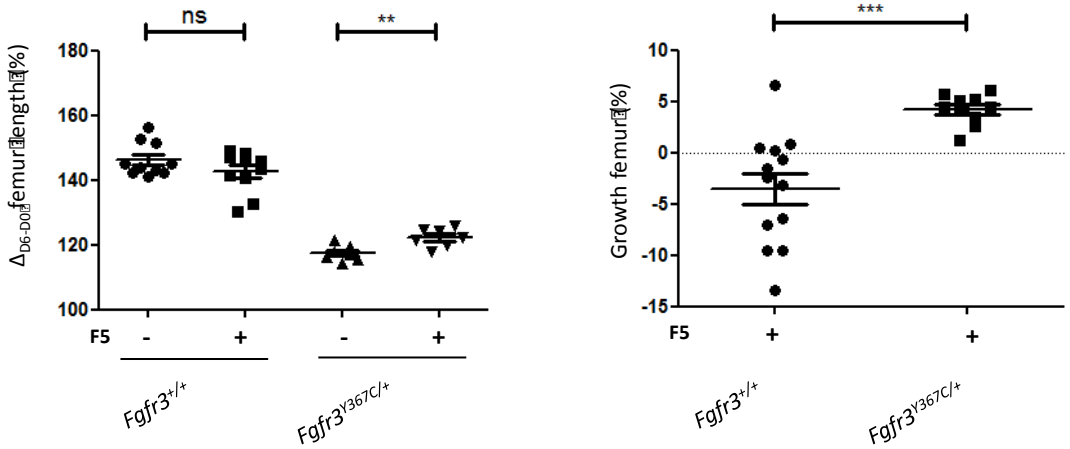


Figure 10. Graphic representation of femur length and % of growth of the femurs treated with F5 (10 µg/ml).

Considering the data of NMR, we evaluated the efficacy of (-)-epicatechin on bone growth using two different concentrations: 1 and 10 µg/mL.

(-)-Epicatechin (10 µg/mL) significantly increased the length (+10%) of the *Fgfr3*$^{Y367C/+}$ femurs comparing to *Fgfr3*$^{+/+}$ femurs (Figure 11). The percentage of femur growth was more important in *Fgfr3*$^{Y367C/+}$ mice than in the *Fgfr3*$^{+/+}$ mice. (-)-Epicatechin (10 µg/mL) did not impair the growth of the *Fgfr3*$^{+/+}$ femurs (Figure 12).

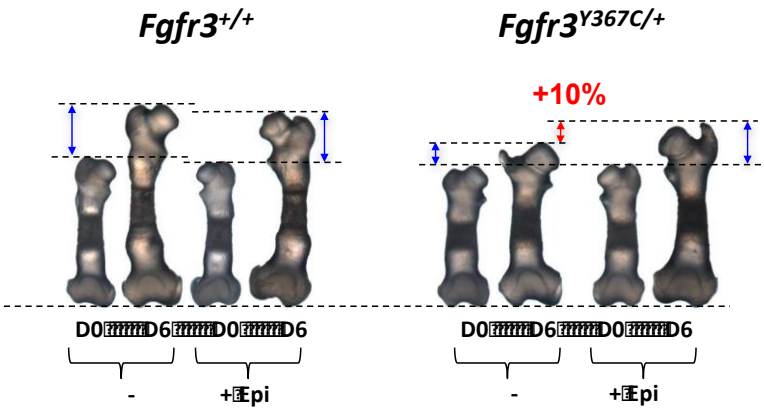


Figure 11. Representative picture of femurs treated with (-)-epicatechin (10 µg/mL).

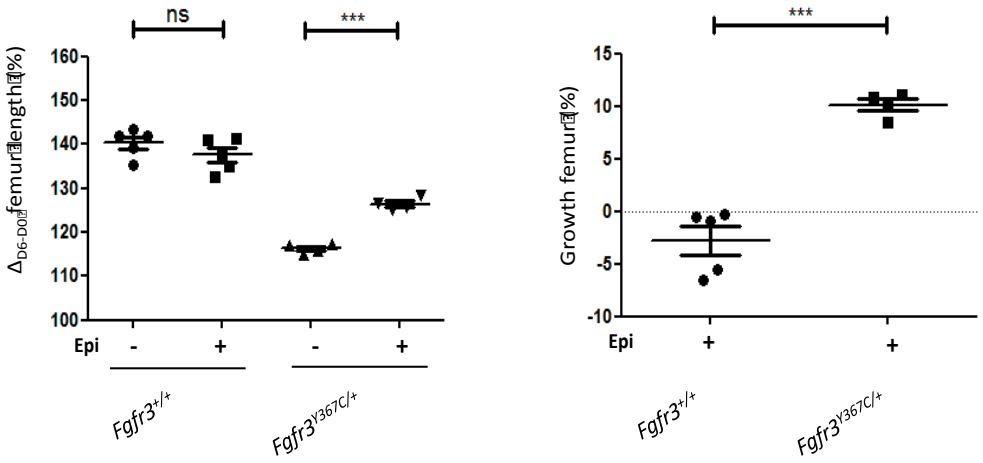


Figure 12. Graphic representation of femur length and % of growth of the femurs treated with (-)-epicatechin (10 µg/mL).

The data obtained with (-)-epicatechin with a lower concentration (1 µg/mL) did not improve the size of the femurs for *Fgfr3*$^{Y367C/+}$ and *Fgfr3*$^{+/+}$ (Figure 13).

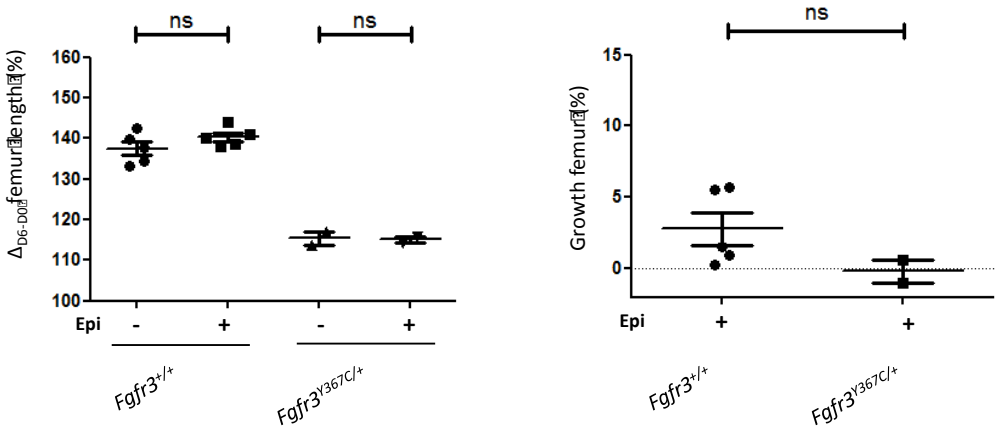


Figure 13. Graphic representation of femur length and % of growth of the femurs treated with (-)-epicatechin (1 µg/mL).

5. Evaluation of the efficacy of F5 from cacao extract and (-)-epicatechin on growth plate cartilage

The immunohistological analyses showed a slight modification of the collagen type X expression in proximal and distal femurs treated with F5 (Figure 14). We noted a decreasing expression of FGFR3 and SOX9 (Figure 14 and Figure 15).

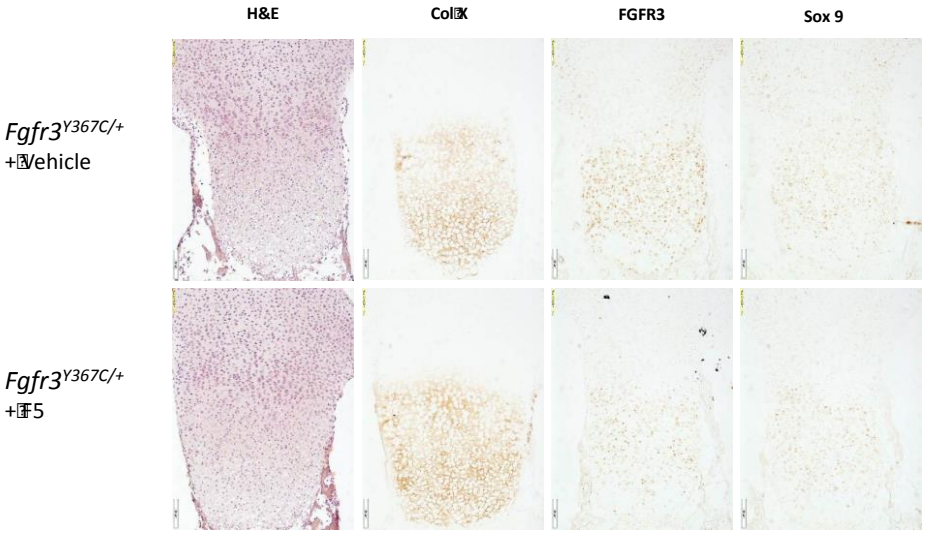


Figure 14. Representative pictures of histological staining and immunohistological labelling of the proximal femurs treated with F5.

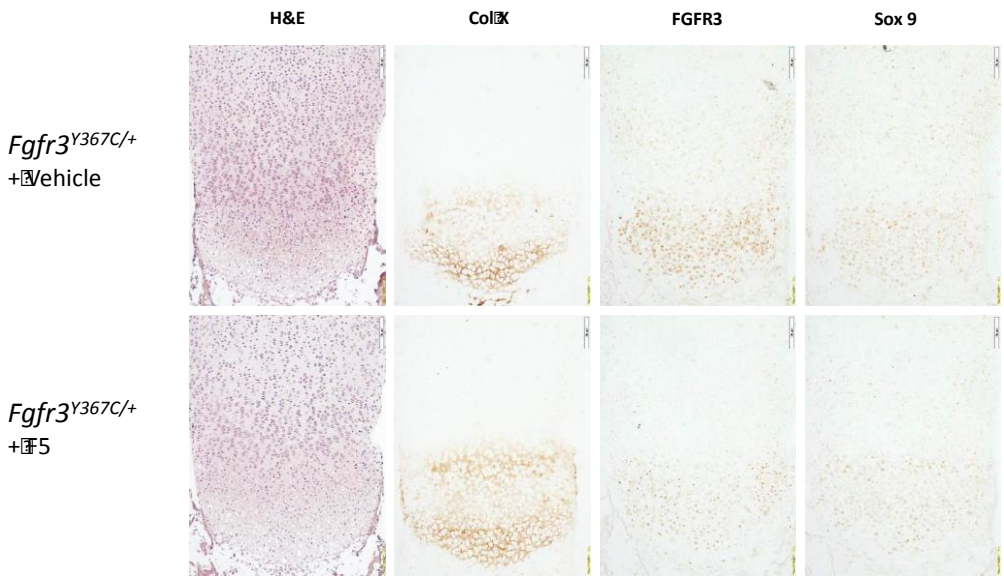


Figure 15. Representative pictures of histological staining and immunohistological labelling of the distal femurs treated with F5.

There was no obvious modification of phosphorylated Erk1/2 expression in the distal and proximal femurs (Figure 16). The analyses of the both proximal and distal femur did not show modification of the epiphysis size (Figure 17).

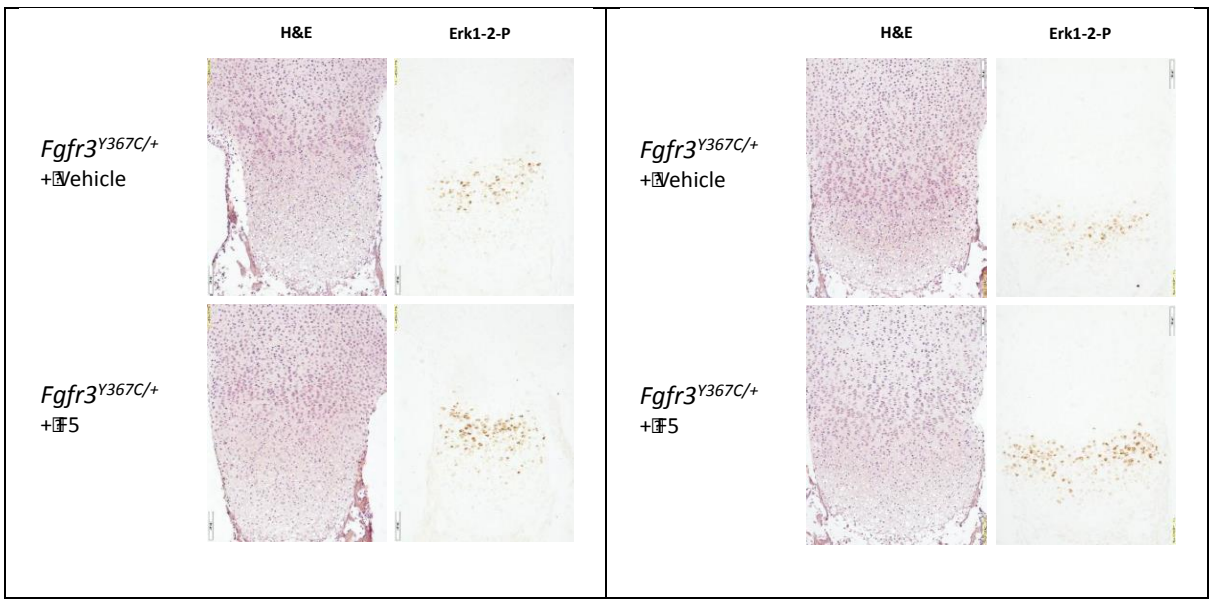


Figure 16. Representative pictures of phosphorylated Erk1/2 immunohistological labelling of the proximal and distal femurs treated with F5.

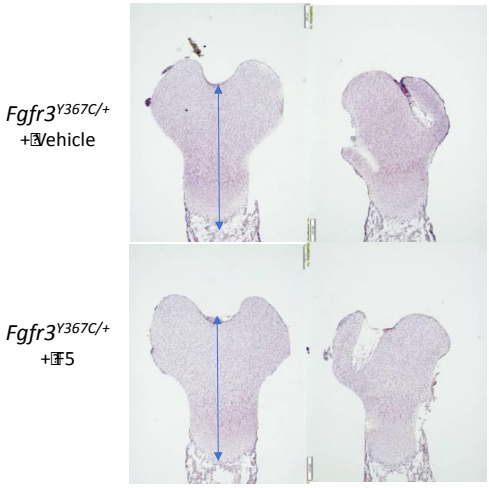


Figure 17. Representative pictures of histological staining proximal of whole femur cartilage growth plate treated with F5.

Studying the H&E staining, we observed that the (-)-epicatechin treatment improved the whole growth plate cartilage. We noted an increase size of the epiphysis as depicted in the Figure 18 with arrows blue and green in the proximal part of the femurs. The gain-of-growth is labelled with red arrows. In the distal part of the femurs, the improvement of the size of the epiphysis was more obvious (Figure 18 and Figure 19). The reserve, proliferative and pre-hypertrophic zones of the growth plate cartilage were increased.

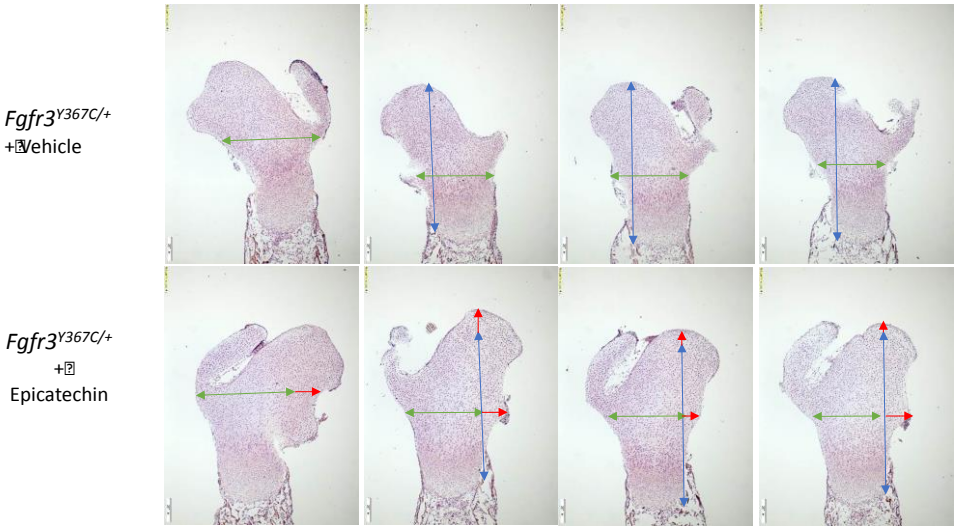


Figure 18. Representative pictures of histological staining proximal of whole femur cartilage growth plate treated with (-)-epicatechin.

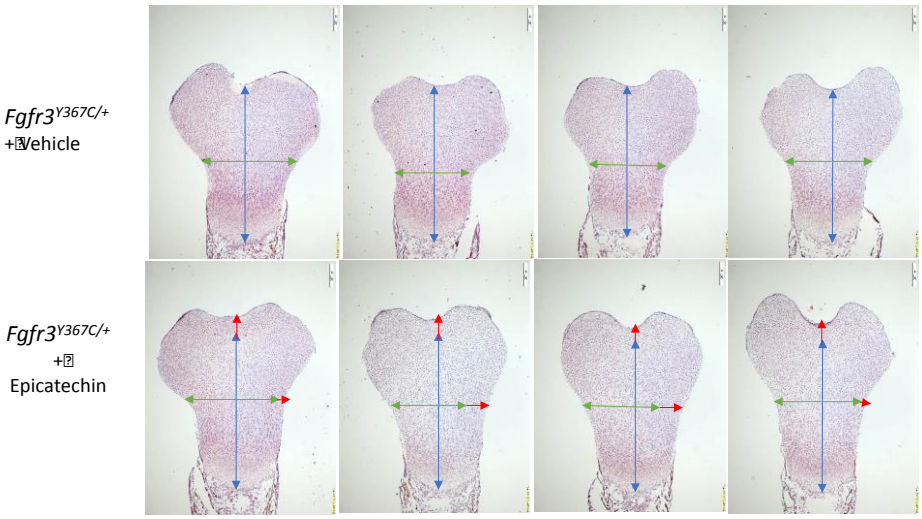


Figure 19. Representative pictures of histological staining distal of whole femur cartilage growth plate treated with (-)-epicatechin.

The expression of collagen type X was not modified in proximal and distal femurs (Figure 20 and Figure 21), while the expressions of FGFR3 and Sox9 were strongly decreased in proximal and distal femurs (Figure 20 and Figure 21). Interestingly, the phosphorylated Erk1/2 expression was slightly decreased in proximal and distal femurs treated with (-)-epicatechin (Figure 22).

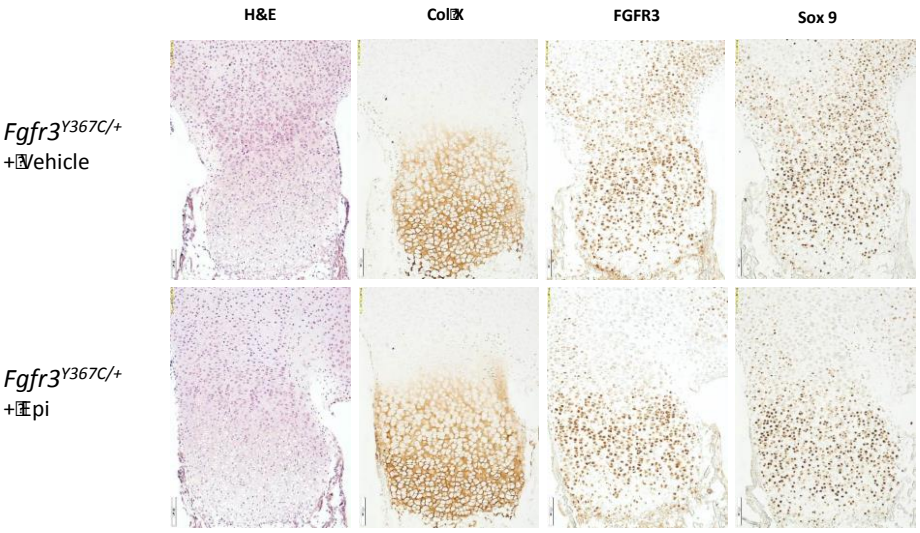


Figure 20. Representative pictures of histological staining and immunohistological labelling of the proximal femurs treated with (-)-epicatechin.

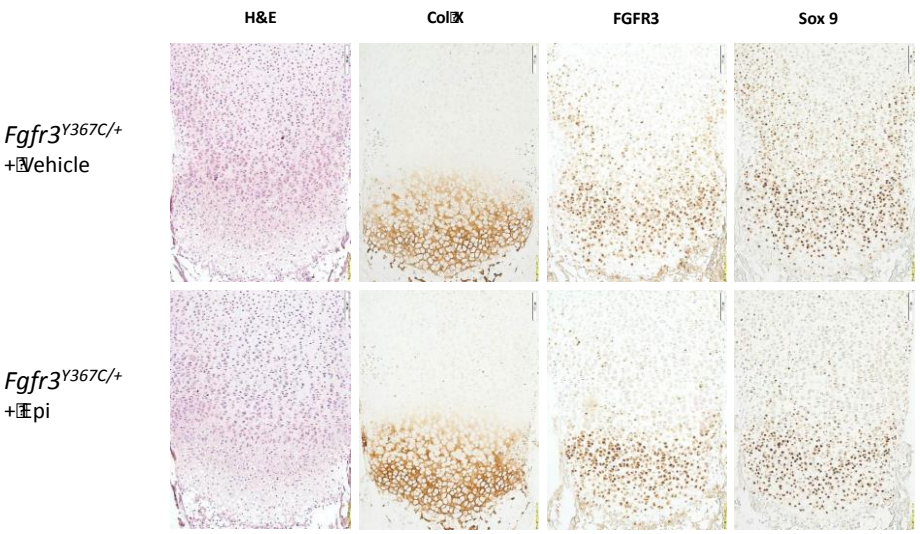


Figure 21. Representative pictures of histological staining and immunohistological labelling of the distal femurs treated with (-)-epicatechin.

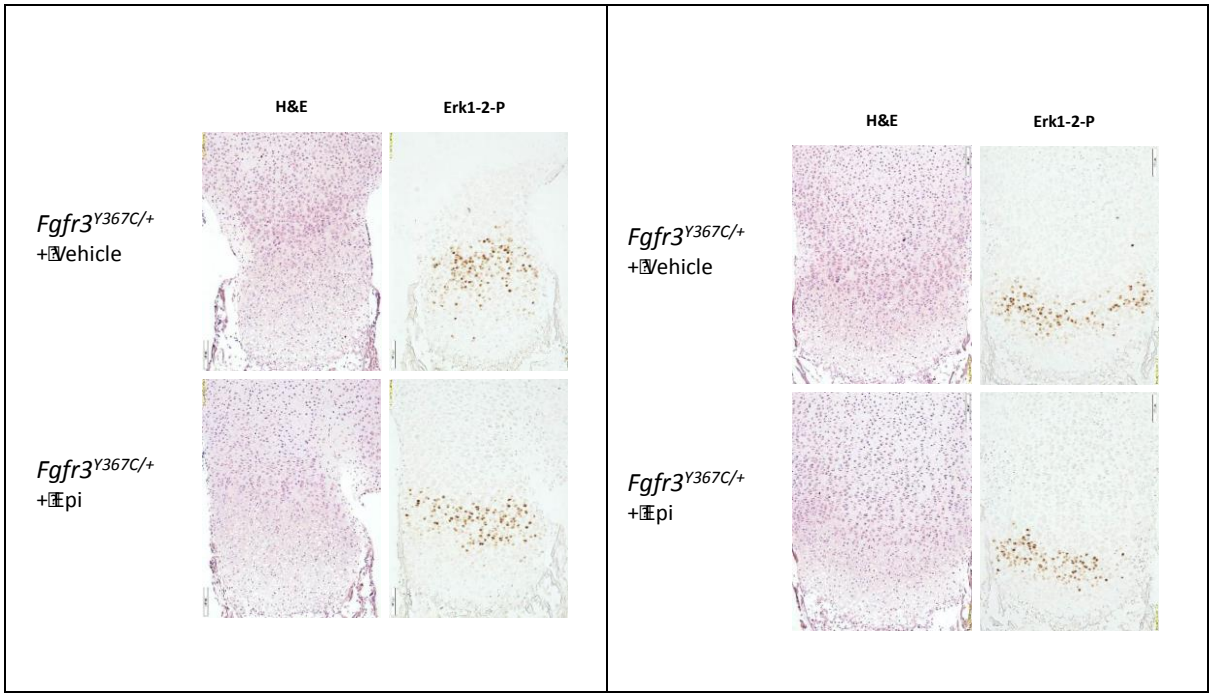


Figure 22. Representative pictures of phosphorylated Erk1/2 immunohistological labelling of the proximal and distal femurs treated with (-)-epicatechin.

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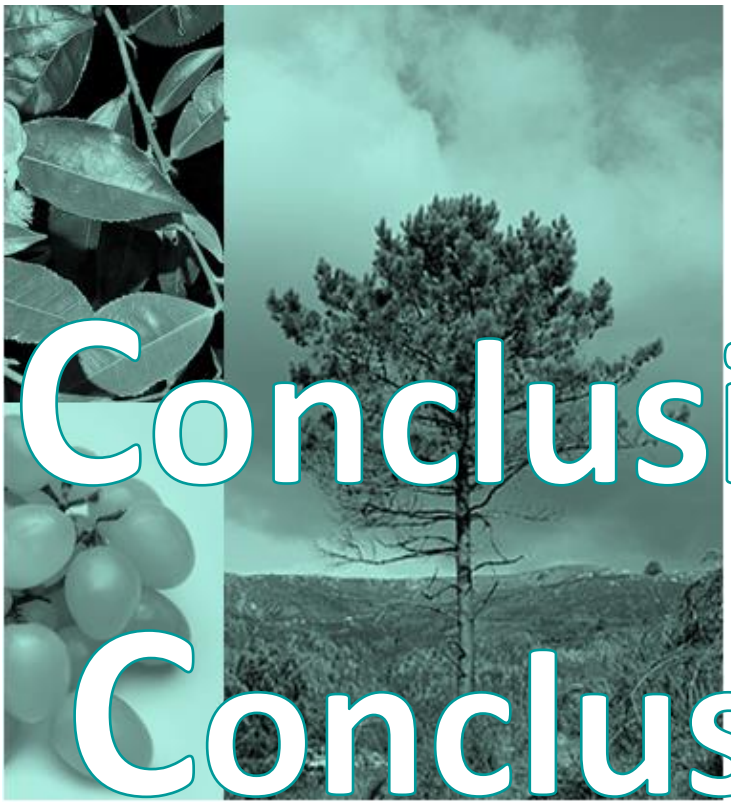

Conclusiones

Conclusions

CONCLUSIONES

1. Mediante la plataforma HPLC-ESI-TOF-MS se pudieron identificar un total de 71 compuestos distribuidos en 5 categorías, de los que un alto porcentaje aparecen de forma conjugada con ácido gálico. Hay que resaltar la presencia de dos compuestos derivados del ácido gálico como son: el dimetoxi-hidrofenil-O-galoil glucopiranosa y el hidroxi-metoxifenil-O-galoil glucopiranosa, que han sido descritos por primera vez en *S. birrea*. Con respecto al proceso de extracción, en términos de rendimiento podemos decir que PLE representa la mejor opción ya que con esta metodología se obtuvieron los mejores resultados en las condiciones cuando la temperatura y el porcentaje de disolvente fueron 176 °C y 50 % (etanol:agua) respectivamente. Además, según los resultados, el mayor número de compuestos fue extraído con PLE, generalmente cuando la temperatura alcanzaba valores bajos, sobre todo para los flavonoides en su forma aglicona. No fue así, para el caso del ácido gálico y algunos derivados de (epi)catequina. Por otro lado, el mayor porcentaje de agua en SLE favorecía la extracción de un mayor número de compuestos derivados del ácido gálico y flavonoides, a excepción de algunos derivados de (epi)cateuquina, que fueron obtenidos con valores cercanos al 50 % de agua:etanol. Por último, la condición 2 de SFE resultó ser muy efectiva para extraer proantocianidinas con residuos galato. En general, las extracciones "green" como PLE representan una poderosa herramienta para obtener compuestos bioactivos de la corteza de *S. birrea* como galato-procianidinas, que pueden ser usadas como suplementos o ingredientes funcionales que permitan la revalorización de este producto.

2. HPLC-ESI-QTOF-MS/MS proporcionó la primera caracterización pormenorizada del perfil fenólico de hojas e inflorescencias de *E. bourgatii*. El uso combinado de la separación por HPLC con una columna con un tamaño pequeño de partícula y la detección mediante espectrometría de masas con analizador QTOF, resultó como una herramienta muy útil para la identificación de metabolitos secundarios de plantas. Un total de 59 compuestos fueron detectados y clasificados en cuatro categorías: ácidos cinámicos y derivados, ácidos hidroxibenzoicos y derivados, flavonoides y otros

compuestos. Además, *E. bourgatii* proporcionó buenos resultados de actividad antinflamatoria *in vitro*, disminuyendo la generación de ROS así como inhibiendo MCP-1 en células HUVEC inducidas con TNF-α. Con respecto a la capacidad antioxidante, se obtuvieron buenos resultados en ensayos tanto de transferencia electrónica como TEAC o FRAP, y de donación de hidrógenos como ORAC.

3. La plataforma analítica HPLC–ESI-QTOF-MS/MS se ha confirmado como una poderosa herramienta para separar y detectar compuestos fenólicos y otros compuestos polares en extractos de corteza de pino y té verde. Con esta metodología, se han conseguido identificar 37 compuestos en el caso de la corteza de pino y 35 en la del té verde en base a sus propiedades cromatográficas como el tiempo de retención, los datos de la espectrometría de masas y el patrón de fragmentación. El grupo de compuestos más representativo en los dos extractos fueron los flavan-3-oles en sus formas oligoméricas. De estos compuestos, isómeros de (epi)fisetinidol-(epi)catequina y otros de chalcan-flavan-3-oles fueron tentativamente identificados por primera vez en la corteza de pino. Con respecto a su bioactividad, demostraron una fuerte capacidad para reducir radicales peroxilo determinada mediante el ensayo ORAC. Además, ambos extractos poseían una gran capacidad para donar electrones que fue evaluada gracias a los ensayos antioxidantes FRAP y TEAC. Adicionalmente, los dos extractos mostraron altos contenidos de polifenoles totales y de Flavan-3-oles.

4. HPLC-ESI-QTOF-MS/MS confirmó la presencia de 36 compuestos en un extracto comercial de semilla de uva, 30 de los cuales fueron también cuantificados con este método. El grupo más representativo de los encontrados fueron las proantocianidinas, principalmente monómeros, dímeros y en sus formas derivadas con ácido gálico. Además de estos compuestos, la floretina y sus derivados fueron identificados por primera vez en las semillas de *V. vinífera*. Estos compuestos han sido mencionados en la literatura científica por poseer actividades frente a enfermedades como efectos antitumorales. Este estudio ha demostrado que extractos de semilla de uva y cacao poseen una significante capacidad antioxidante determinada mediante los ensayos ORAC, FRAP y TEAC. Aunque es el extracto de semilla de uva el que mostró una mayor capacidad para donar electrones y un mayor contenido en polifenoles totales y flavan-3-oles. Por último, según nuestros resultados, el extracto de

cacao parece ser un potente agente para disminuir la expresión de MCP-1 en modelos de inflamación. Su mayor actividad con respecto a la semilla de uva, podría deberse a su contenido de proantocianidinas con un alto grado de polimerización. Este estudio puede aumentar el valor añadido de los subproductos del sector vinícola como son las semillas de uva.

5. HPLC-ESI-QTOF-MS/MS también separó un amplio rango de compuestos fenólicos y simultáneamente proporcionó la caracterización y cuantificación de los principales compuestos de un extracto de *T. cacao*. Además, mediante la cromatografía líquida semi-preparativa se pudieron purificar diferentes fracciones a partir del extracto completo. Un total de 61 compuestos fueron caracterizados tanto en el extracto y en las fracciones. Gracias a este trabajo se abren nuevas posibilidades para el análisis de complejas mezclas de compuestos como procianidinas contenidos en matrices alimentarias o en fuentes naturales más complejas. También se determinó la capacidad antioxidante del extracto completo y de las fracciones, en general exhibieron unos altos valores de actividad para donar electrones (FRAP y TEAC) y para reducir radicales peroxilo (ORAC), lo que podría deberse a la presencia de estas procianidinas tanto en sus formas monoméricas como poliméricas.

6. Se proporcionan datos de gran valor referentes a diferentes metodologías de purificación del extracto de *T. cacao*, además de evaluar la toxicidad de las fracciones obtenidas en el modelo celular HEK293. El uso de cartuchos Sep-Pak C18 permitió separar el extracto completo en distintas fracciones que dependían de la familia o del grado de polimerización de los compuestos, con la intención de reducir la complejidad de esta matriz. Además, la combinación de esta técnica con la HPLC semi-preparativa proporcionó fracciones mucho más purificadas, lo que facilitaría el conocimiento de los efectos sinérgicos o no que pueden afectar a la bioactividad. Por otro lado, el uso de membranas para la separación de este mismo extracto proporcionó una fracción rica en procianidinas en sus formas mono y diméricas en el permeado de la etapa de ultrafiltración debido a la baja capacidad de retención de estos compuestos. Por último, la evaluación de la toxicidad de las fracciones ricas en compuestos fenólicos se realizó mediante el ensayo MTT, que indicó una baja toxicidad de forma general en las fracciones resultantes en este estudio. Aunque algunos valores citotóxicos fueron obtenidos a altas

concentraciones en fracciones ricas en procianidinas como la de UF, y en las que se presentaba mayor grado de pureza como las SP5 y SP9, obtenidas mediante la combinación de la SPE y la HPLC semi-preprarativa.

7. Se ha proporcionado una visión global de las aplicaciones de *L. citriodora* en la prevención de enfermedades y la relación de estos efectos beneficiosos con su composición. En base a los datos de los usos tradicionales terapéuticos de esta planta, los científicos se han visto atraídos a la búsqueda de nuevos productos basados en la hierbaluisa como alternativa a los fármacos. Entre los principales componentes de *L. citriodora*, el verbascósido es el mayor componente y aunque algunos autores sostengan que los efectos que proporciona el extracto podría simplificarse al uso de este compuesto, hay evidencias que revelan que otros compuestos minoritarios podrían influir en las propiedades beneficiosas que aporta la hierbaluisa. Este hecho explica la eficacia que se ha visto que posee el extracto completo frente al compuesto en modelos de obesidad. Por todas las evidencias que se recogen en los estudios preclínicos y clínicos, esta planta merece especial atención a la hora de desarrollar ingredientes naturales o formulaciones a partir de ella que actúen como coadyuvantes en tratamientos actuales. Por último, se ha remarcado la necesidad de estudios que compilen información sobre aproximaciones farmacocinéticas y metabolómicas.

8. Un total de 29 compuestos fueron tentativamente identificados gracias a la plataforma analítica HPLC-ESI-TOF-MS, siendo los fenilpropanoides la mayor clase de compuestos detectada en este extracto. Por primera vez en *L. citriodora* se han identificados compuestos como: un nuevo isómero cafeoil-ramnopiranosil glucopiranósido, shanzisido metil-ester, sacranósido A y diooflavona. Teniendo en cuenta que está demostrado que el extracto completo ha mostrado mayor actividad para activar AMPK en modelos de obesidad frente a su compuesto mayoritario, verbascósido, se ha utilizado la cromatografía líquida semi-preparativa para evaluar esta modulación por parte de compuestos más individualizados. La fracción que contenía solo verbascósido mostró la mayor capacidad para activar AMPK, pero otras fracciones que contenían combinaciones de iridoides y fenilpropanoides o las que contenían terpenos, fenilpropanoides y flavonas, también mostraron altos

valores. Cabe destacar, que fracciones que contenían combinaciones de fenilpropanoides y flavonas aunque activaban AMPK mostraron efectos citotóxicos a mayores concentraciones, probablemente debido a la presencia de algunas flavonas. En base a estos resultados, se ha podido extraer que la combinación de compuestos específicos sería la causa de la fuerte capacidad para activar la AMPK en el extracto completo, haciéndose necesario el estudio de estas combinaciones para encontrar un potente efecto sinérgico en su composición. La importancia de este conocimiento radica en que esta planta puede ser una fuente potencial de ingredientes funcionales.

9. Cada año se generan billones de toneladas de biomasa por la industria agrícola en todo el mundo incluyendo residuos sólidos, líquidos y gaseosos, y podrían ser considerados como una de las fuentes renovables más abundantes y baratas. Muchos de estos residuos pueden causar problemas medioambientales si no se manejan adecuadamente. Es por lo que se presentan distintas estrategias para la utilización de estos residuos en el desarrollo de productos con alto valor añadido. Los compuestos fenólicos con capacidad antioxidante, entre ellos las proantocianidinas, se encuentran en muchos de estos subproductos agroindustriales. Además, estos compuestos han demostrado un gran potencial frente a radicales libres tanto en modelos *in vitro* como *in vivo*. Además, se le han atribuido un amplio rango de efectos farmacológicos como anti-microbianos, anti-cancerígenos y como protectores cardiovasculares, que los hace potencialmente atractivos para el tratamiento y la prevención de enfermedades.

10. El estudio del comportamiento de distintos polifenoles en modelos de oxidación e inflamación a partir de extractos de cacao y de hierbaluisa ha demostrado que las procianidinas típicas del primero ejercen un efecto de recuperación parcial frente al daño producido en el metabolismo energético durante el estado prooxidante en células PON-1 KO. En el caso de *L. citriodora* posee ese mismo efecto pero en el modelo de inflamación, probablemente debido a la acción antiinflamatoria del verbascósido, que inhibiría NF-κB disminuyendo así, la expresión de MCP-1. Además, se ha hecho notable la necesidad de profundizar más en la implicación y el impacto de los polifenoles en la disminución de los niveles de α-KG.

11. El resultado de la invención proporciona un nuevo método para el tratamiento de enfermedades relacionadas con el FGFR-3 como la acondroplasia que consiste en la administración de (-)-epicatequina obtenida de un extracto de *T. cacao*. El método propuesto para la purificación de este compuesto ha sido la combinación de la extracción en fase sólida con cartuchos Sep-Pak C18 y la HPLC semi-preparativa. La composición de cada fracción purificada fue establecida mediante la plataforma analítica HPLC-ESI-TOF-MS. Después de realizar diversos test biológicos preliminares, la fracción 5 fue considerada como candidata para los experimentos en un modelo de acondroplasia. Además, la identificación de los compuestos que estaban contenidos en esta fracción se llevó a cabo tanto por HPLC-ESI-TOF-MS como por RMN, que confirmaron que el compuesto mayoritario era (-)-epicatequina. El tratamiento de líneas de condrocitos TD (discondrodisplasia tibial) con la F5 mostró una fuerte reducción del nivel de fosforilación de ERK1/2, además de en células de acondroplasia. Teniendo en cuenta los resultados obtenidos en la identificación de la fracción y los de bioactividad, se testó tanto la F5 como un estándar comercial de (-)-epicatequina en cultivos de fémur de ratones acondroplásicos. La F5 incrementó notablemente la longitud del fémur $Fgfr^{3Y367C/+}$ en comparación con $Fgfr3^{+/+}$. El estándar comercial incrementó un 10% la longitud del fémur. Además, los análisis inmunohistológicos mostraron modificaciones en la expresión del colágeno tipo X en la región distal y proximal de los fémures tratados con F5. Así como una disminución en la expresión de FGFR3 y SOX9. Los estudios mediante tinción con H&E revelaron que el tratamiento con (-)-epicatequina mejoraba el crecimiento del cartílago en la placa epifisaria. Además, las zonas de reserva, proliferativa y pre-hipertrófica de la epífisis también se vieron aumentadas. Las expresiones de FGFR3 y SOX9 fueron fuertemente disminuidas en la zona proximal y distal del fémur. Por último, también se vio ligeramente reducida la expresión de ERK1/2 fosforilada por la acción del tratamiento con (-)-epicatequina.

CONCLUSIONS

1. HPLC-ESI-TOF-MS provided a total of 71 compounds with a significant large percentage of them as a galloyl form distributed in five major categories from marula bark. Notably, two gallic acid derivatives, dimethoxy-hydroxyphenyl-O-galloyl glucopyranoside and hydroxyl-methoxyphenyl-O-galloyl glucopyranoside, have been described for first time in *S. birrea*. Regarding the extraction process, in terms of yield, PLE represents the best option since this methodology got the highest values for the conditions where the temperature and the percentage of ethanol were set over 176 °C and 50 % (ethanol:water). According the results, the largest number of compounds were extracted with PLE, generally when the temperature achieves low values above all flavonoid aglycones, but not in case of gallic acid and minor (epi)catechin derivatives. On the other hand, the high percentage of water in SLE methodology provided a major number of gallic acid derivatives and flavonoids except in case of some (epi)catequin derivatives that are obtained at around 50 % of water:ethanol. In the end, SFE2 did prove effective as a way of extracting proanthocyanidins with galloyl residues. In general, "green" extractions as PLE represent a powerful tool to obtain bioactive compounds from *S. birrea* bark as galloyl-procyanidins, which can be used as supplements or food ingredients allowing the appreciation of this crop.

2. HPLC-ESI-QTOF-MS/MS platform has been used in the first comprehensive characterization of leaf and inflorescence extracts from *E. bourgatii*. The combined use of HPLC separation with a small particle size column assisted by mass spectrometric detection with mass analyser, QTOF, proved to be a useful tool for identifying secondary metabolites produced by plants. A total of 59 compounds were detected and classified into four groups as cinnamic acids and derivatives, hydroxybenzoic acids and derivatives, flavonoids and other compounds. Moreover, *E. bourgatii* extract showed antioxidant capacity by mechanisms based on electron transfer (TEAC, and FRAP) and hydrogen atom transfer (ORAC), and anti-inflammatory properties, decreasing reactive oxygen species generation as well as inhibiting MCP-1 and their transcripts production, respectively, in TNF-α-induced HUVEC cell line.

3. HPLC–ESI-QTOF-MS/MS platform has been confirmed as a powerful analytical technique for separating and detecting phenolic and other polar compounds in concentrated pine bark and green tea extracts. With this method, 37 compounds were tentatively identified in pine bark extract and 35 compounds in green tea extract based on their chromatographic properties as retention time, MS data and MS/MS fragmentation pattern. The most representative groups of compounds tentatively identified were flavan-3-ols (oligomeric forms). Of these compounds, (epi)fisetinidol-(epi)catechin isomers and other chalcan-flavan-3ol isomers have been tentatively identified for the first time in pine bark. These extracts showed significant antioxidant capacity to reduce peroxyl radicals determinated by the ORAC assay. Moreover, both extracts showed a strong capacity to donate electrons by FRAP and TEAC assays. Additionally, they both had high phenolic and flavan-3-ol contents.

4. HPLC-ESI-QTOF-MS/MS confirmed the presence of 36 compounds in grape seed extract, 30 compounds of them have been quantified. The most representative groups of compounds tentatively identified and quantified were proanthocyanidins (mainly monomers, dimers and galloyl derivatives). Besides these compounds, phloretin and its derivatives have been tentatively identified for the first time in *V. vinifera* seeds. These compounds have been reported to show several activities against diseases, i.e., antitumor effects. Grape seed and cocoa extracts possess a significant antioxidant capacity to reduce peroxyl radicals by ORAC assay. Moreover, grape seed extract shows a stronger capacity to donate electrons by FRAP and TEAC assays, and a higher phenolic and flavan-3-ol contents. Finally, cocoa extract seems to have a better potential decreasing the expression of MCP-1, and therefore to prevent inflammation than grape seed extract due to its content on proanthocyanidins with high-degree of polymerization. This work provides a better understanding of industrial byproduct from the winemaking process such as seeds. The importance of knowledge concerning this byproduct composition and activities is increasing due to its cheap source for the extraction of antioxidant compounds.

5. HPLC-ESI-QTOF-MS/MS separated a wide range of phenolic compounds and simultaneously provided the tentative characterization and the quantification of the major compounds of *T. cacao* extract. HPLC semi-preparative platform permitted to obtain different purified fractions from the whole extract. A total of 61 compounds were characterized and quantified in the *T. cacao* extract and fractions. This work also opens new possibilities for the analysis of such complex compounds as procyanidins in other food and natural complex sources. In addition, *T. cacao* extract and fractions exhibited a strong capacity to donate electrons (FRAP and TEAC) and a significant antioxidant capacity to reduce peroxyl radicals by hydrogen atom transfer (ORAC). It could be due to the presence of procyanidins (monomers and oligomers).

6. Valuable data concerning different methodologies for purification of *T. cacao* compounds and the safety of the obtained fractions in HEK293 cell model was reported in this study. Sep-Pak C18 cartridges permitted to separate the whole extract in fractions depending on the family or polymerization degree in order to reduce the complexity of the matrix. In addition, the combination of this technique and semi-preparative HPLC provided fractions with one or two compounds that would facilitate knowledge of their individual or synergic bioactivities. On the other hand, the use of membrane technology allowed to obtain a monomeric and dimeric PC-rich fraction in the UF permeate due to the low rejection capacity towards these compounds. The assessment of the toxicity of polyphenolic fractions through MTT assay indicated a lack of toxicity. In general, the resulting fractions in this study did not deliver toxic values, more purified compounds exhibited cytotoxic values over 80% as PC-rich fraction (UF) and the most purified, SP5 and SP9, but only at higher concentrations.

7. An overview of the applications of *L. citriodora* in disease prevention has been offered. Reported data provide a basis for reviving the traditional therapeutic uses of *L. citriodora* based on modern scientific knowledge supported by safety issues. This could attract the attention of scientists and researchers looking for new drugs based on *L. citriodora* product which is supposed to be a potential alternative to chemical drugs.

Among the chemical components of *L. citriodora*, verbascoside is the major component detected. Although some authors reasoned that the effects of the extract could be simplified by the use of verbascoside, evidences revealed that other minor components might contribute to the beneficial properties of *L. citriodora*. This fact would explain the higher efficacy of the *L. citriodora* extract in comparison with the isolated verbascoside. Therefore, based on the evidences, *L. citriodora* deserves further attention for additional preclinical and clinical studies in order to develop herbal ingredients or formulations that may act as adjuvants to current medications, studies that will necessarily encompass pharmacokinetic and metabolomic approaches.

8. A total of 29 compounds were tentatively identified in the present study by HPLC-ESI-TOF-MS being phenylpropanoids the major class of compounds found in the extract. For first time in this specie, a new isomer from caffeoyl-rhamnopyranosyl glycopyranoside (peak 14), shanziside methyl ester (peak 16), sacranoside A (peak 20) and diooflavone (peak 25) were detected. Since *Lippia citriodora* extract has exhibited higher capacity to active AMPK than pure verbascoside in insulin-resistant hypertrophic adipocyte model, the bioassay-guided fractionation of *Lippia citriodora* extract has been achieved by HPLC semi-preparative to identify those compounds contributing to such capacity. Fraction containing pure verbascoside (F7) showed the highest activating capacity. Other fractions enriched in a combination of iridoids and phenylpropanoids (F1, F3) or fractions containing terpenes, phenylpropanoids and flavones (F6, F10) also showed a significant activating capacity. Fractions containing a combination of phenylpropanoids and flavones (F8, F9 and F11) activated AMPK but showed a cytotoxic effect when the concentration was increased, probably due to the presence of some flavones. Based on the results, we hypothesized that the combination of specific phenylpropanoids, iridoids and flavones may be the responsible for the strong activating capacity observed in the whole extract. Further research using combinations of pure compounds may be required to delimit the compounds responsible for such activity and to find for a potential synergistic effect. The importance of knowledge concerning this product composition and activities is interesting

due to its potential source for the obtainment of bioactive compounds in order to be used as functional ingredients in food industry.

9. Since billions of metric tons of biomass are generated every year by the agricultural industry worldwide including liquid, solid and gaseous residues, they may be considered one of the most abundant, cheap and renewable resources on earth. Many of these residues can cause environmental problems when they are not managed properly. In this way, different strategies of residue utilization from the agriculture industry have been develope as a source for high value-added products. Phenolic compounds with antioxidant activity, subdivided into the following classes: phenolic acids, flavanones, flavones and flavonols, anthocyanidins, flavan-3-ols and proanthocyanidins, have been identified in several agricultural by-products. Several studies clearly demonstrate that proanthocyanidins from different kinds of agro-industrial by-products have significant potential to scavenge free radicals both *in vitro* and *in vivo* models. Furthermore, a number of pharmacological effects have also been reported, *e.g.*, anti-viral, anti-microbial, antioxidative, anti-tumor promoting properties, as well as cardiotonic and anti-arteriosclerotic activities. The results suggest that these by-products may potentially provide the means for the treatment and prevention of human diseases.

10. The different behaviour of polyphenols as antioxidant and anti-inflammatory molecules has been studied. *T. cacao* extract is responsible to partially return the impairment in the energy management during a pro-oxidant status in PON-1 KO cells maybe through the antioxidant properties of procyanidins. On the contrary, *L. citriodora* extract possess a partial effect on the energy metabolism in a pro-inflammatory scenario probably due to the action of verbascoside which may inhibit NF-κB and, thus, decrease the expression of MCP-1. More investigations are needed to assess the implications and impact of polyphenols to decrease the levels of α-KG.

11. The invention relates to a method of treating a FGFR3-related chondrodysplasia in a patient in need thereof consisting in administering to the subject a therapeutically effective amount of a substantially pure (-)-epicatechin obtained from *T. cacao*. The proposed method by Sep-Pak C18 cartridges permitted separating phenolic compounds from the whole *T. cacao* extract into various

different fractions, which were less complex, enabling their use in order to achieve pure fractions of interested compounds. In addition, eleven fractions were obtained by combination of solid phase extraction by Sep-Pak C18 cartridges and semi-preparative HPLC isolation in order to obtain purified fractions of monomeric and oligomeric proanthocyanidins. The composition of each one was established by the detailed HPLC-ESI-TOF-MS. After preliminary biological tests of the obtained fractions, F5 was selected as a candidate for the biological experiments in achondroplasia model. In addition to HPLC-ESI-TOF-MS identification, NMR experiments confirmed that F5 was mainly composed of (-)-epicatechin. The treatment of the TD chondrocyte lines with F5 (100 µg/ml), showed a strong reduction of the level of Erk1/2 phosphorylation and a significant reduction of Erk1/2 phosphorylation was observed for this fraction in ACH cells. Regarding the data obtained *in vitro* and the data of the NMR analysis, F5 and commercial standard of (-)-epicatechin were tested in femur cultures. F5 significantly increased the length of the *Fgfr3Y367C/+* femurs comparing to *Fgfr3+/+* femurs and (-)-epicatechin (10 µg/ml) significantly increased the length (+10%) of the *Fgfr3Y367C/+* femurs comparing to *Fgfr3+/+* femurs. Moreover, the immunohistological analyses showed a slight modification of the collagen type X expression in proximal and distal femurs treated with F5. We noted a decreasing expression of FGFR3 and SOX9. Studying the H&E staining, we observed that the (-)-epicatechin treatment improved the whole growth plate cartilage. The reserve, proliferative and pre-hypertrophic zones of the growth plate cartilage were increased. The expressions of FGFR3 and Sox9 were strongly decreased in proximal and distal femurs. Interestingly, the phosphorylated Erk1/2 expression was slightly decreased in proximal and distal femurs treated with (-)-epicatechin.