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Grupo de Investigación AGR-274 "Bioactive ingredients"

**NUEVOS ENFOQUES ANALÍTICOS EN LA EVALUACIÓN DEL
POTENCIAL DE COMPUESTOS BIOACTIVOS PROCEDENTES DE
FUENTES VEGETALES PARA EL DESARROLLO DE PRODUCTOS DE
ALTO VALOR AÑADIDO**

Memoria presentada por

María del Carmen Villegas Aguilar

Para optar al grado de

Doctora Internacional por la Universidad de Granada

Tesis doctoral dirigida por

Dr. David Arráez Román

Dra. María de la Luz Cádiz Gurrea

Granada, 2024



UNIVERSIDAD
DE GRANADA



Dpto. de Química Analítica
Prof. Fermín Capitán García

**Nuevos enfoques analíticos en la evaluación del potencial de
compuestos bioactivos procedentes de fuentes vegetales para el
desarrollo de productos de alto valor añadido**

*New analytical approaches in the evaluation of the potential of bioactive
compounds from plant sources for the development of high value-added
products*

Memoria presentada por María del Carmen Villegas Aguilar para optar al Grado de
Doctora Internacional por la Universidad de Granada

Granada, septiembre 2024

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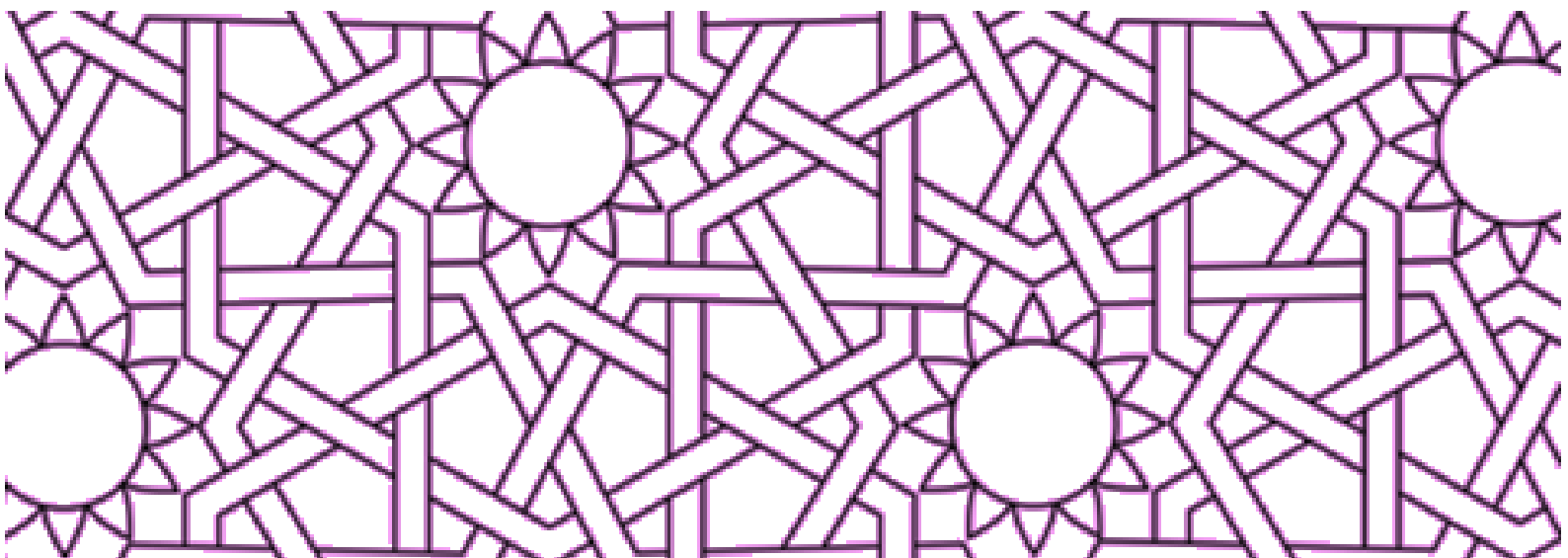


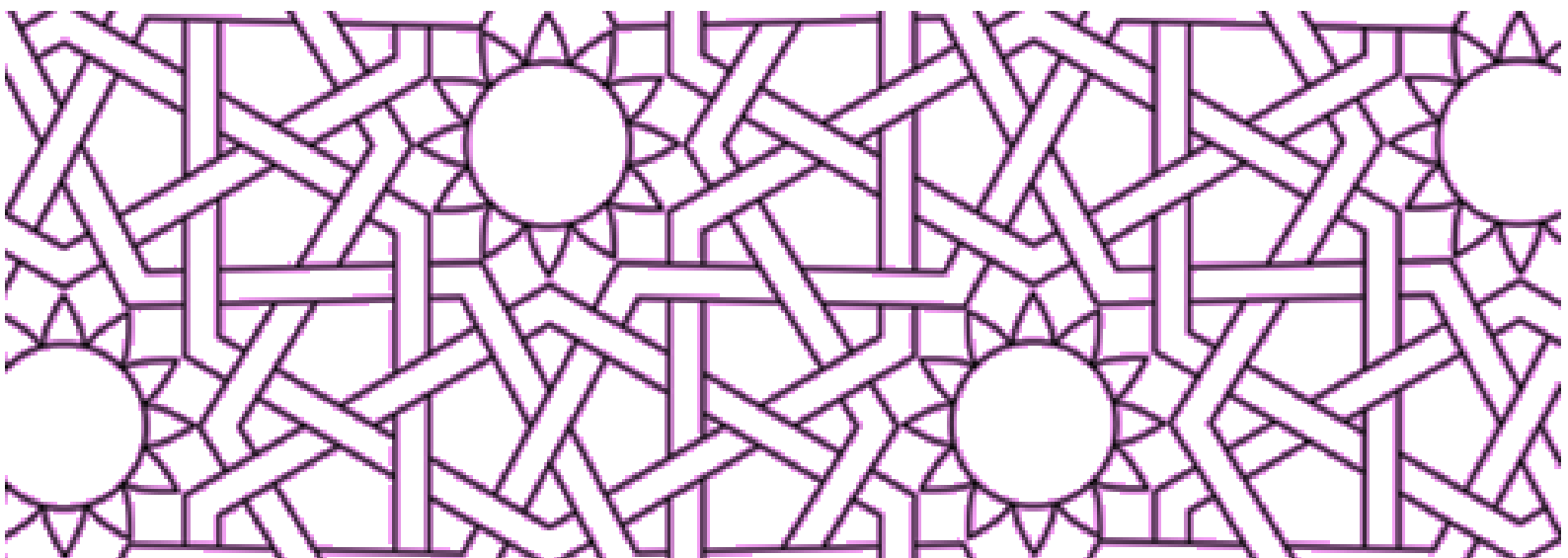
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Abbreviations



List of Abbreviations

¹O₂ (*Singlet oxygen*): Oxígeno singlete

AAPH (*2,2'-azobis(2-amidinopropane) dihydrochloride*): 2,2'-Azobis-(2-aminopropano)-dihidrocloruro

ABTS (*2,2'-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid*): Ácido 2,2'-azinobis(3-etilbenzotiazolina)-6-sulfónico

ACh (*Acetylcholine*): Acetilcolina

AChE (*Acetylcholinesterase*): Acetilcolinesterasa

ANOVA (*Analysis of variance*): Análisis de varianza

ATCI (*Acetylthiocholine*): Acetiltiocolina

COMTs (*Catechol-O-methyltransferases*): Catecol-O-metiltransferasas

DAF-2 (*4,5-diaminofluorescein*): 4,5-diaminofluoresceína

DCF (*2',7'-dichlorodihydrofluorescein*): 2',7'-diclorofluoresceína

DMSO (*Dimethylsulfoxide*): Dimetilsulfóxido

DNA (*Deoxyribonucleic acid*): Ácido desoxirribonucleico

DTNB (*5,5'-Dithio-bis-(2-nitrobenzoic acid)*): Ácido 5,5'-ditiobis(2-nitrobenzoico)

ECM (*Extracellular matrix*): Matriz extracelular

EDTA (*Ethylenediaminetetraacetic acid*): Ácido etilendiaminotetraacético

EE (*Encapsulation efficiency*): Eficiencia de encapsulación

EFSA (*European Food Safety Authority*): Autoridad Europea de Seguridad Alimentaria

ESI (*Electrospray ionisation*): Ionización por electrospray

FALGPA (*N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala*): N-[3-(2-Furil)acrilolil]-Leu-Gly-Pro-Ala

FDA (*United States Food and Drug Administration*): Administración de Alimentos y Medicamentos de los Estados Unidos

FRAP (*Ferric Ion Reducing Antioxidant Power*): Poder antioxidante reductor de iones férricos

H2DCF-DA (*2',7'-dichlorodihydrofluorescein diacetate*): Diacetato de 2',7'-diclorofluoresceína

H₂O₂ (*Hydrogen peroxide*): Peróxido de hidrógeno

HaCaT (*Human immortalised keratinocytes*): Queratinocitos humanos inmortalizados

HMDB (*Human Metabolome Database*): Base de datos del metaboloma humano

HO[•] (*Hydroxyl radical*): Radical hidroxilo

HOCl (*Hypochlorous acid*): Ácido hipocloroso

HPLC (*High-performance liquid chromatography*): Cromatografía de líquidos de alta resolución

HPLC-MS (*High performance liquid chromatography coupled with mass spectrometry*): Cromatografía de líquidos de alta resolución acoplada a espectrometría de masas

IL (*Interleukin*): Interleuquina

LC (*Liquid chromatography*): Cromatografía de líquidos

m/z (*Mass-to-charge ratio*): Relación masa/carga

MAE (*Microwave-assisted extraction*): Extracción asistida por microondas

MS (*Mass spectrometry*): Espectrometría de masas

MTT (*3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide*): Bromuro de 3-(4,5- di-metiltiazol-2-ilo)-2,5-difeniltetrazol

MyD88 (*Myeloid differentiation factor 88*): Factor 88 de diferenciación mieloide

NBT (*Nitroblue tetrazolium*): Nitroazul de tetrazolio

NF- κ B (*Nuclear factor- κ B*): Factor nuclear-kappa B

NO[•] (*Nitric oxide anion*): Anión óxido nítrico

Nrf2 (*Nuclear factor erythroid 2-related factor 2*): Factor nuclear eritroide 2-relacionado con el factor 2

O₂^{•-} (*Superoxide anion*): Anión superóxido

ORAC (*Oxygen Radical Absorbance Capacity*): Capacidad de absorción de radicales de oxígeno

PCA (*Principal component analysis*): Análisis de componentes principales

PLE (*Pressurized liquid extraction*): Extracción con líquidos presurizados

Q (*Quadrupole*): Analizador cuadrupolo

QTOF (*Quadrupole-time-of-flight*): Analizador cuadrupolo-tiempo de vuelo

RNS (*Reactive nitrogen species*): Especies reactivas del nitrógeno

ROS (*Oxygen reactive species*): Especies reactivas del oxígeno

RP-HPLC (*Reversed phase high-performance liquid chromatography*): Cromatografía de líquidos de alta resolución de fase reversa

RT (*Retention time*): Tiempo de retención

S/N (*Signal-to-noise ratio*): Relación señal/ruido

SFE (*Supercritical fluid extraction*): Extracción con fluidos supercríticos

SLE (*Solid-liquid extraction*): Extracción sólido-líquido

SULTs (*Sulfotransferases*): Sulfotransferasas

TEAC (*Trolox-Equivalent Antioxidant Capacity*): Capacidad antioxidante equivalente a Trolox

TNF (*Tumour necrosis factor*): Factor de necrosis tumoral

TOF (*Time-of-flight*): Analizador tiempo de vuelo

UAE (*Ultrasound-assisted extraction*): Extracción asistida por ultrasonidos

UGTs (*Uridine-5'-di-phosphate glucuronosyltransferases*): Uridina-5'-di-fosfato glucuronosiltransferasas

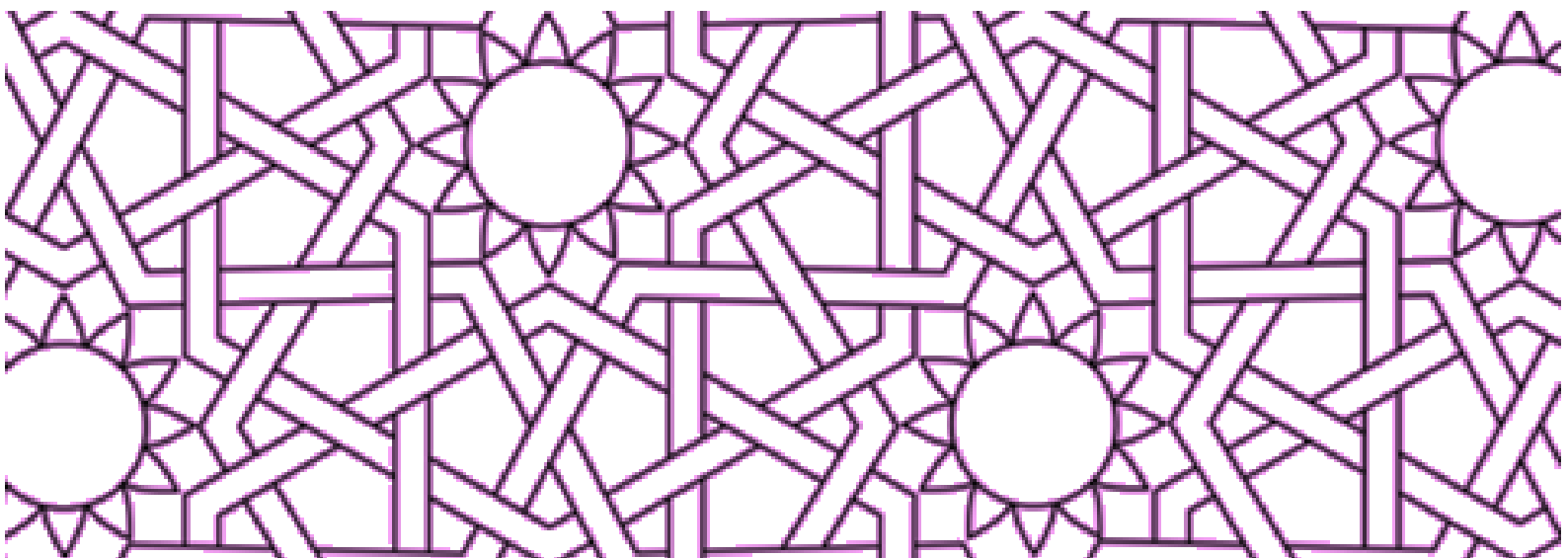
UV (*Ultraviolet*): Ultravioleta

UVA (*Ultraviolet radiation type A*): Radiación ultravioleta tipo A

WHO (*World Health Organization*): Organización Mundial de la Salud

XO (*Xanthine oxidase*): Xantina oxidasa

Figures & Tables



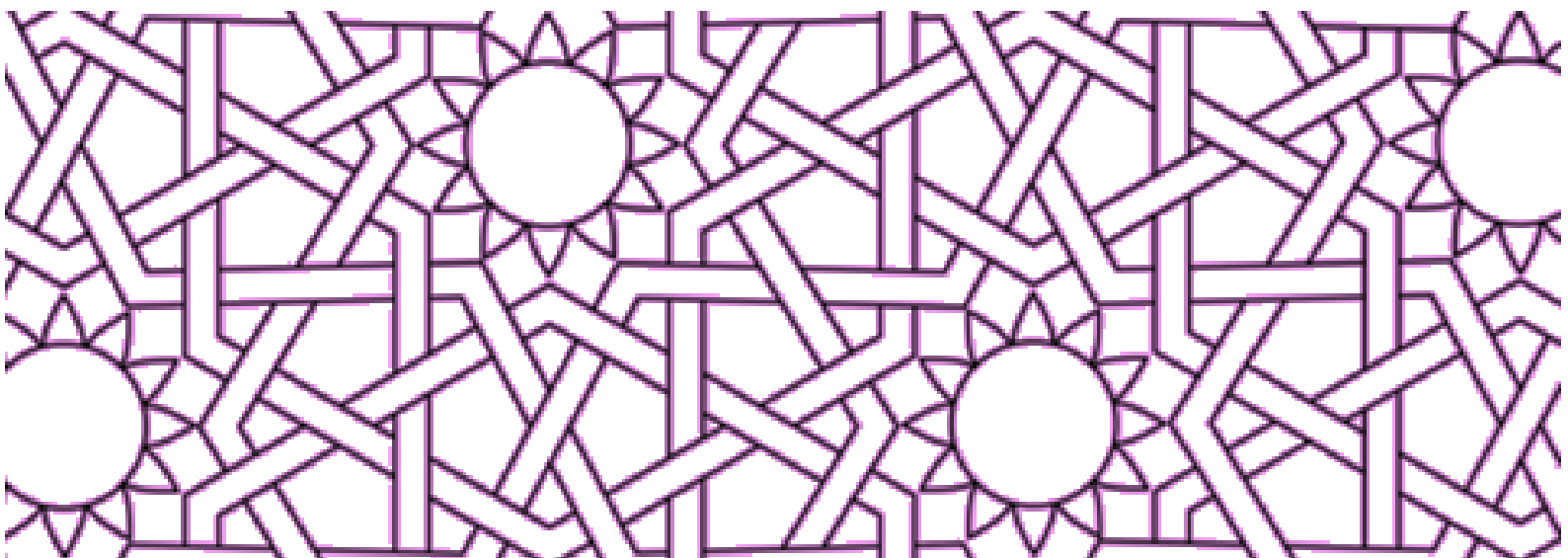
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Summary

Resumen



Summary

The present report presents the results obtained during the preparation of the doctoral thesis entitled **“New analytical approaches in the evaluation of the potential of bioactive compounds from plant sources for the development of high value-added products”**. Both traditional and innovative approaches have been used to study the phenolic profile of different plant extracts and their potential beneficial effect on chronic diseases. This report has been divided into two main sections: introduction and experimental part.

The **INTRODUCTION** section first describes the role of phenolic compounds in the prevention and/or treatment of various chronic diseases, highlighting their antioxidant, anti-inflammatory, neuroprotective and anticarcinogenic potential, as well as their pleiotropic effect. The concept of bioavailability and the main metabolic reactions that these compounds can undergo are also introduced. Furthermore, nanoencapsulation is highlighted as a strategy to optimise their bioavailability. Next, the traditional methodological approach to the study of phenolic compounds is detailed, including the selection of plant matrices, the extraction of phenolic compounds from these matrices, the analytical determination, the evaluation of their bioactive properties both *in vitro* and *in vivo* and finally the development of high added value products. Finally, a new methodological approach for the analysis of phenolic compounds is presented, highlighting the application of metabolomics for the study of the metabolism and bioavailability of these phenolic compounds in nutritional intervention studies. The methodology adopted in this doctoral thesis is based on untargeted metabolomics strategies, and the workflow of this approach is detailed.

The **EXPERIMENTAL PART** presents the studies, organised into chapters, carried out during the doctoral thesis, which is in turn divided into two blocks. **SECTION A** focuses on the use of a traditional methodological approach to study the bioactivity of phenolic compounds present in the plant extracts *Hibiscus sabdariffa*, *Theobroma cacao*, *Silybum marianum*, *Lippia citriodora* and *Olea europaea*, while in **SECTION B** a new methodological approach for the analysis of phenolic compounds is carried out, searching for, identifying and evaluating the anticancer capacity of metabolites derived from phenolic compounds present in plasma after an acute nutritional intervention study.

SECTION A is composed of three chapters. **Chapter 1** compiles information on the effect exerted by the phenolic compounds of the five matrices studied in this PhD thesis and their metabolites on the main metabolic pathways involved in energy metabolism, inflammatory response, ageing and their relationship with the biological properties reported in highly prevalent chronic diseases. The pleiotropic effects of phenolic compounds, as demonstrated in numerous *in vitro* and *in vivo* studies, are highlighted.

Chapter 2 analysed the antioxidant, anti-inflammatory and neuroprotective bioactive potential of the five plant extracts mentioned above, which have different phenolic profiles (determined by HPLC-ESI-QTOF-MS). Additionally, the structure-activity relationship mechanisms of the phenolic compounds present in these plant sources were studied.

In **Chapter 3**, the development of nanovesicles using *O. europaea* leaf extract was carried out in order to explore their potential anticancer properties, specifically in breast cancer, using both tumour and non-tumour cell lines to test their selective antitumour efficacy. This work was conducted in collaboration with the Department of Biochemistry and Molecular Biology I at the University of Granada, thanks to a "Proyectos de Investigación Precompetitivos para Jóvenes Investigadores" granted by the University of Granada.

SECTION B comprises three chapters and focuses on the study of metabolites identified in plasma after the administration of plant extracts from *H. sabdariffa*, *S. marianum*, *T. cacao*, *L. citriodora*, and *O. europaea* in an acute nutritional intervention study in humans within the framework of the National Plan Research Project (RTI2018-096724-B-C22) funded by the Ministry of Science, Innovation and Universities.

In **Chapter 4**, an untargeted metabolomics strategy based on HPLC-ESI-QTOF-MS was employed to investigate the bioavailability and metabolism of the bioactive compounds from *H. sabdariffa*, *S. marianum*, and *T. cacao* in plasma samples collected from an acute nutritional intervention study in humans. This approach enabled the identification and selection of significant variables associated with the exogenous metabolites derived from the consumption of these bioactive compounds for further identification.

In **Chapter 5**, an untargeted metabolomics strategy based on HPLC-ESI-QTOF-MS was also employed to detect common bioavailable metabolites from the extracts of *L. citriodora* and *O. europaea* in plasma samples collected at different time points in an acute nutritional intervention study in humans.

Finally, in **Chapter 6**, an *in vitro* evaluation of the anticancer activity of bioavailable metabolites in pancreatic cancer cell lines was performed based on the results obtained in Chapter 5. A selection of the metabolites identified in Chapter 5 present in plasma derived from an acute nutritional intervention study in humans with *L. citriodora* and *O. europaea* extracts was carried out. In addition, an appendix to this chapter presents the results obtained on the anticancer activity in pancreatic cancer cell lines of other metabolites identified in Chapters 4 and 5. This chapter was carried out in collaboration with the University of Davis (California, USA) thanks to a three-month pre-doctoral stay in 2023 funded by the Spanish Ministry of Universities ('Estancias Breves FPU').

Resumen

La presente memoria recoge los resultados obtenidos durante la elaboración de la tesis doctoral titulada **“Nuevos enfoques analíticos en la evaluación del potencial de compuestos bioactivos procedentes de fuentes vegetales para el desarrollo de productos de alto valor añadido”**. En ella, se ha llevado a cabo tanto un enfoque tradicional como uno innovador para estudiar el perfil fenólico de distintos extractos vegetales y su potencial efecto beneficioso en enfermedades crónicas. Esta memoria se ha dividido en dos grandes secciones: introducción y parte experimental.

En la sección de **INTRODUCCIÓN**, primero se describe el papel de los compuestos fenólicos en la prevención y/o tratamiento de diversas enfermedades crónicas, destacando su potencial antioxidante, antiinflamatorio, neuroprotector y anticancerígeno, así como su efecto pleiotrópico. También se introduce el concepto de biodisponibilidad y las principales reacciones metabólicas que pueden experimentar estos compuestos. Además, se destaca la nanoencapsulación como estrategia para optimizar su biodisponibilidad. A continuación, se detalla el enfoque metodológico tradicional para el estudio de los compuestos fenólicos, incluyendo la selección de matrices vegetales, la extracción de los compuestos fenólicos de estas matrices, la determinación analítica, la evaluación de sus propiedades bioactivas tanto *in vitro* como *in vivo* y finalmente el desarrollo de productos de alto valor añadido. Por último, se expone un nuevo enfoque metodológico para el análisis de los compuestos fenólicos, subrayando la aplicación de la metabolómica para el estudio del metabolismo y la biodisponibilidad de estos compuestos fenólicos en ensayos de intervención nutricional. La metodología adoptada en la presente tesis doctoral se basa en estrategias

metabolómicas no dirigidas, por lo que se detalla el flujo de trabajo asociado a este tipo de aproximación.

En la **PARTE EXPERIMENTAL** se presentan los trabajos, organizados en capítulos, realizados durante la tesis doctoral, y que está a su vez dividida en dos bloques. El **BLOQUE A** se centra en el empleo de un enfoque metodológico tradicional para el estudio de la bioactividad de los compuestos fenólicos presentes en los extractos vegetales *Hibiscus sabdariffa*, *Theobroma cacao*, *Silybum marianum*, *Lippia citriodora* and *Olea europaea*, mientras que en el **BLOQUE B** se lleva a cabo un nuevo enfoque metodológico para el análisis de los compuestos fenólicos, llevando a cabo la búsqueda, identificación y evaluación de la capacidad anticancerígena de los metabolitos derivados de los compuestos fenólicos presentes en el plasma tras un ensayo de intervención nutricional agudo.

El **BLOQUE A** se compone de tres capítulos. El **Capítulo 1** recopila información sobre el efecto ejercido por los compuestos fenólicos de las cinco matrices objeto de estudio de la tesis doctoral y sus metabolitos sobre las principales rutas metabólicas implicadas en el metabolismo energético, la respuesta inflamatoria, el envejecimiento y su relación con las propiedades biológicas reportadas en enfermedades crónicas de alta prevalencia. Se destaca el efecto pleiotrópico que presentan los compuestos fenólicos en numerosos estudios *in vitro* e *in vivo*.

En el **Capítulo 2** se analizó el potencial bioactivo antioxidante, antiinflamatorio y neuroprotector de los cinco extractos vegetales previamente mencionados, los cuales presentan diferentes perfiles fenólicos (determinados mediante HPLC-ESI-QTOF-MS).

Además, se estudiaron los mecanismos de relación entre la estructura y la bioactividad de los compuestos fenólicos presentes en estas fuentes vegetales.

En el **Capítulo 3** se llevó a cabo el desarrollo de nanovesículas utilizando extracto de hoja de *O. europaea*, con el fin de explorar sus potenciales propiedades anticancerígenas, concretamente en cáncer de mama, empleando tanto líneas celulares tumorales como no tumorales para comprobar la eficacia antitumoral selectiva. Este trabajo se realizó en colaboración con el Departamento de Bioquímica y Biología Molecular I de la Universidad de Granada gracias a un “Proyectos de Investigación Precompetitivos para Jóvenes Investigadores” concedido por la Universidad de Granada.

El **BLOQUE B** consta de tres capítulos y se centra en el estudio de los metabolitos identificados presentes en plasma después de la administración de extractos vegetales de *H. sabdariffa*, *S. marianum*, *T. cacao*, *L. citriodora* y *O. europaea*, dentro de un ensayo de intervención nutricional agudo en humanos enmarcado en el Proyecto De Investigación del Plan Nacional (RTI2018-096724-B-C22) financiado por el Ministerio de Ciencia, Innovación y Universidades.

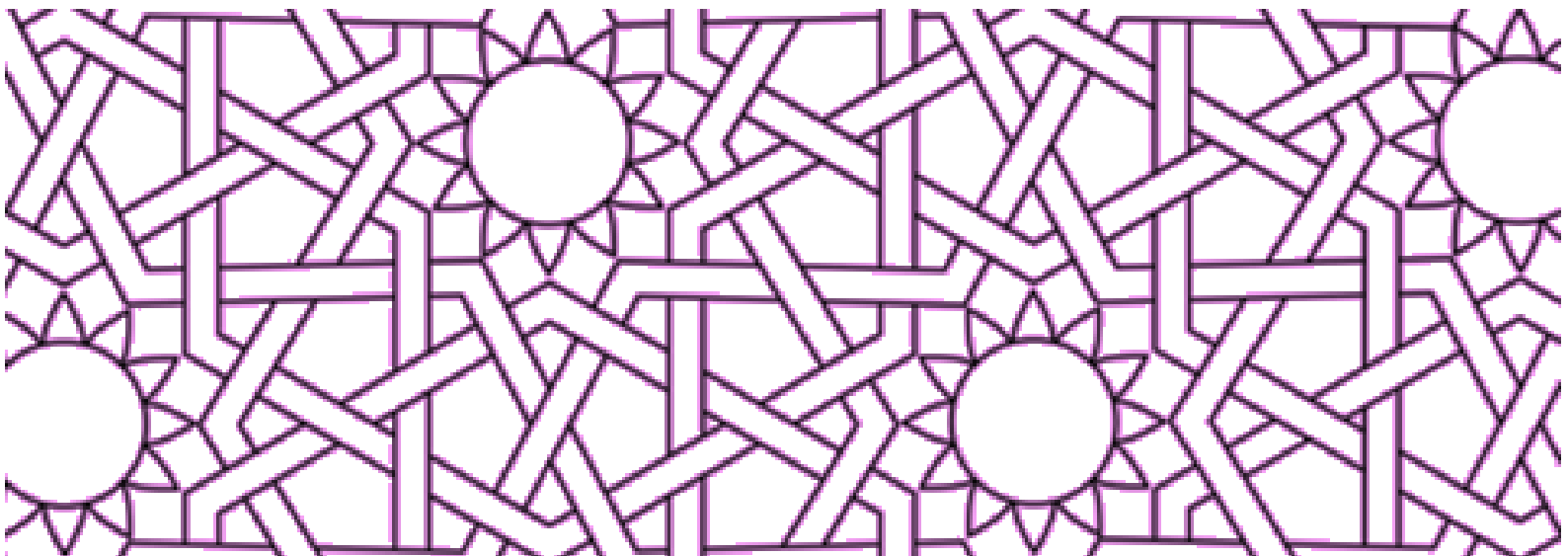
En el **Capítulo 4** se utilizó una estrategia metabolómica no dirigida basada en HPLC-ESI-QTOF-MS para estudiar la biodisponibilidad y el metabolismo de los compuestos bioactivos de las plantas *H. sabdariffa*, *S. marianum* y *T. cacao* en muestras de plasma recogidas de un estudio de intervención nutricional agudo en humanos. Este enfoque permitió identificar y seleccionar las variables significativas asociadas a los metabolitos exógenos derivados del consumo de estos compuestos bioactivos para su posterior identificación.

En el **Capítulo 5** también se empleó una estrategia metabolómica no dirigida basada en HPLC-ESI-QTOF-MS para detectar metabolitos comunes biodisponibles de los extractos de *L. citriodora* y *O. europaea* en muestras de plasma recogidas a diferentes tiempos en un ensayo de intervención nutricional agudo en humanos.

Por último, en el **Capítulo 6** se realizó una evaluación *in vitro* de la actividad anticancerígena en líneas celulares de cáncer de páncreas de los metabolitos biodisponibles en base a los resultados obtenidos en el Capítulo 5. Así, se llevó a cabo una selección de los metabolitos identificados en el Capítulo 5 presentes en plasma derivados de un ensayo de intervención nutricional agudo en humanos con extractos de *L. citriodora* y *O. europaea*. Además, se incluye un anexo a este capítulo en el que se presentan los resultados obtenidos de la actividad anticancerígena en líneas celulares de cáncer de páncreas de otros metabolitos identificados en los Capítulos 4 y 5. Este capítulo se llevó a cabo en colaboración con la Universidad de Davis (California, EEUU) gracias a una estancia predoctoral de tres meses realizada en el año 2023 y financiada por el Ministerio de Universidades (“Estancias Breves FPU”).

Objectives

Objetivos



Objectives

Traditionally, phenolic compounds have been investigated using a bottom-up approach, starting with the extraction of phenolic compounds from plant matrices, characterising and evaluating the bioactivity of phenolic compounds and then testing them in animals and humans.

Therefore, the **main objective** of this doctoral thesis is to employ both a traditional and a new top-down approach to investigate the bioactive potential of phenolic compounds present in various plant sources, such as *Hibiscus sabdariffa*, *Silybum marianum*, *Theobroma cacao*, *Lippia citriodora* and *Olea europaea*, as well as to identify the bioavailable metabolites derived from their consumption and to evaluate their beneficial effects on human health.

In this context, firstly, the characterisation and evaluation of the bioactivity of the extracts will be carried out, allowing these matrices to be analysed from a traditional perspective. Secondly, a new methodological approach to study phenolic compounds will be implemented, in which, following an acute nutritional intervention trial in humans in which nutraceuticals developed with the extracts under study will be administered, bioavailable metabolites will be selected and their bioactivity will be evaluated.

In order to achieve this general objective, the following **specific objectives** were defined:

- To study the effect of the phenolic compounds of the five matrices studied in the thesis and their metabolites on the main metabolic pathways involved in highly prevalent chronic diseases.

- To analytically determine in detail the phenolic compounds present in the different matrices selected for the study by HPLC-ESI-QTOF-MS and *in vitro* evaluation of their antioxidant, anti-inflammatory and neuroprotective capacity.
- Formulation of nanoencapsulations of phenolic compounds to improve the bioavailability of plant extracts.
- To perform an untargeted metabolomics approach based on HPLC-ESI-QTOF-MS to study the bioavailability and metabolism of circulating bioactive compounds after ingestion of nutraceuticals developed with the five selected plant extracts included in an acute human nutritional intervention study.
- To evaluate *in vitro* the anticancer activity of both plant extracts and plasma bioavailable metabolites in breast and pancreatic cancer cell lines.

Objetivos

Tradicionalmente, los compuestos fenólicos se han investigado mediante un enfoque ascendente, en el que se comienza con la extracción de compuestos fenólicos de matrices vegetales y caracterizando y evaluando la bioactividad de los compuestos fenólicos para posteriormente realizar ensayos en animales y seres humanos con estos.

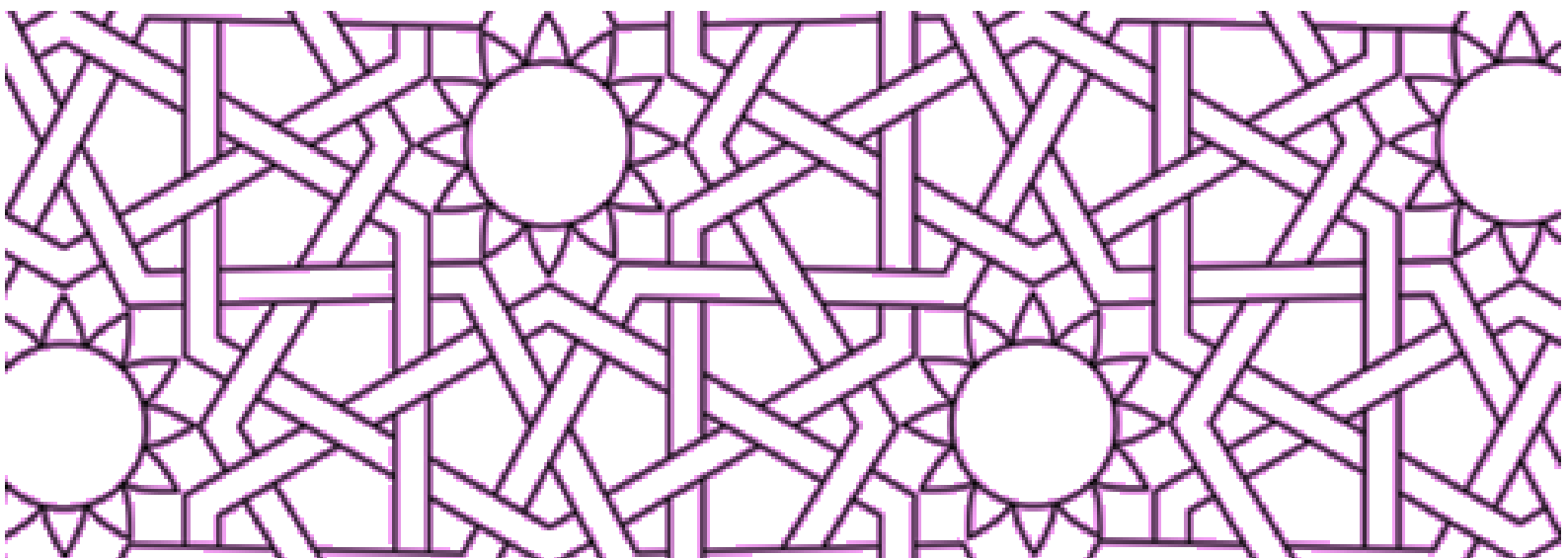
Por esto, el **objetivo principal** de esta tesis doctoral es emplear tanto un enfoque tradicional como un nuevo enfoque descendente para investigar el potencial bioactivo de los compuestos fenólicos presentes en diversas fuentes vegetales, como *Hibiscus sabdariffa*, *Silybum marianum*, *Theobroma cacao*, *Lippia citriodora* y *Olea europaea*, así como para identificar los metabolitos biodisponibles derivados de su consumo y evaluar sus efectos beneficiosos sobre la salud humana.

En este contexto, primero se llevará a cabo la caracterización y evaluación de la bioactividad de los extractos, permitiendo analizar estas matrices desde una perspectiva tradicional. En segundo lugar, se implementará un nuevo enfoque metodológico para estudiar los compuestos fenólicos, en el que, tras un ensayo de intervención nutricional agudo en humanos en el que se administrarán nutracéuticos desarrollados con los extractos objeto estudio, se seleccionaran los metabolitos biodisponibles y se evaluará su bioactividad.

Para la consecución de este objetivo general se establecieron los siguientes **objetivos específicos**:

- Estudiar del efecto que ejercen los compuestos fenólicos de las cinco matrices objeto de estudio de la tesis doctoral y sus metabolitos sobre las principales rutas metabólicas implicadas en enfermedades crónicas de alta prevalencia.
- Determinar analíticamente y pormenorizadamente los compuestos fenólicos presentes en las distintas matrices seleccionadas para el estudio mediante HPLC-ESI-QTOF-MS y evaluación *in vitro* de su capacidad antioxidante, antiinflamatoria y neuroprotectora.
- Formular nanoencapsulaciones de los compuestos fenólicos para la mejora de la biodisponibilidad de los extractos vegetales.
- Llevar a cabo una aproximación metabolómica no dirigida basada en HPLC-ESI-QTOF-MS para estudiar la biodisponibilidad y el metabolismo de los compuestos bioactivos circulantes tras la ingesta de nutraceuticos desarrollados con los cinco extractos vegetales seleccionados incluidos en un ensayo de intervención nutricional agudo en humanos.
- Evaluar *in vitro* la capacidad anticancerígena tanto de extractos vegetales como de metabolitos biodisponibles en plasma en líneas celulares de cáncer de mama y páncreas.

Introduction



Introduction

1. The role of phenolic compounds in the prevention and treatment of chronic diseases

Chronic diseases, such as cardiovascular disease, diabetes, cancer, respiratory diseases, and neurodegenerative disorders, represent a significant global health challenge due to their high prevalence and substantial impact on morbidity and mortality. These diseases constitute the leading cause of global mortality, accounting for **over 74% of all deaths**¹. These chronic conditions represent a significant public health burden, particularly affecting individuals aged 30-69. Cardiovascular diseases are the foremost cause of chronic diseases mortality, claiming the lives of 17.9 million individuals each year. Cancers (9.3 million deaths), respiratory diseases (4.1 million deaths), and diabetes (1.5 million deaths) follow closely behind, highlighting the diverse spectrum of chronic diseases contributing to global mortality rates¹.

Traditional treatments, while often effective, can come with a range of adverse side effects that compromise patients' quality of life and adherence to therapy. Consequently, there is an urgent need to discover and develop **new treatments** that offer therapeutic efficacy with minimal side effects. This search for safer alternatives is driving research into **natural compounds** and plant-based therapies, which are increasingly recognized for their potential to provide health benefits while mitigating the risk of harmful side effects². Examples of natural compounds that are currently employed in the field of medicine include the use of morphine, derived from *Papaver somniferum*, as an anaesthetic agent and the utilisation of paclitaxel, obtained from *Taxus brevifolia*, in the treatment of lung, ovarian and breast cancer². The pursuit of such novel treatments is

crucial not only for improving patient outcomes but also for reducing the long-term healthcare burden associated with managing chronic illnesses. In this sense, **bioactive compounds** present in plants can be a good tool both for prevention and for the new search for alternative treatments to current pharmacological treatments ³.

Bioactive compounds are defined as a set of natural substances, usually found in small amounts in plants and certain foods, that provide health benefits beyond the basic nutritional value of the products by improving physiological functions or reducing the risk of diseases ⁴. Examples include vitamins (B, C, E, K vitamins), essential minerals (iron, zinc, calcium, selenium), and non-nutrient phytochemicals like carotenoids, phenolic compounds, glucosinolates, and phytosterols.

This doctoral thesis will focus on the analysis of **phenolic compounds**, which represent a significant class of bioactive compounds.

1.1 Phenolic compounds

Phenolic compounds are secondary plant metabolites synthesized from the metabolic pathways of shikimic acid, malonate/acetate, pentose phosphate and phenylpropanoids. These compounds stand out due to their extensive variety and **structural diversity**, with numerous compounds exhibiting significant bioactive potential. At present, there are more than 10000 known structures, but all of them share a common skeleton based on an **aromatic ring** with one or more **hydroxyl substituents** ⁵. Such compounds are found in a wide variety of foods and beverages of plant origin, such as vegetables, cereals, legumes, fruits, nuts, seeds, coffee or tea.

These compounds exhibit a spectrum of complexity, ranging from basic phenolic molecules to intricately polymerised forms. Most phenolic compounds are naturally

found in association with mono- and polysaccharides, forming conjugates that incorporate one or more phenolic groups. Additionally, they can be bonded to esters and methyl esters ⁶. Due to the great structural variety of the compounds, they can be classified into different families, which are shown in **Figure 1** ⁷.

It is worth noting that **flavonoids** represent the most significant family within phenolic compounds. Notably, they constitute the most abundant class, with over 5000 identified species. Their structure is characterized by a fundamental C6-C3-C6 carbon skeleton that can undergo modifications and functional group additions, leading to a diverse range of subclasses ⁷.

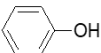
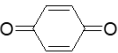
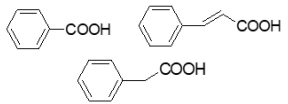
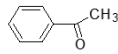
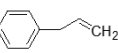
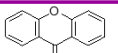
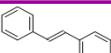
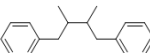
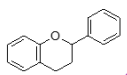
Simple phenols	Phenols 	Benzoquinones 	Coumarins Isocoumarins 
	Phenolic acids 	Acetophenones 	Chromones 
		Phenylpropenes 	Naphthoquinones 
	Lignins <i>Highly cross-linked aromatic polymer</i>	Xanthenes 	Stilbenes 
Polyphenols	Anthraquinones 	Lignans Neolignans 	Hydrolysable tannins <i>Heterogeneous polymer consisting of phenolic acids and simple sugars</i>
	Flavonoids <i>Subfamilies: Flavandiol, Flavanol, Dihydrochalcones, Proanthocyanidins/Condensed tannins, Anthocyanidins, Isoflavonoids, Dihydroflavonols, Biflavonoids, Flavonols, Aurones, Flavones, Chalcones, Flavanones.</i>		

Figure 2. Main families of phenolic compounds and their basic structure.

1.2 Bioactive properties of phenolic compounds against chronic diseases

Phenolic compounds have a **pleiotropic effect**, meaning that they have multiple and varied effects on different biological processes and diseases. Rather than targeting a single pathway or function, these compounds can influence a range of physiological mechanisms. These compounds exert multiple and varied effects on different biological

processes and diseases. In chronic diseases such as neurodegenerative diseases and cancer, phenolic compounds, due to their neuroprotective and anticancer potential (**Figure 2**), can exert their bioactive effects by influencing various metabolic pathways and exerting different mechanisms of action ⁸.

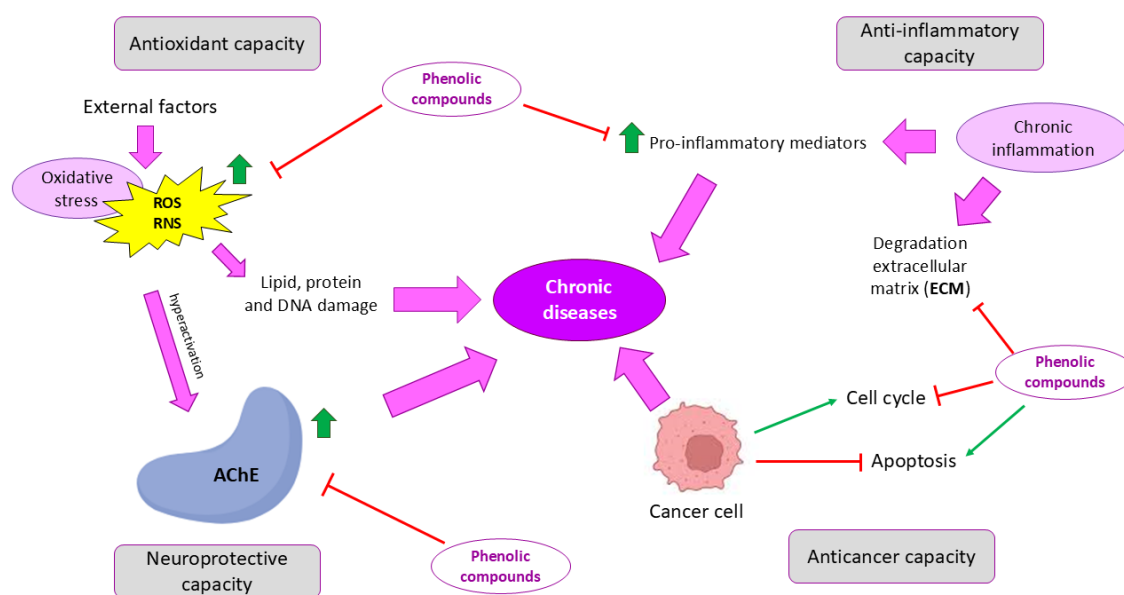


Figure 2. Schematic representation of some examples of the bioactive effect of phenolic compounds against chronic diseases.

Oxidative stress and chronic inflammation are two key features in the development of the pathogenesis of most chronic diseases ^{9,10}. Therefore, the **antioxidant** and **anti-inflammatory properties** of phenolic compounds make them ideal candidates for the prevention and treatment of these diseases (**Figure 2**). However, phenolic compounds also possess other bioactive properties that specifically influence the pathogenesis of certain chronic diseases, such as cardioprotective, neuroprotective, antiatherogenic, and anticancer activities ¹¹. This doctoral thesis focuses on the bioactive role of phenolic compounds against neurodegenerative diseases and cancer. Therefore, the antioxidant,

anti-inflammatory, neuroprotective and anticancer properties of phenolic compounds will be detailed below.

1.2.1 Antioxidant capacity

Although it is widely accepted, that many **chronic diseases** are **multifactorial in origin**, they share **oxidative stress** as a common denominator. Thus, in most of the chronic diseases, oxidative stress plays a key role in the pathology of these diseases ⁹.

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of **oxygen reactive species (ROS)** in cells and tissues and the ability of a biological system to detoxify these reactive products ¹².

Free radicals are molecules that contain an unpaired electron in their outer orbit. The most common free radicals in the human body include **ROS** such as peroxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), and singlet oxygen ($^1O_2$) as well as **reactive nitrogen species (RNS)** such as nitric oxide ($NO^{\bullet}$) ¹³.

Free radicals are naturally produced in the body during **normal metabolic processes**, such as cellular respiration in the mitochondria. However, they can also originate from **external sources**, including ultraviolet (UV) and ionising radiation, environmental pollutants and toxic chemicals, tobacco smoke and alcohol consumption, and infections and inflammations.

Free radicals are integral to a variety of biological processes, many of which **are essential for life**. For instance, they play a key role in the intracellular elimination of bacteria by phagocytes, especially granulocytes and macrophages. Additionally, free radicals are believed to be involved in **cellular signalling pathways** known as redox signalling ¹⁴. At

low to moderate levels, ROS are advantageous for regulating homeostatic processes and a diverse range of cellular functions ¹⁴.

Excessive ROS production can lead to **structural alterations** in cellular proteins, impairing their functions and causing cellular dysfunction and disruption of essential processes ¹⁴. **High ROS levels** result in **lipid, protein**, and deoxyribonucleic acid (**DNA**) **damage** ¹⁴. Excessive free radicals can lead to cellular damage and apoptosis, contributing to a range of serious conditions such as stroke ¹⁵, diabetes, myocardial infarction, cancer, and other significant health issues ¹⁶.

In **chronic diseases** such as **cancer**, ROS, particularly the HO*, can damage both mitochondrial and nuclear DNA, leading to the formation of products such as 8-hydroxy-2'-deoxyguanosine and 8-oxoguanine. Although cells have enzymatic mechanisms to repair this damage, extensive damage that is not adequately repaired can result in mutations that may lead to carcinogenesis. Ageing contributes to the accumulation of oxidative damage and a decline in DNA repair capacity, thereby increasing the risk of chromosomal abnormalities, DNA replication blockages, and cytotoxicity. Furthermore, lipid peroxidation products such as malondialdehyde also have carcinogenic potential. Although all mechanisms are not fully understood, oxidative stress can alter the expression of genes and proteins related to cellular proliferation, thereby contributing to cancer development ¹⁷.

In other chronic diseases such as **neurodegenerative diseases**, ROS have been observed to act as inflammatory neurotoxins produced by microglia and play a key role in neurodegenerative disorders such as Alzheimer's and Parkinson's disease. The detrimental effects of oxidative stress on the brain and nervous system are mainly due

to the diminished ability of the central nervous system to prevent ongoing oxidative damage. Understanding the interplay between disease progression, free radicals and neurotoxicity is crucial for developing treatments ¹⁸.

In this sense, **compounds** with **antioxidant power** can exert **regulatory power** on **oxidative stress**. **Phenolic compounds** exert their antioxidative capacity **through various mechanisms**, including free radical scavenging, chelation of metal cations involved in ROS formation reactions, and donating hydrogen atoms or electrons. Additionally, they can modulate the expression of genes responsible for the synthesis, activation, and inhibition of antioxidant enzymes or related enzymes, and interact with cellular signals ¹⁹. For example, oleuropein, one of the main phenolic compounds in the olive leaf, has been shown to be able to donate hydrogen atoms and electrons and scavenge free radicals ²⁰. *Hibiscus sabdariffa* is another plant matrix with potent antioxidant power. One study evaluated the antioxidant capacity of its aqueous and methanolic extracts, which contain a high content of phenolic compounds. The results demonstrated that both extracts exhibited high capacity to scavenge free radicals, chelate metal cations and donate hydrogen atoms and electrons ²¹. Also, it has been demonstrated that cocoa, a matrix with a high phenolic content, has the capacity to modulate the activities of key enzymes involved in the internal antioxidant defence of the body, including superoxide dismutase, catalase and glutathione peroxidase ²².

1.2.2 Anti-inflammatory capacity

Inflammation is an essential component of the normal repair processes of the organism following injury and is crucial for protection against bacterial and viral infections, as well as harmful environmental agents. However, when inflammation persists over an

extended period, it transforms into **chronic inflammation**. During inflammation, certain cells, mainly macrophages, stimulate the production of pro-inflammatory mediators. These mediators include interleukin (IL)-1 β , IL-6, IL-8, tumour necrosis factor (TNF)- α , ROS, NO * , and prostaglandins. Overproduction of these mediators in chronic inflammation has been linked to the development of several chronic diseases, including Alzheimer's disease and cancer, among others.

In particular, studies on **neurodegenerative diseases** have suggested that **inflammation** is not only a result of neurodegeneration, but is an important factor in the development of the disease. Protein aggregates, which are a very common pathological phenomenon in neurodegeneration, can induce neuroinflammation, which in turn aggravates protein aggregation and neurodegeneration. In fact, inflammation occurs even earlier than protein aggregation ²³.

Chronic inflammation and **cancer** are closely linked. Chronic inflammation contributing to the development and progression of cancer. Immune cells in the inflammatory environment can release substances that promote cell proliferation, inhibit apoptosis, and encourage the formation of new blood vessels, all of which are crucial processes for tumour growth. Additionally, chronic inflammation can create an environment that favours mutations and genetic alterations, increasing the risk of normal cells transforming into cancerous cells ²⁴.

Thus, the reduction of this chronic inflammation can help to halt the progression of chronic diseases. In this regard, phenolic compounds have demonstrated a high anti-inflammatory capacity through various mechanisms of action:

- **Antioxidant activity:** Phenolic compounds scavenge free radicals and reduce oxidative stress, which is a key contributor to inflammation. By neutralising ROS, they prevent oxidative damage to cells and tissues ²⁵.
- **Inhibition of pro-inflammatory enzymes:** Many phenolic compounds inhibit enzymes such as cyclooxygenase and lipoxygenase that are involved in the synthesis of pro-inflammatory mediators, including prostaglandins and leukotrienes ²⁶.
- **Modulation of signalling pathways:** These compounds can modulate key signalling pathways, such as nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor-kappa B (NF-κB) pathways, which are crucial in regulating the expression of inflammatory genes ²⁷.
- **Reduction of pro-inflammatory cytokines and enhancement of anti-inflammatory cytokines:** Phenolic compounds can reduce the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which play a significant role in sustaining inflammation or can enhance the production of anti-inflammatory cytokines, such as IL-10 ²⁸.

Chronic inflammation, in addition to being associated with chronic pathologies, affects numerous organs, including the skin, compromising its integrity and perpetuating the inflammatory response. The **extracellular matrix (ECM)**, a complex assembly of proteins, proteoglycans, and glycosaminoglycans, maintains cellular and tissue architecture. **ECM degradation** plays a significant role in the progression of many **inflammation-related diseases** ²⁹.

An important enzyme in **ECM degradation** is **hyaluronidase**, which depolymerises hyaluronic acid, regulating the size and concentration of hyaluronic acid chains that

influence various pathological processes³⁰. Hyaluronic acid is a polymeric component of the ECM (**Figure 3**) composed of repeating disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine. Under normal conditions, hyaluronic acid forms high molecular weight polymers (over 1000 kDa), but during tissue injury or inflammation, it breaks down into lower molecular weight forms. These smaller hyaluronic acid fragments induce the expression of pro-inflammatory genes in various cell types, including macrophages, and promote the production of pro-inflammatory cytokines such as IL-8 or IL-12³¹. Low molecular weight hyaluronic acid also triggers inflammation via TLR receptor activation and myeloid differentiation factor 88 (MyD88)/NF- κ B signalling, leading to cytokine and chemokine production³². Inhibiting hyaluronidase thus has an anti-inflammatory effect by reducing low molecular weight hyaluronic acid and its pro-inflammatory impact, a property associated with phenolic compounds³¹.

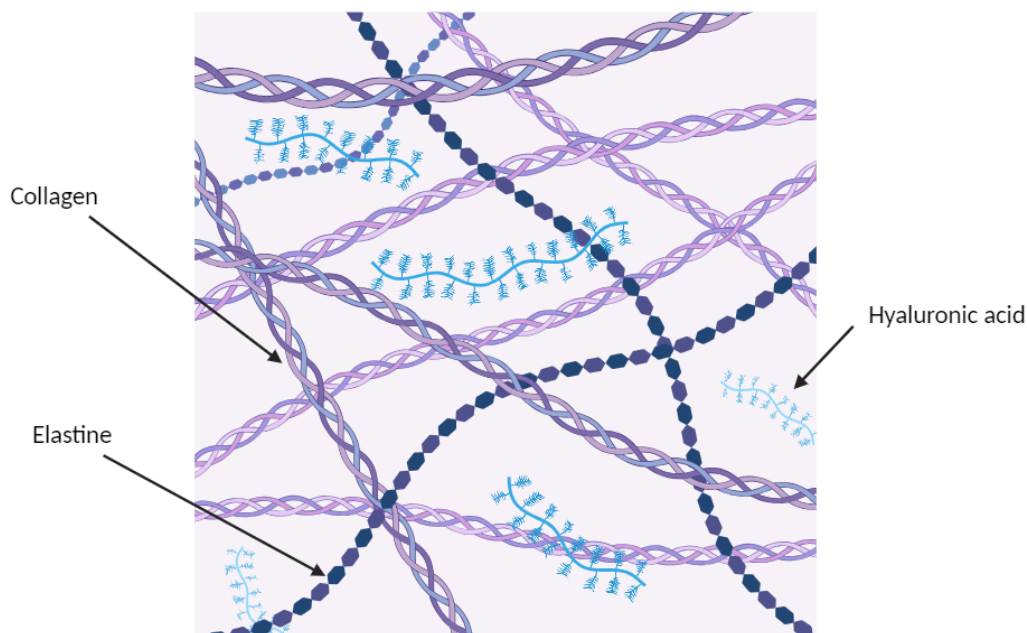


Figure 3. Diagram of some of the components of the extracellular matrix.

Elastase, another enzyme involved in inflammation, is produced by neutrophils in injured tissues. It increases tissue permeability, inflammation, and delays wound healing³³. As a protease, elastase hydrolyses nearly all ECM protein components (**Figure 3**), including elastin, collagen, and fibronectin³⁴. Inhibiting elastase, which is linked to pro-inflammatory activity, reduces inflammation³³.

Collagenase is another enzyme that plays an important role in inflammation by breaking down collagen, a crucial component of the ECM (**Figure 3**) that provides structural support to tissues. During inflammation, collagenase activity can lead to the degradation of collagen fibers, compromising tissue integrity and exacerbating inflammatory processes. This breakdown is associated with conditions where **chronic inflammation** is present, such as rheumatoid arthritis and chronic wounds. In these conditions, excessive collagenase activity contributes to the pathological degradation of ECM proteins, perpetuating tissue damage and impairing healing³⁵.

Inhibiting collagenase activity is a therapeutic strategy aimed at mitigating tissue damage and controlling inflammation-associated disorders. Phenolic compounds, known for their anti-inflammatory properties, have been studied for their potential to modulate collagenase activity and protect ECM proteins from degradation. This approach highlights the intricate relationship between ECM dynamics, collagenase activity, and inflammation management in various pathological contexts³⁵.

In this context, a number of plant matrices with a high content of phenolic compounds have been shown in a range of *in vitro* studies to exhibit **anti-collagenase**, **anti-hyaluronidase** and **anti-elastase** activities. For example, *Citrus unshiu*, a plant belonging to the citrus genus, has demonstrated the ability to inhibit collagenase and elastase

enzymes ³⁶. Similarly, *Podospermum canum*, belonging to the Asteraceae family, has demonstrated the ability to inhibit collagenase, hyaluronidase and elastase ³⁷. The phenolic compounds oleuropein and hydroxytyrosol, which are present in olive leaf, have been demonstrated to inhibit collagenase and elastase ³⁸. Furthermore, the plant *Silybum marianum* has also been shown to possess anti-collagenase and anti-elastase effects ³⁹.

1.2.3 Neuroprotective capacity

The process of **neurodegeneration** is characterised by the persistent malfunction of neural structures and the progressive loss of neurons, leading to a decline in cognitive and motor functions, including memory, learning, decision-making, balance, movement, speech, respiration, and cardiac activity. **Alzheimer's** and **Parkinson's diseases** are the most prevalent neurodegenerative disorders, followed by Huntington's disease and amyotrophic lateral sclerosis. In conditions such as Alzheimer's and Parkinson's diseases, brain neurons are damaged and subsequently destroyed due to the formation of insoluble extracellular amyloid plaques, which result from the aggregation of the A β peptide. These plaques induce an inflammatory response in the brains of affected individuals. As there is **currently no cure**, treatments for neurodegenerative diseases aim to slow cognitive decline. In this sense, there is a need to develop strategies to **prevent** this type of pathology ⁴⁰.

In the case of Alzheimer's disease, one of the **neuroprotective strategies** against the pathogenesis of this disease is the **restoration of acetylcholine** (ACh) levels by inhibiting **acetylcholinesterase** (AChE). Under normal conditions, AChE regulates ACh by breaking it down into choline and acetate molecules. However, overexposure to stressors such as

ROS can increase AChE activity, reducing ACh levels to neurodegenerative thresholds and promoting neurological disorders such as Alzheimer's disease. Additionally, a compelling link has been established between AChE and A β proteins. AChE binds to amyloid proteins via a specific peripheral anionic site, thus accelerating protein misfolding, fibril formation, and the consequent escalation of neurotoxicity ⁴¹.

In this context, phenolic compounds, thanks to their antioxidant power and ability to eliminate ROS, can exert their bioactive effect as they do not allow an increase in AChE activity ⁴². In addition, phenolic compounds have been shown in numerous *in vitro* tests to inhibit this enzyme. For example, Abd El-Aziz et al. (2021) evaluated the antioxidant and **AChE inhibitory power** of an **artichoke extract**, finding that the extract exhibited competitive inhibition of the enzyme ⁴³. In another study, Li et al. (2021) identified and evaluated the phenolic compounds present in **coconut husks** for their **AChE inhibitory potential**. They found that among the identified phenolics, quercetin, kaempferol, and gallic acid showed the highest anti-AChE activity, while catechin, chlorogenic acid, and epicatechin had negative effects ⁴⁴.

1.2.4 Anticancer capacity

The **World Health Organization (WHO)** defines **cancer** a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumours and neoplasms. One defining feature of cancer is the **rapid creation of abnormal cells** that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs; the latter process is referred to as metastasis. Widespread metastases are the primary cause of death from cancer ⁴⁵.

In modern society, cancer management remains a significant **public health challenge**. Despite the availability of various antitumor drugs, issues such as non-specific action, high toxicity, and drug resistance frequently lead to treatment failure. In this regard, **phenolic compounds** stand out for their multifaceted of action and their natural origin, offering both **disease prevention** and a **complementary approach** to existing cancer **therapies** ⁴⁶. They have the potential to improve treatment outcomes while mitigating adverse effects.

Phenolic compounds have demonstrated significant **anticancer potential** through various mechanisms. These bioactive molecules are known for their antioxidant properties, which allow them to **neutralise free radicals** and reduce oxidative stress, a key factor in cancer development ⁴⁶. Additionally, phenolic compounds can modulate multiple **signalling pathways** involved in **cell proliferation** and **apoptosis** ⁴⁶. **Figure 4** shows the stages that cells go through in the process of death by apoptosis.

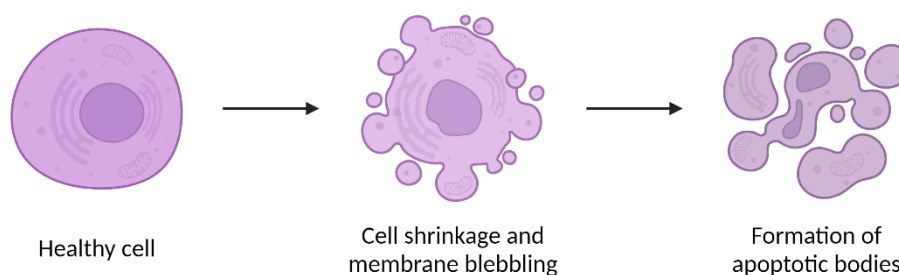


Figure 4. Schematic of the process of programmed cell death by apoptosis.

Several studies have highlighted the ability of **phenolic compounds** to induce **apoptosis** (programmed cell death) in cancer cells. For instance, flavonoids such as **quercetin** ⁴⁷ and **catechins** ⁴⁸ can trigger **apoptosis** by activating **caspases**, enzymes that play a crucial role in the apoptotic process, and by modulating the expression of pro- and anti-apoptotic

proteins. Moreover, **phenolic compounds** can inhibit the proliferation of cancer cells by interfering with the **cell cycle**. Compounds such as **curcumin** ⁴⁹ and **resveratrol** ⁵⁰ have been shown to cause cell cycle arrest at various phases, preventing the replication of cancer cells.

Special attention should be paid to two types of cancer that will be studied in this doctoral thesis, **pancreatic cancer** and **breast cancer**.

Pancreatic cancer is an aggressive disease originating from the cells of the pancreas, an organ located behind the stomach. Known for its challenging early detection and rapid growth and spread before diagnosis, it has one of the poorest prognoses among cancers, with a low survival rate. Authors such as Goldsmith et al. (2018) have evaluated the effect of phenolic compounds present in **olive leaves**, such as **oleuropein** and **hydroxytyrosol**, on pancreatic cancer cells. They observed a reduction in cell proliferation, which was attributed to the activation of **apoptosis** via **caspases** ⁵¹.

On the other hand, **breast cancer** develops in the cells of breast tissue. It is the most common cancer in women, though it can also affect men. Risk factors include a family history of breast cancer, genetic mutations, older age, and exposure to hormones such as oestrogen. Compounds present in **olive leaf** have also been shown to have an anticancer effect on breast cancer by **inhibiting cell proliferation** ⁵².

2. Bioavailability of phenolic compounds

As described above, phenolic compounds exhibit significant bioactive potential against a wide variety of pathologies; however, their **bioavailability** upon ingestion must be considered.

Bioavailability refers to the proportion of a specific compound and/or its metabolite that enters the **systemic circulation**, encompassing processes such as gastrointestinal digestion, absorption, metabolism, and distribution to tissues. It should be noted that only those compounds that reach the target organs can exert a bioactive effect on them. It is important to recognise that the quantity of a compound does not directly correlate with its bioavailability. Some compounds consumed in large amounts have only a small fraction that is bioavailable, whereas others consumed in small quantities may be almost completely bioavailable ⁵³.

Some of the main factors affecting the bioavailability of phenolic compounds are their poor solubility, rapid metabolism and inefficient cellular absorption ⁵³. This means that even if large amounts of phenolic compounds are consumed, only a small fraction can be absorbed and utilised by the body. Improving the **bioavailability of phenolic compounds** is a major research goal, including strategies such as the use of advanced delivery systems like **liposomes** ⁵⁴. Understanding and improving the bioavailability of phenolic compounds is essential to maximise their therapeutic benefits.

2.1 Strategies to improve the bioavailability of phenolics compounds: nanoencapsulation

Improving the **bioavailability** of phenolic compounds is crucial for enhancing their therapeutic efficacy, whether they are **ingested** or applied **topically**. These compounds often face challenges such as poor solubility, stability, and absorption, which limit their effectiveness. For this reason, different strategies should be used to enhance the bioavailability of phenolic compounds for both oral and topical applications. One effective strategy is **nanoencapsulation**, which involves encasing phenolic compounds in

nanoscale carriers. Nanoencapsulation offers several benefits, including protecting phenolic compounds from degradation, enhancing their absorption, and facilitating targeted delivery.

Liposomes are spherical lipid vesicles composed of one or more lipid bilayers, formed by emulsion of natural or synthetic lipids in an aqueous medium. This allows them to encapsulate both hydrophilic and hydrophobic compounds. The membrane formed by the lipid bilayer is arranged with the polar hydrophilic heads facing towards the aqueous medium and towards the interior of the vesicle, and the non-polar hydrophobic tails are oriented within the lipid bilayer, as illustrated in **Figure 5**⁵⁵.

Additionally, solvents like glycerol (**glycerosomes**) or ethanol (**ethosomes**) can be added to improve stability, fluidity, phenolic compound entrapment and target penetration compared to liposomes⁵⁶. To create these liposomes or their derivatives, technologies like probe **sonication** can be employed. This method is straightforward, cost-effective, and rapid, enabling operation with small volumes of reagents.

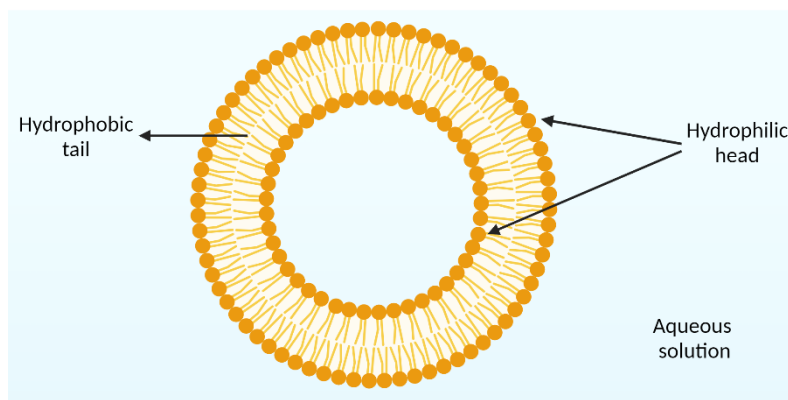


Figure 5. Liposome structure.

For this purpose, it is essential to determine the **encapsulation efficiency (EE)**, which quantifies how effectively the material is trapped within the vesicles. There are several

methodologies available to assess EE, with a straightforward approach involving spectrophotometric assays to compare the total bioactive compound content in both the free extract and the encapsulated form.

Following the determination of the encapsulated content, the stability of these nanovesicles needs to be assessed. This stability can be measured by various methods depending on the specific property to be evaluated ⁵⁷:

- **Particle Size Analysis (Dynamic Light Scattering)**: This technique measures the hydrodynamic size of particles in solution using light scattering. It provides insights into the average size and size distribution of the nanovesicles suspended in solution.
- **Zeta Potential**: This method measures the surface charge of particles dispersed in an aqueous medium. It helps assess the colloidal stability of the nanovesicles and predict their behaviour in solution. A high zeta potential value (either positive or negative) suggests that the particles are sufficiently repelled from each other, reducing the likelihood of aggregation and thus indicating greater stability.

2.2 Metabolic reactions of phenolic compounds

In addition to the bioavailability of phenolic compounds, their metabolism must also be considered. **Metabolism** encompasses the range of transformations and changes that can occur within the organism involving both endogenous compounds and exogenous compounds, such as those derived from food consumption. Phenolic compounds ingested may enter the bloodstream in their native form as present in extracts, or they

may undergo metabolic transformations, resulting in new phenolic compounds with bioactivities differing from the original ones.

It is well established that **phenolic compounds interact** with various **macromolecules**, such as fibres, lipids, proteins, and dietary polysaccharides, during digestion, which affects their bioavailability. The bioavailability of phenolic compounds is influenced by their chemical structure, degree of glycosylation, acylation, conjugation with other phenolic compounds, polymerisation, and solubility. High molecular weight phenolic compounds, such as hydrolysable and condensed tannins, complex flavonoids conjugated with sugars, and those acetylated with hydroxycinnamic acids, are recognised to have lower bioavailability compared to aglycones and low molecular weight phenolic compounds ⁵⁸.

Many phenolic compounds in nature are found in glycosylated forms, which cannot be absorbed directly by epithelial cells. These compounds must be broken down into simpler structures by enzymes in the small intestine or colon. Thus, after ingestion, phenolic compounds that reach the small intestine undergo enzymatic hydrolysis, giving rise to their aglycones ⁵⁹.

These aglycones may either be absorbed into the **bloodstream** unchanged from the small intestine or undergo phase I and phase II metabolic processes within the intestinal tract. Major **phase I metabolic reactions** include **oxidation** and **reduction**, while **phase II reactions** encompass **sulphation**, **glucuronidation**, or **methylation**. These three reactions result in the formation of sulphated, glucuronidated and/or methylated metabolites through the respective action of sulfotransferases (SULTs), uridine-5'-di-

phosphate glucuronosyltransferases (UGTs) and catechol-O-methyltransferases (COMTs)

⁶⁰.

Compounds that are not absorbed in the small intestine pass into the large intestine, where they may be transformed by the **microbiota of the colon** and subsequently absorbed. The colon contains a diverse microbial population of obligate anaerobes and facultative anaerobes, which degrade undigested food matrices and convert components into **microbial metabolites**. The primary reactions occurring in the colon include the formation of **aglycones**, **ring fission**, and various reactions or **cleavages of functional groups**, such as dehydroxylation, demethylation, and decarboxylation ⁶¹. Alternatively, these compounds may be absorbed in the colon or excreted in faeces depending on their chemical nature and the metabolic activity of the microflora ⁶².

All metabolites that have been absorbed into the **bloodstream** can reach the **liver**, where they may again undergo various phase I and phase II metabolic reactions. Following this, the metabolites may be **reabsorbed** into the small intestine via enterohepatic circulation in the bile, eliminated in the **urine** through renal filtration, or distributed to various **tissues** and **cells**, where they may exert a bioactive effect ⁶³.

Figure 6 shows a schematic of the pathway of phenolic compounds during their metabolisation.

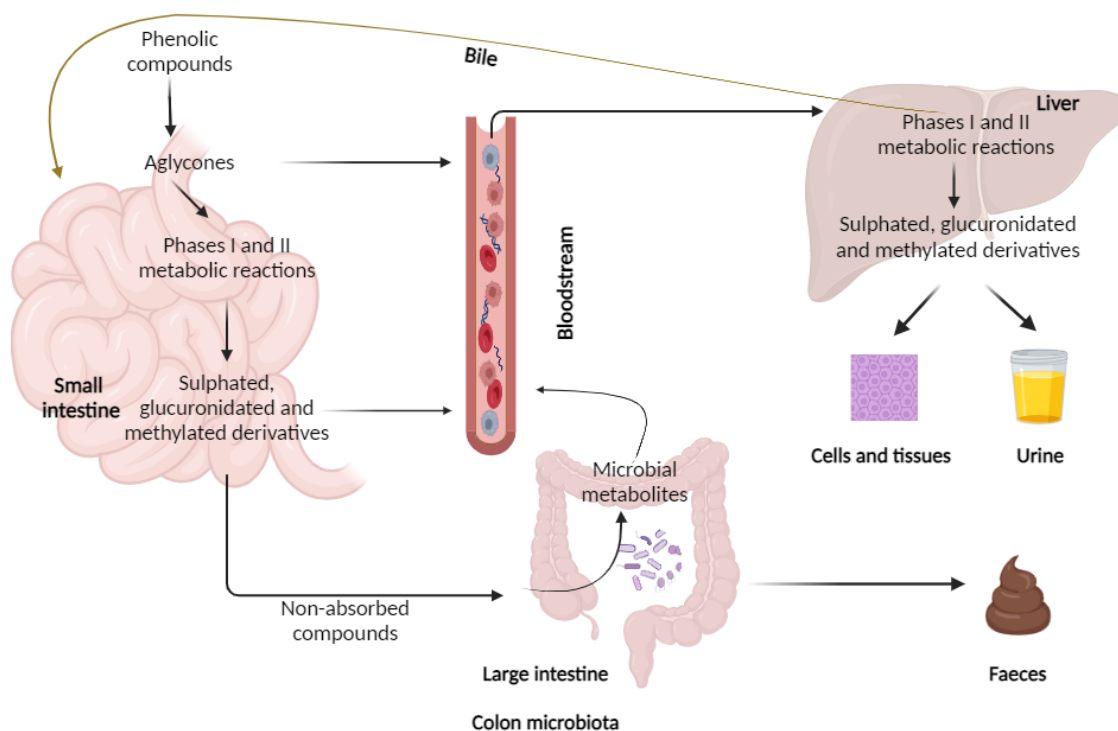


Figure 6. Main metabolisation reactions of phenolic compounds.

3. Traditional methodological approach to the study of phenolic compounds

Traditionally, the **study of phenolic compounds** has been done from a **bottom-up approach** (Figure 7). This workflow begins with the selection of matrices, followed by the extraction and analytical determination of the phenolic compounds present in them. Once extracts rich in phenolic compounds are obtained and characterised, the workflow continues with the *in vitro* evaluation of the bioactivity of the extracts. This is followed by *in vivo* studies, first in animals and then in humans, which can be carried out either on the complete extracts or on selected isolated compounds from these extracts. Ultimately, this process culminates in the development of high-value products such as nutraceuticals and/or cosmeceuticals.

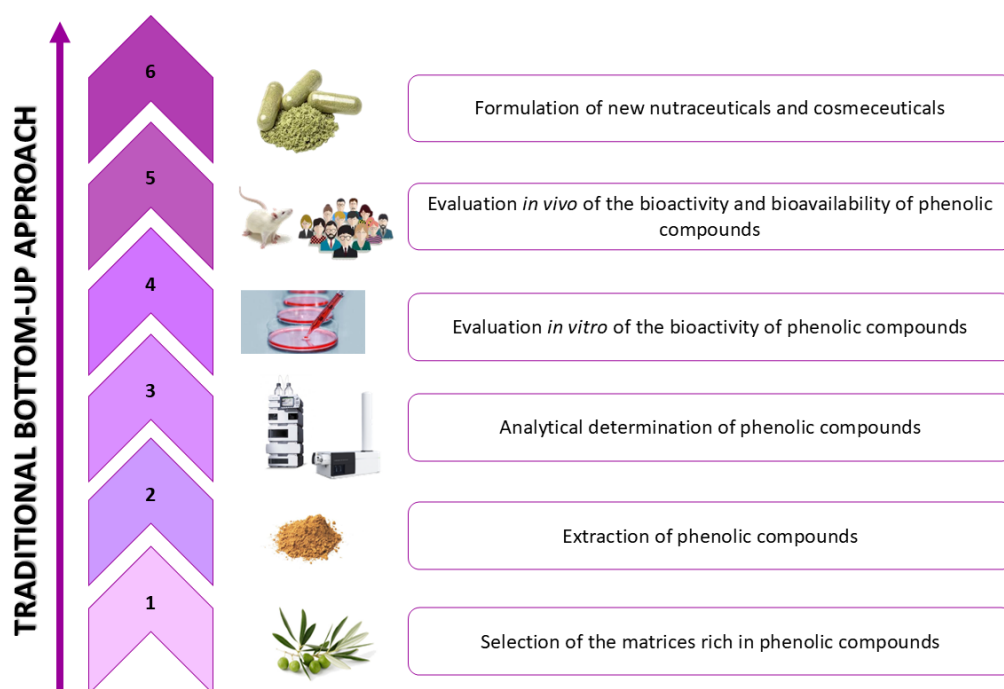


Figure 7. Traditional bottom-up study of phenolic compounds.

There is a wealth of literature that follows this workflow. Numerous studies commence with the extraction and characterisation of phenolic compounds from various matrices^{64–66}, proceed with *in vitro* assays^{67–70}, and finally transition to *in vivo* tests, initially in animals such as rats and mice^{71–74}, and subsequently evaluating phenolic compounds through human intervention studies^{75,76}.

The subsequent steps are comprehensively outlined in a study of phenolic compounds, adopting a bottom-up approach. Moreover, this doctoral thesis encompasses a traditional screening of phenolic compounds, which entails the selection of the matrices under investigation, the analytical characterisation of the studied extracts, and the assessment of their bioactivity using various *in vitro* methodologies. Consequently, the techniques employed in this doctoral thesis are detailed at each of these stages.

3.1 Selection of the matrices rich in phenolic compounds

The **selection of matrices** rich in phenolic compounds is crucial for the study and utilisation of these bioactive compounds. Matrices refer to the different sources from which phenolic compounds can be extracted. These sources are predominantly plant-based and include fruits, vegetables, grains, seeds, leaves, and even certain types of bark. Each of these matrices offers a unique profile of phenolic compounds, contributing to their potential health benefits.

Understanding the **specific phenolic profiles** of these matrices is essential for targeting their application in health-related research and product development. The selection process typically involves evaluating the concentration and diversity of phenolic compounds present, as well as their bioavailability and potential health impacts.

The composition of phenolic compounds depends on both genetic characteristics and environmental factors and varies among **plant species**, even among crops of the same species, with certain species or genera being characteristic of **specific families of phenolic compounds** some of which are exclusive to certain plant species.

In this context, the present doctoral thesis has focused on five plant species (**Figure 8**), each exhibiting a distinct phenolic composition and characterised by their richness in different families of phenolic compounds. Therefore, these **five matrices** have been chosen due to their shared bioactive properties, despite containing distinct families of phenolic compounds. The objective in this context was to investigate the mechanisms by which these **varied families of phenolic compounds** exhibit **similar bioactivity** and demonstrate **pleiotropic effects**. These effects include the modulation of multiple metabolic pathways involved in various mechanisms of action, rendering these matrices

rich in phenolic compounds an ideal alternative for the treatment or prevention of various chronic diseases.



Figure 8. Plant species selected for study in the present doctoral thesis.

It is important to note that these five matrices are authorised by the European Food Safety Authority (EFSA) for use as food supplements and are also included in the BELFRIT list of plants permitted for use in food supplements.

The selected plant species are listed below:

Hibiscus sabdariffa

H. sabdariffa, commonly known as Roselle or Jamaica flower, is a member of the Malvaceae family generating significant research interest due to its diverse applications and purported health benefits. Native to West Africa, this annual or perennial herbaceous shrub is now widely cultivated in **tropical** and **subtropical regions**. The characteristic bell-shaped calyx, with five petals in shades of pink, red, or purple, is the primary part utilized (**Figure 9**). These **calyces** are extensively employed in the

production of a tart beverage known globally under various names such as ‘sorrel’ and ‘agua de jamaica’.

Beyond its culinary applications in teas, jams, jellies, and as a natural food colorant, *H. sabdariffa* exhibits a range of potentially beneficial medicinal properties. These medicinal properties include its use in the treatment of hypertension ⁷⁷ and liver disease ⁷⁸, as well as its neuroprotective ⁷⁹, anti-inflammatory ⁸⁰, anticancer ⁸¹, antioxidant ⁸², and antidiabetic ⁸² effects, and its potential to aid in weight management ⁸³.



Figure 9. *Hibiscus sabdariffa* calyces.

The vast array of **health benefits** attributed to this plant likely stems, in large part, from its unique phenolic composition. This plant is rich in diverse phenolic compounds, including **organic** and **phenolic acids** (e.g., citric acid, hibiscus acid, chlorogenic acid), **flavonoids** (e.g., quercetin, kaempferol, and their corresponding glycosides), and **anthocyanins** (e.g., cyanidin-3-O-glucoside, cyanidin-3-O-sambubioside, delphinidin-3-O-sambubioside).

Theobroma cacao

Theobroma cacao, commonly known as the cacao tree, is a tropical evergreen native to Central and South America, now widely cultivated in **tropical regions**. The cacao tree produces ovoid fruit called **cacao** pods, each containing 20-60 seeds embedded in sweet-sour pulp (**Figure 10**). Requiring a warm, humid climate with consistent temperatures between 20-30°C and well-drained, slightly acidic soils, cacao is usually propagated



Figure 10. *Theobroma cacao* fruit.

from seeds but can also be grown from cuttings or grafting. The seeds, or cocoa beans, are processed to produce cocoa mass, cocoa butter, and cocoa powder, essential for **chocolate** and various confectioneries. The pulp surrounding the seeds is also consumed fresh, used in beverages, or fermented to make alcoholic drinks. Additionally, cocoa butter is widely used in skin care products for its moisturizing properties, and the husks and shells of cocoa pods are utilized as mulch or compost. Nutritionally, cocoa is a good source of magnesium, iron, phosphorus, zinc, and potassium.

From a **medicinal perspective**, cocoa exhibits a range of potentially beneficial properties, including cardiovascular health benefits ⁸⁴, neuroprotective effects ⁸⁵, antioxidant activity ⁸⁶, and potential antidiabetic ⁸⁶ and anticancer effects ⁷⁴. These properties are largely attributed to the richness of the plant in phenolic compounds. The phenolic profile of cocoa encompasses **phenolic acids** such as chlorogenic, caffeic, and ferulic acids. Furthermore, the flavonoid class is well-represented, containing **flavan-3-ols** (e.g.,

epicatechin and catechin), **flavonols** (e.g., quercetin), **procyanidins**, and **anthocyanins**, the latter contributing to the characteristic color of the cocoa and exhibiting antioxidant properties. Notably, cocoa also contains **theobromine**, an alkaloid similar to caffeine but with milder stimulant effects. Theobromine exerts vasodilatory, diuretic, and mood-enhancing properties ⁸⁷.

Silybum marianum

S. marianum, commonly known as **milk thistle**, is a plant belonging to the Asteraceae family famous for its medicinal properties, particularly for liver-related ailments, and has been used for over 2000 years. Native to the **Mediterranean region** but now found globally, milk thistle is a biennial or annual plant that can grow up to 1.5 meters in height with large, spiny, variegated leaves and purple or violet flower heads (**Figure 11**). The seeds, which are the primary medicinal part, are shiny and black. Its most notable medicinal application is in **liver health**, where it aids in treating cirrhosis,



Figure 11. *Silybum marianum* flower.

hepatitis, and fatty liver disease, promoting liver detoxification and regeneration ⁸⁸. The primary active component, **silymarin**, is a complex of **flavonolignans** including silybin A, silybin B, isosilybin A, isosilybin B, silychristin, neosilyhermin, silyhermin and silydianin which exists in its fruit and seeds more than the other parts ⁸⁹. Milk thistle also contains **flavonoids** like taxifolin and quercetin. These phenolic compounds contribute to its hepatoprotective ⁸⁸, antioxidant ⁹⁰, anti-inflammatory ⁹⁰, anticancer ⁹¹,

cardioprotective⁹², neuroprotective⁹³, antidiabetic⁹⁴, renal protective⁹⁵, skin health⁸⁹, immunomodulatory⁸⁹, and antimicrobial effects⁹⁴. Its adaptability and wide range of uses make milk thistle a valuable plant in both traditional and modern medicine.

Lippia citriodora

Lippia citriodora, commonly known as **lemon verbena**, is a perennial shrub native to South America, valued for its aromatic and medicinal properties. It grows up to 2-3 meters tall, with narrow, lance-shaped leaves that emit a strong lemon scent and small, pale lilac or white flowers (**Figure 12**). Its leaves are used to flavour dishes, teas, and beverages, and for their calming and digestive benefits.



Figure 12. *Lippia citriodora* plant.

This plant has been traditionally used due to its medicinal properties, which include antioxidant^{96,97}, anti-inflammatory⁹⁸, antimicrobial⁹⁶, and digestive effects⁹⁹, making it beneficial for treating stress, gastrointestinal issues⁹⁹, and enhancing overall well-being. These properties are attributed to its rich composition of bioactive compounds, such as **hydroxynamic acids**, **flavonoids** like luteolin and apigenin, and **glycosylated iridoids**. Particularly noteworthy is the presence of the **phenylpropanoid** verbascoside, the most abundant phenolic compound in this plant species. In this sense, the therapeutic effects of this plant are supported by both traditional use and scientific research, making it a versatile plant in culinary, cosmetic, and medicinal applications.

Olea europaea

The olive tree (*Olea europaea*) holds significant cultural, economic, and ecological importance in the Mediterranean region and beyond. Botanically classified within the Oleaceae family, it is renowned for its longevity, resilience to harsh climates, and the production of olives, which are utilized for their culinary and industrial properties. Both olives and the oil derived from them, olive oil, have been extensively studied by the scientific community and have been shown to have a wide range of beneficial health effects¹⁰⁰. However, more and more attention is being paid to the study of the bioactive potential of its leaves (**Figure 13**), which are traditionally used in traditional medicine and represent a way of revaluing these products, considered waste products, with an enormous amount of bioactive compounds such as phenolic compounds.



Figure 13. *Olea europaea* leaves.

Olive leaves contain a large variety of phenolic compounds, such as oleuropein, which belongs to the **secoiridoids** group and is the most abundant phenolic compound in this part of the plant. **Flavonoids** both in the aglycone form (quercetin, apigenin, luteolin, diosmetin) or in the glycosylated form (quercetin-7-O-rutinoside, luteolin-7-O-rutinoside, luteolin-7-O-glucoside, luteolin-5-O-glucoside). And **simple phenols**, such as hydroxytyrosol and tyrosol, belonging to the **phenylethanoids** group. Scientific studies have highlighted its antioxidant¹⁰¹, anti-inflammatory¹⁰², antimicrobial¹⁰¹,

cardiovascular ¹⁰³, anticancer ¹⁰⁴, and neuroprotective properties¹⁰⁵, mainly due to the presence of this wide range of phenolic compounds.

3.2 Extraction of phenolic compounds

The **extraction** process consists of the separation of the compounds of interest from the matrix, solid or liquid, where they are contained, based on the differences in their **relative solubilities** compared to the matrix components ¹⁰⁶. Currently, there are different processes for the extraction of phenolic compounds from plant matrices, which can be divided into two main strategies: **conventional** and **non-conventional** or "GREEN" techniques ¹⁰⁷.

- **Conventional extraction methods**: encompasses techniques that rely on ability of the solvents to extract specific compounds and may involve heating and/or agitation ¹⁰⁸. Among conventional methods, **solid-liquid extraction** (SLE) through maceration has been widely described and utilized for extracting phenolic compounds from plant matrices ¹⁰⁶. The effectiveness of any conventional process largely depends on the nature of the solvent, with the solubility and affinity of phenolic compounds in the solvents playing a critical role in extraction efficiency. Solvents commonly used to extract bioactive compounds from plant materials are ethanol, methanol or water.
- **Non-conventional or "green" extraction methods**: these have shorter extraction times, lower solvent and energy consumption and are more environmentally friendly than conventional ones ⁶⁴. Techniques include **ultrasound-assisted extraction** (UAE), **microwave-assisted extraction** (MAE), **supercritical fluid extraction** (SFE), and **pressurized liquid extraction** (PLE).

Each method has its advantages and limitations, and the choice between conventional and non-conventional methods depends on factors such as the target compounds, the desired purity of extracts, cost considerations, and environmental sustainability goals.

3.3 Analytical determination of phenolic compounds

Analytical techniques are essential in the study of plant extracts, as they enable the separation and detection of analytes from complex mixtures, as in the case of plant extracts. These analytical tools can be categorised into two primary groups: **separation techniques**, which are used to resolve the compounds from a mixture, and **spectrometric techniques**, which offer detailed structural information on each analysed compound. Among the separative techniques, **chromatography** is one of the most widely used for the separation of compounds in a mixture. The combination of chromatography and spectrometric analysis allows the qualitative and quantitative characterisation of phenolic compounds within a plant extract.

High performance liquid chromatography (HPLC) as the separation technique and **mass spectrometry (MS)** as the detection technique are described below, as these were the analytical techniques used in the current doctoral thesis for the determination of phenolic compounds.

3.3.1 High-performance liquid chromatography

HPLC is a reliable, rapid, and efficient technique, adept at separating a wide range of mixtures encompassing both low and high molecular weight compounds with varying polarities. This technique is particularly effective for analysing polar and semi-polar compounds such as phenolic compounds¹⁰⁹.

HPLC instrumentation typically comprises a pump, injector, column, detector, and data acquisition system, as illustrated in **Figure 14**. HPLC involves a **stationary phase**, which consists of a porous material packed into a thin metal **column**, and a **mobile phase**, comprising an eluting **solvent** or a mixture of solvents pumped through the column by a high-pressure pump¹¹⁰. The interaction of each compound with the column components varies, causing differences in flow rates that result in the separation of these compounds. The selection of the mobile phase is based on the target analyte, the column used, and the detection system with which it is coupled¹¹¹. For instance, the mobile phase can be modified by introducing acids or bases depending on the target compounds to be separated. Upon elution of all analytes from the column, they are detected, producing an analytical signal. The intensity and duration of this signal depend on the quantity and nature of each analyte. Consequently, each analyte is represented by a peak on the resulting chromatogram, characterised by a specific **retention time (RT)** and **peak area**.

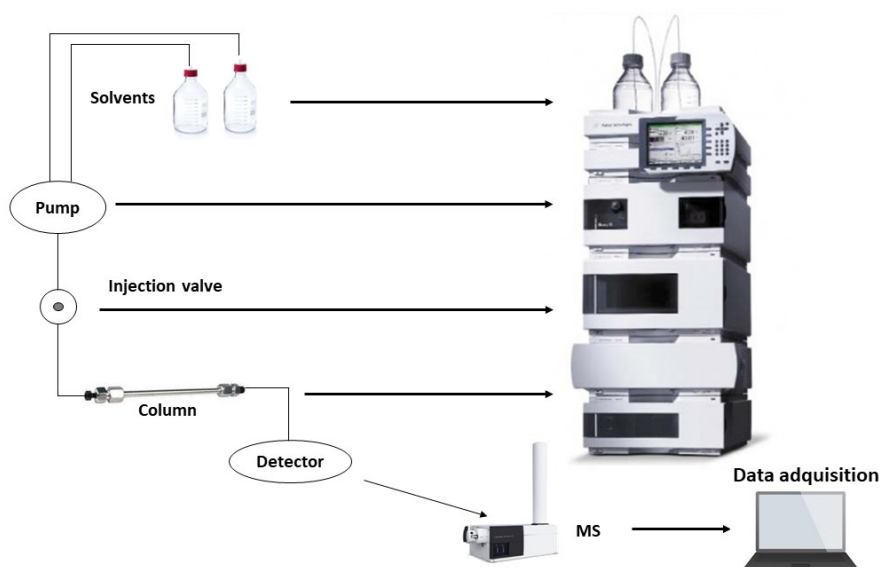


Figure 14. HPLC instrumentation.

The type of stationary phase utilised in liquid chromatography (LC) determines the specific method employed, including partition chromatography, size exclusion chromatography, ion chromatography, and adsorption chromatography ¹¹². Among these, partition chromatography is the most prevalent in phenolic compound research, characterised by a liquid stationary phase retained on a solid support. Within this category, **reversed-phase HPLC (RP-HPLC)** is the preferred technique for separating a wide range of compounds due to its versatility in handling substances with different polarities and molecular masses. In RP-HPLC, the **mobile phase** is **polar**, while the **stationary phase** is **non-polar** or hydrophobic, resulting in higher retention for compounds with more pronounced non-polar characteristics.

The column is the crucial component in HPLC separations. In the case of phenolic compounds analysis, the **C18 column** is most commonly employed, which is packed with octadecylsilyl groups. Regarding column dimensions and particle size, a diverse range is available commercially. C18 columns typically utilise binary systems with acidic **aqueous solutions** and **organic solvents** like methanol or acetonitrile as mobile phases. Acidic conditions in the aqueous mobile phase prevent the ionisation of the phenolic compounds, thereby enhancing their separation within the column ¹¹³.

3.3.2 Mass spectrometry

MS is an **analytical technique** used to measure the **mass-to-charge ratio (m/z)**. It helps identify and quantify the composition of a sample by ionising molecules, separating the ions based on their mass and charge, and detecting them to generate a spectrum ¹¹⁴. The coupling of **high performance liquid chromatography with mass spectrometry** (HPLC-MS) is considered one of the most powerful analytical techniques available. It

facilitates the identification of analytes eluted from the chromatographic column, providing an additional dimension of separation. After the initial separation of analytes in the liquid chromatograph, a subsequent separation occurs in the mass spectrometer according to their **mass-to-charge ratio (m/z)** ¹¹⁵.

MS is an analytical technique that relies on the generation of gas-phase ions, which can be either anions or cations. These ions are separated based on their **m/z ratio** using either electric or magnetic fields. This technique provides crucial information about analytes, including their molecular structure, molecular weight, and purity. Despite the availability of various types of mass spectrometers, they all share a fundamental design that includes an **ionisation source** (which creates ions from the sample), a **mass analyser** (which separates ions according to their m/z ratio), and a **detector** coupled with a **signal processor** to record and interpret the data (**Figure 15**).

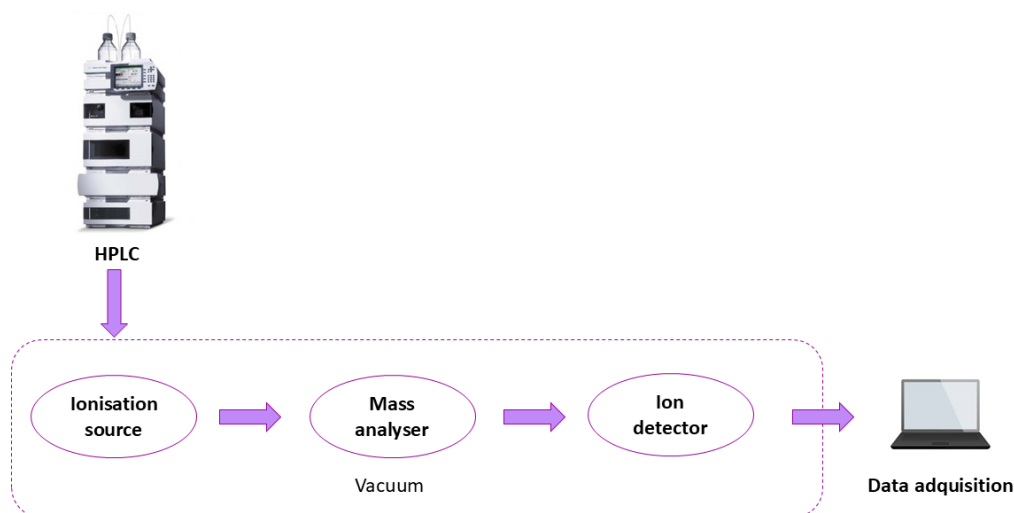


Figure 15. Schematic representation of a mass spectrometer.

In order to combine HPLC with MS, it is necessary to utilise an **ionisation system**. This involves the evaporation and ionisation of the column eluent, which allows the sample molecules to be converted into ionic species in the gas phase prior to separation in the mass spectrometer.

Among various ionisation systems, **electrospray ionisation (ESI)** stands out as the predominant technique for analysing phenolic compounds via HPLC-MS ¹¹⁶. In ESI, the ions are generated at atmospheric pressure by passing a solution-based sample through a fine capillary with an internal diameter of less than 250 μm . This capillary is subjected to a potential difference relative to a counter electrode, typically within the range of +500 to +4,500 volts. The exact voltage required depends on factors such as the inner diameter of the needle and the solvents in the solution.

Regardless of the specific dimensions of the ESI setup, the electrostatic spraying of a sample solution initially produces an aerosol of charged droplets. Often, a concentric flow of gas, such as nitrogen (N_2), is employed to facilitate this nebulisation process. The charged droplets consist of both solvent and analyte molecules, possessing a net **positive** or **negative charge** depending on the polarity of the applied voltage. As the solvent evaporates, the analyte ions are liberated from the droplets and subsequently introduced into the mass analyser of the spectrometer ¹¹⁷.

After this ionisation, the gas-phase ions are directed to the mass analyser. There are different types of mass analysers: quadrupole (Q), ion trap (IT), time-of-flight (TOF), orbitrap, or tandem mass analysers such as **quadrupole-time-of-flight (QTOF)** mass spectrometry. Q and IT analyzers offer low resolution, making them suitable for basic ion filtering and targeted analysis. In contrast, TOF, Orbitrap, and QTOF analysers provide

high-resolution analysis, ideal for complex sample separation and accurate mass measurements¹¹⁸. A QTOF mass spectrometer has been used in the development of this doctoral thesis.

The TOF analyser function is based on the separation of ions following their initial acceleration by an electric field. These ions are exposed to varying velocities as they traverse a free-field region, referred to as a **flight tube**. A detector, located at the terminus of the flight tube, records the arrival time of the charged particles. Consequently, the m/z ratios are ascertained by measuring the duration required for ionised molecules to traverse the free-field region from the source to the detector. The **flight time** for each mass is distinct and is influenced by the acceleration energy of the ion, the distance it must travel, and its m/z ratio. Consequently, ions possessing a higher m/z ratio will travel at a reduced velocity compared to those with a lower m/z ratio¹¹⁹.

Besides the TOF components, a **QTOF mass spectrometer** (**Figure 16**) features a collision cell and a quadrupole situated before the time-of-flight tube. In a QTOF, ion molecules are focused into the quadrupole through a hexapole ion bridge. The analyser also comprises funnels within the ion transmission area, which serve to minimise ion loss during transmission and increase sensitivity.

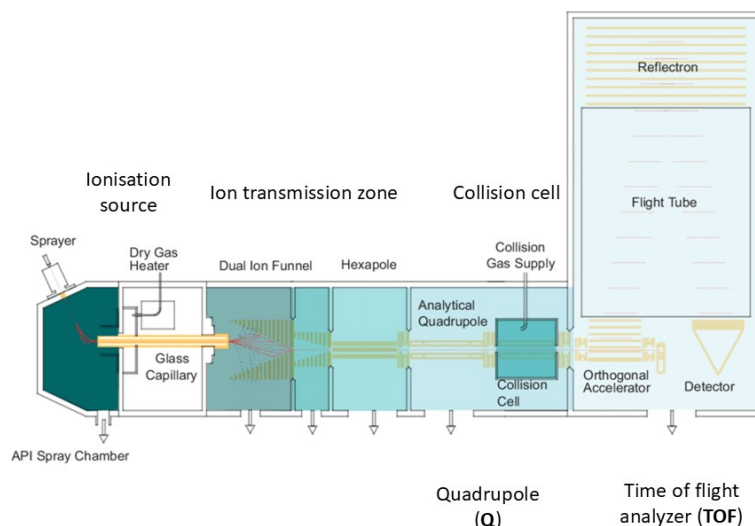


Figure 16. Diagram of the principal components of a QTOF mass spectrometer.

Adapted from Beck et al. (2015) ¹²⁰.

QTOF is capable of operating in both single MS and MS/MS modes. In **MS/MS mode**, ions are selected for collision-induced dissociation within a collision cell using a collision gas, typically nitrogen. The function of the quadrupole is to select specific ions and fragment them in the collision cell with the help of this collision gas. The fragmented ions are then directed into the TOF analyser, where they are separated according to their m/z ratio, as previously described. This combination results in enhanced performance characteristics, such as precise mass measurement and effective fragmentation capability.

Thus, QTOF has proven to be an effective method for the initial characterisation of bioactive compounds in various plant matrices. Additionally, QTOF is a valuable instrument utilised in metabolomic, bioavailability, and pharmacokinetic research ¹²¹.

3.4 Evaluation *in vitro* of the bioactivity of phenolic compounds

In the literature, numerous *in vitro* studies exist both on complete extracts^{122,123} from plant matrices and on isolated compounds^{124,125} from these plant extracts. Some of the most frequently performed *in vitro* assays are those to measure the antioxidant potential of phenolic compounds, as this is one of their most characteristic properties. There are numerous spectrophotometric^{126,127} and cellular assays¹²⁸ to measure this antioxidant potential. The literature also features numerous *in vitro* assays that assess other bioactive properties of phenolic compounds, such as their potential to regulate enzymes associated with various pathologies^{37,129,130}, as well as their anti-cancer potential^{11,131,132}, among other effects. Despite the abundance of these assays, this stage of the workflow is crucial. Before embarking on *in vivo* studies, which are significantly more expensive and time-consuming, *in vitro* assays provide an initial indication of the bioactive potential of the phenolic compounds contained in the extracts. This preliminary evaluation is essential for selecting the most promising candidates for subsequent *in vivo* studies.

The following sections detail the primary *in vitro* assays conducted in this doctoral thesis to evaluate the bioactive properties of the selected matrices under study.

3.4.1 Evaluation of antioxidant capacity

Due to the intricate nature of oxidation processes, no single method can comprehensively capture the antioxidant profile of a sample. Therefore, it is essential to utilise multiple methods to enable effective comparison and interpretation of results.

In this doctoral thesis the evaluation of the antioxidant capacity of phenolic compounds has been determined by different approaches:

- Measurement of the **antiradical capacity**
- Evaluation of free radical and **ROS/RNS scavenging potential**
- Measurement of the antioxidant capacity in **cellular assays**

3.4.1.1 Measurement of the antiradical capacity

Measurements of anti-radical activity can be conducted using two distinct strategies, chosen based on the specific information required:

Direct determination

The radical serves as a quantification parameter, producing an analytical signal. An example of this approach is the “**Trolox-Equivalent Antioxidant Capacity**” (**TEAC**) assay. This involves an electron transfer reaction where the $\text{ABTS}^{\bullet+}$ radical is generated from its precursor, 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) (**Figure 17**). The resulting cation radical is a stable blue-green compound with a visible absorption spectrum. Typically, the $\text{ABTS}^{\bullet+}$ radical is chemically generated using potassium persulphate ¹³³.

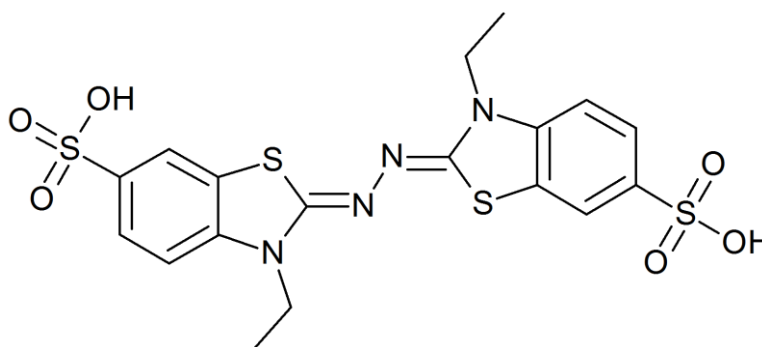


Figure 17. Structure of 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS).

Indirect determination

The presence of free radicals produces the loss or appearance of a reagent and, therefore, the presence of an antioxidant causes an increase or decrease in the signal. An example of this type of electron transfer-based determination is the “**Ferric Ion Reducing Antioxidant Power**” (**FRAP**) assay. This technique depends on the reduction of the ferric tripyridyltriazine complex to its ferrous form by an antioxidant in an acidic medium. Spectrophotometry is employed to measure the absorbance of Fe^{2+} . Therefore, a substance with higher antioxidant activity will result in greater reduction, producing higher concentrations of Fe^{2+} and, consequently, a more pronounced absorbance signal (**Figure 18**)¹³⁴.

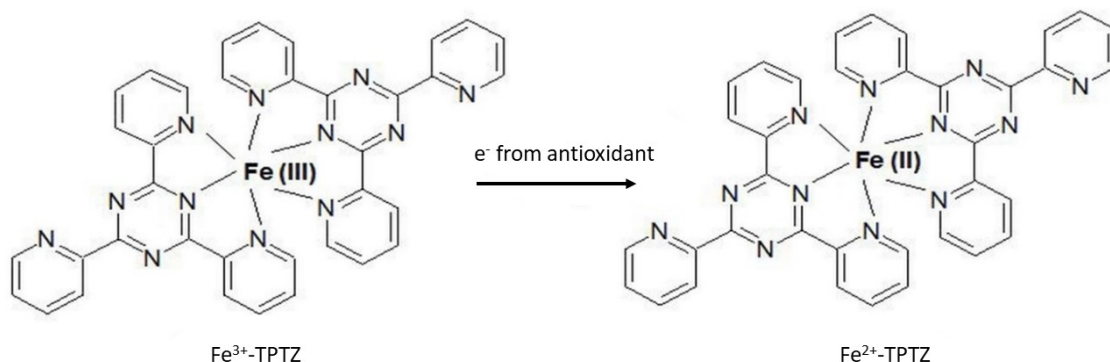


Figure 18. Reaction which occurs in the FRAP method.

Another example of an indirect method is the “**Oxygen Radical Absorbance Capacity**” (**ORAC**) assay, which evaluates the ability of antioxidant compounds to neutralise free radicals through the donation of a hydrogen atom. This method measures the capacity of an antioxidant to inhibit oxidation induced by peroxy radicals, generated by the decomposition of an initiator molecule, typically 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). These radicals attack the fluorescein molecule, resulting in

oxidised fluorescein, which loses its fluorescence, thereby causing a decrease in fluorescence intensity¹³⁵.

3.4.1.2 Evaluation of ROS/RNS scavenging potential

The evaluation of **ROS** and **RNS** scavenging potential is essential for determining the antioxidant capacity of compounds. This process involves assessing the ability of substances to neutralise these reactive species, thereby preventing oxidative stress and protecting cellular components from damage. The evaluation of ROS and RNS scavenging potential typically involves assessing the capacity of a compound to neutralise specific reactive species such as HOCl, $O_2^{\bullet-}$, and $NO^{\bullet}$.

- **HOCl Scavenging Assay:** This assay is based on the fluorescent HOCl-induced oxidation of dihydrorhodamine to rhodamine. Antioxidants inhibit this oxidation, and the decrease in fluorescence indicates their scavenging potential⁷⁰.
- **$O_2^{\bullet-}$ Scavenging Assay:** $O_2^{\bullet-}$ were generated using a nonenzymatic PMS-NADH system, and their scavenging activity was assessed through a colorimetric method. This method involved measuring the reduction of nitroblue tetrazolium (NBT) into a purple-coloured diformazan upon reaction with superoxide anions at 560 nm⁷⁰.
- **$NO^{\bullet}$ Scavenging Assay:** were generated using NOC-5, a nitric oxide donor. The detection and measurement of these anions were facilitated by employing 4,5-diaminofluorescein (DAF-2) as a fluorescent probe. Antioxidants inhibit the formation of nitrite, which is measured by the decrease in absorbance at 546 nm⁷⁰.

Xanthine oxidase (XO) is an enzyme that catalyses the oxidation of hypoxanthine to xanthine and then to uric acid, producing superoxide radicals as a byproduct. These radicals can further generate other ROS such as hydrogen peroxide and hydroxyl radicals. Thus, in this doctoral thesis, in addition to the aforementioned assays for ROS and RNS scavenging, the potential of phenolic compounds to inhibit the enzyme XO has been evaluated. The measurement method relies on the production of resorufin, a highly fluorescent compound, through the oxidation of hypoxanthine by XO, which releases hydrogen peroxide (H_2O_2) as a byproduct. As the reaction progresses, the fluorescence emitted by resorufin can be quantified at excitation/emission wavelengths of 535/587 nm ¹³⁶ (**Figure 19**).

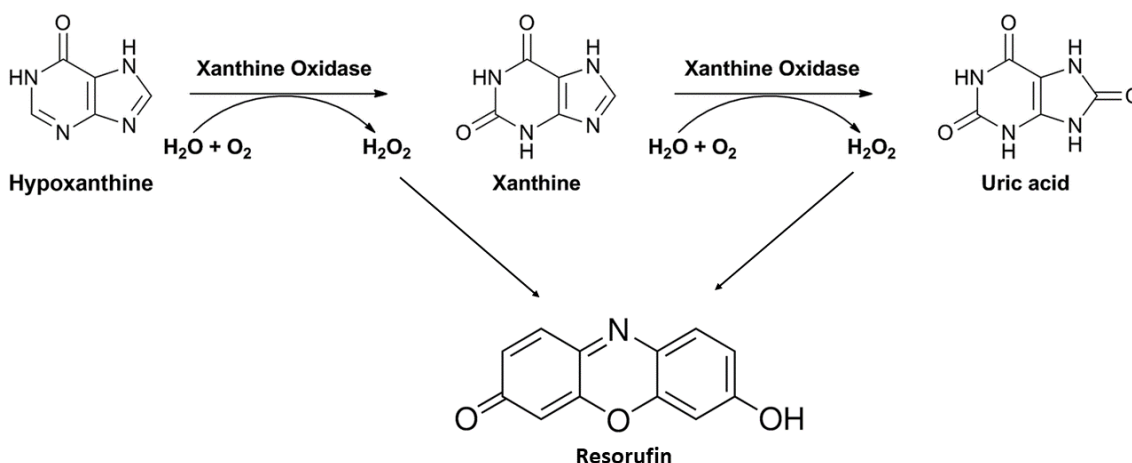


Figure 19. Graphical representation of the XO assay principle.

3.4.1.3 Measurement of the antioxidant capacity in cellular assays

The measurement of antioxidant capacity in **cellular assays** consists of assessing the ability of compounds to neutralise ROS or RNS in a cellular environment.

In this doctoral thesis, the antioxidant capacity of the five selected extracts was evaluated at the cellular level by determining the production of ROS in human

immortalised keratinocytes (HaCaT cell line). To this end, cells treated with the extracts, as well as untreated cells, were exposed to ultraviolet radiation type A (UVA). ROS generation was assessed through the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) (non-fluorescent) to 2',7'-dichlorodihydrofluorescein (DCF) by the action of these radical species ¹³⁷.

3.4.2 Evaluation of anti-inflammatory capacity

The protective capacity of phenolic compounds against chronic inflammation is becoming increasingly important, which has prompted the development of various strategies for their evaluation in this field of research. Among the most widely *in vitro* techniques is the evaluation of the enzymatic activity of several enzymes involved in processes related to the maintenance of ECM integrity, since the disintegration of the ECM triggers a cell signalling cascade that leads to inflammation. Thus, in this doctoral thesis, the capacity of the selected plant extracts to inhibit the enzymes **collagenase**, **elastase**, and **hyaluronidase** has been evaluated.

3.4.2.1 Collagenase inhibition assay

The assay involves pre-incubating the **collagenase** with the inhibitor, such as plant extracts, followed by the addition of a collagen or synthetic peptide substrate. The assay utilised Tricine buffer (pH 7.5), the substrate N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA), and collagenase from *Clostridium histolyticum*. This colorimetric method is based on measuring the degradation of FALGPA after incubation with the enzyme. After incubation, the absorbance was measured at 335 nm ¹³⁸.

3.4.2.2 Elastase inhibition assay

This assay relies on the hydrolysis of the substrate MeOSuc-Ala-Ala-Pro-Val-pNa by **elastase**, which releases p-nitroaniline that can be quantified based on its maximum absorbance at 405 nm. In summary, the substrate, along with an inhibitor (either elastatinal or a plant extract), and the enzyme are prepared in a buffer at pH 7.25. After an incubation period of 30 minutes at 37°C, the absorbance of the solutions is measured at 405 nm to determine the extent of substrate hydrolysis, which correlates with elastase activity inhibition by the tested substances ¹³⁹.

3.4.2.3 Hyaluronidase inhibition assay

This method assesses the reduction in light transmission caused by particles suspended in the solution, which occurs due to the enzymatic degradation of **hyaluronic acid** by the enzyme. In this assay, the enzyme is first incubated with potential inhibitors, such as plant extracts, and then hyaluronic acid substrate is added. The enzymatic reaction results in the production of di- and monosaccharides, as well as smaller fragments of hyaluronic acid. Absorbance readings are then taken at 600 nm to quantify the enzymatic activity and assess the inhibitory effect of the inhibitors ^{138,140}.

3.4.3 Evaluation of neuroprotective capacity

The evaluation of the **neuroprotective capacity** of phenolic compounds involves assessing their ability to protect neurons from damage or degeneration, often caused by oxidative stress, inflammation, or other harmful processes. Phenolic compounds have demonstrated their potential to mitigate neuronal damage through antioxidant and anti-inflammatory mechanisms.

In this thesis, the neuroprotective potential of phenolic compounds was assessed using two distinct measurement techniques. Firstly, an *in vitro* approach was employed to evaluate the ability of the extracts under study to inhibit the **enzyme AChE**. Secondly, an *in vivo* approach was adopted, focusing on assessing the effects of the extracts on the **locomotor activity** of *Drosophila melanogaster* after exposure to hypoxia.

These two complementary approaches provide comprehensive insights into the neuroprotective properties of phenolic compounds, spanning from their direct enzymatic effects *in vitro* to their broader physiological impacts on behaviour and neuronal function *in vivo*.

The *in vitro* assay of **AChE** inhibitory activity was assessed using a photometric colour-based assay. In this method, AChE enzyme was incubated with potential inhibitors, such as phenolic extracts, followed by the addition of a substrate. The reaction commenced with acetylthiocholine (ATCI) serving as the substrate, which AChE cleaved to generate thiocholine. Thiocholine subsequently reacted with 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB), resulting in the formation of the yellow 5-thio-2-nitrobenzoate anion. Enzyme activity was monitored by measuring the rate of thiocholine production, correlating with the increase in yellow colour intensity (**Figure 20**). The absorbance ratio reflecting colour development was recorded every minute at 405 nm wavelength ¹⁴¹.

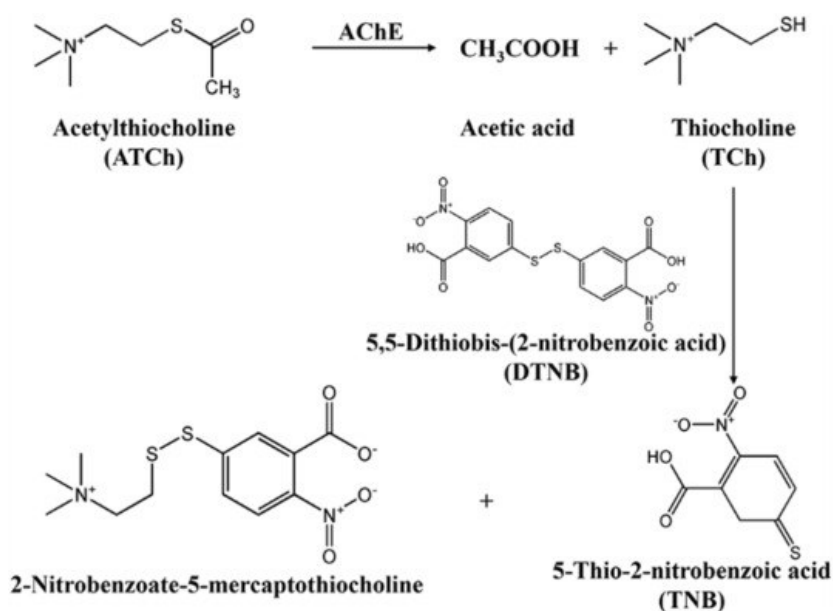


Figure 20. Graphical representation of the AChE assay principle.

Adapted from Liu et al. (2021) ¹⁴².

3.4.4 Evaluation of anticancer capacity

The evaluation of the anticancer capacity of phenolic compounds involves studying their ability to inhibit or mitigate cancer **cell growth** and **proliferation**. Phenolic compounds have shown promise in cancer research due to their antioxidant and anti-inflammatory properties, which can influence cellular pathways involved in tumorigenesis and cancer progression.

To evaluate the anticancer effects of phenolic compounds, **in vitro cell cultures** are commonly employed. These studies typically assess cell proliferation and survival across various cancer models, as well as the mechanisms underlying the **loss of cell viability**. In the present study, cell proliferation assays using **MTT** (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and **crystal violet** assays have been utilised. Additionally, **Western Blot**, evaluation of **ROS production**, **mitochondrial membrane potential assay**, and **apoptosis analysis** have been conducted to elucidate the

mechanisms leading to the loss of cell viability. These methodologies are briefly discussed below.

3.4.4.1 MTT assay

This method is used to determine **cell viability** based on the number of cells in culture. It involves the formation of a coloured compound resulting from a reaction in the mitochondria of viable cells. Specifically, **MTT** is reduced by the mitochondrial enzyme succinate dehydrogenase to form an insoluble formazan compound. The addition of organic solvents, such as dimethylsulfoxide (DMSO), solubilises the formed formazan and releases it into the culture medium, allowing quantification by a colorimetric method that changes colour from yellow to blue/purple (**Figure 21**). The ability of the cells to **reduce MTT** indicates mitochondrial integrity and functional activity, serving as a measure of cell viability. Evaluating this reduction after exposure to a compound provides insights into the toxicity of the compound ¹⁴³. This method is among the most commonly employed assays for assessing the efficacy and interactions of anticancer agents. Nevertheless, it can be influenced by and interfere with certain compounds that alter cell metabolism and reaction conditions. Consequently, the utilisation of alternative methods is occasionally required ¹⁴⁴.

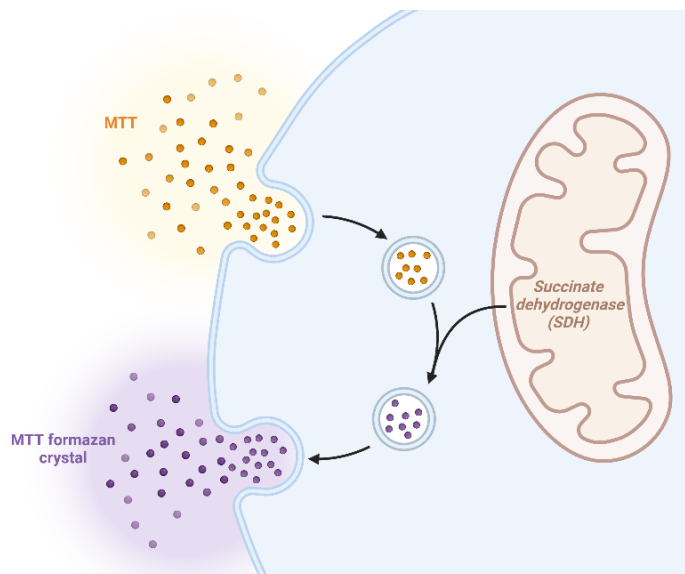


Figure 21. Graphical representation of the MTT assay principle.

3.4.4.2 Crystal Violet Assay

Adherent cells detach from culture plates during cell death. A simple method for detecting the maintenance of cell adhesion involves **staining adherent cells** with **crystal violet** dye, which binds to proteins and DNA. Cells undergoing cell death lose their adherence and are thus removed from the cell population, resulting in a decrease in crystal violet staining within the culture. This protocol outlines a rapid and reliable screening method suitable for studying compounds that impact cell survival and inhibit growth ¹⁴⁵.

3.4.4.3 Western Blot

Western Blot is a powerful and widely utilised tool in molecular biology for detecting and quantifying specific proteins in complex samples, thereby providing crucial information on **protein expression** and **size**. This technique involves cell lysis, protein quantification, separation by SDS-PAGE, and transfer to a nitrocellulose or PVDF membrane. The membrane is blocked and then incubated with a specific primary

antibody, followed by an enzyme-conjugated secondary antibody. Detection is performed via a colour reaction or chemiluminescence, facilitating the visualisation and quantification of the proteins of interest. Although highly specific and sensitive, the technique is laborious and necessitates optimisation of experimental conditions¹⁴⁶. This technique, when applied to **cancer research**, provides an invaluable method for detecting and quantifying specific proteins associated with cancer development, progression, and response to treatment. It enables the identification of cellular mechanisms through which phenolic compounds exert their anticancer effects.

3.4.4.4 ROS production assay

To elucidate the mechanism of action by which phenolic compounds reduce cell viability in cancer cells, assays such as **ROS production** can be performed. One of the mechanisms leading to cell death is the excessive production of ROS, which induces **mitochondrial dysfunction** and subsequent cell death¹⁴⁷.

One of the assays for measuring ROS production is a fluorometric method that detects intracellular ROS, particularly superoxide and hydroxyl radicals, in live cells within one hour of incubation. ROS interact with a cell-permeable sensor, generating a fluorometric signal ($\lambda_{\text{ex}} = 540 \text{ nm}$ / $\lambda_{\text{em}} = 570 \text{ nm}$) that correlates with the ROS levels present. The resulting fluorescence intensity is quantified using spectrophotometry.

3.4.4.5 Mitochondrial membrane potential assay

The **mitochondrial membrane potential** assay is employed to evaluate mitochondrial function within cells. It plays a crucial role in elucidating whether compounds exert anticancer effects by inducing **mitochondrial dysfunction**, thereby leading to cell death.

This assay assesses the electric potential across the inner mitochondrial membrane,

critical for ATP production and various cellular activities. Typically, fluorescent dyes like JC-10 are used, which accumulate in mitochondria based on their membrane potential. Healthy mitochondria with high membrane potential exhibit aggregated JC-10 fluorescence (red), whereas depolarised or dysfunctional mitochondria display green fluorescence due to JC-10 monomers. Analysis of the ratio of red to green fluorescence provides a quantitative indicator of mitochondrial membrane potential, offering insights into mitochondrial health and functionality in biological studies ¹⁴⁸.

3.4.4.6 Apoptosis analysis

In this doctoral thesis, flow cytometry was utilised to identify and quantify apoptotic cells. **Apoptosis** analysis via **flow cytometry** is a powerful method for quantifying and characterising cells undergoing programmed cell death. It relies on fluorescent probes that selectively attach to apoptotic cells based on specific changes associated with apoptosis. The procedure involves culturing and treating cells, staining them with fluorescent probes such as annexin V and 7-AAD, and analysing them using a flow cytometer to detect and quantify apoptotic populations. The flow cytometer provides data on the percentage of cells in different apoptotic stages and overall apoptosis levels in the sample. This approach offers high sensitivity and specificity for studying the response of cancer cells treated with phenolic compounds, allowing for the determination of whether apoptosis is involved in cell death mechanisms ¹⁴⁹.

3.5 *In vivo* models for the study of phenolic compounds

The use of *in vivo* studies with phenolic compounds provides essential insights into their pharmacokinetics, bioavailability, and bioactivity, offering a more comprehensive understanding of their potential health benefits and therapeutic applications beyond the scope of *in vitro* tests. It is important to recognise that while many *in vitro* studies have demonstrated promising bioactive potential for health, some of these findings cannot be fully validated when tested *in vivo*, and vice versa. This discrepancy often arises due to factors such as bioavailability and the metabolic reactions that phenolic compounds undergo upon ingestion. Therefore, *in vivo* studies are crucial for accurately understanding the biological effects of phenolic compounds within a living organism¹⁵⁰.

In this context, two distinct categories of studies can be identified in which *in vivo* models are employed. The first category encompasses studies that assess the bioactivity of phenolic compounds, while the second category comprises studies designed to investigate the bioavailability and metabolism of these compounds.

In both types of study, both animal and human models can be used, but it is common, especially in studies testing the bioactivity of compounds, to start with the animal study and then move on to the human study.

3.5.1 *In vivo* evaluation of the bioactivity of phenolic compounds

To study the bioactivity of phenolic compounds in animal models, there is a wide variety of models that researchers choose depending on the specific biological activity being investigated.

Rodent models, especially mice and rats were used because these species possess well-defined genetics and physiology. These models are essential for exploring antioxidant and anti-inflammatory effects, anticancer activities as well as the potentiality in metabolic disorders such obesity, diabetes or cardiovascular diseases ^{151–153}. The **zebrafish** (*Danio rerio*) is gaining popularity because of its transparent embryos, rapid development, and genetic similarity to humans. It is used to evaluate the toxicity and teratogenic effects of phenolic compounds, as well as to study cardiovascular health and neuroprotection in models of neurodegenerative diseases ¹⁵⁴. The nematode ***Caenorhabditis elegans*** is widely used to study ageing, neurodegeneration and toxicity. It is ideal for studies on the effect of phenolic compounds on lifespan and neuroprotection ¹⁵⁵. The **fruit fly** (*D. melanogaster*) serves as an important model in genetic and neurodegenerative research ¹⁵⁶. **Porcine models** are particularly useful in studying the impact of phenolic compounds on metabolic and cardiovascular conditions ¹⁵⁷. Finally, **non-human primates** are also used in preclinical studies because of their similarity to humans in terms of genetics and physiology. It is therefore useful in models that look at the action of phenolic compounds on chronic diseases like cancer, cardiovascular conditions or neurodegenerative disorders as well as those dealing with cognitive function and behaviour ¹⁵⁸.

As mentioned in the previous section, an *in vivo* study focused on evaluating the effects of extracts on locomotor activity in *D. melanogaster* was carried out in the present doctoral thesis and is detailed below.

This model system is valuable for studying neuroprotective agents as it allows for the observation of behavioural changes indicative of neuronal resilience under hypoxic conditions, a stressor relevant to various neurological disorders.

This screening model provides a straightforward and practical approach for evaluating antioxidant compounds, such as polyphenols. To investigate the effects of hypoxia-reoxygenation injury in *Drosophila*, flies treated with and without the extract were exposed to 2.5 hours of hypoxic stress under controlled conditions (1% O₂; 25°C; 30-40% relative humidity). Subsequently, the **locomotor activity** of the flies was monitored for 4 hours, and the mortality rate following reoxygenation was assessed and expressed as relative mortality compared to the hypoxia group.

Finally, the bioactive effect of phenolic compounds can also be evaluated *in vivo* in **humans**. These types of studies, where bioactive compounds like phenolic compounds are tested, are known as **nutritional intervention studies**. The **main challenges** when conducting studies in humans are the variability of diet, lifestyle, genetics, age, sex and gut microbiota, which can affect the results. As a consequence, it is difficult to maintain similar conditions for all volunteers or patients, in contrast to the situation in animals where conditions can be more easily controlled ¹⁵⁹. Furthermore, the absorption and metabolism of phenolic compounds exhibit considerable inter-individual variability, which may impact their efficacy ¹⁶⁰. Determining the optimal dose and duration for health benefits is also challenging due to the complex nature of phenolic compounds.

In this regard, several aspects need to be taken into account in the design of an intervention study. It should be noted that these aspects are taken into account both in nutritional intervention studies to assess the bioactive potential of phenolic compounds

and in nutritional intervention studies to assess the bioavailability and metabolism of phenolic compounds.

First of all, a solid hypothesis must be the starting point for the development of the nutritional intervention trial. This should start with a **hypothesis based on previous research**, including human, epidemiological, animal and *in vitro* studies ¹⁶¹.

Secondly, it is important to be clear about the type of study to be conducted: single-arm, crossover and parallel. **Single-arm** trials are cost-effective but lack control groups. **Crossover** designs, in which participants receive all interventions, improve precision but require careful planning to avoid carry-over effects. **Parallel designs**, in which each participant receives only one intervention, are preferred for longer-term studies and when returning to baseline is impractical or unethical. The choice of design depends on the study objectives, availability of participants and resource constraints ¹⁶¹.

Other aspects to consider include the **number of participants** and their **selection criteria**, the design of the type of biological samples to be collected, and the frequency of collection ¹⁶¹. Before carrying out a study, it is important to obtain the approval of the relevant local **ethics committee** and to follow the instructions described in the **World Medical Association's Declaration of Helsinki** ¹⁶².

Finally, another key aspect in the design of a human intervention trial is the choice of trial timing, as depending on the **duration of the study**, it can be acute or chronic. **Acute intervention studies** investigate the immediate effects of ingestion, usually within hours to a maximum of 48 hours. They focus on aspects such as absorption, pharmacokinetics, bioavailability or metabolism of ingested compounds. In contrast, **chronic nutritional intervention studies** investigate the long-term effects of substances, foods, or diets over

extended periods, typically ranging from weeks to months or even years. In this regard, the studies of choice for evaluating the **bioactive effects** of phenolic compounds in humans are **chronic intervention studies**. These studies enable the observation of changes in metabolic pathways within the body resulting from the daily intake of the compound, extract, or diet ¹⁶³.

3.5.2 In vivo evaluation of the bioavailability and metabolism of phenolic compounds

One of the major challenges in phenolic research is to understand the absorption and metabolism of phenolic compounds and to find those that actually reach the target organs and exert a biological effect. This type of study, which examines the **bioavailability** and **metabolism** of phenolic compounds, can also be performed *in vitro* by *in vitro digestions*, for example using the INFOGEST protocol ¹⁶⁴. However, most efficacy studies are conducted in animals, using rodent models, in rats and mice, and in humans, by carrying out acute dietary intervention studies.

In the present doctoral thesis, an acute intervention study in humans was conducted to evaluate aspects such as absorption, pharmacokinetics, bioavailability, and metabolism of the ingested compounds present in the extracts under study. In this sense, this type of study is detailed below.

3.5.2.1 Acute intervention studies

Acute intervention studies of phenolic compounds are designed to assess the immediate effect of these compounds on the body following ingestion. They aim to understand several critical aspects of phenolic compounds, such as their absorption, pharmacokinetics, bioavailability and metabolism.

The primary objectives are to determine how quickly and efficiently these compounds are absorbed, analyse their movement through the body, measure their bioavailability at the site of physiological activity, and investigate their metabolic pathways. Methodologically, these studies involve selecting participants, administering controlled doses of phenolic compounds, collecting samples at various time points, and using advanced analytical techniques such as HPLC-MS to identify or quantify the compounds and their metabolites.

In this type of assay, it is important that volunteers adhere to a diet low in foods rich in phenolic compounds, such as fruits and vegetables, in the days preceding the assay and during the hours following it. This precaution helps to prevent interference in the detection of compounds from the administered extracts ¹⁶⁵.

Sample collection is a crucial component, as it provides the material necessary for subsequent studies. Among the biological samples, **blood** (serum and/or plasma), **urine**, and **faeces** are the most commonly chosen for this type of research due to their minimally invasive collection methods and their ability to contain metabolites resulting from the ingestion of phenolic compounds. However, in addition to selecting the appropriate sample type, it is also essential to determine the correct **sampling times**. This is particularly important for plasma and serum samples, as too short or too long times between samples can lead to a loss of information about the pharmacokinetics of the compounds in plasma. **Figure 22** shows an example of the sample collection times typically used for the study of the pharmacokinetics of phenolic compounds in plasma.

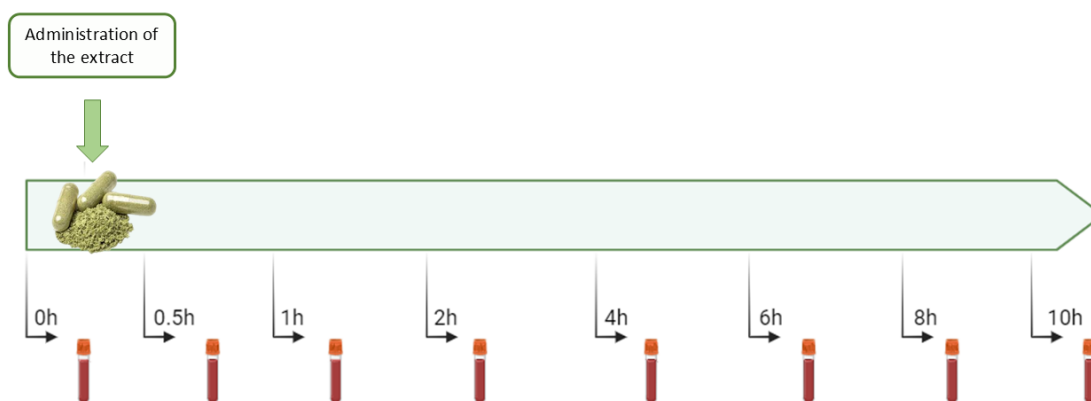


Figure 22. Schematic representation of blood plasma collection times in an acute intervention study.

Before sample analysis, sample treatment is essential to prepare the sample for the chosen analytical platform while preserving the original composition of metabolites as unchanged as possible. This thesis focuses on the analysis of blood plasma, thus outlining the primary strategies for treating this type of sample is crucial.

To obtain **blood plasma** from whole blood, it is essential to first treat the blood with an anticoagulant such as heparin, sodium citrate, or ethylenediaminetetraacetic acid (EDTA). The treated blood is then centrifuged. This process results in the supernatant, which contains the blood plasma, a middle layer that houses the leukocytes, and a bottom layer composed of erythrocytes (**Figure 23**)¹⁶⁶.

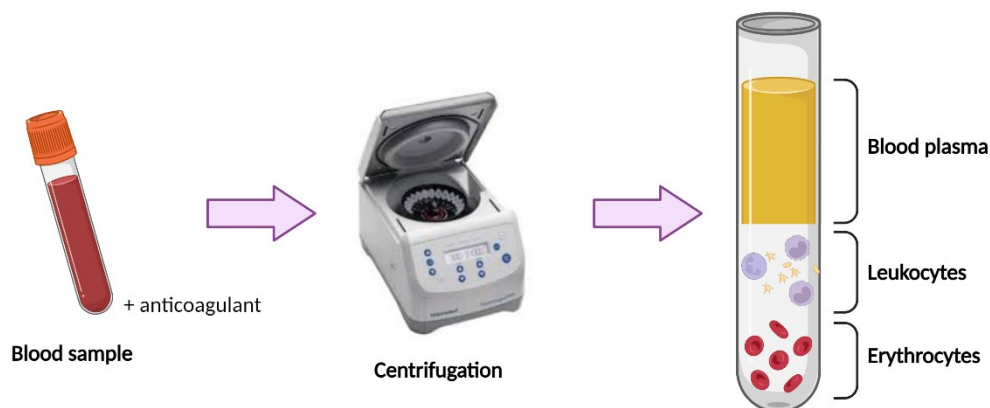


Figure 23. Blood plasma collection process.

The blood plasma samples then have to be prepared for analysis in the analytical platform of choice, which in this case was HPLC-MS. For HPLC-MS analysis, it is essential to **precipitate and remove proteins** from the biological matrix, as they are significant interferents in this type of analysis and incompatible with certain operational modes ¹⁶⁷. Among the different existing methodologies for protein removal, one of the most effective involves the use of **organic solvents** such as methanol, ethanol or a mixture of both ¹⁶⁸. A particularly successful combination is a 1:1 volume ratio of methanol to ethanol (v/v).

The findings of these types of studies provide valuable information on the absorption rate, bioavailability, pharmacokinetic parameters, and metabolic pathways of phenolic compounds, helping to identify effective compounds, guide dosage recommendations, support health claims, and inform long-term studies. Thus, acute intervention studies are essential for understanding the immediate **physiological effects** and **potential health benefits** of **phenolic compounds**, laying the groundwork for further research.

3.5.2.2 Metabolomics as a tool for the study of bioavailability and metabolism of phenolic compounds

The application of **metabolomics** to the study of the bioavailability and metabolism of phenolic compounds offers a comprehensive and detailed approach to understanding how these compounds are processed in the human body.

Conducting a comprehensive metabolomics study is complex; therefore, depending on the object of study, different analytical approaches can be employed. These metabolomics approaches fall mainly into two categories: **targeted** and **untargeted strategies**. The primary distinction between these approaches lies in whether the metabolites are predefined before analysis.

Targeted metabolomics focuses on the analysis a predefined set of **metabolites identified prior to the experiment**. In the study of the absorption, bioavailability, and metabolism of ingested phenolic compounds in both humans and animals, this methodology is the most commonly used. It involves the systematic search for specific predefined metabolites in biological samples, guided by the original composition of bioactive extracts and their anticipated metabolic transformations. Targeted strategies enable the identification and quantification of key metabolites, provided that commercial standards are available.

Several studies in the literature have addressed the use of targeted metabolomics to study the bioavailability and metabolism of phenolic compounds in mice and rat assays with the plant matrices blueberry and grape ¹⁶⁹, *L. citriodora* extract ¹⁷⁰, *S. marianum* ¹⁷¹ or *Epilobium angustifolium* extract ¹⁷². In the case of human studies there are also a

multitude of studies employing this targeted metabolomics for example using matrices such as yerba mate ⁷⁵, rosemary ¹⁶⁵, *H. sabdariffa* ⁸⁰, or *T. cacao* ¹⁷³.

3.6 Formulation of new nutraceuticals and cosmeceuticals

Based on the knowledge of phenolic compounds and the scientific evidence generated by the steps described above, high value-added products such as nutraceuticals and cosmeceuticals are developed. **Nutraceuticals** are medicinal foods, nutritional supplements, dietary supplements, or food components made from herbal or botanical raw materials that can deliver health benefits beyond basic nutrition, modulate immunity, and/or prevent and cure specific diseases and increase life expectancy. On the other hand, a **cosmeceutical** is a hybrid combination of cosmetics and pharmaceuticals containing biologically bioactive ingredients to add medical or drug-like benefits (e.g. cream, lotion, ointment and other similar substances containing botanical and marine extracts such as vitamins, peptides) ¹⁷⁴. Another term related to the aforementioned concepts, and increasingly gaining attention in recent years is **nutricosmetics**. Nutricosmetics refer to products and ingredients that function as nutritional supplements aimed at enhancing the natural beauty of the skin, nails, and hair ¹⁷⁵ (**Figure 24**).

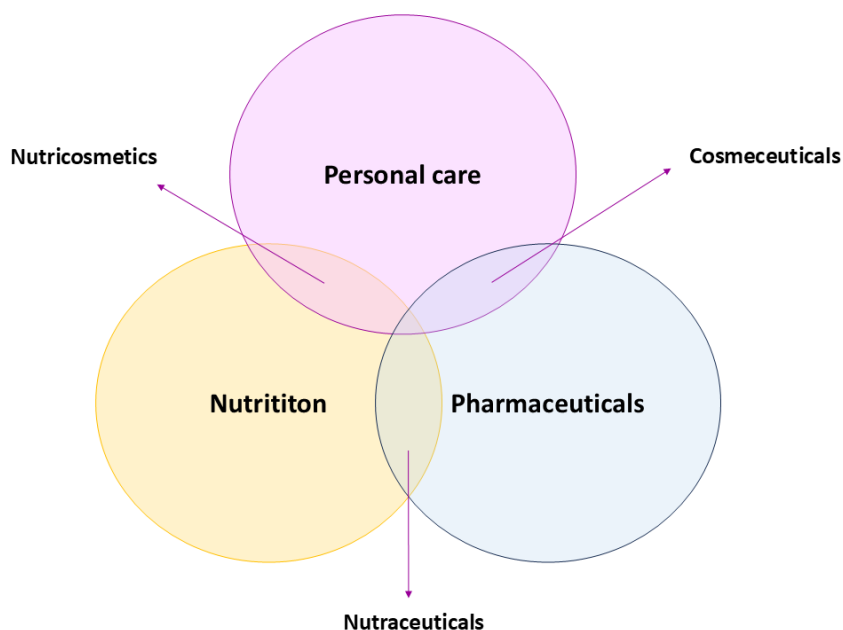


Figure 24. Representation of the interaction between pharmaceuticals, personal care and nutrition.

In nutraceuticals, phenolic compounds can be formulated into capsules, tablets, or powders, often using methods like microencapsulation or nanoparticle delivery systems to enhance bioavailability. For cosmeceuticals, phenolic compounds are incorporated into creams, serums, or gels with advanced delivery systems, such as nanoencapsulations or hydrogels, to improve skin penetration and product stability. Stability and compatibility testing is crucial to ensure the efficacy of the products over their shelf life, and safety assessments, including toxicity studies, are necessary to guarantee the non-toxicity and regulatory compliance of the formulations. Additionally, appropriate packaging that shields the phenolic compounds from environmental factors is essential. The formulations developed have to comply with local and international regulations on nutraceuticals and cosmeceuticals. This may involve compliance with guidelines set by organisations such as the United States Food and Drug Administration (FDA), EFSA or local authorities. Finally, a successful market strategy should highlight the

natural origin and scientifically supported benefits of these compounds, along with consumer education to promote proper usage and maximise effectiveness¹⁷⁶.

4. New methodological approaches for the study of phenolic compounds

The **traditional approach** to studying the bioactive potential of phenolic compounds undoubtedly provides a substantial amount of information; however, as previously mentioned, it has certain **limitations**. The most important of these are the inconsistencies that can arise between bioactivity observed *in vitro* and *in vivo*. This discrepancy is due to the fact that traditional *in vitro* assays are often performed using crude extracts or compounds isolated from these extracts. This approach overlooks the fact that many of these compounds may lack bioavailability when ingested *in vivo*, or that many compounds in the original extracts undergo metabolic transformations resulting in the formation of new metabolites with completely different bioactive properties. Furthermore, another shortcoming of the traditional approach to studying the bioavailability and metabolism of phenolic compounds is its reliance on targeted metabolomics. This approach focuses exclusively on preselected compounds, thus limiting the detection of unexpected or non-preselected metabolites.

In this context, the present doctoral thesis, in addition to a conventional study of phenolic compounds where their bioactive activity has been assessed through various *in vitro* assays and an *in vivo* assay in animals, introduces a novel approach to the study of phenolic compounds in plant extracts. Specifically, a **top-down approach** is proposed, beginning with an *in vivo* assay in humans aimed at identifying metabolites derived from phenolic compounds within the plant matrices under study. This method focuses on the detection of those metabolites that are bioavailable after ingestion by searching

biological samples, in order to subsequently assess the mechanisms of action of these compounds in chronic pathologies. This approach takes into account the bioavailability and metabolic transformations of phenolic compounds in the human body, thus ensuring their ability to effectively reach therapeutic targets and exert their bioactive effects.

This approach shares many of the steps involved in the traditional study of phenolic compounds, but the distinction lies in the sequence in which these steps are conducted. The initial step remains common, but it is directly linked to the formulation of a nutraceutical intended for use in a human intervention study, with the objective of screening for bioavailable compounds and metabolites derived from phenolic compounds that are also bioavailable. Once these metabolites have been identified, they can be selected and obtained either through purification, organic synthesis, or from commercially available sources, and subsequently tested *in vitro* for their effects on therapeutic targets. This approach, therefore, allows for the *in vitro* testing of compounds that are genuinely bioavailable and capable of reaching therapeutic targets *in vivo* (Figure 25).

In the current doctoral thesis, metabolites identified across the entire workflow were **commercially sourced** and subjected to evaluation for their **anticancer potential** using the strategies mentioned above. It is noteworthy that the methodologies employed to assess these anticancer effects are identical for both extracts and isolated metabolites.

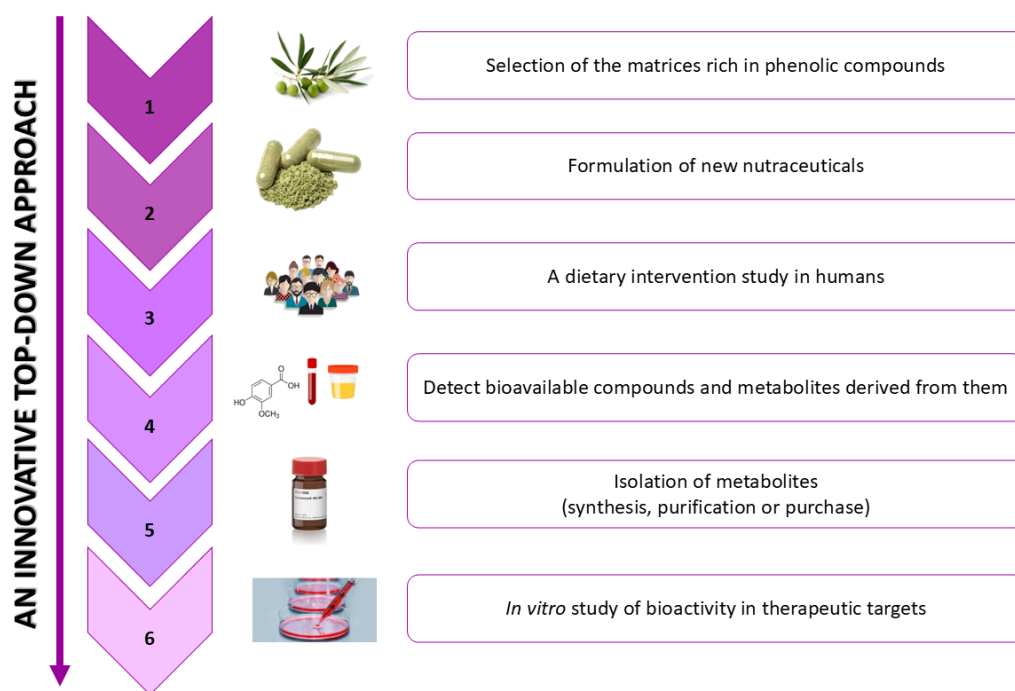


Figure 25. An innovative top-down study of phenolic compounds.

A crucial step in this approach is the search within biological samples for phenolic compounds and their derived metabolites that are bioavailable after human intervention. As mentioned above, these studies have traditionally been carried out using targeted metabolomics strategies, whose main limitation is the inability to identify unexpected or non-preselected compounds, which may lead to the omission of completely novel metabolites. In this context, this doctoral thesis proposes the use of **untargeted metabolomics** as a tool to study the **bioavailability** and **metabolism** of **phenolic compounds**. This approach aims to overcome the limitations of targeted strategies, particularly in plant matrices where there is limited knowledge of metabolites present in plasma or urine.

4.1 Innovative approach based on untargeted metabolomics for the study of the bioavailability and metabolism of phenolic compounds

The development of **untargeted metabolomics** strategies to study the bioavailability and metabolism of phenolic compounds overcomes the main **limitations** of **targeted methods**, but it should be noted that untargeted methods also have limitations and disadvantages compared to targeted methods. **Table 1** shows the main characteristics of the use of both strategies in the study of the bioavailability and metabolism of phenolic compounds.

Table 1. Properties of targeted and untargeted metabolomics approaches strategies in the study of the bioavailability and metabolism of phenolic compounds.

Targeted approach	Untargeted approach
Predefined metabolite selection based on extract composition and possible metabolic reactions	Full metabolomic analysis
Previous known metabolites	Allows finding unexpected metabolites
Absolute quantification	Relative quantification
High sensitivity and specificity	Lower sensitivity and specificity
Metabolite annotation validated against standards	Metabolite annotation depends on information in databases
Simplicity in data processing	Higher complexity in data processing

The key steps of the workflow used in this thesis to investigate the bioavailability and metabolism of phenolic compounds using an untargeted approach to the analysis of

biological plasma samples are outlined below. Each step highlights the key differences compared to the workflow typically used in a targeted metabolomics approach.

4.1.1 Analysis of biological samples

In relation to the analytical determination of metabolites in plasma samples, one of the main differences between targeted and untargeted methods lies in the sensitivity and specificity of the analytical method.

This **targeted approach** offers high **sensitivity** and **specificity**, often employing internal standards and rigorous validation processes to ensure accuracy and reproducibility. Additionally, targeted metabolomics allows for the precise **quantification** of identified metabolites, which is crucial for obtaining pharmacokinetic data from biological samples. The main advantages of this methodology are its high sensitivity and the relative ease of handling and processing the data it generates ¹⁷⁷. Whereas **untargeted metabolomics approaches** examine the comprehensive range of compounds present in a given sample without the a priori identification of specific targets. While untargeted approaches do not initially focus on quantification, they offer a notable advantage in that they can reveal previously unidentified or unexpected metabolites.

In terms of the **analytical instrumentation** used for both types of approach, the targeted metabolomics approach, which has high sensitivity and specificity, uses mass analysers such as the **Orbitrap** mass analyser, which is known for its ability to measure molecular masses with ultra-high resolution and massive accuracy, or the **triple quadrupole** spectrometers, which offer high sensitivity, ideal for accurate quantification of pre-selected compounds. In the case of untargeted metabolomics, **Q-TOF** analysers are widely used as they combine high sensitivity with high resolution, suitable for the

analysis of both known and unknown compounds ¹⁷⁸. In this sense, the analytical platform employed in this doctoral thesis for untargeted analysis of plasma samples to investigate exogenous metabolites from plant extracts is HPLC-ESI-QTOF.

In the study of biological samples using an untargeted approach, the use of **quality control (QC)** samples is of critical importance ¹⁷⁹. QC samples are characterised by their ability to represent the qualitative and quantitative composition of all biological samples under investigation. These samples are essential to monitor and ensure the **reliability of results** throughout the metabolomics workflow, especially when a large number of samples are analysed, which requires their distribution in different sequences or **analytical batches** ¹⁷⁹. This approach is essential to mitigate variability arising not only from biological differences but also from various instrumental factors, making the analysis of QC samples throughout the process crucial to control analytical variability. QC samples can be commercial or synthetic, or can be created by combining aliquots of each individual study sample in equal proportions ¹⁸⁰. After obtaining QC samples, they undergo the same sample treatment procedures as the rest of the samples.

4.1.2 Data processing and statistical analysis

Targeted metabolomics focuses on the **precise quantification** of specific metabolites that have been pre-selected prior to analysis. Because the metabolites of interest are known, data processing is more focused and direct. In contrast, **untargeted metabolomics** generates complex datasets that require advanced bioinformatics tools for analysis, followed by their identification using metabolomic databases.

The main goals of data processing in the workflow of untargeted metabolomics studies are to simplify the data, extract the signals of interest (**molecular features**), and prepare a data matrix suitable for subsequent statistical analysis ¹⁸¹.

The different steps that have been followed from the raw data to process the untargeted metabolomics approach used in this thesis are detailed below.

- **Peak picking:** In this stage, the **detection of signals of interest** is performed, along with baseline correction and noise reduction. It involves identifying and extracting relevant peaks from the chromatographic data, which correspond to the metabolites of interest. This step is essential for isolating true signals from background noise and baseline variations, enabling accurate quantification and identification of metabolites. Typical noise reduction techniques include smoothing algorithms and filtering methods, such as signal-to-noise ratio (S/N) enhancement ¹⁸¹.
- **Peak alignment:** It involves **adjusting the retention times** of detected **peaks across different samples** to ensure that corresponding peaks are correctly aligned. This process accounts for variations in chromatographic conditions that can cause shifts in retention times, thereby enhancing the comparability of data from different runs ¹⁸².
- **Signal intensity normalization:** This process adjusts the **signal intensities** across different samples to account for systematic variations and ensure comparability. Normalisation techniques correct for discrepancies arising from differences in sample concentration, injection volume, or instrument performance. By **normalising signal intensities**, this step enhances the reliability and accuracy of metabolite quantification, allowing for meaningful comparisons between samples.

- **Signal annotation:** This stage aims to consolidate all ionic species originating from the same metabolite into a single molecular entity termed a "**Molecular Feature**". This consolidation reduces the number of signals containing redundant information for statistical analysis ¹⁸³.
- **Statistical analysis:** Prior to the application of various statistical methods to identify patterns and correlations between annotated signals the data have to be transformed, scaled and missing values treated to ensure comparability between samples. Univariate methods, such **Student's t-test** and **analysis of variance (ANOVA)** ¹⁸⁴, are used to identify significant differences in individual metabolites, while multivariate techniques like **principal component analysis (PCA)** (**Figure 26**), which serves dual roles in both exploratory data analysis and quality control by identifying analytical drifts and anomalous data, alongside **cluster analysis**.

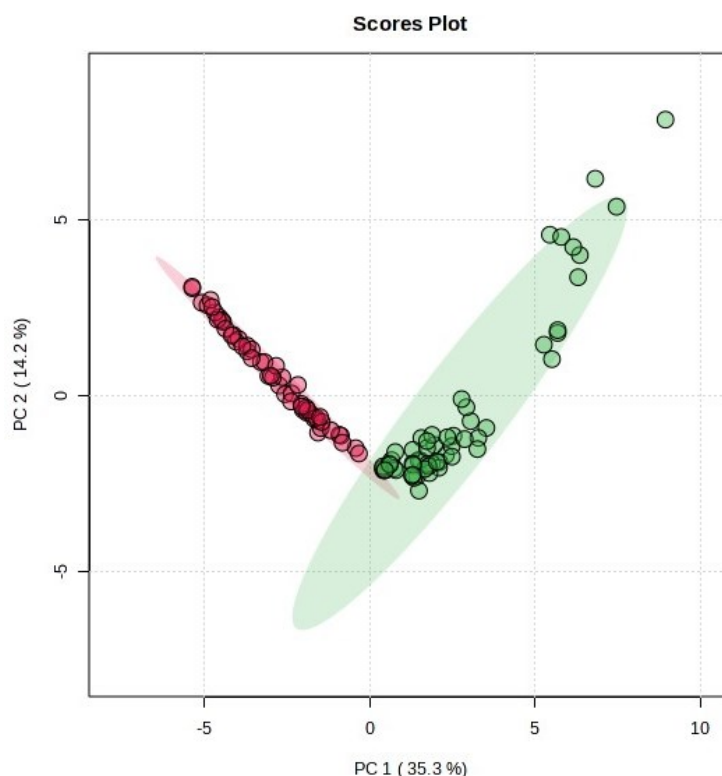


Figure 26. Example of a PCA plot.

4.1.2.1 Tools for data processing

To carry out the various stages of data processing, different software packages can be used. Traditionally, the most commonly employed are **commercial software** developed by analytical instrument manufacturers, as they tend to have more user-friendly interfaces. However, this type of software has limitations when dealing with very large datasets or when performing normalisation based on QC ¹⁸⁴.

In this context, the use of **open-source software** platforms is garnering increased attention. Although these platforms are generally less user-friendly than their commercial counterparts, they offer significant advantages, such as greater versatility and the ability to be tailored to the specific needs of the user. Many of the programs used in metabolomics are developed in the **R language**.

Another widely used open-source programme for data pre-processing is **MZmine**, which offers a more user-friendly and intuitive environment compared to the R environment. Additionally, **MetaboAnalyst** (<https://www.metaboanalyst.ca/>), a web platform, deserves mention for its capability to perform data pre-processing and numerous statistical analyses.

4.1.3 Metabolite Identification

The aim of this step is to determine the precise chemical structures of the detected metabolites. In the case of targeted metabolomics, this step is not necessary as it is based on a predefined list of known metabolites. However, in the case of **untargeted metabolomics**, this metabolite identification step has limitations because it depends on the information available in **databases**. Despite significant advancements in expanding these databases in recent years, the identification of metabolites remains a primary

challenge for this strategy. A considerable portion of signals cannot be matched to known compounds due to the lack of structural information in existing databases. This limitation underlines the continuing need to improve the completeness and accuracy of databases to increase the efficiency and reliability of non-specific metabolomics methods in investigating the bioavailability and metabolism of phenolic compounds ¹⁸⁵.

Various types of information collected throughout the workflow are used to identify the metabolites. These include exact mass values, retention times, isotopic distributions, adducts formed and fragmentation patterns derived from MS/MS analysis ¹⁸⁶.

It is noteworthy that **MS/MS** fragmentation spectra enable the assignment of chemical identities to candidate signals through comparison with entries in **commercial libraries** or freely accessible **online databases** such as the *Human Metabolome Database* (HMDB) (<http://www.hmdb.ca/>).

Challenges in metabolite identification include the great diversity of chemical structures, the presence of unknown or novel compounds not found in existing databases, and the potential for false positives. To address these problems, bioinformatics tools and software are often used to improve accuracy by incorporating additional data such as retention time, isotopic patterns and adduct formation. Examples of such tools are *CEU Mass Mediator* (<http://ceumass.eps.uspceu.es/>) or *Sirius* ¹⁸⁷.

4.2 Assessment of the bioactive potential of identified bioavailable metabolites

As mentioned above, the traditional approach to the study of phenolic compounds has mainly been to evaluate the bioactive potential of whole extracts or phenolic compounds isolated from these extracts to understand their mechanisms of action on various cellular targets. However, the proposed new approach has shifted our attention

to the evaluation of the therapeutic and preventive potential of metabolites that are bioavailable in biological fluids and can reach metabolic pathways to exert bioactive effects. Thus, the bioavailability and metabolic reactions that phenolic compounds undergo upon ingestion are taken into account.

In this context, several studies have already evaluated the **bioactive potential** of **bioavailable metabolites** capable of reaching target organs. For instance, phase II metabolites such as resveratrol glucuronide and resveratrol sulphate, as well as quercetin sulphate and quercetin glucuronide, have been studied for their ability to inhibit carcinogenesis, particularly in breast cancer ^{188,189}. Additionally, quercetin glucuronide has been shown to promote neural stem cell proliferation and migration both *in vitro* and *in vivo*, highlighting a novel mechanism for its positive effects on neurogenesis and suggesting its therapeutic potential in treating neurodegenerative diseases ¹⁹⁰. However, knowledge in this area remains limited, and there is a clear need for further research on the evolution and bioactivity of bioavailable metabolites. More studies are necessary to fully understand how these metabolites behave in the body, their long-term effects, and the mechanisms through which they exert their therapeutic potential.

A significant challenge in this search is the limited availability of these metabolites: their purification from plasma samples is complex due to their minute quantities, many cannot be synthesised, and commercially available options are scarce and very expensive. Therefore, it remains crucial to advance research in this field and explore alternative methods to access a wider range of metabolites.

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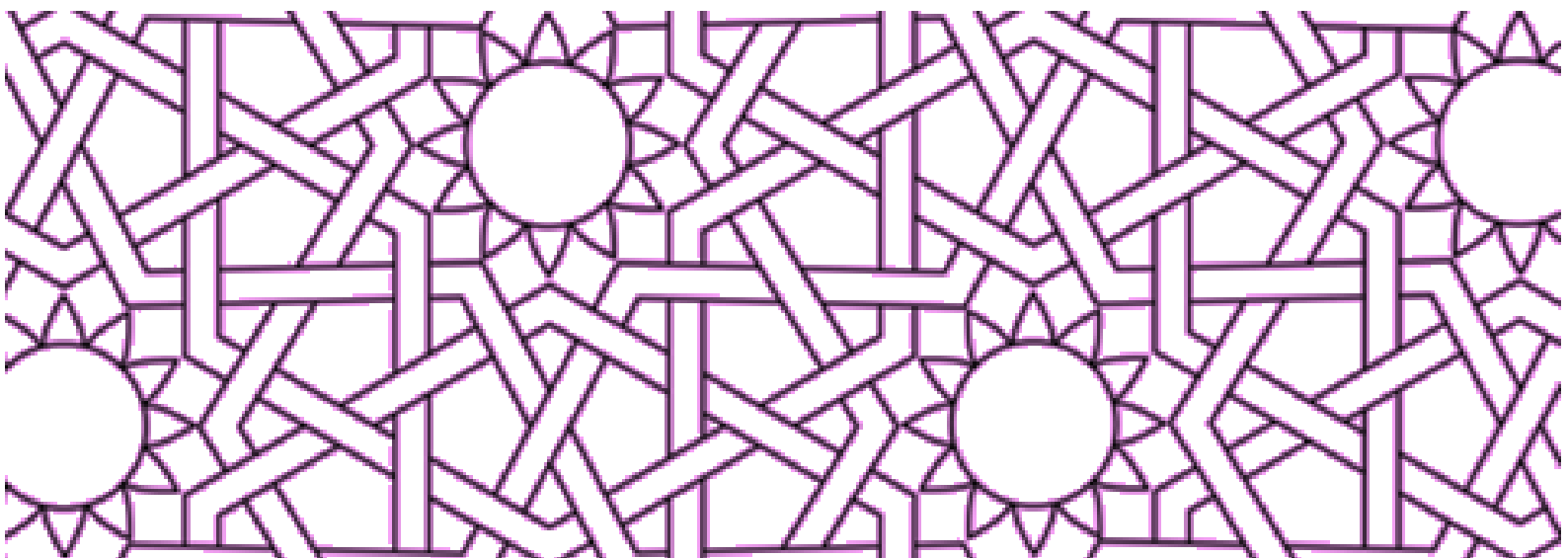
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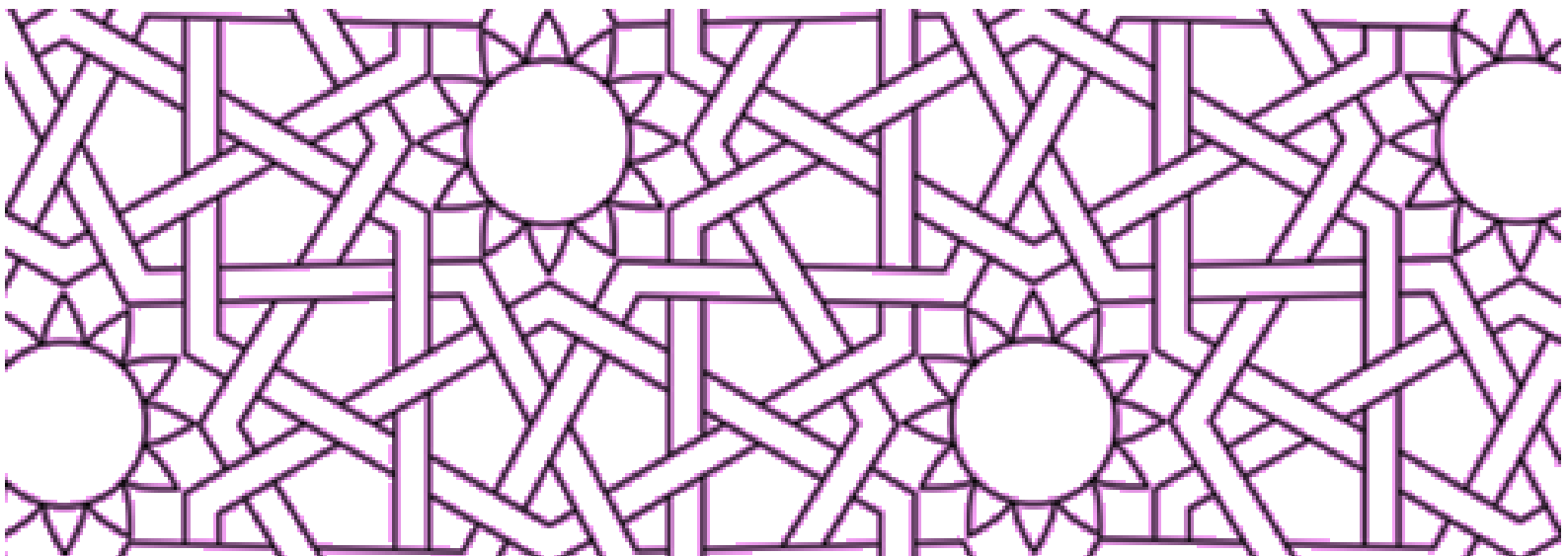
Experimental



***Section A. Traditional
methodological approach for
the study of phenolic
compounds***



Chapter 1



Pleiotropic Biological Effects of Dietary Phenolic Compounds and their Metabolites on Energy Metabolism, Inflammation and Aging

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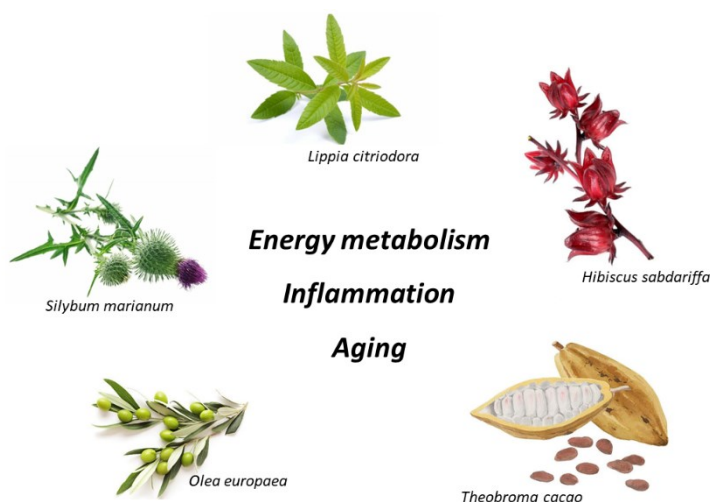
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Abstract

Dietary phenolic compounds are considered as bioactive compounds that have effects in different chronic disorders related to oxidative stress, inflammation process, or aging. These compounds, coming from a wide range of natural sources, have shown a pleiotropic behavior on key proteins that act as regulators. In this sense, this review aims to compile information on the effect exerted by the phenolic compounds and their metabolites on the main metabolic pathways involved in energy metabolism, inflammatory response, aging and their relationship with the biological properties reported in high prevalence chronic diseases. Numerous in vitro and in vivo studies have demonstrated their pleiotropic molecular mechanisms of action and these findings raise the possibility that phenolic compounds have a wide variety of roles in different targets.

Keywords: pleiotropic; phenolic compounds; chronic disorders; metabolites; bioactive compounds; oxidation; inflammation; aging

1. Introduction

Bioactive compounds are substances present as natural sources that have health benefits beyond the basic nutritional value of the product. By consensus, bioactive compounds have been defined as essential and non-essential compounds that are present in nature showing beneficial effects on human health [1]. Among the great variety of bioactive compounds, phenolic compounds stand out for the numerous amounts of compounds described with great bioactive potential and structural diversity. These compounds are secondary metabolites naturally synthesized in plants and present more than 8.000 chemical structures [2].

As mentioned, phenolics have a wide physiological activity in humans, such as an antioxidant [3], antimicrobial [4], anti-allergenic, cardioprotective, antiatherogenic, anti-inflammatory [5–8], and anticancer activities [9]. Due to this diverse and potential bioactivity, phenolic compounds have become functional compounds of great interest to the scientific community [10]. However, when phenolic compounds are ingested, they are usually metabolized suffering various modifications in certain chemical groups by means of glucuronidation, methylation, or sulfation reactions, among others, which can alter the biological activity. Besides that, they can be catabolized by the colonic microflora, which may drastically affect the absorption of these molecules through the gut barrier [11]. For this reason, it is necessary to take into account these metabolic reactions as well as their bioactivity when evaluating the bioactivity of phenolic compounds.

Phenolic compounds have shown a beneficial role against chronic diseases mainly related to metabolic stress, inflammation, and aging. In the literature, extracts and isolated compounds from plants have been reported to act on the main metabolic pathways involved in energy metabolism, inflammatory response, and aging.

In these pathways, there are specific key proteins that act as regulators. The main regulators of energy metabolism are the AMP-protein kinase (AMPK) [12] and the mammalian target of rapamycin (mTOR) [13]. In the case of inflammatory response and aging, the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) and sirtuins are the main regulatory elements, respectively [14,15]. However, these routes do not act in isolation, otherwise they are connected to each other. In addition, results obtained by in vitro and in vivo models support that plant bioactive metabolites exert pleiotropic effects further

than only free radical scavenging capacity [15–19]. They modulate multiple metabolic pathways through a variety of molecular targets, probably due to its molecular promiscuity and character diversity acquired through evolution [15].

In this context, the aim of this review is to gather information on the effects exerted by the phenolic compounds from plant sources and their metabolites in the main metabolic pathways involved in energy metabolism, inflammatory response, aging, and their relationship with the main chronic diseases with high prevalence. Specifically, *Silybum marianum*, *Lippia citriodora*, *Hibiscus sabdariffa*, *Theobroma cacao*, and *Olea europaea* have been selected due to the fact that the main families of phenolic compounds in the diet are fully represented in them.

A literature search of PubMed/MEDLINE using search terms '*Silybum marianum*' '*Lippia citriodora*' '*Hibiscus sabdariffa*' '*Theobroma cacao*' '*Olea europaea*' AND 'energy metabolism' 'inflammation' 'aging', alone or in combination, was done up to 11 December 2019. Papers from the last decade were prioritized, and experimental studies that include the effect of named matrices on named processes were included both in vitro and in vivo in humans and in animal models.

2. Effect of bioactive compounds on energy metabolism

Energy metabolism is the processes that involve food intake, transforming the food to release energy and storing the excess through complex metabolic pathways within the cell. The deregulation of energy homeostasis has been related to numerous chronic diseases, such as cancer, inflammation, obesity, diabetes, etc. [20]. In this sense, it has been shown that AMPK, which is a highly conserved serine/threonine protein kinase formed by a heterotrimer with an α -catalytic subunit, a scaffolding β -subunit, and a

regulatory γ -subunit, presenting each subunit multiple isoforms [12], is an important regulator of this cellular energy homeostasis [21].

AMPK is activated allosterically in cellular conditions with high concentration of AMP (ischemia, hypoxia, exercise, metabolic waste, and nutrient starvation) and is inhibited when the ratio ATP:AMP is high (nutrient-rich conditions) [22]. AMPK is also activated either by phosphorylation of Thr172 in the AMPK α by liver kinase B1 (LKB1) [23] or by Ca²⁺/calmodulin-dependent protein kinase β (CaMKK β) in response to elevated intracellular Ca²⁺ concentrations, independently of the energetic state of the cell [24].

AMPK takes part in the regulation of carbohydrate and lipid metabolism, and its activation resulting in the inhibition of ATP-consuming anabolic pathways, including fatty acid (FA) synthesis, hepatic gluconeogenesis, cholesterol, and isoprenoid synthesis, and mTOR-mediated protein translation. AMPK activation also stimulates ATP production through the increase of FA oxidation, muscle glucose transport, caloric intake and mitochondrial biogenesis [12,21]. In addition to the AMPK, there are other proteins involved in modulation of insulin effects on lipid metabolism as protein kinase B (Akt/PKB) and protein kinase C (PKC)- ζ [25].

Adipogenic transcription factors as PPAR γ and C/EBP- α induce gene expression changes characteristic of mature adipocytes and remain elevated for the life of the cells [26]. These factors positively regulate each other's expression since PPAR γ can promote adipogenesis in C/EBP α -deficient cells [27]. Adipogenesis is the process by which undifferentiated precursor cells differentiate into fat cells and occurs in different stages. First, the growth-arrested preadipocytes initiate mitotic clonal expansion (MCE), this allows reentry into the cell cycle for another two rounds of division. After the MCE, the

adipocyte differentiation is divided into the early, intermediate, and late stages. In the early stage, C/EBP β and C/EBP δ increase, producing changes in the expression of PPAR γ and C/EBP α that increase their levels until the beginning of the late stage when their levels start to lower gradually. Finally, the late stage is also regulated by PPAR γ and C/EBP α which have a synergistic effect [28]. On the other hand, there are several proteins that act in the regulation of the lipogenesis, such as fatty acid synthase (FAS) and sterol regulatory element-binding proteins (SREBPs). FAS is a multi-enzyme protein that catalyzes fatty acid synthesis. The expression of this protein is regulated by multiple transcription factors. For example, SREBPs is one of these transcription factors, which also regulates the expression of proteins that participate in processes such as synthesis of cholesterol, triacylglycerols, and phospholipids [29].

It is also known that AMPK can inhibit the de novo synthesis of FAs either by direct phosphorylation and inhibition of the enzymes acetyl-CoA carboxylase (ACC) [30] and 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase [31] or by the inhibition of the SREBP-1c [29].

Regarding energy stress the AMPK can be activated by phosphorylation, then the mTOR pathway is negatively regulated by directly phosphorylating the regulatory-associated protein of mammalian target of rapamycin (raptor) and tuberous sclerosis complex protein-2 [12,13]. In the plasma membrane, numerous receptors such as G protein-coupled receptor (GPCR); insulin-like growth factor receptor (IGF-R) and insulin receptor (IR) catch the signal that chemokines and growth factors wield on the cell that have a positive effect on mTOR through PI3K/Akt, since a serine/threonine protein kinase is activated by Akt [32]. Moreover, mTOR interacts with certain proteins to form two

distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 responds to energy, stress, amino acids, oxygen, and growth factors and is sensitive to rapamycin. It promotes cell growth by inducing anabolic processes and inhibiting catabolic processes. In the case of mTORC2, it responds to growth factors and regulates cell survival and metabolism, as well as the cytoskeleton, and this is not sensitive to rapamycin [33].

In summary, mTOR participates in the regulation of several processes such as protein synthesis and degradation, cell survival, longevity, and proliferation. Also, mTOR signaling pathway plays a fundamental part in the regulation of adipose tissue functions such as lipid metabolism, adipogenesis, and thermogenesis [34]. The deregulation or the chronic activation of mTOR may contribute to the pathogenesis of chronic diseases, such as obesity, non-alcoholic fatty liver disease, tuberous sclerosis and Alzheimer's disease [32]. In this sense, it has been discussed the mechanisms by which polyphenols can delay the molecular pathogenesis of oxidative stress via inhibition of mTOR-signaling pathways. Besides the main mentioned pathways, the mammalian sirtuin protein family (comprising SIRT1-SIRT7) are enzymes with an important role in the control of the metabolic status. Sirtuins are nicotinamide adenine dinucleotide dependent (NAD) enzymes that regulate the action of target transcription factors and other enzymes by deacetylation at acyl-lysine residues or ADP-ribosylation that allows the control of the organismal health-span and life-span [35]. In addition, several studies have shown the benefits of phenolic compounds in neurodegenerative and metabolic disorders by activating certain sirtuins [36].

Table 1 shows different types of trials related to the effect of the selected plants on the main metabolic pathways related to energy metabolism.

Table 1. Main parameters of the tests that show the effect of selected plant on the main metabolic pathways related to energy metabolism.

Assay	Model Type	Source	Effective Dose	Parameters	References
In vitro	Huh7.5.1 human hepatoma and Jurkat T cells	Silymarin (<i>S. marianum</i> extract)	80 µM for 4, 8 and 24 h	AMPK and mTOR pathways	[13]
	Activated T lymphocytes	Silymarin (<i>S. marianum</i> extract)	100 µM for 96 h	Cell cycle and PI3K/Akt/mTOR signaling pathway	[37]
	Hypertrophic adipocytes	Verbascoside (isolated from <i>L. citriodora</i> extract) and <i>L. citriodora</i> extract	400 µg/mL for 48 h of <i>L. citriodora</i> 108 µg/mL for 48 h of Verbascoside	Expression of PPARα, FAS, and AMPK	[38]
	3T3-L1 Hypertrophic adipocytes	29 compounds of the <i>L. citriodora</i> extract	200 and 400 µg/mL of the <i>L. citriodora</i> fractions for 48 h	AMPK activation	[39]
	3T3-L1 mature adipocytes	<i>L. citriodora</i> extract Verbascoside, luteolin-7-diglucuronide and loganic acid isolated from <i>L. citriodora</i> extract	100, 200 and 400 µg/mL of the whole extract for 24 h 25, 50 and 100 µg/mL of each isolated compound for 24 h	AMPK activation	[40]
	PC12 cells	Verbascoside (isolated from <i>L. citriodora</i> extract)	30 µM for 1, 3, 6, 12, 24 h	PI3K/Akt/mTOR signaling pathway	[41]
	Colorectal cancer cells	Verbascoside (isolated from <i>L. citriodora</i> extract)	100 µM for 72 h	PI3K/Akt/mTOR signaling pathway	[42]
	3T3-L1 preadipocytes	<i>H. sabdariffa</i> extract	250, 500, 1000, 2000 and 5000 µg/mL for 36 h	Adipocyte differentiation	[26]
	3T3-L1 preadipocytes	Theobromine (isolated from <i>T. cacao</i>)	50, 100, and 150 µg/mL for 6 days	Adipocyte differentiation	[28]
	Human HepG2 cells	Cocoa flavanol epicatechin (isolated from <i>T. cacao</i>)	10 µM for 24 h	Lipid metabolism	[25]
	Rat Müller cells	Cocoa enriched with polyphenols	100 ng/mL, 1 µg/mL and 10 µg/mL for 24 h	Sirtuin pathway	[43]

	Human HepG2 hepatocytes	<i>O. europaea</i> fruit pulp extract	10, 20, 40 and 80 µg/mL for 24 h	AMPK and SREBP-1c activation	[29]
	Primary-cultured rat-hepatocytes	Oleuropein, hydroxytyrosol and tyrosol (isolated from <i>O. europaea</i>)	10 µM for 24 h of each phenol	Lipid synthesis	[44]
	Breast cancer cells	Secoiridoids from extra virgin olive oil	-	AMPK and mTOR activation	[17]
	Vascular adventitial fibroblasts	Hydroxytyrosol (isolated from <i>O. europaea</i>)	200 and 400 µM for 24 h	Autophagy	[45]
	3T3-L1 adipocytes	Hydroxytyrosol (isolated from <i>O. europaea</i>)	1.0 and 10 µM for 72 h	AMPK and genes involved in fatty acid oxidation activation	[46]
In vivo	Induced inflammation rats	<i>H. sabdariffa</i> extract	10, 20, and 40 mg/kg daily for 5 days	Oxidative stress and NF-κB translocation	[47,48]
	Obese mice	<i>H. sabdariffa</i> extract	33 mg of total anthocyanins/kg three times a week for 8 weeks	PPARγ and C/EBP-α transcription	[49]
	Obese mice	MetA (mixture of <i>L. citriodora</i> and <i>H. sabdariffa</i> extracts)	50 and 100 mg/kg once daily for 8 weeks	Adipogenesis-related genes, oxidation-related genes, lipogenesis-related genes expressions and AMPK activation	[50]
	Diabetic rats	Theobromine (isolated from <i>T. cacao</i>)	5 mg/kg daily for 12 weeks	NAD ⁺ /SIRT1 activity	[51]
	Obese rats	Epicatechin (isolated from <i>T. cacao</i>)	1 mg/kg daily for 2 weeks	Levels of skeletal muscle and abdominal tissue SIRT1 and UCP1	[52]
	SAMP8 mice	Phenolic compounds of olive oil	-	SIRT1 expression	[53]
In vitro/In vivo	HK2 cells	Silybin (isolated from <i>S. marianum</i> extract)	50 µM for 24 h (in vitro)	Mitochondrial function	[54]
	SIRT3 knockout mice		200 mg/kg for 7 days (in vivo)		
	Glioblastoma cells	Theobromine (isolated from <i>T. cacao</i>)	10 µM for 72 h (in vitro)	Akt/mTOR pathway NF-κB pathway	[32,55]
	Rats		0.05% w/w for 40 days (in vivo)		
	SH-SY-5Y neuroblastoma cells	Hydroxytyrosol (isolated from <i>O. europaea</i>)	10 µM for 24h (in vitro)	AMPK activation	[56]
	<i>db/db</i> mice		10 and 50 mg/kg per day for 8 weeks (in vivo)		

3T3-L1 Hypertrophic adipocytes	Polyphenols derived from <i>L. citriodora</i>	500 µg/mL for 72h (in vitro)	AMPK activation	[57]
Obese/overweight subjects	and <i>H. sabdariffa</i> extracts	500 mg/day for 2 months (in vivo)		

2.1 *Silybum marianum*

The action of several compounds or the entire extract of *S. marianum* have been evaluated by in vitro models in different cell lines such as human liver cells, T lymphocytes and HK2 cells.

Lovelace et al. (2015) studied the role of silymarin in the cellular metabolism of both human liver and T cells (Huh7.5.1 human hepatoma and Jurkat T cells). Silymarin is a flavonoid extract that contains silibinin, isosilybin, silydianin, and silychristin, obtained from the plant *S. marianum*. This study showed that nontoxic doses of silymarin first induced energy stress responses and then, the cells respond to these stresses with reparative and adaptive changes instead of dying. Moreover, silymarin modulates metabolic pathways, like AMPK, resulting in the suppression of mTOR and inflammatory signaling [13]. This action can be divided into two phases. The first phase consists in the induction of the stress response when the activation of AMPK signaling. In addition, the induction of DNA-damage-inducible transcript 4 mRNA and protein expression result in the inhibition of mTOR signaling translating to endoplasmic reticulum stress induction. The second phase results in the inhibition of the inflammation because the activation of AMPK signaling inhibits the nuclear factor- κ B (NF- κ B) signaling. This fact shows that the AMPK presents a major role in transducing the signal from silymarin-induced stress to anti-inflammatory effects. The effect of silymarin in the inflammation has also been explained for the Forkhead box O3 inhibition [58], a key conduit between cell metabolism, growth, and inflammation, and which is associated with suppression of many inflammatory genes such as C-X-C motif chemokine 10, whose expression has been associated with many autoimmune diseases [59].

As mentioned earlier, mTOR has several regulatory routes, for example PI3K/Akt/mTOR signaling route. Gharagozloo et al. (2012) investigated the effect of silymarin on the cell cycle and the PI3K/Akt/mTOR signaling pathway of activated T lymphocytes. They showed that silymarin inhibited cell proliferation through the suppression of the mTOR signaling pathway and also through G1 cell cycle arrest in human activated T lymphocytes in vitro [37].

The effect of some compounds of *S. marianum* have also been evaluated in both sirtuin 3 (SIRT3) knockout mice and diabetic Wistar rats. In this case, silybin, the major pharmacologically active compound of the *S. marianum* fruit, has also showed the ability to protect against cisplatin-induced acute kidney injury and tubular cell apoptosis both in vitro (HK2 cells) and in vivo (SIRT3 knockout mice) by improving mitochondrial function through the elevation of SIRT3 expression [54]. Besides, it has also been reported that silymarin extracts have an effect on sirtuin 1 (SIRT1). The administration of silymarin causes a positive regulation of SIRT1 mRNA expression, and this overexpression is related to a decrease in lipid concentrations, a higher glycogen content and a negative regulation of the SREBP-1c gene in the diabetic Wistar rats liver. This is a very important achievement in the field of diabetes research, since diabetes causes a significant increase in fasting blood sugar and in total cholesterol and triglycerides in the liver [60].

As well as silymarin induces a stress response and suppresses inflammation, there are other natural phytochemicals derived from plants with this capacity, for example, curcumin and epigallocatechin have demonstrated the same effects in AMPK pathways [61,62].

2.2 *Lippia citriodora*

There are several studies that prove the effect of *L. citriodora* extract or some of its compounds on energy metabolism, for example, this effect has been studied in hypertrophic adipocytes, PC12 cells, and colorectal cancer cells.

Verbascoside is the most abundant phenylpropanoid glycoside compound in *L. citriodora*. This plant is known to possess multiple biological activities including anti-inflammatory, antioxidant, and anticancer as well as anti-obesity effects with AMPK activation [50]. For example, the incubation of high glucose-induced hypertrophic adipocytes with extract of *L. citriodora* or verbascoside produces a change in mRNA expression and protein levels of PPAR α , a major regulator of lipid metabolism, FAS, lipogenic-related gene, and the central metabolic sensor AMPK. The result of the incubation is an increase in PPAR α , a decrease in FAS and a significant activation of AMPK [38].

29 compounds from *L. citriodora* obtained by a HPLC semi-preparative purification method were tested in 3T3-L1 hypertrophic adipocyte model to prove their influence in the activation of AMPK. This activation was measured by immunofluorescence microscopy, resulting in that flavonoids, phenylpropanoids and iridoids, mainly luteolin-7-diglucuronide, verbascoside, and gardoside, respectively, were the compounds with greater ability to activate AMPK. It was also observed that several compounds showed a synergic behavior, which provided a greater capacity of activation of the AMPK [39]. Similarly, Olivares-Vicente et al. (2019) also observed this synergic effect being verbascoside, luteolin-7-diglucuronide and loganic acid the compounds with greatest capacity to activate AMPK in mature adipocytes [39,40]. The mechanism of action by

which these compounds exerted this activating action on AMPK could be explained by their direct actuation as agonists of AMPK, linking to the AMP binding sites of the gamma subunit and/or the different sites of the interaction zones between the beta and gamma subunits [40]. Since these results showed the ability of *L. citriodora* compounds to activate the AMPK pathway, this mechanism could be considered a strategy against obesity-related metabolic disorders [39,40].

On the other hand, the effect of verbascoside on mTOR has also been tested showing the ability of this compound to inactivate PI3K/Akt and activate the PI3K/Akt/mTOR signaling pathway in PC12 cells and colorectal cancer cells. [41,42].

The effect of the extract or isolated compounds of *L. citriodora* has also been evaluated by different authors in rabbits and obese/overweight human. In this scenario, verbascoside demonstrated the ability to activate SIRT1 activity in rabbit's heart and liver. This SIRT1 can regulate antioxidant genes and participates in the regulation of certain signaling pathways, such as AMPK activation, associated with metabolic disorders and obesity. Therefore, the antioxidant effect of verbascoside could be regulated by increasing SIRT1 activity, leading to treatment of obesity and related metabolic disorders through AMPK activation [63]. Herranz-López et al. (2019) conducted a trial in 56 obese/overweight subjects for 2 months in which a dietary supplement containing 500 mg of a combination of phenolic extracts from *L. citriodora* and *H. sabdariffa* (LC-HS) was administered. The consumption of this dietary supplement showed a reduction in symptoms associated with obesity-related diseases. In addition, the LC-HS extract significantly reduced lipid content and increased AMPK activity in a model of hypertrophied adipocyte cells. Therefore, the results of this study suggested

that this dietary supplement causes a modulation of fat metabolism in adipose tissue, probably mediated by the activation of AMPK [57].

2.3 *Hibiscus sabdariffa*

H. sabdariffa is known for presenting antioxidant, antigenotoxic and biomodulatory effects in animals exposed to toxic agents. This plant also has diuretic, antimicrobial, and antihypertensive effects [64]. In this sense, Kim et al. (2007) showed that *H. sabdariffa* acts as a mitogen-activated protein kinase (MEK) inhibitor. This inhibition blocks significantly the ability of MEK 1/2 to phosphorylate extracellular signal-regulated kinase 1/2 (ERK1/2) and the expression of PPAR γ and C/EBP- α during adipocyte differentiation induction [26].

Moreover, there are numerous studies conducted in different animal models in which *H. sabdariffa* has demonstrated an effect on energy metabolism. For example, phenolic compounds of *H. sabdariffa* inhibited inflammation processes by down-regulating cyclooxygenase-2 (COX-2) and inhibiting the activation of p38 kinase and c-Jun N-terminal kinase (JNK), postulating a relationship between *H. sabdariffa* polyphenols, oxidative stress and suppression of nuclear factor- κ B (NF- κ B) translocation in a lipopolysaccharide-induced inflammation rat model [47,48].

Regarding, PPAR γ and C/EBP- α , when the gene expression of both transcription factors PPAR γ and SREBP-1c is observed, the last one is regulated by C/EBP- α [65]. In fact, in obese mice, the supplementation with *H. sabdariffa* extract reduced the transcription of both factors [49]. However, the inactivation of these transcription factors occurs with the activation of the AMPK, for example, by the phenolic compounds present in *H. sabdariffa*

extracts. So, these results suggested that the effects of *H. sabdariffa* over adipogenesis are mediated by SREBP-1c and PPAR γ regulation through AMPK activation [49].

On the other hand, AMPK and PPARs also participate in the pathogenesis of both alcoholic liver and non-alcoholic fatty liver diseases, in which fatty acid oxidation is impaired. It has been checked that the ameliorating of both diseases can be achieved with the administration of AMPK or PPAR α activators both in vitro and in vivo. Phenolics compounds have also demonstrated their effects by increasing PPAR γ mediated adiponectin expression, along with AMPK activation [47].

In addition, synergistic effects of *L. citriodora* and *H. sabdariffa* extract, Metabolaid® (MetA), on the metabolism of obese mice fed a high-fat diet were analyzed by Lee et al. (2018). In this study, with the administration of MetA, it was observed that body-weight gain, white adipose tissue (WAT), liver weight, serum, and hepatic lipid profiles and serum glucose levels were reduced [50]. Besides, MetA affected to numerous metabolic pathways more significant than that of with the extracts alone. For example, MetA reduced significantly the expression of the adipogenesis-related genes, CEBP/ α , PPAR γ , and SREBP-1c and their target genes, aP2/FABP4 and FAS, whereas increased the thermogenesis-related genes as uncoupling protein 1 (UCP1) and uncoupling protein 2 (UCP2) in WAT. Moreover, this extract increased the activation of AMPK and fatty oxidation-related genes, PPAR α and ACS1, while the lipogenesis-related genes were decreased [50].

2.4 *Theobroma cacao*

The action of several phytochemicals of *T. cacao* has been evaluated in different cell lines such as 3T3-L1 preadipocytes, high-glucose treated HepG2 cells or rat Müller cells.

Theobromine is a methylxanthine from the *T. cacao*. This compound has shown to suppress the activation of the Akt/mTOR and NF- κ B pathways by in vitro and in vivo models [32,55]. In addition, theobromine has also shown the ability to inhibit adipocyte differentiation in 3T3-L1 preadipocytes through down-regulation of PPAR γ , C/EBP α , aP2 and leptin. Specifically, this compound exerts its inhibitory effect at the early stage of adipogenesis through AMPK activation and suppression of the c-Jun N-terminal kinase (JNK) and ERK signaling pathways in 3T3-L1 adipocytes [28].

Regarding flavonoids, epicatechin, a cocoa flavanol, decreases total lipid included in high glucose-exposed hepatic cells via Akt, AMPK, and PKC ζ [25]. This compound also reduces the levels of SREBP1-c and FAS and increases the levels of PPAR α in high-glucose treated HepG2 cells through the inhibition of PKC ζ [26].

Duarte et al. (2015) evaluated the possible protective effects of cocoa on the diabetic retina. In this study, rat Müller cells were treated with cocoa extract enriched in polyphenols. It was observed that the activation of PARP-1 and the decrease of the levels of NAD $^{+}$ and SIRT1 in diabetes were dose-dependent with oral administration of polyphenolic cocoa extract. This study showed that the cocoa extract improved the SIRT1 retinal pathway, allowing the protection of the retina in diabetic patients [43].

The effect of some compounds of *T. cacao* have also been evaluated in different animal models such as hypertensive rats with induced diabetes and obesity rat model induced by a high-fat diet. In this regard, Papadimitriou et al. (2014) evaluated the effect of theobromine treatment in spontaneously hypertensive rats with induced diabetes. They observed that in diabetes mellitus, SIRT1 activity was reduced by PARP-1 activation and NAD $^{+}$ depletion due to low concentration of AMPK increasing NOX4 expression. This is

associated with extracellular matrix (ECM) accumulation in the diabetic kidney. Therefore, an increase in the activation of SIRT1 can be achieved by theobromine administration, revealing its therapeutic potential for diabetic nephropathy [51].

Gutiérrez-Salmeán et al. (2014) conducted a study in which epicatechin was administered to obesity rats induced by a high-fat diet. A decrease in the rate of weight gain, blood glucose, and hypertriglyceridemia were detected. In addition, it was observed that epicatechin restored the levels of SIRT1 and UCP1 in the skeletal muscle and abdominal tissue of obesity-induced rats [52].

2.5 *Olea europaea*

Several studies have demonstrated that the fruit and leaf extracts of *O. europaea* possess antihypertensive, antithrombotic, anticancer, antiatherogenic hypoglycemic, anti-inflammatory and antimicrobial properties [29]. These biological activities have been evaluated in different cell models to assess the effects on energy metabolism in human HepG2 hepatocytes, rat liver cells, adventitious vascular fibroblasts, breast cancer cells, SH-SY5Y neuroblastoma cells and 3T3-L1 adipocytes. On this scenario, *O. europaea* fruit pulp extract promotes AMPK phosphorylation in human HepG2 hepatocytes. This phosphorylation causes an activation of AMPK which leads to the inhibition of lipid accumulation due to the activation of low regulation of FAS expression through SREBP-1c inactivation [29].

Concerning main phenolic compounds of extra virgin olive oil, oleuropein, hydroxytyrosol, and tyrosol have been reported to their biochemical and pharmacological properties. The effect of these compounds on the synthesis of lipids in primary cultured rat liver cells has been studied. The results showed the inhibition of the

synthesis of *de novo* cholesterol and fatty acids without an effect on the cell viability. In order to clarify the lipid-lowering mechanism of these compounds, the key enzymes in these processes were evaluated, obtaining as a result the reduction of ACC and HMG-CoA reductase activities after 2 h of 25 μ M phenol treatment. It was also concluded that the ACC inhibition appears to be mediated by phosphorylation of AMPK [44].

In addition, some secoiridoids from extra virgin olive oil have been reported to activate AMPK, which results in the inhibition of mTOR in breast cancer cells [17]. This inhibition may be important in the action against chronic diseases because in these pathologies there are an overactivation of the nutrient-sensing mTOR due to the loss of responsiveness to active AMPK, a suppressor of mTOR [21].

Hydroxytyrosol has shown a positive regulatory effect on AMPK allowing the prevention of type 2 diabetes brain damage [66]. Wang et al. (2018) studied the hydroxytyrosol effect in autophagy in adventitious vascular fibroblasts, observing that it regulated the expression of the SIRT1 mRNA and protein and that it finally caused the suppression of Akt/mTOR. The authors concluded that hydroxytyrosol regulated the autophagy of adventitious vascular fibroblasts through the suppression of Akt/mTOR mediated by SIRT [45]. Following this positive regulatory effect of hydroxytyrosol on AMPK, other studies have determined in high glucose-induced neuroblastoma SH-SY5Y cell damage an increase of neuroprotection in db/db mice, which showed hyperglycemia and obesity [56]. Moreover, in 3T3-L1 adipocytes, this compound is able to act positively in AMPK and on genes involved in fatty acid oxidation such as PPAR α , Cpt1, and PPAR γ [46].

Regarding in vivo studies, the effect of *O. europaea* have been evaluated in model 8 prone to senescence accelerated mice (SAMP8) and db/db mice. In this sense, it has

been observed that the high intake of phenolic compounds of olive oil, i.e., a high consumption of hydroxytyrosol, positively increased SIRT1 in SAMP8 mice. This positive effect on SIRT1 led to the stimulation of an antioxidant response and a decrease in oxidative stress in the heart of SAMP8 mice [53].

3. Effect of bioactive compounds on inflammation process

Inflammation is a process that is part of the normal repair process when there is damage to the individual and is essential for protection against bacterial and viral infections and harmful environmental agents. When the inflammation persists over time, it becomes chronic inflammation which has been related to several age-related chronic diseases such as Alzheimer's disease, atherosclerosis, diabetes, cancer, rheumatoid arthritis, among others [67].

Numerous studies have shown that Nrf2 contributes to the anti-inflammatory process by organizing the arrest of inflammatory cells and regulating gene expression through the antioxidant response element (ARE). This signaling pathway allows the regulation of the expression of anti-inflammatory genes and inhibits the progression of inflammation [14]. Nrf2 is a transcription factor that plays an important role in the control of the response to oxidative stress.

Under basal conditions, Nrf2 is locked by kelch-like ECH-associated protein (Keap1) by its two motifs (ETGE and DLG) leading to CUL3-mediated ubiquitination and finally its proteasome degradation. While under oxidative stress conditions, Nrf2 dissociates from Keap1, thus Nrf2 accumulates in the nucleus and activates the ARE-gene battery [68]. One of this ARE-gene is HO-1, upregulated HO-1 transform the heme into CO, bilirubin and free iron. This CO produced acts as an inhibitor of the NF- κ B pathway which

decreases the expression of pro-inflammatory cytokines as interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α), while bilirubin also acts as an antioxidant. Besides, HO-1 inhibits the activation of pro- and anti-inflammatory cytokines as IL-10 [14].

Although Keap1 is the major regulator Nrf2 activation, Nrf2 can be also activated by additional signal transduction pathways, e.g., JNK, p38 MAPK, AMPK or PI3K/Akt, promoting anti-oxidative effects [68]. Both genetic and pharmacological studies propose the existence of a functional cross-talk between Nrf2 and NF- κ B pathways. Nrf2 and NF- κ B are key pathways involved in controlling the balance of cellular redox status and responses to inflammation and stress [69]. When a situation of oxidative stress occurs, I κ B kinase (IKK β) is activated raising the release and nuclear translocation of NF- κ B. At the nuclear site, NF- κ B causes the transcription of pro-inflammatory mediators such as IL-1, IL-6, TNF- α , inducible nitric oxide synthase (iNOS) and intracellular adhesion cyclooxygenase-2 (COX-2) [14]. Excessive increase in IL-1, IL-6 and TNF- α occurs frequently in an acute inflammatory response and in several chronic inflammatory diseases [70].

The activity of NF- κ B influences Keap1/Nrf2/ARE signaling pathway in different ways. One of these is Keap1, which degrades IKK β by ubiquitination, resulting in the inhibition of NF- κ B activity. Furthermore, inflammatory mediators like COX-2 are produced in the inflammatory processes reacting with Keap1 and activating Nrf2, thereby initiating gene transcription with simultaneous inhibition of NF- κ B activity [14].

Numerous *in vivo* studies have demonstrated that Nrf2 plays an important effect in several inflammatory diseases such as gastritis, arthritis or neurodegenerative disease. In this sense, Ahmed et al. (2017) demonstrated that the absence of Nrf2 reflected

greater symptoms of inflammation [14]. **Table 2** shows the effects of the selected matrices on the main metabolic pathways related to inflammation process.

3.1 *Silybum marianum*

Isosilybin, a compound present in *S. marianum* extract presents pharmacological activities which have been reported on in vitro studies. For example, Zhou et al. (2016) reported that isosilybin could alleviate the A β ₂₅₋₃₅-induced oxidative stress damage, which is considered a direct cause of Alzheimer's disease, in HT-22 hippocampal cells. They determined the mechanism of action of isosilybin that significantly increased the protein and mRNA expression of antioxidases, such as HO-1, glutathione S-transferase (GST), and aldo-keto reductases 1C1 and 1C2 (AKR1C2), through the activation of NRF2/ARE signaling. This activation induces inhibition of ROS accumulation and ultimately alleviating A β ₂₅₋₃₅-induced oxidative stress damage in HT-22 cells [71].

Table 2. Main parameters of the tests that show the effects of different plant species on the main metabolic pathways related to inflammation.

Assay	Model type	Source	Effective dose	Parameters	References
In vitro	HT-22 hippocampal cells	Isosilybin (isolated from <i>S. marianum</i> extract)	10 μ M for 18 h	Activation of NRF2/ARE signaling	[71]
	3T3-L1 hypertrophic adipocytes	<i>L. citriodora</i> extract	400 μ g/mL for 48 h	NF- κ B expression	[38]
	LPS-stimulated macrophage RAW 264.7 cells	Essential oil from <i>H. sabdariffa</i>	50, 100 and 200 μ g/mL for 24 h	NF- κ B and MAPK signaling pathways activation	[70]
	Lymph node carcinoma of the prostate cells	<i>H. sabdariffa</i> leaf extracts	0.5 mg/mL for 24 h	Akt/NF- κ B/MMP-9 pathway regulation	[72]
	wild-type and Nrf2 KO astrocytes	(-)-epicatechin (isolated from <i>T. cacao</i>)	100 μ M for 1 h	Nrf2 regulation	[73]
	LPS-stimulated peritoneal murine macrophages	Extra virgin olive oil polyphenolic extract	25 and 50 mg/mL for 18 h	AMPK phosphorylation and NF- κ B nuclear translocation	[74]
	Primary astrocytes	Maslinic acid (isolated from <i>O. europaea</i>)	1 and 10 μ M for 24 h	NF- κ B nuclear translocation	[75]
	CD31+/VEGFR-2+ cells	Oleacein and oleuropein (isolated from <i>O. europaea</i>)	1, 2, 5 and 10 μ M for 3 h	HO-1 and Nrf2 levels	[76]
In vivo	Rats with arsenic treatment	Silybin (isolated from <i>S. marianum</i> extract)	75 mg/kg per day for 4 weeks	Nrf2 and NF- κ B expressions	[77]
	NASH mice	Silybin (isolated from <i>S. marianum</i> extract)	105 mg/kg per day for 8 weeks	NF- κ B nuclear translocation	[78]
	Induced gastric injury rats	Silymarin (<i>S. marianum</i> extract)	50 mg/kg for 5 days	Nrf2 and NF- κ B expressions and pro-inflammatory cytokines levels	[79]
	Zebrafish	Verbascoside (isolated from <i>L. citriodora</i> extract)	400 μ g/mL per day for 4 days	Nrf2/ARE signaling pathway activation	[80]

	Rats with induced hepatotoxicity	<i>H. sabdariffa</i> extract	100 mg/kg/per day for 4 weeks	NF-κB expression and levels of inflammatory mediators	[64]
	TPA-induced mouse skin	Cocoa polyphenols	40 and 200 mg/kg in a dose	MAPKs and NF-κB signaling pathways activation	[81]
	Rats with induced colon carcinogenesis	Cocoa polyphenols	Cocoa-rich diet for 8 weeks	MAPKs and NF-κB signaling pathways activation	[82]
	Obese rats	Cocoa proteins	150 mg/kg per day for 8 weeks	TNF-α protein and mRNA levels	[83]
	Mice with associated colitis	Cocoa	5 and 10% cocoa in the diet for 2 months	Nrf2 levels and COX-2 expression	[84]
	Autoimmune myocarditis rats	Oleuropein (isolated from <i>O. europaea</i>)	20 mg/kg per day for 4 weeks	MAPKs and NF-κB pathways regulation	[85]
	Rats with induced renal injury	<i>O. europaea</i> leaf extract	100 and 200 mg/kg for 15 days	NF-κB nuclear translocation and Nrf2, HO-1 and NQO-1 expressions	[86]
	Rats with induced testicular damage	<i>O. europaea</i> leaf extract	300 mg/kg daily for 5 days	NF-κB nuclear translocation and Nrf2, HO-1 and NQO-1 expressions	[87]
In vitro/In vivo	PC12 cells Sprague–Dawley rats	Verbascoside (isolated from <i>L. citriodora</i> extract)	10, 30 and 10 μM for 12 h (in vitro) 25 mg/kg in three doses at 3, 6 and 12 h (in vivo)	HO-1 expression	[41]
	RAW264.7 macrophage cells/Mice	Dp3-Sam (isolated from <i>H. sabdariffa</i>)	50-200 mM for 30 min (in vitro) 15 mM/kg for 4 days (in vivo)	MAPK and NF-κB pathways regulation and levels of inflammatory mediators	[88]

Furthermore, the anti-inflammatory effects of certain compounds present in *S. marianum* have been evaluated by in vivo studies. In this sense, silybin has shown a potential anti-inflammatory effect in rats exposed to an arsenic treatment, an environmental and an industrial pollutant [77]. Milton Prabu and Muthumani (2012) observed that the silybin diet supplementation with arsenic treatment causes the expression of Nrf2 mRNA in the kidney tissue of rats to reverse at the level of the control group. In addition, the administration of this compound to the rats without arsenic treatment increases mRNA even above the group control. In this study, the administration of arsenic caused an over-expression of NF- κ B in the kidney tissue that could be reduced to baseline levels with the administration of silybin [77].

The administration of silybin in a nonalcoholic steatohepatitis (NASH) mouse model showed that this compound decreased significantly the elevation of p-IKK α / β which is produced by the NASH in liver [78], allowing the blocking of NF- κ B.

Other studies revealed that silymarin up-regulated gastric tissue Nrf2 expression, down-regulated gastric tissue NF- κ B p65 protein expression, that is necessary to activate the NF- κ B [89], and decreased gastric tissue levels of pro-inflammatory cytokines like TNF- α and IL-6 in rats with indomethacin-induced gastric injury [79].

In general, most of the experimental studies suggest that dietary *S. marianum*/silybin activates antioxidant pathways such as Nrf2/HO-1 and downregulates NF κ B.

3.2 *Lippia citriodora*

L. citriodora extract has demonstrated the ability to downregulate NF- κ B expression in 3T3-L1 hypertrophic adipocytes. In this cell line, the gene expression of some cytokines

was measured observing that the plant extract significantly reduced the expression levels of IL-1 β , IL-6, TNF- α and CCL2/MCP-1 [38].

Verbascoside has been shown to have an anti-inflammatory effect in in vivo studies using zebrafish or Sprague–Dawley rats. Verbascoside stands out for its anti-inflammatory power, since it exerts a regulation on the Keap1/Nrf2/ARE signaling pathway [41,80,90]. In addition, this phenylpropanoid has been considered an activator of Nrf2 and an inducer of HO-1 expression in both in vitro (PC12 cells) and in vivo (Sprague–Dawley rats) assays through activation of the ERK and PI3K/Akt signal pathways [41]. In another study, the protective capacity of verbascoside in the treatment of Parkinson's disease was evaluated in a zebrafish model. It was observed that this compound penetrated the blood brain barrier showing a potential therapeutic value in the treatment of Parkinson's disease since it prevents movement disorders and activates the Nrf2/ARE signaling pathway to attenuate oxidative stress and inflammation [80].

3.3 *Hibiscus sabdariffa*

The anti-inflammatory effect of *H. sabdariffa* extract has been evaluated in different cell lines, such as macrophages, lymph node carcinoma of prostate cells and macrophage cells RAW264.7; while there are in vivo studies that have evaluated the effect of this matrix in mice and rats with hepatotoxicity.

The anti-inflammatory activity of the essential oil extracted from *H. sabdariffa* was evaluated in LPS-stimulated macrophage RAW 264.7 cells showing an inhibition of the activation of NF- κ B and MAPK (JNK and ERK1/2) signaling pathways that resulted in a decrease of NO and pro-inflammatory cytokine (IL-1, IL-6, TNF- α , COX-2, and iNOS) production [70].

Chiu et al. (2015) evaluated the action of *H. sabdariffa* leaf extracts in lymph node carcinoma of the prostate cells resulting in an inhibitory effect on the activity and expressions of matrix metalloproteinase-9 (MMP-9). The MMP-9 inhibition is a consequence of NF- κ B inactivation by the *H. sabdariffa* leaf extracts mediated by Akt [72].

Anthocyanins are the major phenolic compounds in *H. sabdariffa*, suggesting that the anti-inflammatory capacity of this plant is caused by these compounds. For example, delphinidin 3-sambubioside (Dp3-Sam) showed an anti-inflammatory effect by in vivo and in vitro models. The administration of Dp3-Sam in mice inhibited the production of inflammatory factors, such as IL-6 and TNF- α , through the downregulation of cellular signaling including MAPK and NF- κ B pathways. On the other hand, the levels of inflammatory mediators, such as iNOS, NO, IL-6, MCP-1, and TNF- α induced by lipopolysaccharide in RAW264.7 macrophage cells, decreased after the administration of Dp3-Sam [88].

Moreover, *H. sabdariffa* extract has shown in rats with thioacetamide (TAA)-induced hepatotoxicity the capacity to inhibit the expression of NF- κ B and to decrease the levels of TNF- α , IL-6 and IFN- γ , which would indicate a hepatoprotective effect [64].

3.4 *Theobroma cacao*

The anti-inflammatory effect of the compounds of *T. cacao* have been evaluated in vitro in primary astrocytes isolated from wild-type and Nrf2 KO mice and in vivo studies.

Several studies support the idea of the anti-inflammatory capacity of cocoa phenolics. For instance, the mechanism of action of (-)-epicatechin in this pathway was evaluated in primary astrocytes isolated from wild-type and Nrf2 KO mice. In addition, the results

revealed that this compound protected astrocytes against hemoglobin toxicity through the positive regulation of Nrf2 and the inhibition of the activity of activator protein 1 (AP-1). Both cases were mediated by the suppressive action of the compound in the phosphorylation of JNK and by the nuclear expression of JNK, c-jun, and c-fos. Besides that, the inhibitory action of (-)-epicatechin on AP-1 activity was independent of Nrf2, since it occurred in both wild-type and Nrf2 KO astrocytes. Moreover, the positive action of (-)-epicatechin in Nrf2 did not alter the expression of HO-1, which could be the result of a balance between the signaling pathways of Nrf2 activation and AP-1 inhibition [73]. On this scenario, Lan et al. (2017) reported the effect of (-)-epicatechin on the reduction of hemorrhagic stroke injury through the Nrf2 signaling pathway [73].

Cocoa polyphenols have shown the capacity to inhibit the expression activation of MAPKs and NF- κ B signaling in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse skin that cause superoxide-anion generation [81] and in rats model of azoxymethane (AOM)-induced colon carcinogenesis, respectively [82].

Both TNF- α protein and mRNA levels thereof measured in white adipose tissue and the pro-inflammatory chemokine MCP-1 decreased when cocoa protein was administered in rats after a high fat diet [83]. Besides that, the secretion and the mRNA levels of pro-inflammatory cytokines as TNF- α and IL-6 were inhibited by theobromine treatment [28].

Pandurangan et al. (2015) explored the antioxidant properties of cocoa in a mouse model with colitis-associated cancer induced by azoxymethane (AOM)/dextran sulfate sodium (DSS). This study revealed that the administration of 5 and 10% cocoa in the diet caused

a higher Nrf2 accumulation in the nucleus of colon cells and reduced the COX-2 expression, an inflammatory mediator that is increased during AOM/DSS-induction [84].

3.5 *Olea europaea*

The possible anti-inflammatory action of the extract of *O. europaea* or isolated phenolic compounds of this matrix has been evaluated by various authors in both in vitro and in vivo assays. In the case of in vitro assays, peritoneal murine macrophage models stimulated with LPS, astrocytes induced by lipopolysaccharide and different types of cells with induced inflammation have been used.

In this way, Cárdeno et al. (2014) evaluated the potential anti-inflammatory mechanisms of the phenolic extract from EVOO on LPS-stimulated peritoneal murine macrophages. They obtained that this extract decreased LPS-induced inflammatory responses through the reduction of AMPK phosphorylation and the prevention of the NF- κ B translocation [74].

Some of the compounds responsible for the anti-inflammatory capacity of the olive leaf and fruit, such as maslinic acid [75], oleuropein [76,91], oleacein [76], hydroxytyrosol and tyrosol [92], have been isolated and tested to verify their effects on the main metabolic pathways involved in the inflammatory response. For example, maslinic acid, an oleanane-type triterpenoid, has shown an anti-inflammatory effect in astrocytes induced by LPS. The results demonstrated that maslinic acid attenuated LPS-induced translocation of NF- κ B p65 subunit to the nucleus and avoided LPS-induced I κ B α phosphorylation in a concentration-dependent manner [75].

Furthermore, hydroxytyrosol and tyrosol have shown an anti-inflammatory effect in several studies. These compounds were capable of decreasing the NF- κ B pathway and

downregulating genes and pathways related to inflammation, such as COX-2, IL-1 β , TNF- α , Nrf2, HO-1, in different cell lines with induced inflammation [92–94]. Another study revealed that both oleuropein and oleacein caused a strongly increased expression of HO-1 and Nrf2 levels in CD31⁺/VEGFR-2⁺ cells decreased by angiotensin II treatment [76]. In addition, the effect of oleuropein was tested in experimental autoimmune myocarditis rats, resulting in a decrease in serum production of TNF- α , IL-1 β and IL-6 and an attenuation of the MAPKs and NF- κ B pathways [85].

Finally, other parts of the plant besides the fruit also have bioactive compounds that can exert beneficial effects on health. Thus, olive leaf extract has been tested in rats with cyclophosphamide-induced renal injury [86] and in rats with testicular damage induced by intraperitoneal injection [87]. In both models, the inflammation caused by the injury was reduced by the effect of olive leaf extract. This anti-inflammatory response was exerted due to the capacity of the compounds present in the olive leaf extract to inhibit the translocation of NF- κ B, suppress pro-inflammatory markers and increase the expression of Nrf2, HO-1 and NQO-1.

4. Effect of bioactive compounds on aging

Aging is a complex process characterized by the accumulation of molecular, cellular and organ damage. All these damages lead to loss of function and increase vulnerability to disease and death [95]. The hallmarks of aging can be organized into three key features. The primary hallmarks consist of harm to cellular functions: telomere attrition, genomic instability, loss of proteostasis and epigenetic alterations. Due to these damages, a series of antagonistic responses are produced: deregulated nutrient detection, altered mitochondrial function and cellular senescence. Finally, integrative hallmarks give rise to

the characteristic clinical phenotype of the aging that ends in the physiological reserve loss, the decrease of organs and the reduced function [96].

Several studies have shown that dietary or calorie restriction increase the lifespan of diverse organisms, furthermore in monkey rhesus and humans this restriction protects against age-related pathologies like cancer, diabetes, Alzheimer's disease and cardiovascular disease [95].

The extension of lifespan mediated by the calorie restriction is mediated by mechanisms that end in autophagy. Furthermore, calorie restriction downregulates the mTOR pathway and upregulates SIRT1 deacetylase and the AMPK pathway, all of these pathways also upregulate autophagy [96]. Autophagy decreases with age, improving this degradation route of misfolded proteins, damaged organelles and intracellular pathogens which will prevent their accumulation and, therefore, related diseases. If attention is paid to the effect of metabolic regulation on aging, the downregulation of mTOR pathway, one of the main nutrient sensors and metabolic regulation, either with rapamycin or deletion of ribosomal S6 protein kinase 1 (S6K1) has shown an increase of mouse lifespan [95]. Another important protein in the detection of nutrients is AMPK, this kinase is activated with nutrient starvation, caloric restriction, and exercise, since there is a decrease in ATP and an increase in AMP in these processes. AMPK activation has been reported to promote cell survival under environmental stress and this is related to autophagy [96]. In this sense, a high level of AMP has been also linked to the accumulation of NAD that participates in caloric restriction. High levels of NAD activate sirtuins in situations of nutrient deprivation or growth inhibitory signaling. There is

evidence that sirtuins participate in the regulation of insulin signaling and that they act as regulators of longevity [96]. Nevertheless, the role of sirtuins is still unclear.

On the other hand, the mitochondrial free radical theory of aging states that continued exposure to reactive oxygen species causes mitochondrial DNA to increase susceptibility to oxidative damage and aging [97]. Consequently, any damage that affects mitochondrial DNA, dynamics, biogenesis or integrity resulting in deregulated metabolism that is associated with aging.

Another highlight is the ability of different homeostasis maintenance mechanisms in mammals. This responds to different nutrients and hormones to maintain physiological blood glucose levels [96]. With age, many of these sensors and molecular targets are lost or decreased, and this is related to diseases associated with old age, such as cardiovascular and metabolic disorders, muscular atrophy, neurodegenerative diseases and cancer susceptibility [96].

Besides, aging is also related to an increase in pro-inflammatory cytokines, which is known to interfere with the insulin action. These cytokines result in both the secretion and production of pro-inflammatory cytokines by increasing the number of senescent cells or by the greater accumulation of visceral fat associated with age, respectively [109]. Certain of these cytokines and inflammatory markers are leptin, TNF- α , IL-6 and plasminogen-1 activation inhibitor. All of them have been related to chronic conditions associated with age [109]. In **Table 3**, it can be observed different in vivo studies that show the effect of the matrices included in this review on the main metabolic pathways related to aging.

Table 3. In vivo studies of the included sources on the main metabolic pathways related to aging.

Model Type	Source	Effective Dose	Parameters	References
Aging mice	<i>S. marianum</i> protein hydrolysate	400, 800 and 1.200 mg/kg daily for 7 weeks	Liver mitochondria damage	[98]
<i>C. elegans</i>	Silymarin (<i>S. marianum</i> extract)	25 and 50 μ M	Lifespan	[99]
Rabbits	<i>L. citriodora</i> extract	2.2 g per 100 kg of feed	Oxidative damage markers	[100]
Rabbits	Verbascoside (isolated from <i>L. citriodora</i>)	5 mg/kg for 80 days	Sirt1 activity and antioxidant levels	[101]
Yeast BY4741	<i>H. sabdariffa</i> extract	100 μ L of 300 ppm	Lifespan	[102]
Medaka fish	Phenolic compounds-enriched cocoa extract	1, 4 and 8 mg/mL for 4 days	Lifespan and oxidative stress	[103]
Aged rats	<i>O. europaea</i> leaf extract	1000 mg/kg daily for 2 months	Antioxidant parameters	[104]
<i>C. elegans</i>	Oleuropein aglycone (isolated from <i>O. europaea</i>)	-	Aggregation of proteins in amyloid diseases	[105,106]
Type 2 diabetic patients	Silymarin (<i>S. marianum</i> extract)	140 mg thrice daily for 45 days	Antioxidant indices	[107]
Elderly individuals	<i>T. cacao</i> flavanol consumption	993, 520 and 48 mg daily for 8 weeks	Cardioprotective effect	[108]

4.1 *Silybum marianum*

The action of *S. marianum* on metabolic pathways related to aging and its effect on increasing lifespan have been evaluated by different authors using in vivo studies. As an example, d-galactose-induced aging mice were used to prove the antioxidant and anti-aging effects of *S. marianum* protein hydrolysate (SMPH). The results showed that this compound exerted a beneficial effect against aging since it restored the damage induced by d-galactose in liver mitochondria [98]. In this sense, silymarin and 2,3-dehydrosilybin A/B (DHS) from *S. marianum* have also been shown to promote lifespan in *C. elegans* [99].

On the other hand, it is known that several neurodegenerative diseases related to age occur when there is an increase in oxidative stress, so it is a key aspect to be analyzed. Silymarin supplementation improves antioxidant indices, like the activity of SOD, GPX activity and total antioxidant capacity (TAC) whereas it reduces inflammatory markers in type 2 diabetic patients [107].

4.2 *Lippia citriodora*

Palazzo et al. (2019) evaluated the effect of *L. citriodora* extract in rabbits, observing that there were improvements in the functions of the liver and kidney in the markers of oxidative damage and in the lipid profile of the animals by the action of the extract. Due to the relationship between the antioxidant effect and caloric restriction with the anti-aging effect, these effects exerted by the extract may be related to protection against aging [100].

Other results that support the anti-aging effect of *L. citriodora* are those obtained by Corbi et al. (2018). This study proved the effect of verbascoside in rabbits, demonstrating a relationship between the compound administration and the SIRT1 activation in the rabbit's heart and liver tissue. This suggested that SIRT1 could mediate the antioxidant effects of this extract [63]. In addition, the activation of SIRT1 is related to an increase in autophagy, which in turn increases animal's lifespan [96].

4.3 *Hibiscus sabdariffa*

The anti-aging effect of *H. sabdariffa* was evaluated by Sarima et al. (2019) observing that the *H. sabdariffa* petal extract extended the lifespan of yeast BY4741 under oxidative stress and normal conditions. The extract was able to induce intracellular mechanisms against oxidative stress such as modification of mitochondrial activity and

upregulation of genes involved in tolerance mechanism opposed to oxidative stress and key genes in aging pathway like SIR2 [102]. Moreover, the antigenotoxic effect associated with the hibiscus extracts has been related to its antioxidant capacity and therefore to the anti-aging effect [110,111].

4.4 *Theobroma cacao*

The effect of compounds present in the *T. cacao* extract on aging has been evaluated in both in vitro and in vivo assays. Phenolic compounds-enriched cocoa extract has also shown to participate in the regulation of the expression of several genes involved in oxidative stress and prolonged lifespan in medaka fish. The extract induced the activation of the antioxidant defense when an oxidative stress situation is produced. Regarding the lifespan extension, in medaka fish, the activation of the SIRT1 protein did not occur directly but was done indirectly in a similar way to that produced in mammals. The extract activated Epac1, a cAMP effector protein, which leads to high levels of cAMP and indirectly activates the enzymatic function SIRT1 [103].

On the other side, one of the diseases with a high prevalence in old aging is cardiovascular disease. Cocoa phenolics have shown cardioprotective effects in different studies that were carried out both in cell and in human trials through the antioxidant, anti-inflammatory and antihypertensive properties of these phenolic compounds from cocoa [108,112,113].

4.5 *Olea europaea*

Olive phenolic compounds have been linked to an anti-aging effect due to several capacities to produce protective effects. The administration of *O. europaea* leaf extract at a dose of 1000 mg/kg in aged rats showed a decrease in high levels of hepatic

malondialdehyde (MDA), diene conjugate (DC) and carbonyl protein (PC) that appears in aged rats. This anti-aging effect presented by the extract may be related to its strong radical scavenging action, acting as an antioxidant itself without affecting the antioxidant system [104].

Moreover, protective effects from polyphenols of *O. europaea* have been proven in both *C. elegans* and in humans. Examples of these effects are the interference with the aggregation of proteins in amyloid diseases, the protection of cells and tissues against aging-associated functional disturbance or the transcriptional modulation through epigenetic modifications [105,106]. Olive phenolics have also been linked to anti-aging and cardioprotective properties because they present similar effects to caloric restriction in tissues and organs such as muscle, brain, fatty tissue and kidneys in several ways, but specifically through increased levels and activation of sirtuins [114,115].

5. Bioavailability and pharmacokinetic properties of phenolic compounds

The pharmacological potency of the phenolic compounds is important for the effect that they exert in biological systems. However, their efficacy does not depend only on this potency but also their bioavailability and pharmacokinetic parameters are important. For example, there are phenolic compounds that are rapidly absorbed by the intestinal barrier and plasma in their native state, while others are absorbed in small quantities and may be highly metabolized or rapidly excreted [18]. Therefore, understand the bioavailability, absorption and metabolism of plant polyphenols is essential to determine their mode of action and their final active metabolites.

Accordingly, the metabolites that reach the circulating blood and target tissues may differ from their native forms and the gastrointestinal tract plays a crucial role in this.

Phenolic compounds suffer modifications throughout the gastrointestinal tract, first the compounds are hydrolyzed by gastric fluid in the stomach and then metabolized by enzymes of the intestinal cells or catabolized by the microflora of the colon, which can greatly affect the absorption of these compounds [18]. In addition, phenolic compounds can undergo phase I and phase II reactions in the liver. The modifications that the compounds suffer most frequently are glucuronidation, sulfation, and methylation. [11,116].

As mentioned earlier, the five matrices selected to be the subject of this review contain several types of phenolic compounds. As mentioned, whole phenolic extracts or isolated phenolic compounds are responsible for the attributed effect of these matrices on the metabolic pathways related to energy metabolism, inflammation, and aging. However, as indicated in this point, it is important to take into account the metabolites resulting from the ingestion of these matrices on the mentioned metabolic pathways.

In *L. citriodora* verbascoside and isoverbascoside have an effect within the studied metabolic pathways, so it is interesting to consider their bioavailability and pharmacokinetic properties. In this way, the effect of the consumption of an oral acute dose of *L. citriodora* extract (1440 mg/kg) on the antioxidant response of blood cells was further studied in rats using high-resolution mass spectrometry in order to determine other potential metabolites in plasma [117]. In the study, these compounds were found at high levels intact in plasma, suggesting that both compounds could be absorbed in their native forms. However, five other metabolites derived from verbascoside and isoverbascoside by deglycosylation (hydrolysis), methylation or glucuronidation were also found in plasma, namely hydroxytyrosol, ferulic acid, caffeic acid, ferulic acid

glucuronide, and homoprotocatechuic acid, together with another eight phenolic compounds [117]. In other study, Wistar rats were orally treated with a dosage of 2000 mg/kg of *L. citriodora* extract that contained a 25% of verbascoside. Blood samples were taken at different times after ingestion and verbascoside was found in plasma samples with a maximum concentration at 20 min. This study concluded that there was a fast absorption of verbascoside in the gut barrier but a very low bioavailability, which may compromise to assign the observed effects to verbascoside. However, there was evidence of the bioactivity of phenylpropanoids at very low concentrations in cell models (at the micromolar range), so low micromolar concentrations of verbascoside over a long-term in plasma could be responsible for some of the effects of the plant [118].

Concerning *H. sabdariffa* polyphenol-enriched extract, the bioavailability and pharmacokinetic parameters were evaluated in Wistar rats after an acute oral dose of 1200 mg/kg [119]. In this study, it was detected in plasma a total of 17 compounds, 11 of these were metabolites. Phenolic acids appeared in plasma without any modification while most of flavonols were found as kaempferol or quercetin glucuronide conjugates, reaching maximum concentration at 120 min. Of all the quercetin and kaempferol derivatives in plasma, both quercetin glucuronide and aglycone had the highest concentrations. These compounds showed higher removal values, revealing an accumulation of tissue and a probable long-term effect [119].

Cocoa polyphenols include a subclass of flavonoids, namely flavan-3-ols, occurring as monomers, mainly epicatechin and catechin, oligomers (procyanidins B1, B2, and C1) and polymers (up to ten units), known as procyanidins [120]. The metabolism of flavan-3-ols has been frequently studied because they have experienced being more

bioavailable compared to other cocoa polyphenols. The metabolic changes of frequent flavan-3-ols cocoa have been studied after consumption as well as the physiological levels of epicatechin and catechin [116]. For example, Actis-Goretta et al. (2012) clarified that after the consumption of dark chocolate, (–)-epicatechin-3′-β-d-glucuronide, (–)-epicatechin 3′-sulfate and 3′-O-methyl epicatechin sulfates substituted at positions 4′, 5 and 7 were the most relevant metabolites of (–)-epicatechin found in plasma. In addition, the total urine excretion of (–)-epicatechin was 20% of the amount ingested [121].

Taking into account hydroxytyrosol from *O. europaea*, the bioavailability of this simple phenol has been explored in humans, for example in the trial conducted by Miro-Casas et al. where quantified hydroxytyrosol and its main metabolite such as 3-O-methylhydroxytyrosol in plasma and urine after a dose of 25 mL of virgin olive oil in healthy humans. The results showed that hydroxytyrosol and 3-O-methyl-hydroxytyrosol in plasma increased as a response to virgin olive oil administration, reaching maximum concentrations at 32 and 53 min and that approximately 98% of hydroxytyrosol was present in conjugated forms, mainly glucurono-conjugates. With these results, the authors suggested that ingested hydroxytyrosol can be extensively metabolized for the first time in the intestine and liver and that the biological activity of this compound may be probably derived from its metabolites [122]. In another study, nine healthy volunteers were randomized to receive encapsulated or liquid olive leaf extract as a single lower dose (51.1 mg of oleuropein, 9.7 mg of hydroxytyrosol) or higher (76.6 mg of oleuropein, 14.5 mg of hydroxytyrosol). Then, the opposite dose (but the same formulation) one week later was administered. In the study, fifteen phenolic olive compounds were rapidly found in plasma and urine. They were mainly metabolites derived from phase II; three of them were metabolites derived from hydroxytyrosol, four from oleuropein aglycone

and two from homovanilic acid, being hydroxytyrosol glucuronide found in greater quantity. In addition, new metabolites derived from oleuropein in the urine were identified, such as elenolic acid, homovanilic alcohol sulfate and glucuronide of elenolic acid. These results confirmed the rapid absorption of phenolic compounds from the extract of *O. europaea* and the extensive biotransformation of hydroxytyrosol and oleuropein into metabolites, mainly as glucuronidated conjugates, as mentioned above [123].

In the case of the *S. marianum*, no bioavailability and pharmacokinetic studies have been found to comment on the main metabolic transformations of its main phenolic compounds.

Finally, in this section, some of the transformations suffered by the main phenolic compounds of the matrices under study have been summarized. It can be seen that many of these appear in plasma natively and, therefore, can exert the bioactive effect, but also that many of them undergo modifications that make them transform into new compounds with different bioactive capacities from their native form. These results suggest that the aforementioned effects of the natural sources on metabolic pathways (**Figure 1**) may be due to native forms of the phenolic compounds or their metabolic transformations.

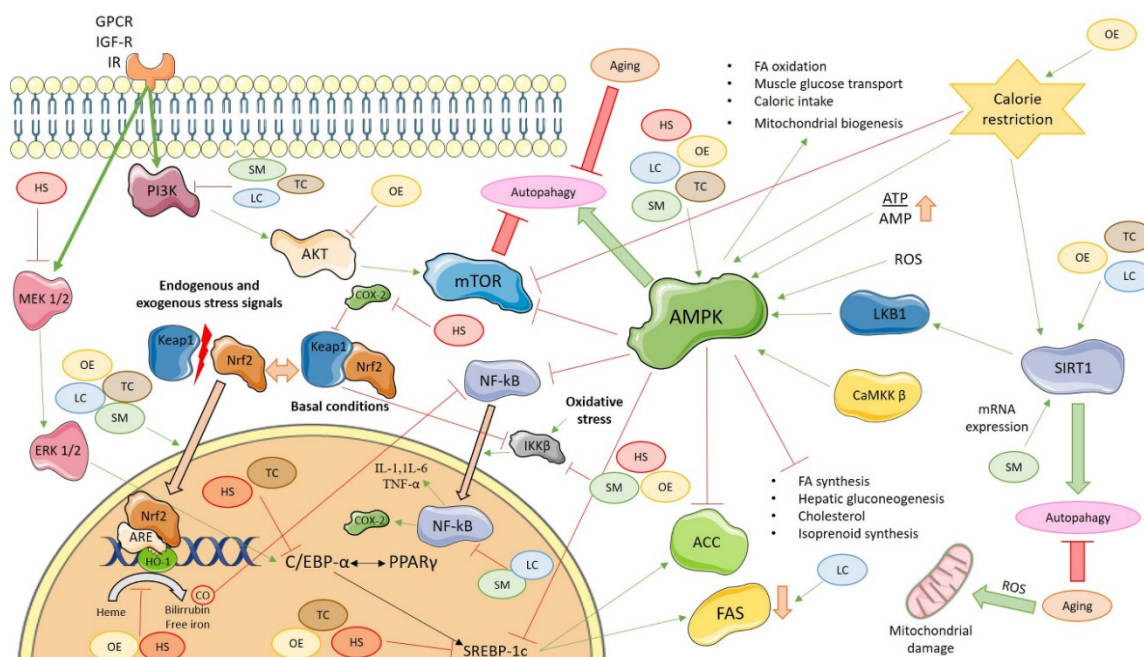


Figure 1. Molecular signaling pathways that are activated or inactivated by dietary phenolics or their metabolites in oxidative stress, inflammation process, and aging. SM: *Silybum marianum*; LC: *Lippia citriodora*; HS: *Hibiscus sabdariffa*; TC: *Theobroma cacao*; OE: *Olea europaea*; AMPK: AMP-activated protein kinase; mTOR: the mammalian target of rapamycin; ATP: Adenosine triphosphate; AMP: Adenosine monophosphate; ROS: Reactive oxygen species; LKB1: liver kinase B1; CaMKK β: Ca²⁺/calmodulin-dependent protein kinase β; SIRT1: Sirtuin 1; ACC: Acetyl-CoA carboxylase; FAS: Fatty acid synthase; NF-κB: Nuclear factor-κB; IKKβ: IκB kinase; COX-2: Cyclooxygenase-2; Akt: Protein kinase B; PI3K: Phosphoinositide 3-kinase; Keap1: Kelch-like ECH-associated protein; Nrf2: Nuclear factor-erythroid 2 p45-related factor 2; MEK 1/2: Mitogen-activated protein kinase 1/2; ERK 1/2: Extracellular signal-regulated kinase 1/2; GPCR: G protein-coupled receptor; IGF-R: Insulin-like growth factor receptor; IR: Insulin receptor; ARE: Antioxidant response element; HO-1: Heme oxygenase 1; CO: Carbon monoxide; C/EBP-α: CCAAT/enhancer-binding protein α; PPAR-γ: Proliferator Peroxisome Activated Receptor-γ; SREBP-1c: Sterol regulatory element-binding protein-1c; IL-1: Interleukin-1; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor α. Thin green arrows indicate the activation of a molecule; thin red lines indicate the inactivation of a molecule; thick green arrows indicate the promotion of a process; thick red lines indicate the reduction of a process; thick orange lines indicate the translocation of molecules.

6. Conclusions

Beyond the potential of scavenging free radicals of dietary phenolic compounds, multiple cellular and molecular pathways have been shown to be involved in their roles on different chronic disorders, showing their pleiotropic nature.

As can be seen throughout this review, the metabolic pathways related to energy metabolism, inflammation, and aging are closely related and share central molecules in

their regulation. Despite this intimate relationship, there are key molecules in any one of them, which are subject to the regulatory effect of phenolic compounds.

Focusing attention on the metabolic pathways involved in energy metabolism, it can be seen that AMPK exerts an effect as a central regulator, since its activation or inhibition can trigger an effect on other important molecules of energy metabolism. In addition, the activation of AMPK leads to the inhibition of ATP-consuming anabolic pathways, which translates into the inhibition of molecule synthesis processes and the activation of reserve cell degradation. Another important molecule in energy metabolism is mTOR, whose negative regulation is directly related to the activation of AMPK. Several studies, both in vitro and in vivo have shown that entire extracts from natural sources have a positive regulatory effect on AMPK and negative on mTOR, highlighting *L. citriodora* extract or its main compound verbascoside isolated, *S. marianum* extract and secoiridoids from extra virgin olive oil.

In the case of the inflammation-related metabolic pathways, there are two main molecules on which the phenolic compounds have been shown to have a regulatory effect, Nrf2, and NF- κ B. Nrf2 plays an important effect in several inflammatory diseases because the absence of Nrf2 in the nucleus has reflected greater symptoms of inflammation while the presence of NF- κ B in the nucleus triggers an inflammatory response since it causes the transcription of pro-inflammatory mediators. Both Nrf2 and NF- κ B have been shown to be the target of the pleiotropic effect of phenolic compounds in both in vitro and in vivo studies. Some examples noted are the effect of silybin and isosilybin, isolated from *S. marianum* extract, whole *L. citriodora* extract and (-)-epicatechin, isolated from *T. cocoa*.

As for the aging-related metabolic pathways, there are many common intermediaries to the two mentioned above, so many of the aforementioned effects of phenolic compounds on energy metabolism and inflammation would also have an effect on the regulation of aging. Note the central regulator of metabolism, AMPK, and sirtuins. The effect of sirtuins on the regulation of longevity has been studied by various authors although their role on aging today is not entirely clear. Because the metabolic pathways related to aging share many molecules with the other two mentioned, the vast majority of the aforementioned effects of the revised phenolic compounds on energy metabolism and inflammation could also be considered a regulatory effect of aging. Therefore, it should be taken into account that verbascoside (isolated from *L. citriodora*) has shown an effect on certain sirtuins, which makes it potentially effective in aging.

From the data summarized in this review, it is very clear that different pathways are involved to trigger their potential even in different pathologies. However, further studies are still needed to assess the therapeutic and pharmacological potentials of these bioactive compounds due, as mentioned in Section 5, to the pharmacological potency of the phenolic compounds also depend on their bioavailability. For this reason, it is necessary to elucidate if the effects of the studied compounds are effective in their native form or if the metabolic transformations thereof are those that actually exert the bioactive effect.

With this review, it is possible to obtain an integrative image of the existing relationships of the metabolic pathways related to energy metabolism, inflammation, and aging. It also highlights the pleiotropic effect of different types of phenolic compounds on key

molecules of these metabolic pathways and their relationship with certain chronic diseases.

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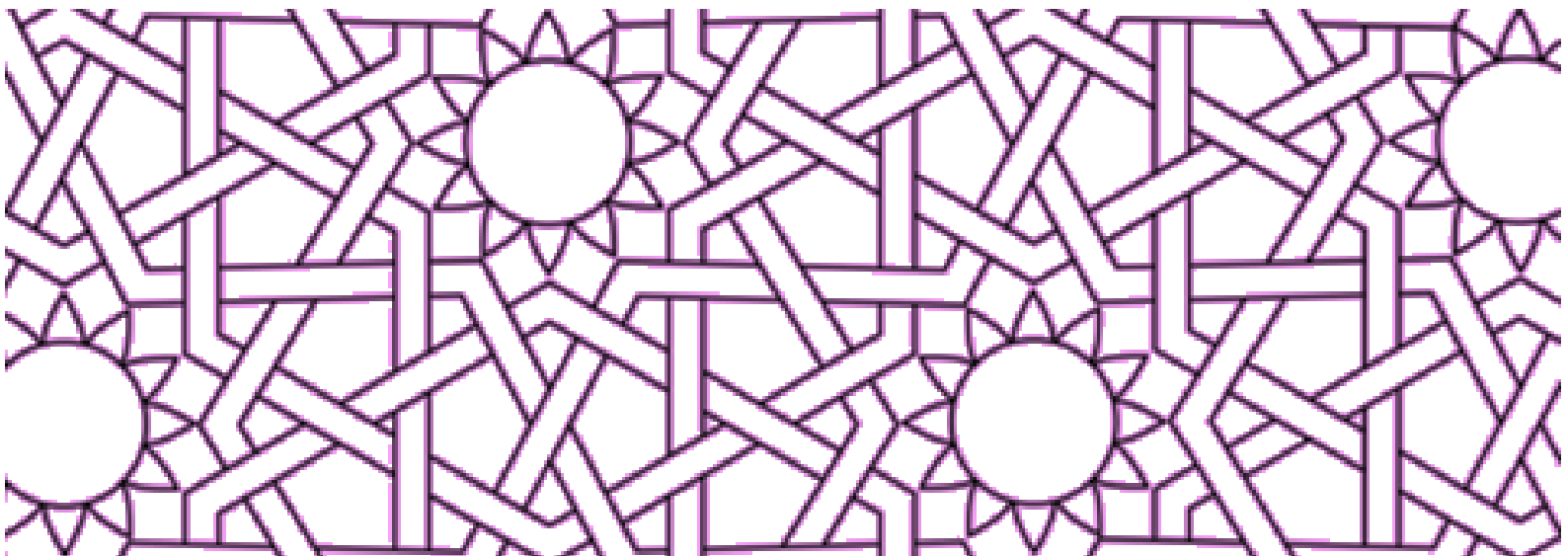
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Chapter 2





Evaluation of Bioactive Effects of Five Plant Extracts with Different Phenolic Compositions against Different Therapeutic Targets

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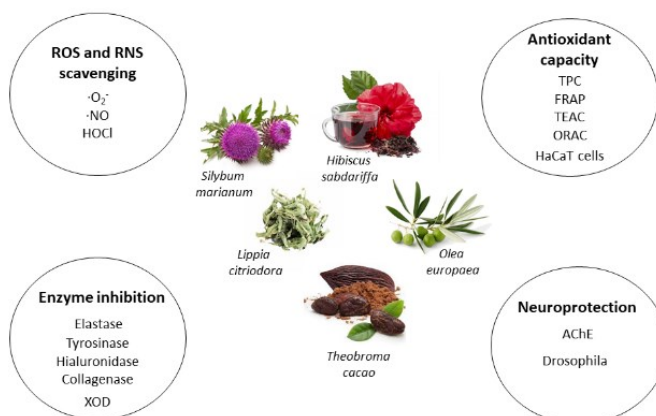
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Abstract

Plant extracts rich in phenolic compounds have been reported to exert different bioactive properties. Despite the fact that there are plant extracts with completely different phenolic compositions, many of them have been reported to have similar beneficial properties. Thus, the structure–bioactivity relationship mechanisms are not yet known in detail for specific classes of phenolic compounds. In this context, this work aims to demonstrate the relationship of extracts with different phenolic compositions versus different bioactive targets. For this purpose, five plant matrices (*Theobroma cacao*, *Hibiscus sabdariffa*, *Silybum marianum*, *Lippia citriodora*, and *Olea europaea*) were selected to cover different phenolic compositions, which were confirmed by the phytochemical characterization analysis performed by HPLC-ESI-qTOF-MS. The bioactive targets evaluated were the antioxidant potential, the free radical scavenging potential, and the inhibitory capacity of different enzymes involved in inflammatory processes, skin aging, and neuroprotection. The results showed that despite the different phenolic compositions of the five matrices, they all showed a bioactive positive effect in most of the evaluated assays. In particular, matrices with very different phenolic contents, such as *T. cacao* and *S. marianum*, exerted a similar inhibitory power in enzymes involved in inflammatory processes and skin aging. It should also be noted that *H. sabdariffa* and *T. cacao* extracts had a low phenolic content but nevertheless stood out for their bioactive antioxidant and anti-radical capacity. Hence, this research highlights the shared bioactive properties among phenolic compounds found in diverse matrices. The abundance of different phenolic compound families highlights their elevated bioactivity against diverse biological targets.

Keywords: phenolic compound; HPLC-MS; free radical scavenging capacity; enzyme inhibition; antioxidant; neuroprotection; structure–activity relationship

1. Introduction

Phenolic compounds are a class of compounds present in plants that have been reported to have enormous bioactive potential [1]. These compounds are secondary metabolites naturally synthesized in plants, and more than 8000 different chemical structures have been reported [2]. Based on their basic chemical structures, these compounds have been classified into at least 10 different classes [3]. It should be noted that, even though they show so much structural variability, they are widely studied for their antioxidant [4,5], anti-inflammatory [6], anti-aging [7], neuroprotective [8], and anticarcinogenic [9] potentials, among others. In fact, plant sources with different phenolic contents have been demonstrated to be involved in multiple pathways in the pathogenesis of different diseases [10,11]. In this scenario, there is emerging evidence on the neuroprotective activity of different phenolic compounds present in various plant sources regarding inhibition of acetylcholinesterase (AChE), A β aggregation, proinflammatory markers, and 1-methyl-4-phenyl-1-1,2,3,6-tetrahydropyridine (MPTP)-induced apoptosis, among others [12]. Thus, phenolic compounds from different matrices have been shown to have neuroprotective capacity, such as *Eucommia ulmoides* Oliver (rich in phenolic acids, flavonoids, and iridoid glycosides), herbaceous peony (with paeoninflorin as the main active ingredient), and *Sanghuangproux vaninii* (an extract rich in caffeic acid); they may ameliorate the dopaminergic deficiency in the MPTP-induced model in the zebrafish brain [12–14]. However, common phenolics present in different sources, for example, quercetin, which is contained in different matrices such as apple, chocolate, or cherry [15], have been shown to have both antioxidant and tyrosinase inhibitory actions [16].

Another example is kaempferol, another flavonoid, which is also present in a wide variety of plants and has a high antioxidant capacity [17]. When compared to other groups—for example, verbascoside, which belongs to the phenylpropanoids and is present in different matrices such as *Lippia citriodora* and *Olea europaea*—they have also been shown to have a high antioxidant capacity [18]. In this context, the structure–bioactivity relationship mechanisms are not yet known in detail for specific classes of phenolic compounds present in different plant sources.

Hence, the aim of this study is to evaluate the bioactive potential of different plant extracts with different phenolic profiles and to analyse the structure–bioactivity relationship mechanisms of phenolic compounds present in different plant sources. For this purpose, five plant matrices (fruits of *Theobroma cacao* (TC), calyxes of *Hibiscus sabdariffa* (HS), fruits of *Silybum marianum* (SM), leaves of *L. citriodora* (LC), and leaves of *O. europaea* (OE)) were selected. On these selected plants, a hydroalcoholic mixture extraction was carried out, and in order to know the phenolic content, a characterization by HPLC-ESI-TOF-MS was performed.

2. Materials and Methods

2.1 Chemicals

All chemicals used in this study were of analytical reagent grade and used as received. LC-MS grade acetonitrile and formic acid for mobile phases were purchased from Riedel-Haën (Honeywell, Charlotte, NC, USA). For solutions, ultrapure water was obtained with a Milli-Q system Millipore (Bedford, MA, USA), and absolute ethanol was purchased from VWR chemicals (Radnor, PA, USA). The chemical standards (verbascoside, myrecetin-3-glucoside, quercetin, epigallocatechin, gardoside, sylibin, oleuropein,

kaempferol, catechin, quercetin glucoside, quinic acid, and procyanidin B1) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The following reagents were used for the different in vitro assays—sulfuric acid and absolute ethanol—they were obtained from Riedel-de-Haën (Honeywell, NC, USA). Acetic acid, sodium carbonate, sodium hydroxide, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine and hydrochloridic acid were purchased from Fluka (Honeywell, NC, USA). Folin reagent, gallic acid (GA), sodium phosphate monobasic and dibasic, potassium persulfate, ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonate)), fluorescein, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Tris (tri(hydroxymethyl)aminomethane), sodium acetate, heptahydrate ferrous sulphate, ferric chloride, DMF (dimethylformamide), NADH (β -nicotinamide adenine dinucleotide), DAF-2 (diaminofluorescein diacetate), DHR (dihydrorhodamine), potassium dihydrogen phosphate anhydrous, NBT (nitrotetrazolium blue chloride), Cayman's xanthine oxidase fluorometric assay kit, tyrosinase inhibitor screening kit (colorimetric), acetylcholinesterase from Electrophorus, acetylthiocholine iodide, neutrophil elastase colorimetric kit, 1-10 phenantroline, sodium chloride, hyaluronidase from sheep testes, hyaluronic acid, FALGPA (*N*-[3-(2-furyl)acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine), and collagenase from *Clostridium histolyticum* were purchased from Sigma-Aldrich (St. Louis, MO, USA). NOC-5 was purchased from Chemcruz (Santa Cruz Biotechnology, Dallas, TX, USA).

Human keratinocytes (the spontaneously immortalized cell line HaCaT) were obtained from Cell Lines Service (CLS) GmbH (Eppelheim, Germany). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin solution (10,000

U/mL penicillin and 10 mg/mL streptomycin in citrate buffer) were purchased from Gibco™/Thermo Fisher Scientific (Waltham, MA, USA). Hoechst 33342 and 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) probes were acquired from Molecular Probes™ (Invitrogen™/Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Extraction procedure from plant matrices

The pre-industrial extracts of the selected plant matrices were provided by NATAC Biotech S.L. (Cáceres, Spain). These extracts were obtained by ethanol/water mixture extraction, which is considered a favorable solvent for the extraction of polar substances such as phenolic compounds, in addition to being environmentally friendly and non-toxic to humans (GRAS (Generally Recognized As Safe) solvent).

The extraction parameters were optimized for each plant matrix individually and are described below. For all extracts, a solid–liquid extraction (maceration) was carried out using a hydroalcoholic mixture for two hours. A mixture of EtOH:H₂O (80:20; v:v) was used for all samples except for SM, which was EtOH 96%. The extraction temperatures were set at 45 °C for the OE and HS extracts and 55 °C for the rest of the extracts. A solvent:plant ratio of 20:1 was used for all extracts except for SM, which was 25:1. The obtained extracts were dried using a vacuum drying, stored at room temperature, and protected from light until their analysis. For the different analytical and bioactive analyses, the extracts were reconstituted with the same solvents and mixture used for the extraction procedure.

2.3 HPLC-ESI-TOF-MS analysis

TC, HS, SM, LC, and OE extracts at 5000 mg/L were analysed by high-performance liquid chromatography (Agilent 1290 HPLC, Agilent Technologies, Palo Alto, CA, USA) coupled to mass spectrometry with a quadrupole time-of-flight analyser (Agilent 6545 QTOF Ultra High Definition, Agilent Technologies, Palo Alto, CA, USA). Chromatographic analysis was carried out in reversed phase with a C18 ACQUITY UPLC BEH column (1.7 μm , 2.1 mm, 150 mm, 130 Å, Waters Corporation, Milford, MA, USA). The working temperature of the column was 60 °C. The mobile phases were (A) acidified water with 0.1% of formic acid (v/v) and (B) acetonitrile. The following mobile phase gradient was used for optimal separation: 0.00 min [A:B 100/0], 5 min [A:B 90/10], 18 min [A:B 15/85], 24 min [A:B 0/100], 25.50 min [A:B 0/100], 26.50 min [A:B 95/5], and 32.50 min [A:B 95/5]. A mobile phase flow rate of 0.4 mL/min and an injection volume of 5 μL was used.

MS acquisition was performed in electrospray negative ionization (ESI) mode in a mass range between 50 and 1200 m/z . Other parameters were as follows: gas flow rate 10 L/min; gas temperature 200 °C; nebulizer 20 psig, enveloping gas temperature 350 °C, enveloping gas flow rate 12 L/min, VCap 4000 V, nozzle voltage 500 V.

Finally, the acquired data were processed through Qualitative Analysis of MassHunter workstation software version B.06.00 (Agilent Technologies), Mzmine 2.53, and Sirius 2.0. The compounds were annotated by comparison of the MS/MS spectra with those from analytical standards or published in the literature and databases, such as SciFinder®, CEU Mass Mediator, Human Metabolome Data Base (HMDB), and Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.4 In vitro assays for bioactive determination of phenolic compounds in extracts

The assays described below were adapted to a 96-well polystyrene microplate, and absorbance and fluorescence measurements were performed on a Synergy H1 Monochromator-Based Multimode Microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

2.4.1 Total phenolic content and antioxidant capacity measurements

Total phenolic content (TPC) was measured by the Folin–Ciocalteu method using gallic acid as a reference compound for the standard curve [19]. Measurements were carried out in triplicate.

The FRAP assay was performed following the method described by Benzie and Strain (1996) [20]. The reduction of the radical cation of ABTS was performed by TEAC assay using a method previously described by Zulueta et al. (2022) [21]. To test the ability of the extracts to scavenge peroxy radicals, an ORAC method was used with some modifications [21]. In all assays, measurements were performed in triplicate.

2.4.2 Evaluation of free radical and ROS/RNS scavenging potential

All free-radical scavenging assays were performed and adapted according to Rojas-García et al. (2022) [22]. To measure the scavenging capacity of the radical superoxide anion ($\cdot\text{O}_2^-$), a colorimetric method was used. A fluorometric-based method was used for the nitric oxide ($\cdot\text{NO}$) and HOCl assays. The results for the three assays were expressed as the concentration of the different extracts needed to inhibit ROS/RNS formation by half (IC_{50}).

2.4.3 Evaluation of enzymatic inhibition potential

The activity of the extracts to inhibit the enzyme tyrosinase was performed using the Tyrosinase Inhibitor Screening Kit (Colorimetric). The Xanthine Oxidase (XO) inhibitory activity of the extracts was measured using the Cayman's XO Fluorometric Assay Kit. The elastase inhibition assay was measured using the Neutrophil Elastase Inhibitor Screening Kit. For the measurement of hyaluronidase inhibition, the test performed by Nema et al. (2013) [23] was used with some modifications. The inhibitory effect against collagenase of the extracts was performed following the methodology performed by Kumar et al. (2019) [24], but some parameters were modified. Finally, acetylcholinesterase (AChE) inhibitory activity was measured using a photometric assay described by Ellman et al. (1961) [25]. All assays were performed in triplicate, and the result was expressed by calculating the IC_{50} using different concentrations of the extracts except for the tyrosinase and elastase assays, where the % inhibition of the enzymes was calculated at 500 and 1000 mg/L concentration of the extracts, respectively.

2.4.4 Cellular assays to measure antioxidant capacity

Cytotoxic activity *in vitro*

The cytotoxic activity of the extracts was assessed on human immortalized keratinocytes (HaCaT cell line). Cells were maintained following the manufacturer's indications. DMEM with 4.5 g/L glucose and 1 mM pyruvate was supplemented with 10% (v/v) of fetal bovine serum and 1% (v/v) penicillin–streptomycin solution. Cells were grown at 37 °C in a humidified 5% CO₂ incubator and were passed every 2–3 days. Assays were carried out in 96-well plates with 12,000 seeded cells in each well.

Cells were seeded and were treated after 24 h with each extract at different concentrations (5–800 $\mu\text{g/mL}$). Cells were incubated for 24 h, and nuclei were stained by adding Hoechst 33342 fluorescent probe during the last 30 min (4.5 μM final concentration). Extracts were freshly prepared at 100 mg/mL in DMSO, and corresponding DMSO controls were included in the assay to evaluate cytotoxic effects caused by this solvent.

Fluorescence measurements were carried out in PBS 1x using a Cytation 3 Cell Imaging Multimode reader (BioTek, Winooski, VT, USA) with 377 nm excitation and 447 nm emission filters. Data were expressed as a percentage of cellular viability compared to nontreated cells. IC_{50} values were calculated through nonlinear regression of the algorithm-transformed concentrations and the normalized responses.

Antioxidant activity *in vitro*

To evaluate the antioxidant effectiveness of the extracts, HaCaT cells were cultured for 24 h as described above and then treated with noncytotoxic concentrations (10, 20 y 40 $\mu\text{g/mL}$) of the extracts. After 24 h of treatment, cultures were washed twice with PBS 1x. Cells were maintained with a thin layer of PBS 1x while were exposed to solar ultraviolet radiation type A (UVA) radiation (8 J/cm^2) emitted by a Bio-Link Crosslinker BLX-E312 (Vilber Lourmat, Collégien, France). To prevent excessive heating due to the UVA exposure, plates were meanwhile put on ice as described previously by Cooper et al. (2009) [26]. In parallel, treated cells were manipulated in the same manner but were covered during UVA exposure (nonirradiated controls). Subsequently, cells were incubated with Hoechst 33342 (4.5 μM) and H2DCF-DA (30 μM) in fresh medium for 30 min. H2DCF-DA (nonfluorescent) to monitor ROS generation through its oxidation to

fluorescent 2',7'-dichlorodihydrofluorescein (DCF) by those radical species and cell viability (Hoechst). Both fluorescent signals were measured by the Cytation 3 reader as described above for Hoechst 33342 and using 485 nm excitation and 535 nm emission filters for DCF. DCF signals were normalized with the nuclei number determined for each well by Hoechst staining, and data were expressed as percentage of ROS (%) compared to nontreated and nonirradiated cells.

2.5 *In vivo* neuroprotection capacity

2.5.1 *Drosophila* stock and exposure to hypoxia

Drosophila melanogaster has been commonly employed as a neurodegeneration model system due to its minimal resource demands and notable conservation, including the response to alterations in oxygen levels, which is similar to the human. Hypoxia-induced injury is a crucial mechanism in several medical conditions, including ischemic stroke, cardiac infarction, and renal disease, among others. In fact, *drosophila* has been proposed by several authors to study the impact of hypoxia-reperfusion [27,28] and represents a good screening model for neurovascular disease [29].

Drosophila melanogaster stock (Oregon R strain) was kindly provided by Dr. Luisma Escudero. Flies were bred in polystyrene tubes on a standard medium at constant temperature and humidity (25 °C; 50% humidity) and a 12 h light/dark cycle. Three days after emergence, male flies were sexed under CO₂ anesthesia, and vials containing 10–15 male flies were prepared. Treated flies received instant food formulation (Genesee Scientific, Morrisville, NC, USA) prepared in water containing the plant extracts at the indicated concentrations. After hatching, male flies were maintained in standard food for 5 days, and a set of flies were supplemented in the food with the plant extracts at

0.05, 0.2, and 0.3 mg/mL. The control group was kept on media prepared in vehicle (0.5% ethanol in water). Treatment media was refreshed once during the experimental procedure. After 5 days, flies were subjected to 2.5 h of hypoxia (1% O₂, 25 °C, 30–40% humidity) by introducing the vials in a hypoxic glove box (Coy, Grass Lake, MI, USA) where the environmental oxygen was displaced by N₂. Then, flies were monitored for locomotor activity for 4 h and mortality rate was assessed after reoxygenation and represented as relative mortality to the hypoxia group. Each experiment consisted of three tubes per condition (10–15 flies/vial) and was repeated at least three times.

2.5.2 *Drosophila locomotor activity monitoring*

To study the effect of hypoxia exposure on fly behavior, flies were transferred into a 25 mm empty polycarbonate tube and placed in the *Drosophila* Activity Monitoring (DAM) system v3.11.1.35 (LAM25H-3, Trikinetics Inc., Waltham, MA, USA). Locomotor activity was recorded for 4 h by registering the infrared light beam crosses in each tube at 3 different heights. The DAMSystem3 Data Collection Software was used for data acquisition, and raw data were grouped into 30-minute intervals using FileScan Software v1.13. Live flies were counted at the beginning and the end of the assessment period. Relative mean beam crosses were calculated by normalizing mean movement counts per fly to the hypoxia group and represented as a heatmap using GraphPad Prism v7.

2.6 Statistical analysis

The results were presented as mean $\pm$ standard deviation (SD) of at least three replicates. IBM SPSS Statistics 24.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of antioxidant, free radical, and enzyme data. The differences between samples

were statistically analysed by one-way ANOVA, and post hoc comparisons of the means were performed with Tukey's HSD and T3 de Dunnett tests.

GraphPad Prism version 8.01 (GraphPad Software, San Diego, CA, USA) was used for the representation and analysis of cellular assays. Data were expressed as the mean $\pm$ SD of 5–10 replicates depending on the assay. Statistical differences were determined by one-way ANOVA and statistical comparisons with Tukey's test.

3. Results

3.1 Characterisation of the extracts by HPLC-ESI-qTOF-MS

The TC, HS, SM, LC, and OE extracts were tentatively characterized by HPLC-ESI-qTOF-MS. Base peak chromatograms (BPCs) of the five extracts are shown in Figure S1. The characterization was carried out based on retention times, fragments, mass spectra, predictions from different software, and other studies previously published in the literature. According to the identification guidelines proposed by Sumner et al. (2007) [30], compounds were annotated at level 1 with commercial standards, at level 2 by comparing the MS/MS spectra with those present in the databases, at level 3 based on the molecular formulation and MS1 spectra, and at level 4 where the molecules remain as unknowns. All this information is provided in Tables S1, S2, S3, S4, and S5 for TC, HS, SM, LC, and OE extracts, respectively.

In total, 292 compounds were characterized, specifically 52 compounds in TC, 40 compounds in HS, 67 compounds in SM, 85 compounds in LC, and 98 compounds in OE. It was worth noting the difference between the matrices in phenolic richness, with SM, LC, and OE having more than 65 compounds in each, while TC and HS have less than 60 compounds. This fact may be mainly due to the extraction conditions and the polarity of

the majority of phenolic compounds in each matrix. Among the five extracts, LC and OE contain the highest number of annotated compounds. Briefly, the flavan-3-ols was the class with the highest richness in the TC extract since epigallocatechin, quinic acid, and gluconic acid were the main compounds present in this extract. The HS extract has a high presence of hibiscus acid, hibiscus acid lactone, and glycosylated flavonoids, such as quercetin 3-O-rutinoside and quercetin 7-glucoside. The SM extract stood out for its high flavonoid presence, especially silybin and its isomers such as silycystin, isosilybin b, or its modified forms such as dehydrosilybin, silybin hydrogenated or acetylsilybin A/B. The LC extract was characterized by a particularly high presence of phenylpropanoids. Among the phenylpropanoids, verbascoside presented the highest presence. In addition, a high presence of iridoids and secoiridoids, such as shanziside, and glycosylated compounds of this type, such as gardoside, was detected. In the OE extract, the presence of the oleuropein aglycone was particularly high. The parental form of this compound, oleuropein, and other modifications of oleuropein, such as oleuropein-glucoside, were also found in high concentrations.

Table 1 shows the common compounds among the five matrices under study. The high presence of fatty acids shared by the five matrices is noteworthy. The matrices with the highest number of compounds in common were LC and OE, with verbascoside, malic acid, gluconic acid, and fatty acids such as linolenic acid and palmitic acid standing out among the compounds in common.

Table 1. Common compounds that have been identified in more than one of the matrices.

Compound	Rt (min)	[M-H] ⁻	Mol. Formula	T. <i>cacao</i>	H. <i>sabdariffa</i>	S. <i>marianum</i>	L. <i>citriodora</i>	O. <i>europaea</i>
Gluconic acid	1.01	195.0519	C ₆ H ₁₂ O ₇	x			x	x
Malic acid	1.07	133.0140	C ₄ H ₆ O ₅				x	x
Quinic acid	1.13	191.0292	C ₇ H ₁₂ O ₆	x				x
Chlorogenic acid	4.02	353.0867	C ₁₆ H ₁₈ O ₉		x	x		
Epigallocatechin	9.18	305.0690	C ₁₅ H ₁₄ O ₇	x				x
Verbascoside	9.33	623.1978	C ₂₉ H ₃₆ O ₁₅				x	x
Quercetin 3-O-rutinoside	9.60	609.1458	C ₂₇ H ₃₀ O ₁₆		x			x
Quercetin glucoside	9.85	463.0878	C ₂₁ H ₂₀ O ₁₂	x	x	x		
Unknown	10.23	539.1751	C ₂₅ H ₃₂ O ₁₃		x			x
Quercetin	12.16	301.0339	C ₁₅ H ₁₀ O ₇	x	x			
Unknown	12.95	329.0654	C ₁₇ H ₁₄ O ₇		x		x	
Dihydrocapsiate	13.53	307.192	C ₁₈ H ₂₈ O ₄				x	x
Eupatorin	13.56	343.0818	C ₁₈ H ₁₆ O ₇		x		x	
Gingerol	13.92	293.1748	C ₁₇ H ₂₆ O ₄	x	x		x	x
Hydroxylinolenic acid	15.74	293.2113	C ₁₈ H ₃₀ O ₃		x		x	x
Unknown	16.30	540.3288	C ₂₉ H ₄₉ O ₉	x		x	x	x
Unknown	16.60	566.3453	C ₃₁ H ₅₁ O ₉			x	x	x
Ethyl vanillate	17.77	195.0686	C ₁₀ H ₁₂ O ₄	x				x
Linolenic acid	18.46	277.2159	C ₁₈ H ₃₀ O ₂	x	x		x	x
10'-Apo-beta-carotenal	18.73	375.2712	C ₂₇ H ₃₆ O	x	x	x	x	x
Linoleic acid	19.16	279.2328	C ₁₈ H ₃₂ O ₂	x	x	x	x	x
Palmitic acid	19.82	255.2325	C ₁₆ H ₃₂ O ₂	x			x	x
Unkwnon	19.87	403.3052	C ₂₂ H ₄₄ O ₆	x	x	x		x
Oleic Acid	19.95	281.2482	C ₁₈ H ₃₄ O ₂	x	x	x	x	x
Unknown	20.98	383.1934	C ₁₆ H ₃₂ O ₁₀	x	x		x	x
Ginsenoside Rh2	20.98	621.4417	C ₃₆ H ₆₂ O ₈			x	x	

x: presence of the compound in the extract

3.2 Evaluation of the antioxidant and anti-inflammatory capacities of the extracts

3.2.1 Evaluation of TPC, antioxidant capacity and ROS scavenging potential

In **Table 2**, we can find the TPC values obtained by the Folin–Ciocalteu method and the results of the FRAP, TEAC, and ORAC tests for the five matrices under study. As a result, all the matrices under study show antioxidant capacity, so they can all be considered bioactive against oxidative stress. It is worthwhile to highlight the case of the SM extract,

which obtained the highest values for the four tests. In addition, a general trend showing that the higher the content of TPC, the greater the capacity for the transfer of electrons and H atoms can be observed, confirming previous studies [31]. This is also in agreement with the findings in the case of the HS extract, which presented the lowest values in the four assays. It is important to note that there is no universal method for measuring antioxidant capacity, as different methods can measure various mechanisms of action. For instance, assays like FRAP and TEAC are based on single electron transfer (SET), employing indirect and direct approaches, respectively. On the other hand, the ORAC method relies on hydrogen atom transfer (HAT), which is similar to both electron transfer and hydrogen atom transfer. Choosing different assays to evaluate antioxidant activity can offer a comprehensive prediction of this bioactive potential, providing complementary information [32].

Table 2. Evaluation of total phenolic content and antioxidant capacity of extracts.

Sample	TPC (mg GAE/g DE)	FRAP (mmol Fe ²⁺ /g DE)	TEAC (mmol TE/g DE)	ORAC (mmol TE/g DE)
<i>T. cacao</i>	255 ± 12 ^a	1.38 ± 0.09 ^{b,c}	1.25 ± 0.08 ^a	2.35 ± 0.09 ^c
<i>H. sabdariffa</i>	51 ± 1 ^d	0.47 ± 0.05 ^d	0.24 ± 0.02 ^c	1.16 ± 0.03 ^d
<i>S. marianum</i>	617 ± 8 ^b	1.4 ± 0.3 ^{a,b,c,d}	1.3 ± 0.1 ^a	11.7 ± 0.3 ^b
<i>L. citriodora</i>	344 ± 15 ^c	2.4 ± 0.2 ^a	1.15 ± 0.08 ^a	5.9 ± 0.2 ^a
<i>O. europaea</i>	216 ± 28 ^a	1.65 ± 0.10 ^{a,b}	0.84 ± 0.05 ^b	6.0 ± 0.2 ^a

TPC: Total Polar Compounds; FRAP: Ferric Reducing Antioxidant Power Assay; TEAC: Trolox Equivalent Antioxidant Capacity; ORAC: Oxygen Radical Absorbance Capacity; GAE: Gallic Acid Equivalent; DE: Dry Extract; TE: Trolox Equivalent. Data are means ± standard deviation ($n = 3$). Different letters represent statistically significant differences at $p < 0.05$ level.

Table 3 reveals the amount of extract required to inhibit half the concentration of reactive species (IC₅₀).

Table 3. Evaluation of radical scavenging of controls and extracts.

Sample	$\cdot\text{O}_2^-$ (mg/L) ¹	$\cdot\text{NO}$ (mg/L) ¹	HOCl (mg/L) ¹
<i>T. cacao</i>	29.7 ± 0.4 ^c	0.42 ± 0.02 ^b	0.71 ± 0.02 ^b
<i>H. sabdariffa</i>	50 ± 2 ^a	10.3 ± 1.0 ^c	1.32 ± 0.04 ^d
<i>S. marianum</i>	57 ± 6 ^a	5.0 ± 0.8 ^a	0.70 ± 0.01 ^b
<i>L. citriodora</i>	n.d.	3.76 ± 0.08 ^a	3.5 ± 0.4 ^a
<i>O. europaea</i>	269 ± 17 ^d	3.0 ± 0.2 ^a	16 ± 2 ^c
Gallic acid	50 ± 3 ^a	1.4 ± 0.3 ^b	3.8 ± 0.3 ^a
Epicatechin	70 ± 5 ^b	0.87 ± 0.02 ^b	0.18 ± 0.01 ^e

Data are means ± standard deviation ($n = 3$). ¹ Inhibitory Concentration at 50%. Different letters represent statistically significant differences at $p < 0.05$ level. n.d.: no data.

The intracellular accumulation of ROS, which occurs in cells under oxidative stress, is responsible for several chronic pathologies, including cancer, neurodegenerative or cardiovascular pathologies [33]. Thus, Reuter et al. (2010) revealed that oxidative stress can activate several transcription factors, which can lead to the expression of more than 500 different inflammation-related genes [34]. The activation of this entire cascade can lead to chronic inflammation, which in turn may mediate most chronic diseases, including cancer, diabetes, cardiovascular, neurological, and pulmonary diseases [34]. In this regard, phenolic compounds have been shown to have a potent antioxidant effect because their chemical structure means they can eliminate ROS, and their antioxidant capacity is therefore related to the other properties of this type of compound, such as anti-inflammatory and neuroprotective properties.

In relation to radical scavenging assays, with the exception of LC and OE for the $\cdot\text{O}_2^-$ assay, all showed bioactivity. TC presented the best IC_{50} value for all the radical scavenging tests, being significantly better than the other extracts. This indicates that the types of phenolic compounds present in TC have a higher anti-radical power than those present in OE for the $\cdot\text{O}_2^-$ and HOCl test and that the HS for the $\cdot\text{NO}$ test presented the highest IC_{50} values. For these tests, epicatechin (EPI) and gallic acid (GA) were used as standard

controls, listed in **Table 3**. It is observed that, especially in the HOCl assay, EPI has significantly better values compared to the five plant matrices, which is related to the TC extract being one of the lowest as this extract has a high EPI content.

3.2.2 Evaluation of enzymatic inhibition capacity

Table 4 presents the inhibitory effect of the five extracts under study on the hyaluronidase, XOD, tyrosinase, elastase, and collagenase enzymes and the positive control used for each enzyme. As mentioned above, phenolic compounds are involved in the regulation of the level of reactive species. In this sense, the excess of these reactive species can lead to excessive activation and dysregulation of different enzymes studied in this work. For instance, the enzyme XOD is a dehydrogenase responsible for catalyzing hypoxanthine to xanthine and subsequently to the oxidation of uric acid. However, when oxidative stress is present, XOD is transformed into an oxidase, which leads to the production of superoxide radicals and causes many inflammatory diseases [35].

Table 4. Evaluation of enzymatic inhibition capacity of controls and extracts.

Sample	Hyaluronidase (mg/L) ¹	XOD (mg/L) ¹	Tyrosinase (% inh.) ²	Elastase (% inh.) ³	Collagenase (mg/L) ¹	AChE (mg/L) ¹
<i>T. cacao</i>	12 ± 2 ^a	n.d.	28 ± 8 ^{a,b,c}	23 ± 4 ^{b,c,d}	156 ± 1 ^b	244 ± 9 ^a
<i>H. sabdariffa</i>	66 ± 6 ^d	n.d.	5 ± 2 ^c	44.1 ± 0.5 ^{a,b,c,d}	1190 ± 35 ^b	n.d.
<i>S. marianum</i>	4.9 ± 0.4 ^a	4.4 ± 0.4 ^a	39 ± 4 ^a	n.d.	56 ± 3 ^b	1259 ± 53 ^b
<i>L. citriodora</i>	87 ± 5 ^c	3.2 ± 0.4 ^{a,b}	17 ± 2 ^{b,c}	48 ± 3 ^a	633 ± 27 ^a	316 ± 4 ^c
<i>O. europaea</i>	187 ± 5 ^b	2.3 ± 0.3 ^b	28 ± 3 ^{a,b}	25 ± 3 ^{b,c,d}	618 ± 9 ^a	373 ± 6 ^d
Gallic acid	102 ± 4 ⁴	n.d.	n.d.	n.d.	n.d.	n.d.
Epicatechin	167 ± 6 ⁴	9 ± 1 ^c	n.d.	n.d.	n.d.	n.d.
Physostigmine	n.d.	n.d.	n.d.	n.d.	n.d.	0.043 ± 0.004 ⁶
1,10-phenanthroline	n.d.	n.d.	n.d.	n.d.	83 ± 2 ⁵	n.d.
Elastatinal (51.26 ppm)	n.d.	n.d.	n.d.	53 ± 5	n.d.	n.d.
Kojic acid (21.3 ppm)	n.d.	n.d.	49 ± 6	n.d.	n.d.	n.d.

Data are means ± standard deviation ($n = 3$). ¹ Inhibitory Concentration at 50%. ² At 500 mg/L. ³ At 1000 mg/L. ⁴ Inhibitory Concentration at 20%. ⁵ % Inhibition. ⁶ Inhibitory Concentration at 90%. Different letters represent statistically significant differences at $p < 0.05$ level. n.d.: no data.

In the case of the hyaluronidase assay, all matrices under study show bioactivity. Nevertheless, SM has been shown to be the most bioactive, while OE is significantly less so. For the enzyme XOD, the lowest IC₅₀ value corresponds to OE, while TC and HS did not even reach this 50% inhibition. Furthermore, when compared to the positive control used, EPI, both SM and LC, and OE have a more significant ability to inhibit the enzyme. The tyrosinase inhibition assay shows the % inhibition of the enzyme at 500 mg/L, with SM showing the highest activity and HS the lowest and with the other three extracts being similar in terms of inhibition. In the elastase assay, the results were expressed as % inhibition of the enzyme at 1000 mg/L, with the LC extract having the highest inhibitory power, while SM is not able to inhibit. Thus, in this case, with the exception of SM, all the extracts showed bioactivity. Finally, in the collagenase assay, the five matrices under study were able to inhibit collagenase, highlighting, in this case, SM since, when

compared to HS, which is the least bioactive, there is a big difference in the dose needed to inhibit the enzyme at 50%.

3.2.3 Cellular assays to measure antioxidant capacity

The cytotoxic effects of the extracts in HaCaT cells were plotted in **Figure 1** (Statistical significance for **Figure 1** was included in **Table S6**). DMSO was used as a vehicle to solubilize the extracts and not alter cell viability, even at the highest used concentration (**Table S7**).

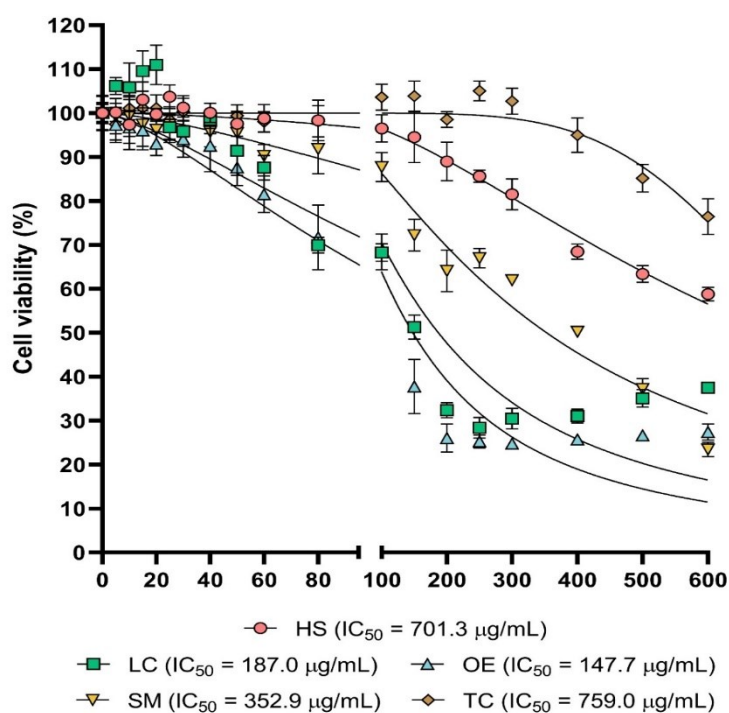


Figure 1. Cytotoxic activity. Dose–response cell viability plots for the five extracts tested. Cell viability values were obtained as described in methods section. Statistical significance was included in **Table S6**.

LC and OE extracts exhibited the highest cytotoxicity, and both treatments resulted in a statistically significant reduction in cell viability from 40 $\mu\text{g/mL}$. IC_{50} was estimated as 187 $\mu\text{g/mL}$ for LC and 147.4 $\mu\text{g/mL}$ for OE. In the case of the SM extract, cytotoxic effects were statistically significant from 60 $\mu\text{g/mL}$, and IC_{50} was 352.9 $\mu\text{g/mL}$. With the HS treatment, cell viability was decreased from 200 $\mu\text{g/mL}$ with statistical significance, and

calculated IC_{50} was 701.3 $\mu\text{g/mL}$ for this extract, which did not reduce cell viability in a significant manner up to 400 $\mu\text{g/mL}$ and whose IC_{50} was 759 $\mu\text{g/mL}$.

The effectiveness of the extracts as antioxidant ingredients was explored in the HaCaT model due to the significant role of oxidative stress on skin health and aging. UVA was chosen as a well-known inductor of ROS generation and oxidative stress in the skin [36]. On the one hand, as shown in **Figure 2**, none of the extracts were able to reduce the basal oxidative stress in the absence of UVA. On the other hand, UVA radiation increased the presence of ROS in a significant way in all the conditions, and only the SM extract was able to decrease these ROS levels in a dose-dependent manner. Pretreatment with 20 $\mu\text{g/mL}$ of SM reduced the increment in ROS levels from 146% (untreated but irradiated condition) to 129%. A statistically significant reduction to 105% ROS was evidenced for 40 $\mu\text{g/mL}$ (**Figure 2D**, ####, $p < 0.0001$). Apparently, the rest of the extracts (HS, OE, LC, and TC) did not prevent the oxidative action of UVA. Furthermore, the LC extract exhibited a significant prooxidant effect at 20 and 40 $\mu\text{g/mL}$, probably related to a phototoxic effect of some of its components.

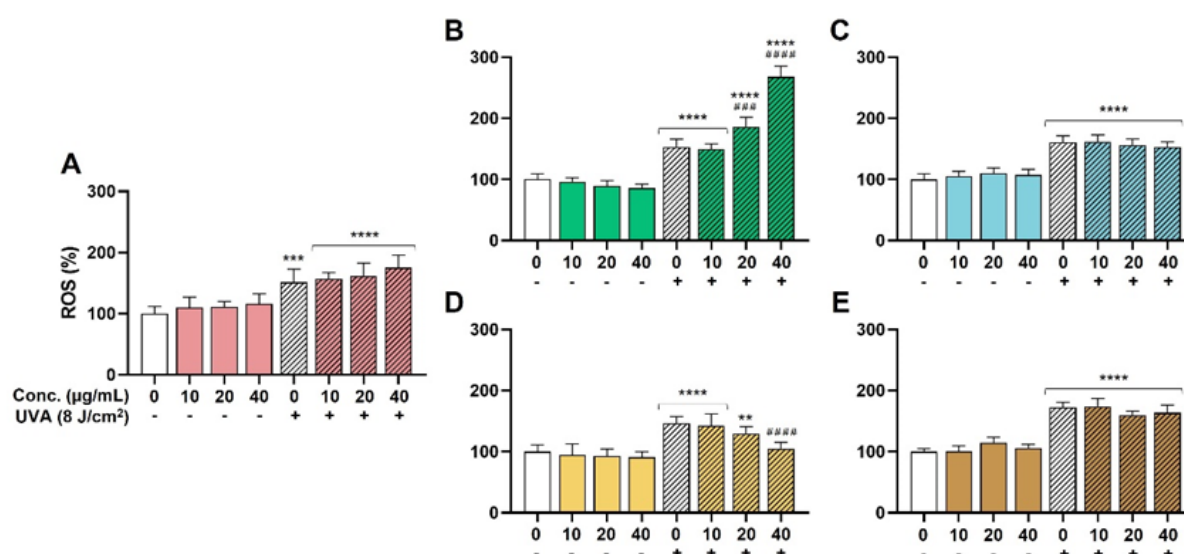


Figure 2. Antioxidant activity. ROS levels were measured as described in methods section. Significance was established at $p < 0.05$. ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$) are in the figures and indicate statistically significant differences compared to the nontreated and nonirradiated control. ### ($p < 0.001$), and ##### ($p < 0.0001$) indicate statistically significant differences compared to the nontreated irradiated control. (A): *Hibiscus sabdariffa*; (B): *Lippia citriodora*; (C): *Olea europaea*; (D): *Silybum marianum*; (E): *Theobroma cacao*.

3.3 Evaluation of the neuroprotective effect of the extracts

There is a close link between the ability of phenolic compounds to exert their neuroprotective effect through their antioxidant and free radical scavenging action and their ability to inhibit enzymes involved in neurodegenerative diseases, such as AChE [8].

3.3.1 Evaluation of acetylcholinesterase (AChE) inhibition capacity

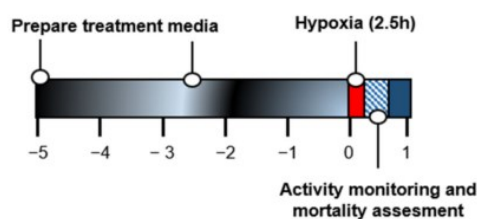
Table 4 shows the inhibitory effect of the extracts on AChE, showing that the lowest extract concentration to inhibit 50% of the enzymatic activity was for the TC extract, followed by LC, OE, and SM extracts. The HS extract did not even reach 50% of the enzymatic activity at very high doses of concentration. Thus, with the exception of HS, all showed bioactivity against enzyme inhibition.

3.3.2 Effect of supplementation with different plant extracts on hypoxia–reoxygenation injury in *D. melanogaster*

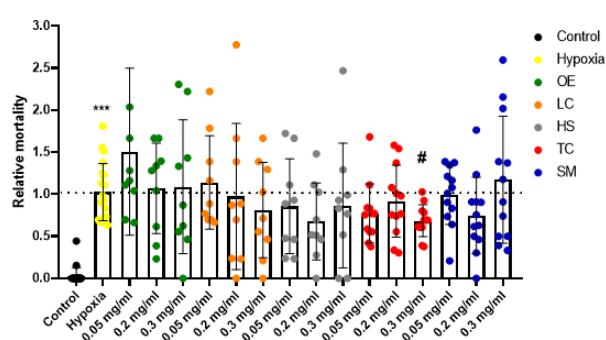
Hypoxic stress is known to produce injury in flies. The *Drosophila* model does not reproduce some aspects of human brain ischemia because of its primitive blood system (blood vessels are lacking, and there is no lymphoid blood cell lineage). However, the model recapitulates important pathogenic features when subjected to hypoxia, such as increased activation of brain caspases, locomotor deficiencies, and mortality [37]. Moreover, increases in several oxidative stress markers and changes in metabolic activity have been observed in flies subjected to hypoxia [27]. Therefore, the use of this screening model provides an easy and convenient way of testing antioxidant compounds, such as polyphenols. To investigate the influence of hypoxia–reoxygenation injury in *Drosophila*, treated flies were subjected to hypoxic stress for 2.5 h under controlled conditions (1% O₂; 25 °C; 30–40% relative humidity) (**Figure 3a**). As expected, flies in the hypoxia group showed a significant increase in mortality compared to the control group 4 h after reoxygenation. Treatment with the extract TC at the higher concentration resulted in a significant increase in fly survival after the hypoxia challenge (**Figure 3b**).

We also studied fly behavior by quantifying animal movement for 4 h after hypoxia. While control flies moved uniformly over time, flies in the hypoxia group showed a reduction in locomotor activity, indicated by fewer beam crosses, which were more evident after 120–150 min of reoxygenation, revealing that the reperfusion injury worsened fly behavior. However, treatment with TC could not recover the loss of locomotor activity induced by hypoxia (**Figure 3c**).

(a)



(b)



(c)

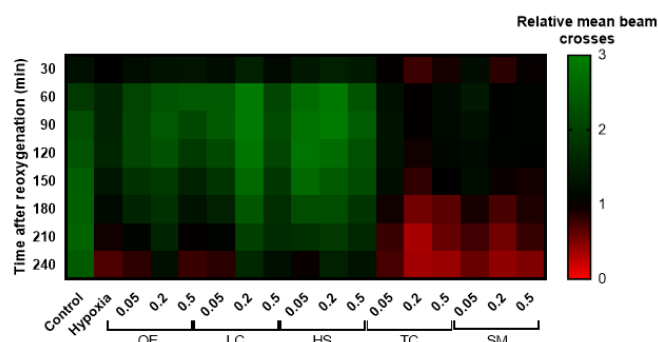


Figure 3. Effect of plant extract supplementation on *Drosophila melanogaster* exposed to severe hypoxia. (a) Schematic illustration of the hypoxia protocol in flies. (b) Effect of the treatment on fly survival after exposure to 1% O₂ for 2.5 h. Data were analyzed using one-way ANOVA test followed by Dunnett's multiple comparisons test *** $p < 0.001$ vs. control; # $p < 0.05$ vs. hypoxia. (c) Heatmap displaying the locomotor activity of treated flies. Flies were transferred into the monitor immediately after hypoxia and the activity was recorded for 240 min after reperfusion. Each cell shows the mean beam crosses per fly in the group.

The results obtained show that there is no single matrix that stands out for its bioactivity in all the tests, but rather, depending on the bioactive target and the assay in question,

there are matrices that stand out. Still, the rest, with a few exceptions, also show bioactivity. This shows that despite the structural diversity present in the different families of phenolic compounds, they all show high bioactivity; it is the combination of these compounds that gives them their pleiotropic character.

4. Discussion

The extracts under study have been characterized, giving a wide range of phenolic compounds, some of which are characteristic of each of the matrices and a minority of which are common among the different matrices (**Table 1**). Although most of the compounds are not common, all matrices have bioactive potential against the targets under study, so the differences in potential between them may be mainly due to these specific compounds being in each of the matrices and also due to the differences in the ratio between them.

When we pay attention to the results of the antioxidant tests for TPC and FRAP, TEAC, and ORAC (**Table 2**), the higher the TPC, the greater the antioxidant power shown in the tests. Thus, HS has the lowest TPC and the lowest value in the other antioxidant test values, while SM has the highest TPC content and high values in the rest of the tests measuring antioxidant power. This relationship is consistent with the tests carried out by Aroso et al. (2017) [38], in which there is also a positive relationship between the TPC content and the tests measuring antioxidant power.

In the case of the other three remaining plant matrices, they have intermediate values for the results of the TPC and FRAP, TEAC, and ORAC assays. For the FRAP assay, the matrices TC, SM, and OE showed very similar values. This may be due to the fact that there are similar compounds in their composition. For example, both TC and OE have

epigallocatechin [39], a compound that has been shown to have a potent action in the FRAP assay. In contrast, SM, with its high content of flavolignans such as silybin A and B, has also been shown to have a potent action in the FRAP assay [40]. In the case of the ORAC assay, the matrices LC and OE obtained a similar and high value, which may be due to the fact that both matrices share the most compounds in their composition, like verbascoside, which has been shown to be potent in proton transfer, the mechanism of action on which this antioxidant capacity test is based [41].

In the case of cellular assays in HaCaT cells, TC, OE, and SM extracts were able to decrease ROS levels; however, the only one that achieved this in a dose-dependent manner was SM. This is in agreement with Svobodová et al. (2007) [42], who found that flavonolignans present in the SM extract suppress UVA-induced oxidative stress in HaCaT cells, making this extract potentially useful in the treatment of UVA-induced skin damage.

In relation to the tests to measure the capacity to eliminate specific ROS and RNS, for the $\cdot\text{O}_2^-$ radical test, the HS and SM matrices obtained a similar IC_{50} value, which is also similar to the value obtained for the gallic acid standard. **Table 1** shows that the compounds that these two matrices have in common include chlorogenic acid and quercetin glucoside. In this sense, there are studies that demonstrate the high power of chlorogenic acid [43], quercetin glucoside [44], and gallic acid [45] for the uptake of the superoxide radical, which is in agreement with the results obtained.

In the test to eliminate HOCl, TC and SM were the ones that obtained the lowest IC_{50} . Despite the difference in phenolic composition, they achieved similar results. TC extract has a high epigallocatechin and epicatechin content. As shown in **Table 3**, epicatechin

has a high free radical scavenging power, with its power in the HOCl scavenging test standing out, where it has more power than even the whole TC extract. This is supported by different studies showing the high power of TC extract to scavenge free radicals [46]. Furthermore, He et al. (2018) evaluated the power of different catechins against radicals, and they found that epigallocatechin gallate possessed the highest radical scavenging power, followed by epigallocatechin, epicatechin, and catechin in descending order of power. This suggests that the effect of these compounds is strongly related to the structure of catechins, mainly due to the hydroxyl and galloyl groups [47]. For SM, silybin has shown potent action for HOCl removal [48].

In the case of the XOD enzyme, the OE and LC extracts have been shown to have potent inhibitory power. Both extracts share compounds such as verbascoside, the main bioactive compound present in LC. This compound was shown to be a potent inhibitor of the XOD enzyme, as verbascoside is able to enter the active site of XOD and form hydrogen bonds with amino acid residues (such as Lys-1045, Arg-880, Arg-912, Glu-1261, and Gln-1194) [49].

Within the enzymes involved in maintaining skin firmness, hyaluronidase, elastase, and collagenase, there is no single matrix that stands out for all of them. For both hyaluronidase and collagenase, the TC and SM matrices have the lowest IC₅₀ values. Thus, in the case of TC, the content of compounds from the flavan-3-ols group was shown to be potent inhibitors of these three enzymes involved in the loss of elasticity and firmness of the skin [50]. For SM, the main group of compounds are flavolignans, which have also been shown to have a potent inhibitory action on the aforementioned

enzymes. This shows that phenolic compounds from different groups and, therefore, with different chemical properties can exert the same bioactive effect [51].

The mechanism of action by which the polyphenols present in our matrices have the ability to inhibit enzymes would be the next step in our research. There are *in silico* studies—using different phenolic compounds that have been tested on the enzymes—for example, the inhibition of rosmarinic acid against human hyaluronidase. Molecular docking studies revealed that rosmarinic acid is bounded to the hyaluronidase binding pocket with four binding interactions [52]. In our study, the hyaluronidase used was not human but bovine, but these are highly phylogenetically conserved proteins [53]. Another compound that has been tested *in silico* against collagenase, elastase, and tyrosinase enzymes is caffeine, which was shown to form a stable protein–ligand complex validated by molecular dynamics simulation. Thus, the potential of phenolic compounds in the inhibitory action of these enzymes is shown [54].

Finally, the neuroprotective effect of the different extracts was evaluated in both *in vitro* and *in vivo* assays. In the case of the *in vitro* AChE enzyme inhibition assay, the extract with the highest enzyme inhibitory capacity was TC, followed by LC and OE extracts with a similar IC_{50} value. In the case of the *in vivo* assay in which the survival of flies after hypoxia challenge was measured, it was TC that significantly improved survival. The TC extract is high in epigallocatechin and epicatechin, compounds that have been shown to be potent inhibitors of AChE [55,56]. The similar inhibition of LC and OE at doses slightly higher than TC may be due to their high presence of flavonoid glycosides, which are considered essential for AChE inhibition [57].

The results highlight the importance of reporting the bioactive properties of plant extracts according to their phenolic composition and not simply their total phenolic content, as many of the bioactive properties can be related to a particular phenolic type or phenolic family or to the synergistic action of them.

In this context, when exploring the potential use of bioactive compounds, such as phenolic compounds, for health improvement, utilizing combined plant extracts becomes intriguing. A rich array of phenolic compounds from diverse sources could offer broader benefits to the organism, leveraging the potential synergistic effects arising from different types of phenolic compounds [58]. Therefore, plant extracts with this bioactive potential could be used for the development of nutraceuticals for the prevention of diseases related to metabolic stress and inflammation [59]. Additionally, these extracts could be employed in nutricosmetics, as the main enzymes evaluated—such as tyrosinase, collagenase, and hyaluronidase—are associated with maintaining skin color, elasticity, and hydration, respectively [60].

5. Conclusions

In this study, the antioxidant, anti-inflammatory, and neuroprotective effects of five plant extracts showing different phenolic compositions have been evaluated by means of different assays in order to assess the relationship between the presence of different types of phenolic compounds and their bioactivity. This study shows that not all extracts that are rich in phenolic compounds show the same bioactivities in the assays used, but, depending on their phenolic composition, there are extracts with greater or lesser bioactive potential against different targets. In general, it is observed that when there are common phenolic compounds in the different extracts, some of the bioactive

capacities are similar, as has been observed in the case of LC and OE. Nevertheless, the greatest bioactive difference has been observed when comparing the richness of the families of phenolic compounds, so the SM extract, rich in flavolignans such as silybin, are shown to possess a high antioxidant capacity in both spectrophotometric and cellular assays. It also proved to be a potent inhibitor of tyrosinase, hyaluronidase, and collagenase. In contrast, in other bioactivity tests measuring free radical scavenging capacity and neuroprotective effect through inhibition of the enzyme AChE and survival in flies subjected to hypoxia stress, it was the TC extract that stood out, possibly due to its high presence in compounds of the flavan-3-ol family, such as epigallocatechin and epicatechin. In future studies, it would be interesting to study the relationship between the phenolic composition of the matrices and the macronutrients present in the same matrices, with the aim of evaluating whether these interactions affect the hypoactivity of the phenolic compounds. Thus, this work demonstrates that phenolic compounds present in different matrices have common bioactive properties and that the abundance of different families of phenolic compounds makes them stand out with higher bioactivity against different biological targets.

Supplementary Materials: The following supporting information can be downloaded at www.mdpi.com/xxx/s1, Figure S1. Base peak chromatogram from the extracts. A. *T. cacao*. B. *H. sabdariffa*. C. *S. marianum*. D. *L. citriodora*. E. *O. europaea*; **Table S1**. Identification of phytochemical compounds in *T. cacao* extract by HPLC-ESI-qTOF-MS; **Table S2**. Identification of phytochemical compounds in *H. sabdariffa* extract by HPLC-ESI-qTOF-MS; **Table S3**. Identification of phytochemical compounds in *S. marianum* extract by HPLC-ESI-qTOF-MS; **Table S4**. Identification of phytochemical compounds in *L. citriodora* extract by HPLC-ESI-qTOF-MS; **Table S5**. Identification of phytochemical

compounds in *O. europaea* extract by HPLC-ESI-qTOF-MS; **Table S6**. Numerical results (mean and SD) and statistical significance for **Figure 1** results; **Table S7**. Equivalent DMSO concentrations for each extract concentration.

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Supplementary Material

Figure S1. Base peak chromatogram from the extracts. A. *T. cacao*. B. *H. sabdariffa*. C. *S. marianum*. D. *L. citriodora*. E. *O. europaea*.

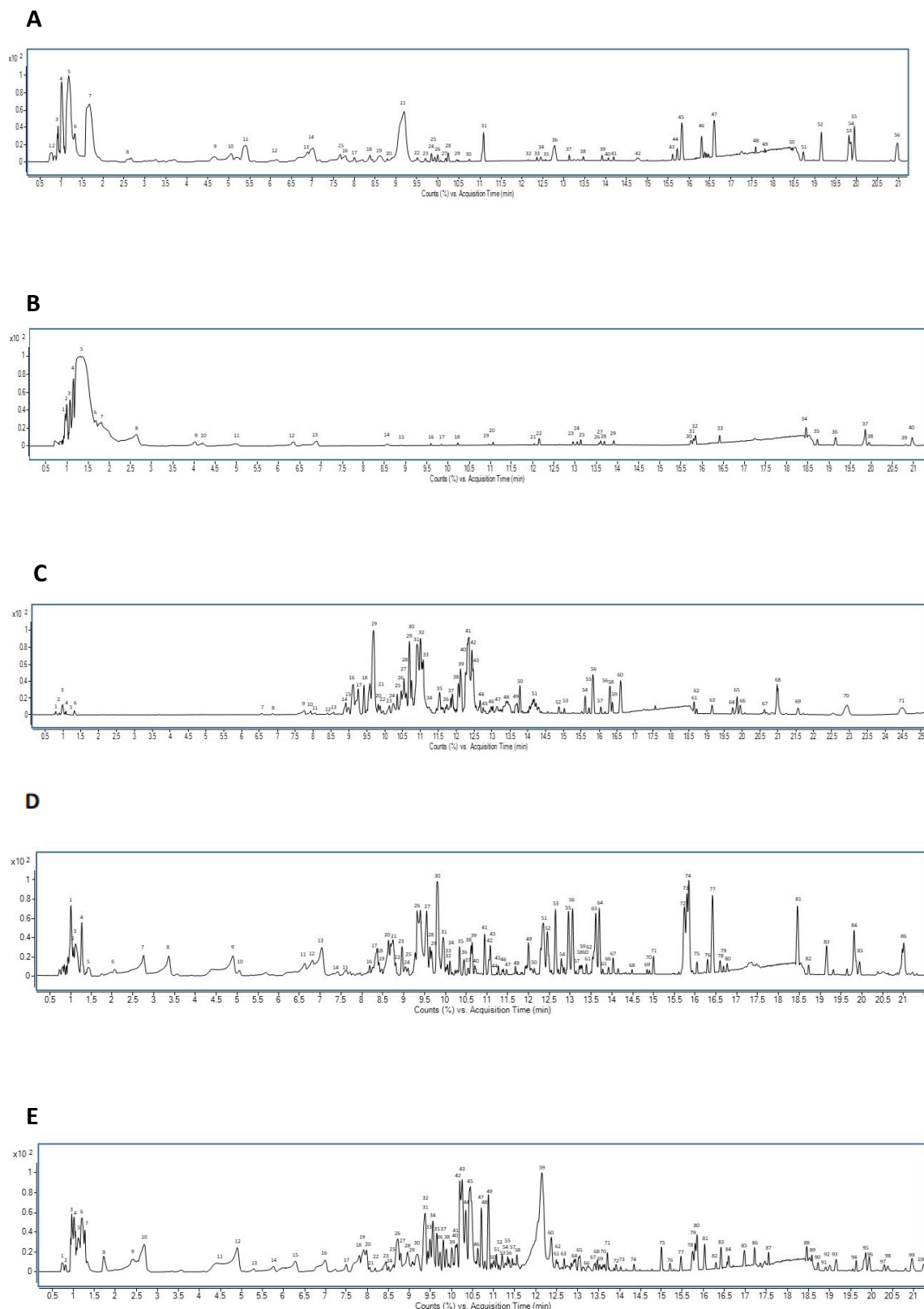


Table S1. Identification of phytochemical compounds in *T. cacao* extract by HPLC-ESI-qTOF-MS.

Peak	RT (min)	Observed [M-H]-	Theoretical [M-H]-	Mass error (ppm)	Mol. Formula	Level of annotation	Compounds	MS/MS fragments	References
1	0.75	272.9512	-	-	-	4	Unknown	-	-
2	0.91	203.1035	203.1037	-0.98	C ₈ H ₁₆ N ₂ O ₄	2	Valylserine	74/141/173	Pubchem: 18218236
3	0.92	217.1187	217.1194	-3.22	C ₉ H ₁₈ N ₂ O ₄	2	Leucylserine	74/129/173	Pubchem: 3621685
4	1.01	195.0519	195.0510	4.61	C ₆ H ₁₂ O ₇	2	Gluconic acid	75/129/177	HMDB0000625
5	1.13	191.0207	191.0197	5.24	C ₆ H ₈ O ₇	2	Citric acid isomer 1	87/111	HMDB0000094
6	1.33	191.0228	191.0197	16.23	C ₆ H ₈ O ₇	2	Citric acid isomer 2	87/111	HMDB0000094
7	1.69	96.9605	96.9601	4.13	H ₂ O ₄ S	3	Sulfate	-	HMDB0001448
8	2.67	117.0554	117.0557	-2.56	C ₅ H ₁₀ O ₃	2	3-Hydroxyvaleric acid	71/99	HMDB0000531
9	4.66	294.0606	294.0619	-4.42	C ₁₃ H ₁₃ NO ₇	2	N-caffeoyl-L-aspartate isomer 1	88/132/179	(M. L. Cádiz-Gurrea et al., 2014)
10	5.05	294.0610	294.0619	-3.06	C ₁₃ H ₁₃ NO ₇	2	N-caffeoyl-L-aspartate isomer 2	88/132/179	(M. L. Cádiz-Gurrea et al., 2014)
11	5.40	131.0704	131.0714	-7.63	C ₆ H ₁₂ O ₃	2	L-Leucate	85	Pubchem: 69955
12	5.61	407.1554	407.1559	-1.23	C ₁₇ H ₂₈ O ₁₁	3	Secoiridoid derived	99/263/305	(M. de la L. Cádiz-Gurrea et al., 2020)
13	6.88	278.0670	278.0670	0.00	C ₁₃ H ₁₃ NO ₆	2	L-Aspartic acid. N-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]	71/93/119	(M. L. Cádiz-Gurrea et al., 2014)
14	7.01	165.0554	165.0557	-1.82	C ₉ H ₁₀ O ₃	2	Dihydro-p-coumaric acid	72/103/147	HMDB0002199
15	7.60	289.0716	289.0718	-0.69	C ₁₅ H ₁₄ O ₆	1	Catechin	161/163/174/192	HMDB0002780
16	7.80	275.1006	-	-	-	4	Unknown	-	-
17	8.01	577.1352	577.1352	0.00	C ₃₀ H ₂₆ O ₁₂	1	Procyanidin B1	-	(M. L. Cádiz-Gurrea et al., 2014)
18	8.37	437.2040	437.2028	2.74	C ₁₉ H ₃₄ O ₁₁	2	Ebracteatoside D isomer 1	57/99/161	-
19	8.61	167.0366	167.0350	9.58	C ₈ H ₈ O ₄	3	Vanillic acid	-	HMDB0000484

20	8.79	865.1988	865.1985	0.35	C ₄₅ H ₃₈ O ₁₈	2	Procyanidin C	289/577	(M. L. Cádiz-Gurrea et al., 2014)
21	9.18	305.0690	305.0667	7.54	C ₁₅ H ₁₄ O ₇	1	Epigallocatechin	225/67/59	(Zhang et al., 2018)
22	9.51	437.2057	437.2028	6.63	C ₁₉ H ₃₄ O ₁₁	2	Ebracteatoside D isomer 2	57/99/161	-
23	9.70	737.1750	737.1723	3.66	C ₃₆ H ₃₄ O ₁₇	2	(Epi)catechin dimer hexose	449/611/539/289	(M. L. Cádiz-Gurrea et al., 2014)
24	9.85	463.0878	463.0882	-0.86	C ₂₁ H ₂₀ O ₁₂	1	Quercetin glucoside	149/242/271/285/300/335	HMDB0037362
25	9.93	707.1787	707.1828	-5.80	C ₃₂ H ₃₆ O ₁₈	2	Kalambroside A	449/539/581/289	Pubchem: 10349838
26	9.99	516.2476	516.2450	5.04	C ₂₄ H ₃₉ NO ₁₁	4	Unknown	-	(M. L. Cádiz-Gurrea et al., 20) 14
27	10.18	326.1075	326.1034	12.57	C ₁₈ H ₁₇ NO ₅	2	Deoxyclovamide (N-[(2E)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]-L-tyrosine)	206/282/119	(M. L. Cádiz-Gurrea et al., 2014)
28	10.24	433.0806	433.0776	6.93	C ₂₀ H ₁₈ O ₁₁	2	Quercetin arabinoside	300/271	(M. L. Cádiz-Gurrea et al., 2014)
29	10.45	238.1091	238.1085	2.52	C ₁₂ H ₁₇ NO ₄	3	Dihydroisoflavipucine	-	Pubchem: 38354263
30	10.75	272.0935	272.0928	2.57	C ₁₅ H ₁₅ NO ₄	2	L-Thyronine	94/124	HMDB0000667
31	11.09	421.2064	421.2079	-3.56	C ₁₉ H ₃₄ O ₁₀	2	1-Octen-3-yl primeveroside	57/97/277	HMDB0032960
32	12.16	301.0339	301.0354	-4.98	C ₁₅ H ₁₀ O ₇	1	Quercetin	151/121/65	HMDB0005794
33	12.36	391.1196	391.1187	2.30	C ₂₃ H ₂₀ O ₆	2	Dehydrodeguelin	117/62	(Bini et al., 2023)
34	12.46	329.2329	329.2333	-1.21	C ₁₈ H ₃₄ O ₅	2	5,8,12-Trihydroxy-9-octadecenoic acid isomer 1	211	HMDB0030936
35	12.57	329.2358	329.2333	7.59	C ₁₈ H ₃₄ O ₅	2	5,8,12-Trihydroxy-9-octadecenoic acid isomer 2	211	HMDB0030936
36	12.79	357.1215	357.1191	6.72	C ₁₆ H ₂₂ O ₉	2	Sweroside	97/198	(M. L. Cádiz-Gurrea et al., 2014)
37	13.13	394.2954	394.2963	-2.28	C ₂₃ H ₄₁ NO ₄	4	Unknown	-	-
38	13.47	276.1227	276.1241	-5.07	C ₁₅ H ₁₉ NO ₄	3	Barmumycin	-	(Lorente et al., 2010)
39	13.92	293.1748	293.1758	-3.41	C ₁₇ H ₂₆ O ₄	2	Gingerol	236/221	HMDB0005783
40	14.07	333.1815	333.1820	-1.50	C ₁₈ H ₂₆ N ₂ O ₄	3	Hydroxyhuperzine	-	(Jiang et al., 2010)

41	14.20	367.1671	367.1663	2.18	C ₂₁ H ₂₄ N ₂ O ₄	3	3,4-dimethoxy-N-[4-[oxo(1-piperidinyl)methyl]phenyl]benzamide	-	CHEBI:114263
42	14.77	195.0686	195.0663	11.79	C ₁₀ H ₁₂ O ₄	2	Ethyl vanillate	123	(Schwarz et al., 2021)
43	15.61	564.3295	-	-	-	4	Unknown	-	-
44	15.71	476.2780	476.2780	0.00	C ₂₇ H ₄₁ O ₇	2	Sinapoyloxypalmitate	279	Pubchem: 90657145
45	15.83	564.3292	-	-	-	4	Unknown	-	-
46	16.30	540.3288	-	-	-	4	Unknown	-	-
47	16.60	566.3492	-	-	-	4	Unknown	-	-
48	17.58	299.2585	299.2592	-2.34	C ₁₈ H ₃₆ O ₃	3	Hydroxyoctadecanoic acid	-	HMDB0112182
49	17.81	297.2424	297.2435	-3.70	C ₁₈ H ₃₄ O ₃	2	Oxoctadecanoic acid	253/155	HMDB0030981
50	18.46	277.2159	277.2173	-5.05	C ₁₈ H ₃₀ O ₂	2	Linolenic acid	205/97	HMDB0001388
51	18.73	375.2712	375.2752	-10.66	C ₂₀ H ₄₀ O ₆	2	Myristyl glucoside	291/311	Pubchem: 6453025
52	19.16	279.2328	279.2330	-0.72	C ₁₈ H ₃₂ O ₂	3	Linoleic acid	-	HMDB0000673
53	19.82	255.2325	255.2330	-1.96	C ₁₆ H ₃₂ O ₂	2	Palmitic acid	190/110	HMDB0000220
54	19.87	403.3052	403.3065	-3.22	C ₂₂ H ₄₄ O ₆	3	Hexadecyl D-glucoside	-	Pubchem: 171356
55	19.95	281.2482	281.2486	-1.42	C ₁₈ H ₃₄ O ₂	2	Oleic Acid	211/101	HMDB0000207
56	20.98	383.1934	383.1923	2.87	C ₁₆ H ₃₂ O ₁₀	3	Hexanedioic acid derivative	-	Pubchem: 88032455

RT: Retention Time; Mol. Formula: Molecular Formula

Table S2. Identification of phytochemical compounds in *H. sabdariffa* extract by HPLC-ESI-qTOF-MS.

Peak	RT (min)	Observed [M-H]-	Theoretical [M-H]-	Mass error (ppm)	Mol. Formula	Level of annotation	Compounds	MS/MS fragments	References
1	0.96	132.0302	132.0302	0.00	C ₄ H ₇ NO ₄	2	Aspartic Acid	88/71	HMDB0000191
2	0.99	294.0807	294.0831	-8.16	C ₁₀ H ₁₇ NO ₉	2	Fructose-aspartic acid	132/88	(Luo et al., 2020)
3	1.07	207.0150	207.0146	1.93	C ₆ H ₈ O ₈	2	Hibiscus acid isomer 1	189/127	HMDB0031159
4	1.15	207.0159	207.0146	6.28	C ₆ H ₈ O ₈	2	Hibiscus acid isomer 2	189/127	HMDB0031159
5	1.32	189.0078	189.0041	19.58	C ₆ H ₆ O ₇	2	Hibiscus acid lactone	127	(Rodríguez-Medina et al., 2009)
6	1.66	203.0215	203.0197	8.87	C ₇ H ₈ O ₇	2	Hibiscus acid monomethyl ester isomer 1	142/157/201	(Hifnawy et al., 2020)
7	1.82	203.0215	203.0197	8.87	C ₇ H ₈ O ₇	2	Hibiscus acid monomethyl ester isomer 2	142/157/201	(Hifnawy et al., 2020)
8	2.63	217.0332	217.0354	-10.14	C ₈ H ₁₀ O ₇	2	Hibiscus acid dimethylester	155/125	(Villegas-Aguilar et al., 2020)
9	4.02	353.0867	353.0878	-3.12	C ₁₆ H ₁₈ O ₉	2	Chlorogenic acid isomer 1	191/135	HMDB0003164
10	4.20	297.0266	297.0252	4.71	C ₁₂ H ₁₀ O ₉	2	Methyl gallate derivative isomer 1	125/82	(Pierson et al., 2014)
11	4.98	297.0266	297.0252	4.71	C ₁₂ H ₁₀ O ₉	2	Methyl gallate derivative isomer 2	125/82	(Pierson et al., 2014)
12	6.34	353.0867	353.0878	-3.12	C ₁₆ H ₁₈ O ₉	2	Chlorogenic acid isomer 2	191/135	HMDB0003164
13	6.89	353.0867	353.0878	-3.12	C ₁₆ H ₁₈ O ₉	2	Chlorogenic acid isomer 3	191/135	HMDB0003164
14	8.56	335.0771	335.0772	-0.30	C ₁₆ H ₁₆ O ₈	2	5-O-Caffeoylshikimic acid	161/191/133	(Villegas-Aguilar et al., 2020)
15	8.90	367.1030	367.1035	-1.36	C ₁₇ H ₂₀ O ₉	2	3-O-Feruloylquinic acid	135/179	HMDB0030669
16	9.60	609.1458	609.1461	-0.49	C ₂₇ H ₃₀ O ₁₆	3	Quercetin 3-O-rutinoside	-	HMDB0037934
17	9.84	463.0884	463.0882	0.43	C ₂₁ H ₂₀ O ₁₂	2	Quercetin 7-glucoside	301/463	HMDB0302151
18	10.23	539.1751	539.1770	-3.52	C ₂₅ H ₃₂ O ₁₃	3	Oleuropein	-	HMDB0035872

19	10.96	317.0294	317.0303	-2.84	C ₁₅ H ₁₀ O ₈	2	Myrecetin	151/107	HMDB0002755
20	11.07	312.1239	312.1241	-0.64	C ₁₈ H ₁₉ NO ₄	2	N-Feruloyltyramine	148/190	Pubchem: 6440659
21	12.04	522.3276	522.3283	-1.34	C ₂₅ H ₄₉ NO ₁₀	4	Unknown	-	-
22	12.15	301.0339	301.0354	-4.98	C ₁₅ H ₁₀ O ₇	1	Quercetin	151/121/65	HMDB0005794
23	12.95	329.0654	329.0667	-3.95	C ₁₇ H ₁₄ O ₇	2	3,7-Dimethylquercetin	285/299/314	HMDB0029263
24	13.05	299.0538	299.0561	-7.69	C ₁₆ H ₁₂ O ₆	3	Kaempferide	-	Pubchem: 5281666
25	13.13	394.2945	394.2936	2.28	C ₂₀ H ₄₃ O ₇	4	Unknown	-	-
26	13.56	343.0821	343.0823	-0.58	C ₁₈ H ₁₆ O ₇	3	Eupatorin isomer 1	-	HMDB0252128
27	13.61	343.0818	343.0823	-1.46	C ₁₈ H ₁₆ O ₇	3	Eupatorin isomer 2	-	HMDB0252128
28	13.69	313.0684	313.0718	-10.86	C ₁₇ H ₁₄ O ₆	3	Cirsimaritin	-	Pubchem: 188323
29	13.92	293.1767	293.1758	3.07	C ₁₇ H ₂₆ O ₄	3	Gingerol	236/221	HMDB0005783
30	15.74	293.2107	293.2122	-5.12	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 1	-	HMDB0011108
31	15.81	293.2119	293.2122	-1.02	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 2	-	HMDB0011108
32	15.85	293.2113	293.2122	-3.07	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 3	-	HMDB0011108
33	16.41	295.2265	295.2279	-4.74	C ₁₈ H ₃₂ O ₃	3	Hydroxylinoleic acid	-	HMDB0247599
34	18.46	277.2159	277.2173	-5.05	C ₁₈ H ₃₀ O ₂	2	Linolenic acid	205/97	HMDB0001388
35	18.73	375.2712	375.2752	-10.66	C ₂₀ H ₄₀ O ₆	2	Myristyl glucoside	291/311	Pubchem: 6453025
36	19.15	279.2328	279.2330	-0.72	C ₁₈ H ₃₂ O ₂	3	Linoleic acid	-	HMDB0000673
37	19.86	403.3052	403.3065	-3.22	C ₂₂ H ₄₄ O ₆	3	Hexadecyl D-glucoside	-	Pubchem: 171356
38	19.94	281.2508	281.2486	7.82	C ₁₈ H ₃₄ O ₂	2	Oleic Acid	211/101	HMDB0000207
39	20.81	443.2490	443.2439	11.51	C ₂₆ H ₃₆ O ₆	3	Bufotalin	-	Pubchem: 12302120
40	20.97	383.1916	383.1923	-1.83	C ₁₆ H ₃₂ O ₁₀	3	Hexanedioic acid derivative	-	Pubchem: 88032455

RT: Retention Time; Mol. Formula: Molecular Formula

Table S3. Identification of phytochemical compounds in *S. marianum* extract by HPLC-ESI-qTOF-MS.

Peak	RT (min)	Observed [M-H]-	Theoretical [M-H]-	Mass error (ppm)	Mol. Formula	Level of annotation	Compounds	MS/MS fragments	References
1	0.80	112.9866	112.9880	-12.39	C ₄ H ₂ O ₄	3	Acetylenedicarboxylic acid	-	HMDB0247933
2	0.90	134.0479	134.0472	5.22	C ₅ H ₅ N ₅	3	Adenine	-	HMDB0000034
3	0.99	179.0556	179.0561	-2.79	C ₆ H ₁₂ O ₆	3	Fructose	-	HMDB0000660
4	1.06	266.0883	266.0894	-4.13	C ₁₀ H ₁₃ N ₅ O ₄	3	Adenosine	-	HMDB0000050
5	1.09	117.0130	-	-	-	4	Unknown	-	-
6	1.32	197.8088	-	-	-	4	Unknown	-	-
7	6.57	303.0492	303.0510	-5.94	C ₁₅ H ₁₂ O ₇	2	Dihydroquercetin	285/125	(Ferysiuk et al., 2020)
8	6.87	353.0881	353.0878	0.85	C ₁₆ H ₁₈ O ₉	2	Chlorogenic acid	191/161	HMDB0003164
9	7.75	373.1487	373.1504	-4.56	C ₁₇ H ₂₆ O ₉	2	Deoxyloganin	165/135	Pubchem: 440906
10	7.93	441.1949	441.1978	-6.57	C ₁₈ H ₃₄ O ₁₂	2	Hexo-Glucose	395/249	Pubchem: 129630443
11	8.05	505.1915	505.1927	-2.38	C ₂₂ H ₃₄ O ₁₃	2	Oleuropeic acid derivative isomer 1	459/293	(Tian et al., 2009)
12	8.41	475.1843	475.1821	4.63	C ₂₁ H ₃₂ O ₁₂	3	Cistanoside E	-	Pubchem: 21632979
13	8.58	505.1935	505.1927	1.58	C ₂₂ H ₃₄ O ₁₃	2	Oleuropeic acid derivative isomer 2	459/293	(Tian et al., 2009)
14	8.92	459.1856	459.1872	-3.48	C ₂₁ H ₃₂ O ₁₁	2	Apiosylepirhododendrin	89/59	Pubchem: 101287073
15	8.99	163.0389	163.0401	-7.36	C ₉ H ₈ O ₃	2	4-Hydroxycinnamic acid	119	HMDB0002035
16	9.12	193.0141	193.0142	-0.52	C ₉ H ₆ O ₅	2	Trihydroxycoumarin	137/93	(Waseem et al., 2021)
17	9.26	481.1161	481.1140	4.36	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 1	125/178	MoNA:VF-NPL-QTOF009680
18	9.42	455.2130	455.2134	-0.88	C ₁₉ H ₃₆ O ₁₂	3	Alkyl glycoside	-	(Vidal & Ccapatinta, 2018)
19	9.58	303.0512	303.0510	0.66	C ₁₅ H ₁₂ O ₇	2	Dihydroquercetin	285/125	(Ferysiuk et al., 2020)
20	9.73	607.1081	607.1093	-1.98	C ₃₀ H ₂₄ O ₁₄	2	(Epi)gallocatechin-A-(epi)gallocatechin	285/303	(Singh et al., 2018)
21	9.82	187.0957	187.0976	-10.16	C ₉ H ₁₆ O ₄	3	Azelaic acid	-	HMDB0000784
22	9.87	433.1123	433.1140	-3.93	C ₂₁ H ₂₂ O ₁₀	2	Naringenin 4'-O-glucoside	271/151	Pubchem: 42607906

23	10.12	481.1125	481.1140	-3.12	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 2	125/178	MoNA:VF-NPL-QTOF009680
24	10.25	483.1293	483.1297	-0.83	C ₂₅ H ₂₄ O ₁₀	2	Silybin hydrogenated	125/151/285	(Vrba et al., 2018)
25	10.35	813.3162	813.3187	-3.07	C ₃₈ H ₅₄ O ₁₉	2	Tricrocin	767/473	Pubchem: 22833598
26	10.46	383.1604	383.1612	-2.09	C ₂₁ H ₂₄ N ₂ O ₅	3	Alscomine	-	Pubchem: 11969856
27	10.54	675.3212	675.3174	5.63	C ₃₉ H ₄₈ O ₁₀	2	Gambogic acid A	629/293	ChemBK: 1592842-93-7
28	10.61	481.1120	481.1140	-4.16	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 3	125/178	MoNA:VF-NPL-QTOF009680
29	10.69	287.0516	287.0561	-15.68	C ₁₅ H ₁₂ O ₆	2	Aromadendrin	125/259	HMDB0030847
30	10.75	317.0655	317.0667	-3.78	C ₁₆ H ₁₄ O ₇	2	(+)-Dihydroisorhamnetin	245/259	HMDB0037501
31	10.96	481.1114	481.1140	-5.40	C ₂₅ H ₂₂ O ₁₀	2	Silychrestin isomer 1	151/125	MoNA:RIKENPlaSMA007844
32	11.00	481.1139	481.1140	-0.21	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 3	125/178	MoNA:VF-NPL-QTOF009680
33	11.03	481.1150	481.1140	2.08	C ₂₅ H ₂₂ O ₁₀	2	Silychrestin isomer 2	107/125/151/325/463	MoNA:RIKENPlaSMA007844
34	11.08	481.1146	481.1140	1.25	C ₂₅ H ₂₂ O ₁₀	2	Silychrestin isomer 3	125/151/178/325/355/433	MoNA:MetaboBASE0687
35	11.54	677.1892	677.1876	2.36	C ₃₅ H ₃₄ O ₁₄	4	Unknown	-	-
36	11.85	685.3060	685.3077	-2.48	C ₃₃ H ₅₀ O ₁₅	2	Pterocidine B	477/639	Pubchem: 122228272
37	11.90	659.1765	659.1770	-0.76	C ₃₅ H ₃₂ O ₁₃	2	Phylloflavanine	178/125	(Foo, 1987)
38	12.06	481.1130	481.1140	-2.08	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 4	125/152/301	MoNA:VF-NPL-QTOF009680
39	12.12	481.1176	481.1140	7.48	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 5	125/152/301	MoNA:VF-NPL-QTOF009680
40	12.26	481.1172	481.1140	6.65	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 6	125/152/273	MoNA:VF-NPL-QTOF009680
41	12.34	481.1158	481.1140	3.74	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 7	125/152/301	MoNA:VF-NPL-QTOF009680
42	12.44	481.1120	481.1140	-4.16	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 8	125/152/301	MoNA:VF-NPL-QTOF009680
43	12.48	481.1156	481.1140	3.33	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 9	125/152/301	MoNA:VF-NPL-QTOF009680
44	12.67	381.2269	381.2282	-3.41	C ₂₁ H ₃₄ O ₆	3	Sarcosin	-	Pubchem: 46173994
45	12.94	477.0837	477.0827	2.10	C ₂₅ H ₁₈ O ₁₀	3	Pradinone I	-	Pubchem: 460846
46	13.01	477.2489	477.2494	-1.05	C ₂₆ H ₃₈ O ₈	3	Adenanthin B isomer 1	-	(Hu et al., 2019)
47	13.12	811.1896	811.1938	-5.18	C ₃₅ H ₄₀ O ₂₂	3	Kaempferol 3-[2''-glucosyl-6''-acetylgalactoside] 7-glucoside	-	FDB016214

48	13.42	989.2502	989.2510	-0.81	C ₅₂ H ₄₆ O ₂₀	4	Unknown	-	-
49	13.70	523.1249	523.1246	0.57	C ₂₇ H ₂₄ O ₁₁	3	Acetylsilybin A/B	-	Pubchem: 10236891
50	13.78	477.2522	477.2493	6.08	C ₂₆ H ₃₈ O ₈	3	Adenanthin B isomer 2	-	(Hu et al., 2019)
51	14.14	479.0975	479.0984	-1.88	C ₂₅ H ₂₀ O ₁₀	2	Dehydrosilybin	125/177/179/283 /327/449	HMDB0040513
52	14.87	627.1569	627.1567	0.32	C ₂₇ H ₃₂ O ₁₇	2	Vitexia-glucoside	152/271/435/463	Pubchem: 56933064
53	15.02	313.2378	313.2384	-1.92	C ₁₈ H ₃₄ O ₄	3	Octadecanedioic acid	-	Pubchem: 70095
54	15.61	564.3351	-	-	-	4	Unknown	-	-
55	15.71	476.2772	476.2780	-1.68	C ₂₇ H ₄₁ O ₇	3	Sinapoyloxypalmitate	-	Pubchem: 90657145
56	15.83	564.3364	-	-	-	4	Unknown	-	-
57	16.04	540.3289	540.3304	-2.78	C ₂₉ H ₄₉ O ₉	3	Steroid compound	-	(Hendriani & Yulinah Sukandar, 2016)
58	16.30	540.3306	540.3304	0.37	C ₂₉ H ₄₉ O ₉	3	Steroid compound	-	(Hendriani & Yulinah Sukandar, 2016)
59	16.37	566.3453	-	-	-	4	Unknown	-	-
60	16.60	566.3530	-	-	-	4	Unknown	-	-
61	18.66	591.4117	591.4114	0.51	C ₃₁ H ₆₀ O ₁₀	4	Unknown	-	-
62	18.73	375.2712	375.2752	-10.66	C ₂₀ H ₄₀ O ₆	2	Myristyl glucoside	291/311	Pubchem: 6453025
63	19.16	279.2328	279.2330	-0.72	C ₁₈ H ₃₂ O ₂	3	Linoleic acid	-	HMDB0000673
64	19.74	577.3730	577.3746	-2.77	C ₃₃ H ₅₄ O ₈	3	Timosaponin A	-	(Wang et al., 2021)
65	19.85	403.3052	403.3065	-3.22	C ₂₂ H ₄₄ O ₆	3	Hexadecyl D-glucoside	-	Pubchem: 171356
66	19.94	281.2508	281.2486	7.82	C ₁₈ H ₃₄ O ₂	2	Oleic Acid	211/101	HMDB0000207
67	20.62	605.4040	605.4059	-3.14	C ₃₅ H ₅₈ O ₈	3	Deoxybafilomycin A1	-	CAS: 1883587-79-8
68	20.98	621.4417	621.4372	7.24	C ₃₆ H ₆₂ O ₈	3	Ginsenoside Rh2	-	Pubchem:119307
69	21.56	633.4375	633.4371	0.63	C ₃₇ H ₆₂ O ₈	3	Karaviloside III	-	CHEMBL2335924
70	22.92	371.2592	371.2592	0.00	C ₂₄ H ₃₆ O ₃	3	Pelandjauic acid	-	Pubchem: 178575
71	24.49	373.2751	373.2748	0.80	C ₂₄ H ₃₈ O ₃	3	Dehydrolithocholic acid	-	Pubchem: 4446994

RT: Retention Time; Mol. Formula: Molecular Formula

Table S4. Identification of phytochemical compounds in *L. citriodora* extract by HPLC-ESI-qTOF-MS.

Peak	Rt (min)	Observed [M-H]-	Theoretical [M-H]-	Mass error (ppm)	Mol. Formula	Level of annotation	Compounds	MS/MS fragments	References
1	1.01	195.0511	195.0510	0.51	C ₆ H ₁₂ O ₇	2	Gluconic acid	75/105/177	HMDB0184581
2	1.07	133.014	133.0142	-1.50	C ₄ H ₆ O ₅	3	Malic acid isomer 1	-	CAS: 6915-15-7
3	1.14	133.0141	133.0142	-0.75	C ₄ H ₆ O ₆	3	Malic acid isomer 2	-	CAS: 6915-15-7
4	1.27	317.0452	317.0514	-19.56	C ₁₂ H ₁₄ O ₁₀	4	Unknown	-	-
5	1.47	303.8787	-	-	-	4	Unknown	-	-
6	2.07	391.1232	391.1246	-3.58	C ₁₆ H ₂₄ O ₁₁	2	Shanziside	229/185/167/149/123	(Quirantes-Piné et al., 2010)
7	2.73	373.1101	373.114	-10.45	C ₁₆ H ₂₂ O ₁₀	1	Gardoside	123/149/211	(Quirantes-Piné et al., 2010)
8	3.35	461.1642	461.1665	-4.99	C ₂₀ H ₃₀ O ₁₂	2	Verbasoside	135/315	(Quirantes-Piné et al., 2010)
9	4.90	389.1091	389.1089	0.51	C ₁₆ H ₂₂ O ₁₁	2	Theveside	121/69	(Quirantes-Piné et al., 2009)
10	5.06	487.1446	487.1457	-2.26	C ₂₁ H ₂₈ O ₁₃	3	Cistanoside F	-	(Quirantes-Piné et al., 2009)
11	6.61	387.1617	387.1661	-11.36	C ₁₈ H ₂₈ O ₉	2	Tuberonic acid glucoside	59/89	Pubchem: 5281204
12	6.81	431.1916	431.1923	-1.62	C ₂₀ H ₃₂ O ₁₀	3	Sacranoside A	-	Pubchem: 102094959
13	7.01	433.2064	433.2079	-3.46	C ₂₀ H ₃₄ O ₁₀	2	Monoterpene derivative	387/89	(Li et al., 2019)
14	7.41	225.1136	225.1132	1.78	C ₁₂ H ₁₂ O ₄	2	Eugenitin	59	Pubchem: 3083581
15	7.63	307.1405	307.1398	2.28	C ₁₃ H ₂₄ O ₈	4	Unknown	-	-
16	8.19	593.1497	593.1512	-2.53	C ₂₇ H ₃₀ O ₁₅	3	Kaempferol 3-O-rutinoside	-	HMDB0302426
17	8.25	641.2095	641.2087	1.25	C ₂₉ H ₃₈ O ₁₆	3	Yadanzioside I	-	Pubchem: 10484290
18	8.37	435.2226	435.2236	-2.30	C ₂₀ H ₃₆ O ₁₀	3	Deniose	-	Pubchem: 10741574
19	8.48	377.1814	377.1817	-0.80	C ₁₇ H ₃₀ O ₉	4	Unknown	-	-
20	8.64	639.1863	639.1931	-10.64	C ₂₉ H ₃₆ O ₁₆	3	Plantamajoside	-	Pubchem: 5281788

21	8.75	377.1814	377.1817	-0.80	C ₁₇ H ₃₀ O ₉	4	Unknown	-	-
22	8.80	553.1575	553.1563	2.17	C ₂₅ H ₃₀ O ₁₄	2	Lippioside II	161/135	131751542
23	8.96	353.1822	353.1817	1.42	C ₁₅ H ₃₀ O ₉	3	Hydroxy-PEG6-acid	-	Pubchem: 60146229
24	9.28	653.2077	653.2087	-1.53	C ₃₀ H ₃₈ O ₁₆	2	Campneoside I	621	(Quirantes-Piné et al., 2009)
25	9.33	623.2	623.1981	3.05	C ₂₉ H ₃₆ O ₁₅	1	Verbascoside isomer 1	153/161/179/487	HMDB0034843
26	9.40	623.1913	623.1981	-10.91	C ₂₉ H ₃₆ O ₁₅	1	Verbascoside isomer 2	153/161/179/487	HMDB0034843
27	9.56	623.1921	623.1981	-9.63	C ₂₉ H ₃₆ O ₁₅	1	Verbascoside isomer 3	153/161/179/487	HMDB0034843
28	9.64	417.2128	417.213	-0.48	C ₂₀ H ₃₄ O ₉	3	Maryal	-	Pubchem: 5320270
29	9.67	651.1231	-	-	-	4	Unknown	-	-
30	9.82	623.2007	623.1981	4.17	C ₂₉ H ₃₆ O ₁₅	1	Verbascoside isomer 3	153/161/179/487	HMDB0034843
31	9.96	637.2131	637.2138	-1.10	C ₃₀ H ₃₈ O ₁₅	3	Verbaspinoside isomer 1	-	Pubchem: 23928137
32	10.02	521.1645	521.1665	-3.84	C ₂₅ H ₃₀ O ₁₂	3	Linearoside	-	Pubchem: 102066680
33	10.05	551.1693	551.1770	-13.97	C ₂₆ H ₃₂ O ₁₃	2	Durantocide I	175/151/235	Pubchem: 95223135
34	10.11	463.2523	463.2549	-5.61	C ₂₂ H ₄₀ O ₁₀	3	Methyladoligose E	-	Pubchem: 101921401
35	10.34	467.2105	467.2075	6.42	C ₂₇ H ₃₂ O ₇	3	Mexicanolide	-	Pubchem: 267328
36	10.45	637.2162	637.2138	3.77	C ₃₀ H ₃₈ O ₁₅	3	Verbaspinoside isomer 2	-	23928137
37	10.55	635.1270	635.1254	2.52	C ₂₈ H ₂₈ O ₁₇	3	Acacetin 7-diglucuronide	-	Pubchem: 11968442
38	10.62	651.2308	651.2294	2.15	C ₃₁ H ₄₀ O ₁₅	3	Martynoside	-	HMDB0254342
39	10.64	335.1708	335.1711	-0.90	C ₁₅ H ₂₈ O ₈	4	Unknown	-	-
40	10.71	595.1654	595.1668	-2.35	C ₂₇ H ₃₂ O ₁₅	2	Neoeriocitrin	163/87	Pubchem: 114627
41	10.94	591.2074	591.2083	-1.52	C ₂₉ H ₃₆ O ₁₃	2	Osmanthuside B	161/113	(Leyva-Jiménez et al., 2018)
42	11.04	569.2231	569.2240	-1.58	C ₂₇ H ₃₈ O ₁₃	2	Sesinoside	161/188/359	Pubchem: 102502279
43	11.09	337.1870	337.1868	0.59	C ₁₅ H ₃₀ O ₈	4	Unknown	-	-
44	11.28	681.2200	681.2189	1.61	C ₃₅ H ₃₈ O ₁₄	2	PinoresinolO-[6-O-(E)-caffeoyl]- β-D-glucopyranoside	323/161	Pubchem: 17410
45	11.37	361.1878	361.1868	2.77	C ₁₇ H ₃₀ O ₈	3	Glyceryl octyl ascorbic acid	-	Pubchem: 54588053
46	11.40	895.3534	-	-	-	4	Unknown	-	-

47	11.48	181.0875	181.0870	2.76	C ₁₀ H ₁₄ O ₃	3	3,4-Dimethoxyphenethyl alcohol	-	Pubchem: 81911
48	11.67	579.1697	579.1719	-3.80	C ₂₇ H ₃₂ O ₁₄	2	Naringin	163/137	HMDB0002927
49	11.99	315.0493	315.0510	-5.40	C ₁₆ H ₁₂ O ₇	2	Isorhamnetin isomer 1	271/300	HMDB0002655
50	12.29	481.1147	481.1140	1.45	C ₂₅ H ₂₂ O ₁₀	1	Silybin isomer 1	125/152/179/180/301	MoNA:FIO01021
51	12.34	481.1124	481.1140	-3.33	C ₂₅ H ₂₂ O ₁₁	1	Silybin isomer 2	125/152/179/180/302	MoNA:FIO01022
52	12.44	481.1131	481.1140	-1.87	C ₂₅ H ₂₂ O ₁₂	1	Silybin isomer 3	125/152/179/180/303	MoNA:FIO01023
53	12.64	329.0647	329.0667	-6.08	C ₁₇ H ₁₄ O ₇	2	3,7-Dimethylquercetin isomer 1	285/299/314	HMDB0029263
54	12.79	599.2689	599.2709	-3.34	C ₂₉ H ₄₄ O ₁₃	2	Jatamanvaltrate C	162/243/291/389	-
55	12.96	329.0668	329.0667	0.30	C ₁₇ H ₁₄ O ₇	2	3,7-Dimethylquercetin isomer 2	285/299/314	HMDB0029263
56	13.03	299.0546	299.0561	-5.02	C ₁₆ H ₁₂ O ₆	2	Chrysoeriol	183	HMDB0030667
57	13.14	394.2954	-	-	-	4	Unknown	-	-
58	13.22	309.2061	309.2071	-3.23	C ₁₈ H ₃₀ O ₄	3	13(S)-Hydroperoxylinoic acid isomer 1	-	HMDB0003871
59	13.26	315.0502	315.0510	-2.54	C ₁₆ H ₁₂ O ₇	2	Isorhamnetin isomer 2	271/300	HMDB0002655
60	13.28	309.2058	309.2071	-4.20	C ₁₈ H ₃₀ O ₄	3	13(S)-Hydroperoxylinoic acid isomer 2	-	HMDB0003871
61	13.39	297.1698	297.1707	-3.03	C ₁₆ H ₂₆ O ₅	3	Dihydroartemisinin methyl ether	-	Pubchem: 68911
62	13.53	307.1917	307.1915	0.65	C ₁₈ H ₂₈ O ₄	2	Dihydrocapsiate	235/185/121	HMDB0034781
63	13.61	343.0813	343.0823	-2.91	C ₁₈ H ₁₆ O ₇	2	Quercetin 3',4',7-trimethyl ether	313/163	Pubchem: 5748558
64	13.70	313.0697	313.0717	-6.39	C ₁₇ H ₁₄ O ₆	2	Cirsimaritin isomer 1	313	HMDB0250276
65	13.78	373.0924	373.0929	-1.34	C ₁₉ H ₁₈ O ₈	2	Chrysosplenetin	315/256	Pubchem: 5281608
66	13.92	293.1747	293.1758	-3.75	C ₁₇ H ₂₆ O ₄	3	Gingerol	-	HMDB0005783
67	14.03	305.1731	305.1758	-8.85	C ₁₈ H ₂₆ O ₄	2	9,16-Dioxo-10,12,14-octadecatrienoic acid	135/206/249	Pubchem: 18546963
68	14.49	313.0697	313.0717	-6.39	C ₁₇ H ₁₄ O ₆	2	Cirsimaritin isomer 2	313	HMDB0250276
69	14.84	413.2567	413.2545	5.32	C ₂₂ H ₃₈ O ₇	3	Ascorbyl palmitate isomer 1	-	HMDB0039883
70	14.90	413.2524	413.2545	-5.08	C ₂₂ H ₃₈ O ₇	3	Ascorbyl palmitate isomer 2	-	HMDB0039883

71	14.99	721.3661	721.3652	1.25	C ₃₄ H ₅₈ O ₁₆	4	Unknown	-	-
72	15.74	293.2107	293.2122	-5.12	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 1	-	HMDB0011108
73	15.80	293.2119	293.2122	-1.02	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 2	-	HMDB0011108
74	15.85	293.2113	293.2122	-3.07	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 3	-	HMDB0011108
75	16.05	699.3800	699.3809	-1.29	C ₃₂ H ₆₀ O ₁₆	3	Propargyl-PEG14-acid	-	Pubchem: 102514849
76	16.30	540.3306	-	-	-	4	Unknown	-	-
77	16.42	295.2282	295.2279	1.02	C ₁₈ H ₃₂ O ₃	3	Hydroxylinoleic acid	-	HMDB0247599
78	16.60	566.3530	566.3460	12.36	C ₃₁ H ₅₁ O ₉	4	Unknown	-	-
79	16.68	293.2109	293.2122	-4.43	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 4	-	HMDB0011108
80	16.77	293.2120	293.2122	-0.68	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 4	-	HMDB0011108
81	18.46	277.2159	277.2173	-5.05	C ₁₈ H ₃₀ O ₂	2	Linolenic acid	205/97	HMDB0001388
82	18.73	375.2712	375.2693	5.06	C ₂₇ H ₃₆ O	2	10'-Apo-beta-carotenal	116	HMDB0059605
83	19.16	279.2328	279.2330	-0.72	C ₁₈ H ₃₂ O ₂	3	Linoleic acid	-	HMDB0000673
84	19.82	255.2325	255.2330	-1.96	C ₁₆ H ₃₂ O ₂	2	Palmitic acid	190/110	HMDB0000220
85	19.95	281.2482	281.2486	-1.42	C ₁₈ H ₃₄ O ₂	2	Oleic Acid	211/101	HMDB0000207
86	20.98	621.4417	621.4372	7.24	C ₃₆ H ₆₂ O ₈	3	Ginsenoside Rh2	-	Pubchem: 119307

RT: Retention Time; Mol. Formula: Molecular Formula

Table S5. Identification of phytochemical compounds in *O. europaea* extract by HPLC-ESI-qTOF-MS.

Peak	RT (min)	Observed [M-H]-	Theoretical [M-H]-	Mass error (ppm)	Mol. Formula	Level of annotation	Compounds	MS/MS fragments	References
1	0.94	194.0424	194.0426	-1.03	C ₆ H ₁₀ O ₇	3	Glucuronic acid	-	HMDB0000127
2	0.96	181.0721	181.0718	1.66	C ₆ H ₁₄ O ₆	2	Sorbitol	89/101	HMDB0000247
3	1.02	195.0512	195.0510	1.03	C ₆ H ₁₂ O ₇	2	Gluconic acid	75/105/177	HMDB0184581
4	1.05	383.1200	383.1195	1.31	C ₁₄ H ₂₄ O ₁₂	3	Acetylsaccharose	-	Pubchem: 129641662
5	1.13	133.0143	133.0142	0.75	C ₄ H ₆ O ₅	3	Malic acid	-	(Nergiz & Ergönül, 2009)
6	1.20	191.0545	191.0561	-8.37	C ₇ H ₁₂ O ₆	1	Quinic acid	85/127	HMDB03072
7	1.27	317.0535	-	-	-	4	Unknown	-	-
8	1.72	96.9605	96.9601	4.13	H ₂ O ₄ S	3	Sulfate	-	HMDB0001448
9	2.42	153.0551	153.0557	-3.92	C ₈ H ₁₀ O ₃	2	Hydroxytyrosol isomer 1	123/93	HMDB0005784
10	2.69	153.0549	153.0557	-5.23	C ₈ H ₁₀ O ₃	2	Hydroxytyrosol isomer 2	123/93	HMDB0005784
11	4.39	389.1113	389.1089	6.17	C ₁₆ H ₂₂ O ₁₁	2	Oleoside isomer 1	121/59/69	(Fu et al., 2023)
12	4.91	389.1078	389.1089	-2.83	C ₁₆ H ₂₂ O ₁₁	2	Oleoside isomer 2	121/59/69	(Fu et al., 2023)
13	5.31	611.1881	611.1829	8.51	C ₂₄ H ₃₆ O ₁₈	3	2-[2-hydroxy-3-[4-[2-hydroxy-3-(1,2,3-tricarboxypropan-2-yloxy)propoxy]cyclohexyl]oxypropoxy]propane-1,2,3-tricarboxylic acid	-	Pubchem: 59999877
14	5.75	447.1487	447.1508	-4.70	C ₁₉ H ₂₈ O ₁₂	2	Hydroxytyrosol derivative	285	(Garcia-Aloy et al., 2020)
15	6.29	403.1208	403.1246	-9.43	C ₁₇ H ₂₄ O ₁₁	2	Oleoside 11-methyl ester isomer 1	165/223	Pubchem: 10692563
16	7.01	377.1453	377.1453	0.00	C ₁₆ H ₂₆ O ₁₀	2	Aldehydic form of decarboxymethyl elenolic acid glucoside	153/197	(Garcia-Aloy et al., 2020)
17	7.48	519.1702	519.1719	-3.27	C ₂₂ H ₃₂ O ₁₄	3	Segetoside A	-	Pubchem: 102316547
18	7.81	403.1239	403.1246	-1.74	C ₁₇ H ₂₄ O ₁₁	2	Oleoside 11-methyl ester isomer 2	165/223	Pubchem: 10692563

19	7.91	461.1678	461.1664	3.04	C ₂₀ H ₃₀ O ₁₂	2	Verbasoside isomer 1	135/161/315	(Quirantes-Piné et al., 2010)
20	7.98	461.1657	461.1664	-1.52	C ₂₀ H ₃₀ O ₁₂	2	Verbasoside isomer 1	135/161/315	(Quirantes-Piné et al., 2010)
21	8.05	555.1823	555.1719	18.73	C ₂₅ H ₃₂ O ₁₄	2	Hydroxyoleuropein isomer 1	151	(Katsinas et al., 2021)
22	8.18	593.1550	593.1512	6.41	C ₂₇ H ₃₀ O ₁₅	2	Vicenin 2	353/383/473	(Bouaziz et al., 2005)
23	8.42	401.1802	401.1817	-3.74	C ₁₉ H ₃₀ O ₉	2	Methyl 7-epi-12-hydroxyjasmonate glucoside	221/59	Pubchem: 131751189
24	8.51	581.2795	581.2815	-3.44	C ₂₆ H ₄₆ O ₁₄	4	Unknown	-	(Baker & Regg, 2018)
25	8.62	609.1487	609.1461	4.27	C ₂₇ H ₃₀ O ₁₆	2	Quercetin 3-O-rutinoside	271/300/301	HMDB0257021
26	8.72	195.0660	195.0663	-1.54	C ₁₀ H ₁₂ O ₄	2	Hydroxytyrosol Acetate	59	Pubchem: 155240
27	8.79	557.2455	557.2426	5.20	C ₂₇ H ₄₂ O ₁₀ S	3	Thiocladospolide J	-	(Salvatore et al., 2021)
28	8.96	555.1679	555.1719	-7.20	C ₂₅ H ₃₂ O ₁₄	2	Hydroxyoleuropein isomer 2	151	(Katsinas et al., 2021)
29	9.07	525.1620	525.1614	1.14	C ₂₄ H ₃₀ O ₁₃	2	Demethyloleuropein	61/121/165	Pubchem: 6450302
30	9.19	305.0662	305.0667	-1.64	C ₁₅ H ₁₄ O ₇	2	Epigallocatechin	125	HMDB0038361
31	9.37	623.1978	623.1981	-0.48	C ₂₉ H ₃₆ O ₁₅	1	Verbascoside isomer 1	161/315/461	(Attia et al., 2018)
32	9.43	551.2700	551.2709	-1.63	C ₂₅ H ₄₄ O ₁₃	3	Norisoprenoid derivative	-	(Cebrián-Tarancón et al., 2021)
33	9.49	701.2317	701.2298	2.71	C ₃₁ H ₄₂ O ₁₈	2	Oleuropein-glucoside isomer 1	139/357/481/566	Pubchem: 102031346
34	9.55	701.2300	701.2298	0.29	C ₃₁ H ₄₂ O ₁₈	2	Oleuropein-glucoside isomer 2	139/357/481/566	Pubchem: 102031346
35	9.65	447.0934	447.0933	0.22	C ₂₁ H ₂₀ O ₁₁	3	Luteolin 3'-glucoside	-	Pubchem: 12309350
36	9.72	701.2291	701.2298	-1.00	C ₃₁ H ₄₂ O ₁₈	2	Oleuropein-glucoside isomer 3	139/357/481/566	Pubchem: 102031346
37	9.81	623.1947	623.1981	-5.46	C ₂₉ H ₃₆ O ₁₅	1	Verbascoside isomer 2	161/315/461	(Attia et al., 2018)
38	9.88	607.1936	607.2032	-15.81	C ₂₉ H ₃₆ O ₁₄	3	Isosyringalide 3'-rhamnoside	-	Pubchem: 21629997
39	10.01	539.1779	539.1770	1.67	C ₂₅ H ₃₂ O ₁₃	1	Oleuropein isomer 1	139/205/275/307/377	HMDB0035872
40	10.10	569.1834	569.1876	-7.38	C ₂₆ H ₃₄ O ₁₄	2	Methoxyoleuropein isomer 1	151	HMDB0035445
41	10.13	569.1876	569.1876	0.00	C ₂₆ H ₃₄ O ₁₄	2	Methoxyoleuropein isomer 2	151	HMDB0035445
42	10.20	539.1808	539.1770	7.05	C ₂₅ H ₃₂ O ₁₃	1	Oleuropein isomer 2	139/205/275/307/377	HMDB0035872

43	10.26	539.1811	539.1770	7.60	C ₂₅ H ₃₂ O ₁₃	1	Oleuropein isomer 3	139/205/275/307/377	HMDB0035872
44	10.34	539.1736	539.1770	-6.31	C ₂₅ H ₃₂ O ₁₃	1	Oleuropein isomer 4	139/205/275/307/377	HMDB0035872
45	10.46	539.1746	539.1770	-4.45	C ₂₅ H ₃₂ O ₁₃	1	Oleuropein isomer 5	139/205/275/307/377	HMDB0035872
46	10.63	539.1781	539.1770	2.04	C ₂₅ H ₃₂ O ₁₃	1	Oleuropein isomer 5	139/205/275/307/377	HMDB0035872
47	10.71	601.2208	601.2138	11.64	C ₂₇ H ₃₈ O ₁₅	3	Rubricauloside	-	Pubchem: 196862
48	10.80	555.2057	555.2083	-4.68	C ₂₆ H ₃₆ O ₁₃	3	11-Hydroxyiridodial glucoside pentaacetate	121/69/183	CSID391583
49	10.88	523.1812	523.1821	-1.72	C ₂₅ H ₃₂ O ₁₂	2	Ligstroside	361	HMDB0034751
50	11.01	793.2908	793.2924	-2.02	C ₃₈ H ₅₀ O ₁₈	4	Unknown	-	(Garcia-Aloy et al., 2020)
51	11.07	377.1218	377.1242	-6.36	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 1	95/139	HMDB0301749
52	11.14	553.1924	553.1927	-0.54	C ₂₆ H ₃₄ O ₁₃	3	4''-Methyloleuropein	-	C00056345
53	11.20	377.1233	377.1242	-2.39	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 2	95/139	HMDB0301749
54	11.28	377.1208	377.1242	-9.02	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 3	95/139	HMDB0301749
55	11.34	393.1162	393.1191	-7.38	C ₁₉ H ₂₂ O ₉	2	10-hydroxyoleuropein aglycone isomer 1	137/181	(Serrano-García et al., 2022)
56	11.38	377.1223	377.1242	-5.04	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 4	95/139	HMDB0301749
57	11.44	623.1403	623.1406	-0.48	C ₃₁ H ₂₈ O ₁₄	3	Isorhamnetin-3-O-β-D-(6-p-coumaroyl) glucoside	-	(Romero-Márquez et al., 2023)
58	11.55	393.1150	393.1191	-10.43	C ₁₉ H ₂₂ O ₉	2	10-hydroxyoleuropein aglycone isomer 2	137/181	(Serrano-García et al., 2022)
59	12.15	377.1266	377.1242	6.36	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 3	95/139	HMDB0301749
60	12.38	377.1236	377.1242	-1.59	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 4	95/139	HMDB0301749
61	12.54	377.1225	377.1242	-4.51	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 5	95/139	HMDB0301749
62	12.58	377.1231	377.1242	-2.92	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 6	95/139	HMDB0301749
63	12.59	287.2218	287.2228	-3.48	C ₁₆ H ₃₂ O ₄	2	Dihydroxyhexadecanoic acid	99/134	HMDB0037798
64	12.95	315.0487	315.0510	-7.30	C ₁₆ H ₁₂ O ₇	3	Isorhamnetin isomer 1	-	Pubchem: 5281691

65	13.06	315.0492	315.0510	-5.71	C ₁₆ H ₁₂ O ₇	3	Isorhamnetin isomer 2	-	Pubchem: 5281691
66	13.41	377.1241	377.1242	-0.27	C ₁₉ H ₂₂ O ₈	2	Oleuropein aglycone isomer 7	95/139	HMDB0301749
67	13.47	391.1384	391.1398	-3.58	C ₂₀ H ₂₄ O ₈	2	Methyl-oleuropein aglycone	179/211/151	(Garcia-Aloy et al., 2020)
68	13.53	899.2973	899.2979	-0.67	C ₄₄ H ₅₂ O ₂₀	4	Unknown	-	-
69	13.58	307.1926	307.1915	3.58	C ₁₈ H ₂₈ O ₄	2	Dihydrocapsiate	235/185	Pubchem: 9873754
70	13.61	513.1774	513.1766	1.56	C ₂₇ H ₃₀ O ₁₀	2	Baohuoside I	173/217/366	HMDB0248860
71	13.71	659.2274	659.2345	-10.77	C ₃₃ H ₄₀ O ₁₄	2	2''-O-Rhamnosylcariside II	366	Pubchem: 5318987
72	13.92	293.1754	293.1758	-1.36	C ₁₇ H ₂₆ O ₄	3	Phytuberin	-	(Sánchez-Martínez et al., 2022)
73	14.03	305.1746	305.1758	-3.93	C ₁₈ H ₂₆ O ₄	3	Dioxooctadecatrienoic acid	-	Pubchem: 18546963
74	14.35	513.1798	513.1766	6.24	C ₂₇ H ₃₀ O ₁₀	3	Icariin II	-	Pubchem: 123134739
75	15.00	721.3721	721.3746	-3.47	C ₄₅ H ₅₄ O ₈	4	Unknown	-	-
76	15.21	485.3277	485.3272	1.03	C ₃₀ H ₄₆ O ₅	2	Quillaic acid	439/409	Pubchem: 101810
77	15.46	647.3255	647.3226	4.48	C ₃₈ H ₄₈ O ₉	3	8,8a-Dihydro-8-hydroxygambogenic acid	-	(Tao et al., 2009)
78	15.74	293.2135	293.2122	4.43	C ₁₈ H ₃₀ O ₃	2	Hydroxylinolenic acid isomer 1	275/235	HMDB0011108
79	15.80	293.2120	293.2122	-0.68	C ₁₈ H ₃₀ O ₃	2	Hydroxylinolenic acid isomer 2	275/235	HMDB0011108
80	15.84	293.2115	293.2122	-2.39	C ₁₈ H ₃₀ O ₃	2	Hydroxylinolenic acid isomer 3	275/235	HMDB0011108
81	16.03	487.2899	487.2913	-2.87	C ₂₅ H ₄₄ O ₉	3	Lankolide	-	Pubchem: 11465948
82	16.30	540.3291	-	-	-	4	Unknown	-	-
83	16.42	295.2278	295.2279	-0.34	C ₁₈ H ₃₂ O ₃	3	Hydroxylinoleic acid	-	HMDB0247599
84	16.60	566.3530	566.3460	12.36	C ₃₁ H ₅₁ O ₉	4	Unknown	-	-
85	16.97	469.3303	469.3323	-4.26	C ₃₀ H ₄₆ O ₄	3	Glycyrrhetic acid	-	Pubchem: 73398
86	17.21	471.3464	471.3480	-3.39	C ₃₀ H ₄₈ O ₄	3	Maslinic acid	-	HMDB0002392
87	17.55	799.3973	799.3910	7.88	C ₄₃ H ₆₀ O ₁₄	2	Oleuropein derivative (oleuropein + C18H28O)	539/277/307/377	(Contreras et al., 2021)
88	18.46	277.2167	277.2173	-2.16	C ₁₈ H ₃₀ O ₂	3	Linolenic acid	-	HMDB0001388

89	18.58	777.4053	777.4067	-1.80	C ₄₁ H ₆₂ O ₁₄	2	Oleuropein derivative (oleuropein + C ₁₆ H ₃₀ O)	539/275/307/377	(Contreras et al., 2021)
90	18.73	375.2712	375.2693	5.06	C ₂₇ H ₃₆ O	2	10'-Apo-beta-carotenal	116	HMDB0059605
91	19.00	617.3837	617.3848	-1.78	C ₃₉ H ₅₄ O ₆	3	3-beta-O-(trans-p-Coumaroyl)maslinic acid	-	Pubchem: 14335962
92	19.16	279.2328	279.2330	-0.72	C ₁₈ H ₃₂ O ₂	3	Linoleic acid	-	HMDB0000673
93	19.64	933.6920	-	-	-	4	Unknown	-	-
94	19.82	255.2325	255.2330	-1.96	C ₁₆ H ₃₂ O ₂	2	Palmitic acid	190/110	HMDB0000220
95	19.85	403.3052	403.3065	-3.22	C ₂₂ H ₄₄ O ₆	3	Hexadecyl D-glucoside	-	Pubchem: 171356
96	19.95	281.2482	281.2486	-1.42	C ₁₈ H ₃₄ O ₂	2	Oleic Acid	211/101	HMDB0000207
97	20.30	391.2838	391.2854	-4.09	C ₂₄ H ₄₀ O ₄	4	Unknown	-	-
98	20.39	621.2719	621.2705	2.25	C ₃₅ H ₄₂ O ₁₀	3	1-Hydroxy taxuspine C	-	Pubchem: 15923424
99	20.97	383.1916	383.1923	-1.83	C ₁₆ H ₃₂ O ₁₀	3	Hexanedioic acid derivative	-	Pubchem: 88032455
100	21.24	758.5444	-	-	-	4	Unknown	-	-

RT: Retention Time; Mol. Formula: Molecular Formula

Table S6. Numerical results (mean and SD) and statistics significance for **Figure 1** results.

Conc. (µg/mL)	Cell viability (%)														
	HS			LC			OE			SM			TC		
	Mean	SD	Sign.	Mean	SD	Sign.	Mean	SD	Sign.	Mean	SD	Sign.	Mean	SD	Sign.
0	100.00	2.41		100.00	1.92		100.00	3.75		100.00	4.04		100.00	2.25	
5	100.19	2.09	ns	106.16	1.92	ns	97.30	4.02	ns	99.31	4.03	ns	97.47	3.62	ns
10	97.37	5.69	ns	105.84	5.54	ns	96.71	3.60	ns	99.36	4.69	ns	101.09	2.62	ns
15	103.05	1.87	ns	109.53	4.59	***	96.04	4.41	ns	97.40	5.00	ns	101.14	5.97	ns
20	99.77	1.30	ns	110.97	4.49	****	93.02	2.71	ns	96.18	4.06	ns	101.10	3.03	ns
25	103.70	2.71	ns	96.77	2.68	ns	96.81	2.99	ns	97.35	4.10	ns	98.46	1.04	ns
30	101.25	2.15	ns	95.82	1.29	ns	94.06	4.12	ns	95.38	2.93	ns	100.30	3.67	ns
40	100.05	2.21	ns	98.48	1.99	ns	92.53	5.88	ns	95.74	3.42	ns	97.28	1.96	ns
50	97.57	2.83	ns	91.44	5.59	****	87.62	4.11	****	95.51	4.65	ns	99.41	2.51	ns
60	98.77	3.16	ns	87.56	2.78	****	81.51	4.19	****	90.24	2.59	**	98.03	2.30	ns
80	98.32	4.63	ns	69.95	1.77	****	71.72	7.37	****	91.84	5.65	*	98.11	3.60	ns
100	96.49	3.11	ns	68.24	1.99	****	68.43	4.10	****	87.71	3.33	****	103.62	2.97	ns
150	94.50	5.82	ns	51.30	2.74	****	37.77	6.16	****	72.20	3.60	****	103.84	3.41	ns
200	88.98	4.37	****	32.41	1.73	****	26.04	3.20	****	64.08	4.72	****	98.51	1.79	ns
250	85.61	1.41	****	28.39	2.35	****	25.26	1.50	****	67.01	2.21	****	105.05	2.22	ns
300	81.52	3.51	****	30.46	2.33	****	24.75	0.81	****	61.96	0.56	****	102.69	2.95	ns
400	68.47	1.75	****	31.05	1.60	****	25.75	1.18	****	50.24	1.12	****	94.96	3.91	ns
500	63.38	1.88	****	35.06	2.02	****	26.65	0.75	****	37.23	2.36	****	85.16	3.16	****
600	58.82	1.56	****	37.52	1.00	****	27.40	1.83	****	23.45	1.64	****	76.42	4.09	****

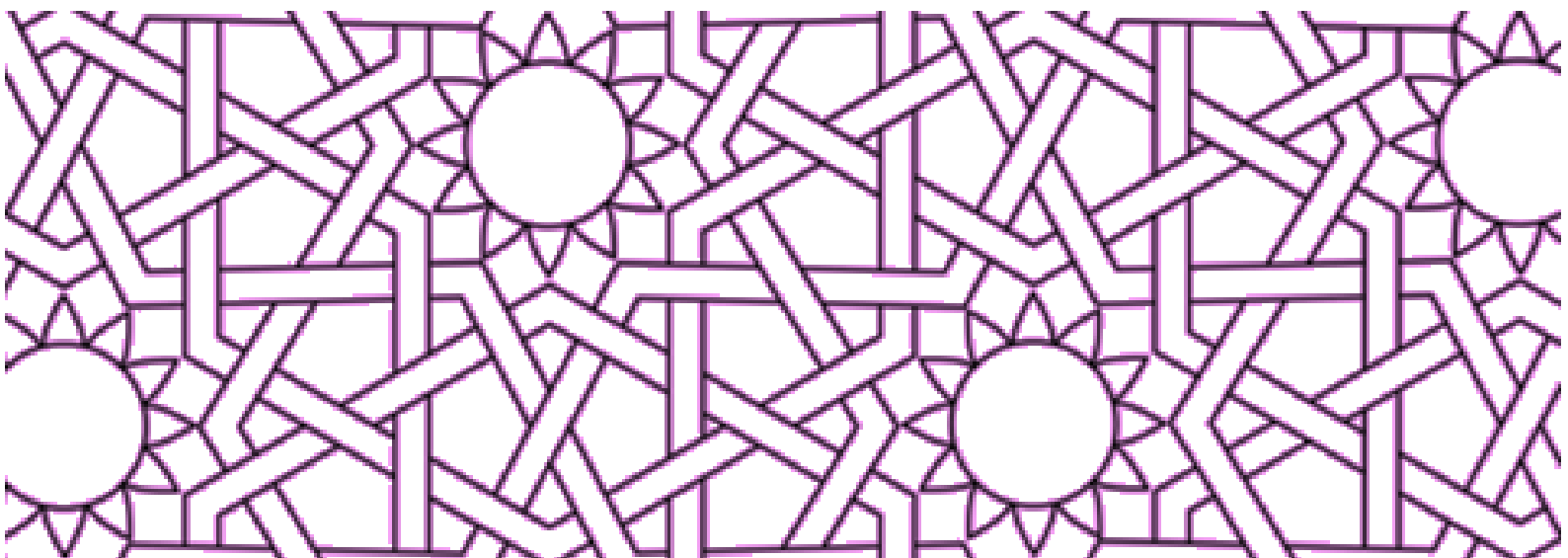
ns (non statistical significance), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Table S7. Equivalent DMSO concentrations for each extract concentration.

Equivalent extract conc. (µg/mL)	DMSO (%)	Cell viability (%)		
		Mean	SD	Sign.
0	0	100.00	5.65	ns
300	0.3	99.37	2.17	ns
400	0.4	99.78	8.00	ns
500	0.5	97.62	3.66	ns
600	0.6	98.16	4.17	ns
800	0.8	91.22	3.69	****

ns (non statistical significance) and **** ($p < 0.0001$).

Chapter 3



CHAPTER 3

Targeted Breast Cancer Therapy Using Novel Nanovesicle Formulations of *Olea europaea* extract

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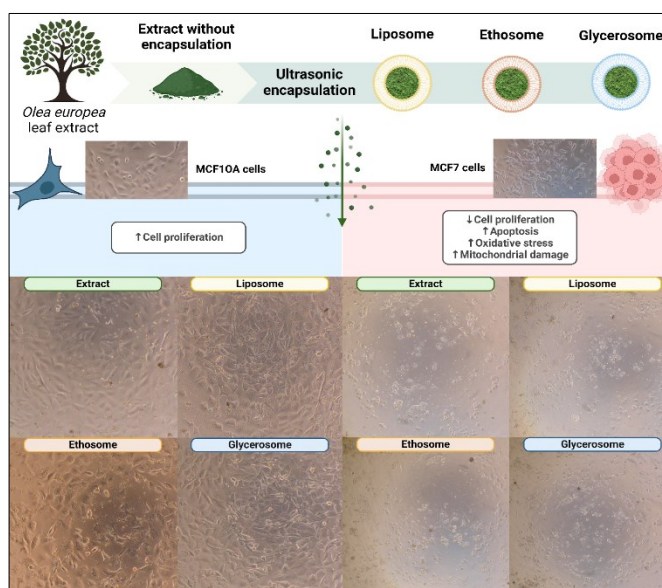
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Abstract

Olive leaf is a byproduct of the olive tree that is rich in phenolic compounds with potential anticarcinogenic effects against various cancers, including breast cancer. Nevertheless, the ingestion or topical application of such plant extracts face certain limitations. These can be addressed by encapsulating the extracts in nanovesicles to enhance their release and bioavailability. This study aims to develop nanovesicles using *Olea europaea* leaf extract to exploit its potential anti-cancer properties. Soy lecithin was used to form liposomes for encapsulation of the olive leaf extract. In addition, ethanol and glycerol were added to form ethosomes and glycerosomes, respectively. The antiproliferative effect of both the free extract and the three formed nanovesicles was tested in MCF7 and MCF10A cell lines. To comprehend the mechanisms leading to reduced cell viability after exposure to olive leaf extract and its nanovesicles, levels of reactive oxygen species (ROS), mitochondrial membrane potential, and apoptotic stage were evaluated. The results suggest that both the nanovesicles and the free extract are antiproliferative agents against MCF7 tumour cells. However, when examining the impact of olive leaf extract and the formulated nanovesicles on MCF10A cells, there was no reduction in cell viability. Our findings indicate that the anti-tumour effect of the extract and its nanovesicles may be due to increased oxidative stress mediated by mitochondrial damage. The mechanism through which olive leaf extract exerts its antiproliferative effect on the breast cancer tumour line implies that apoptosis may be induced by the extract via the involvement of a mitochondria-dependent ROS-mediated pathway.

Keywords: Nanovesicles; Olive leaf extract; Antitumour; MCF7; MCF10A; Apoptosis

Abbreviations

7-AAD, aminoactinomycin D; ATCC, American type culture collection; ABS, absorbance; DMEM F12, Dulbecco's modified eagle medium: nutrient mixture f12; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DPPH INH, DPPH inhibition; EE, entrapment efficiency; ESI-QTOF-MS, electrospray ionization quadrupole time-of-flight mass spectrometry; FBS, foetal bovine serum; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; FITC, fluorescein isothiocyanate; GI₂₅, 25% growth inhibitory effect; GI₅₀, 50% growth inhibitory effect; GI₇₅, 75% growth inhibitory effect; H₂O₂, hydrogen peroxide; HPLC, high performance liquid chromatography; LC-MS, liquid chromatograph mass spectrometer; LDH, lactate dehydrogenase; MMP, mitochondrial membrane potential; PDI, polydispersity index; ROS, reactive oxygen species; RPMI-1640, Roswell Park memorial institute 1640; SEM, standard error of the mean

1. Introduction

Breast cancer stands as the most prevalent malignancy diagnosed in women, with 2.3 million new cases and 670,000 deaths reported in 2020 globally [1]. It manifests as a heterogeneous disease with well-defined risk factors, and post-diagnosis survival rates are contingent upon the tumour's hormonal profile [2]. The aetiology of breast cancer is multifaceted, with hormonal, genetic, and environmental factors seemingly interplaying in its pathogenesis [3]. In this context, it has been observed that, among others, breast cancer has a lower incidence in Mediterranean regions compared to other European countries [4]. The primary explanation for this phenomenon may be attributed to the Mediterranean diet, which is rich in vegetables and characterised by a lower intake of meat, as well as a high consumption of olive oil and fish [4]. The compounds found in these foods, particularly olive oil, appear to exhibit potential anticarcinogenic properties [5]. Specifically, olive oil contains phenolic compounds known for their significant bioactive properties, which include antitumoural effects [5]. Beyond olive oil, other parts of the olive tree that are usually discarded, like the olive leaf, contain even higher concentrations of these bioactive compounds, especially the major phenolic compounds most characteristic of this matrix, such as oleuropein and hydroxytyrosol [6]. This provides novel opportunities in the search for active, cancer-targeted, and effective compounds [7]. Among the various compounds, the phenolic compounds stand out. They exhibit anticancer potential against tumour cells and offer beneficial effects on non-cancerous cells, making them promising candidates for new cancer drugs. Besides their anticarcinogenic effects, these compounds are also being explored as natural chemopreventives due to their low side effects and toxicity, and their ability to neutralize carcinogens and affect cellular processes [8].

Despite the therapeutic potential of phenolic compounds in olive leaf extract, several limitations should be considered. Some of the phenolic compounds present in this type of extract can have low aqueous solubility, can easily undergo oxidation and are sensitive to changes in temperature and light when dissolved [9]. Additionally, upon ingestion, these compounds may undergo metabolic reactions such as methylation, sulphation, or glucuronidation, leading to the formation of other compounds with properties that differ from those of the original extract, which limit its potential effects [10]. To address these limitations and facilitate the incorporation of these bioactive compounds into food supplements or topical applications such as creams or gels, they could be encapsulated in appropriate nanocarriers for delivery [11]. Phospholipid vesicles stand out among the various nanocarriers suitable for food supplements and topical creams and gels, as they primarily consist of phospholipids, which naturally occur as major components of cell membranes [12,13]. In addition to phospholipids, other compounds such as ethanol and glycerol can be combined with phospholipids to modify the chemical properties of the nanovesicles, resulting in the formation of liposomes, ethosomes, and glycerosomes, respectively [14].

In this context, the objective of this study is the development of nanovesicles utilizing *Olea europaea* leaf extract, with the aim to exploit its potential anticancer properties. Specifically, we focus on breast cancer, a disease that significantly impact in our society. The results of our research support the selective antitumor efficacy of these extract against cancer cells (MCF7 breast tumour cell line). Furthermore, we show the cytocompatibility of these leaf extracts with the non-tumoral phenotype (MCF10A non-tumoral cell line).

2. Material and methods

2.1 Materials

Soy lecithin was purchased from Acofarma (Madrid, Spain). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). Glycerin was purchased from Guinama (Valencia, Spain). Ethanol and methanol of LC-MS grade were sourced from Fisher Scientific (Madrid, Spain). Foetal Bovine Serum (FBS), horse serum, RPMI-1640 (Roswell Park Memorial Institute 1640) and Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM F12) were obtained from Biowest (Nuaillé, France). Epidermal Growth Factor, hydrocortisone, insulin, cholera toxin, Lactate Dehydrogenase (LDH) Activity Assay Kit, Fluorimetric intracellular reactive oxygen species (ROS) kit and mitochondrial membrane potential kit were from Sigma-Aldrich (Madrid, Spain). The Muse Annexin V & Dead Cell Assay was from Merck Chemicals & Life Science (Madrid, Spain). All other reagents were of analytical grade.

2.2 Plant material

The commercial olive leaf extract was supplied by NATAC S.L., derived from solid-liquid extraction of ground leaves using 80% ethanol as the extraction solvent. The extraction process involved a solvent-to-sample ratio of 20:1, a temperature of 45°C, and an extraction duration of 2 hours.

2.3 Vesicle Preparation

Soy lecithin and the olive leaf extract were weighed in a glass vial and hydrated with 2 mL of bi-distilled water to obtain liposomes. To optimize the preparation of liposome nanovesicles, various tests were conducted involving different concentrations of soy lecithin (210, 180, 150 and 125 mg/mL), extract concentration (60, 50 and 30 mg/mL),

and varying repetitions of the 15 cycles of 5 seconds on and 2 seconds off (7, 6, 5, 4 and 3 repetitions). Ethanol (6.25%) was mixed with the lipid and the extract to obtain ethosomes. Glycerosomes were prepared adding glycerin (6.25%) to liposomes. Finally, the dispersions were sonicated (15 + 15 + 15 + 15 + 15 cycles, 5 s on and 2 s off, 13 % of probe amplitude, allowing the sample to cool between each sonication), using a SONOPULS HD 2200 (BANDELIN electronic GmbH & Co. KG, Berlin, Germany). A vial containing the extract at the same concentration and water was also prepared, and it underwent the same sonication cycles. Liposomes, ethosomes and empty glycerosomes without extract were also carried out.

2.4 Vesicle Characterization

The average diameter and polydispersity index of the vesicles were measured using photon correlation spectroscopy with a Zetasizer nano-ZS (Malvern Instruments, Worcestershire, UK). The surface charge of the vesicles (ζ -potential) was determined by converting the electrophoretic mobility through the Smoluchowski approximation of the Henry equation with the same instrument. Each sample was diluted with distilled water (1:100) to ensure optical clarity and prevent attenuation of the laser beam by particles, as well as reduce scattered light that could interfere with measurements. Data are presented as means $\pm$ standard deviation of three measurements.

The entrapment efficiency was determined by calculating the percentage of antioxidant activity in the vesicle dispersions before and after purification through dialysis to eliminate untrapped extract. Vesicle dispersions (1 mL) were loaded into dialysis tubes (Spectra/Por® 2 Dialysis Membrane Standard RC Tubing, 12–14 kDa MW cut-off; Spectrum Laboratories Inc., DG Breda, The Netherlands) and maintained at room

temperature in water (2 L), with constant stirring for 2 hours. The water was refreshed after 1 hour. Antioxidant activity of dispersions before and after dialysis was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) colorimetric assay.

The antioxidant activity of the samples was measured before and after dialysis. Different sample dilutions with methanol (20 µL) were mixed with 980 µL of the DPPH methanolic solution (70 µg/mL), and incubated for 30 min at room temperature in the dark. The absorbance (ABS) was measured at 517 nm against blank. The DPPH inhibition (DPPH INH %) was calculated according to Eq. (1):

$$DPPH\ INH\ \% = 100 - \frac{ABSS_{sample} \times 100}{ABS_{blank}} \quad (1)$$

The entrapment efficiency (EE%) of active molecules was determined by comparing the antioxidant activity found after dialysis with the initially measured activity, expressed as a percentage (Eq. (2)).

$$EE\ \% = \frac{DPPH\ INH\ dyalised}{DPPH\ INH\ ininitial} \times 100 \quad (2)$$

2.5 Stability Study

The formulations were stored in the dark at 4°C, and their stability was assessed by measuring the average size of the nanovesicles, polydispersity index, and ζ-potential on a weekly basis for a period of six months.

2.6 Cell culture

The ATCC-originated MCF7 cells were maintained in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The ATCC-originated MCF10A cells were maintained in DMEM F-12 supplemented with 5% horse serum, 10 ng/mL Epidermal Growth Factor, 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, 10 ng/mL of cholera toxin and 100 U/mL penicillin and 100 µg/mL streptomycin. All cells were passaged or changed with fresh medium every other day and grown in 37°C humidified incubator supplemented with 5% CO₂. Due to its hormone sensitivity, MCF7 is a crucial in vitro model for certain breast cancer types [15], whereas MCF10A acts as a model for normal breast cells [16] providing a basis for comparison with cancerous cells.

2.7 Viability assay

To evaluate the effects of the natural leaf extract and its encapsulated forms in various nanovesicle preparations (i.e., liposomes, ethosomes, and glycerosomes), MCF7 and MCF10A cells were seeded onto 96-well plates (10,000 cells/well) in their respective media. Following 24 hours of seeding, the cells were incubated with different concentrations of the extract and its encapsulated forms. Specifically, concentrations ranging from 0.1 to 0.5 mg/mL were used to explore the GI₅₀ values (concentration required to inhibit cell growth by 50%). The evaluation of the antiproliferative activity of these formulations was conducted utilizing the crystal violet staining assay, in conjunction with a standard curve based on cell number, as previously described [17]. The optical density of crystal violet within each well was quantified at 590 nm directly from the plates using an HTX Microplate Reader (BioTek Instruments, USA). Additionally,

the cytotoxicity of the different preparations was assessed by quantifying the release of LDH.

2.8 Evaluation of reactive oxygen species production

The assessment of intracellular ROS was performed using the fluorometric intracellular ROS kit. Briefly, cells were plated in 96-well black plates with transparent bottoms designed for fluorometric evaluation (20,000 cells/well) and cultured with or without the addition of the natural extract and the three types of encapsulations at GI₂₅, GI₅₀ and GI₇₅. Hydrogen peroxide (H₂O₂) at a concentration of 0.5 mM served as the positive control. Fluorescence intensity, proportional to the amount of ROS present, was measured $\lambda_{ex} = 490/\lambda_{em} = 520$ nm.

2.9 Mitochondrial membrane potential assay

The collapse of the mitochondrial membrane potential (MMP) as leading process in apoptotic cascade was assayed by using JC-10 dye following the manufacturer's recommendations. Briefly, cells were exposed to 0.1 or 0.2 units of the natural extract, liposome, ethosome and glycosome during 24 hours. After this incubation period, JC-10 dye solution was added and the fluorescence intensity ($\lambda_{ex} = 490/\lambda_{em} = 525$ nm) and ($\lambda_{ex} = 540/\lambda_{em} = 590$ nm) was monitored. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

2.10 Apoptosis

The Muse[®] Annexin V & Dead Cell Kit was used to determine and quantify the apoptotic cells using flow cytometry. Briefly, cells were plated in 12-well plates (400,000 cells per well) and incubated with or without the extract and three types of encapsulations at GI₅₀. Staurosporine 0.5 μ g/mL was used as positive control. After treatment, the

collected cells were resuspended in the supplied binding buffer, and then stained with FITC-conjugated annexin V and 7-Aminoactinomycin D (7-AAD) at room temperature for 20 min in the dark. The fluorescent intensities of the cells were detected using flow cytometry (Muse® Cell Analyzer, Merck, Germany). The Annexin V+/7-AAD- and Annexin V+/7-AAD+ cell populations were representative of apoptotic cells.

2.11 Statistical analyses

The results are expressed as means $\pm$ SEM (standard error of the mean). To assess the presence of significant differences in experiments, the Student's t-test for two independent samples was conducted. For more than two groups One-way analysis of variance (ANOVA) was applied. Following a significant result from the ANOVA, a Tukey's Honestly Significant Difference (HSD) test was conducted as a post-hoc analysis to identify which specific treatment groups differed significantly from each other. Graphpad Prism 8 was used for statistical analyses. When multiple comparisons are made p-values < 0.05 are considered statistically significant and are indicated by letters when multiple comparison. The results indicated statistically significant differences as denoted as well by * $p < 0.05$; ** $p < 0.001$.

3. Results and discussion

3.1 Optimisation of nanovesicle preparation

After assessing the conditions listed in Material and methods, the mean diameter, polydispersity index (PDI), and ζ -potential values of the liposomes were measured.

Mean diameter values ranged from 86 to 142 nm, PDI values ranged from 0.22 to 0.44 and ζ -potential values ranged from -41.7 to -49 mV. It was concluded that the optimal

values were achieved with a combination of 150 mg/mL soy lecithin, 50 mg/mL extract, and 5 repetitions of the 15 cycles.

The olive leaf extract utilized for the encapsulation process was tentatively characterized by HPLC-ESI-QTOF-MS in the previous study by [18]. In the *O. europaea* extract, the content of the glycosylated secoiridoid oleuropein was particularly high, as well as some derivative forms of these compounds such as methoxyoleuropein, oleuropein-glucoside, hydroxyoleuropein and oleuropein aglycone.

After optimizing the concentrations for forming liposomes with olive leaf extract, 6.25% ethanol was introduced to the existing formulation to facilitate ethosome formation. Similarly, the same percentage of glycerol was added to the optimized liposome formulation for glycosome formation. The selection of this percentage was informed by previous experiments involving the nanovesicles of other extracts, prioritizing a minimal concentration to mitigate any potential impact on cell viability posed by the presence of ethanol and glycerol [19].

3.2 Nanovesicle characterization

3.2.1 Size and ζ -potential determination

The mean diameter, the PDI and the ζ -potential values of empty and loaded liposomes, ethosomes and glycosomes were investigated and the results are reported in **Table 1**.

As shown in **Table 1** all nanovesicles show monomodal size distributions characterized by dimensions ranging between 107 nm and 127 nm. As can be seen for all three types of nanovesicles, the presence of olive leaf extract induces a slight increase in hydrodynamic diameter with respect to their respective empty nanovesicles. This suggests that the charged compounds induce a different organisation of the lipid

membrane, thus modifying its properties [11]. Additionally, it is noteworthy that the size of the ethosomes, whether empty or containing the extract, is the smallest in comparison to the other nanovesicles types. Several studies in the literature have indicated that increasing ethanol concentration tends to decrease the size of ethosomes [20,21]. However, exceeding the optimal ethanol concentration can result in bilayer leakage, causing a slight increase in vesicle size and a significant reduction in trapping efficiency. Moreover, higher ethanol concentrations may lead to vesicle solubilization [22]. It is crucial to consider the subsequent application of the encapsulation and potential interference from ethanol, as observed in our case.

Table 1. Mean diameter (MD), polydispersity index (PDI), ζ -Potential and Entrapment Efficiency (EE%) of empty and loaded with olive leaf extract liposomes, ethosomes and glycerosomes.

	MD (nm)	PDI	ζ -Potential (mV)	EE (%)
Empty liposomes	117 $\pm$ 4 ^a	0.25 $\pm$ 0.04 ^a	-43 $\pm$ 2 ^a	-
Empty ethosomes	107 $\pm$ 3 ^a	0.28 $\pm$ 0.01 ^a	-40 $\pm$ 1 ^a	-
Empty glycerosomes	113 $\pm$ 2 ^a	0.24 $\pm$ 0.02 ^a	-39 $\pm$ 3 ^a	-
Liposomes	127 $\pm$ 6 ^a	0.27 $\pm$ 0.01 ^a	-41 $\pm$ 1 ^a	54 $\pm$ 5 ^a
Ethosomes	119 $\pm$ 6 ^a	0.33 $\pm$ 0.01 ^b	-39 $\pm$ 2 ^a	53 $\pm$ 1 ^{a,b}
Glycerosomes	122 $\pm$ 3 ^a	0.31 $\pm$ 0.03 ^b	-41 $\pm$ 1 ^a	40 $\pm$ 1 ^b

Data are presented as mean $\pm$ standard error of the mean (SEM) of three independent experiments conducted in triplicate. Statistically significant differences as determined by Tukey's HSD post-hoc test ($p < 0.05$). Treatments with the same letter within each column are not significantly different from each other.

The PDI values of all nanovesicles, in the range 0.24-0.33, reveal the homogeneity and uniformity of the investigated liposomes, ethosomes and glycerosomes. Significant disparities in the PDI values are evident when contrasting empty glycerosomes with those infused with the extract.

The highly negative values of ζ -potential in the nanovesicles can be attributed to the negative groups present in phosphatidylcholines [23]. This characteristic is beneficial for the storage stability of vesicles in dispersion over time, as it enhances electrostatic

repulsion among vesicles. This, in turn, inhibits their agglomeration and fusion, contributing to the preservation of the integrity of the formulations [24].

3.2.2 *Entrapment efficiency of extract*

As shown in **Table 1**, the encapsulation efficiency for olive leaf extract was approximately 50% in both liposomes and ethosomes, whereas in glycerosomes, it was significantly lower at 40% compared to the other two nanovesicles.

There are studies where the encapsulation efficiency using this type of nanovesicles is higher [14,25]; however, the efficiencies around 50% observed in this study may be attributed to the more hydrophilic nature of the phenolic compounds present in olive leaf extract [11,26]. Thus, with an encapsulation efficiency of around 50% in the formulation, we would have both free and encapsulated extract, which could be an advantage for the potential use with bioactive properties, as we would have both nanovesicles and free form.

3.2.3 *Stability of nanovesicles over time*

Table 2 shows the mean diameter, PDI and ζ -potential of the three types nanovesicles with the extract on day 1 and day 166 (6 months) of storage at 4 °C protected from light sources.

Table 2. Mean diameter (MD), polydispersity index (PDI), ζ -Potential of liposomes, ethosomes and glycosomes loaded with olive leaf extract measured at day 0 and day 166.

	Day 0			Day 166		
	MD (nm)	PDI	ζ -Potential (mV)	MD (nm)	PDI	ζ -Potential (mV)
Liposomes	127 $\pm$ 6 ^a	0.27 $\pm$ 0.01 ^a	-41 $\pm$ 1 ^a	127 $\pm$ 1 ^a	0.35 $\pm$ 0.02 ^b	-39 $\pm$ 1 ^a
Ethosomes	119 $\pm$ 6 ^a	0.33 $\pm$ 0.01 ^a	-39 $\pm$ 2 ^a	118 $\pm$ 1 ^a	0.41 $\pm$ 0.02 ^b	-42 $\pm$ 2 ^a
Glycosomes	122 $\pm$ 3 ^a	0.31 $\pm$ 0.03 ^a	-41 $\pm$ 1 ^a	118 $\pm$ 1 ^a	0.36 $\pm$ 0.01 ^a	-39 $\pm$ 1 ^a

Data are presented as mean $\pm$ standard error of the mean (SEM) of three independent experiments conducted in triplicate. Statistically significant differences as determined by Tukey's HSD post-hoc test ($p < 0.05$). Treatments with the same letter between the day 0 and day 166 columns do not differ significantly from each other.

When comparing the mean measured values between day 0 and day 166, there were no significant changes in the mean diameter and ζ -potential for any of the three types of nanovesicles. However, significant differences were observed in the PDI when comparing liposomes and ethosomes between day 0 and day 166.

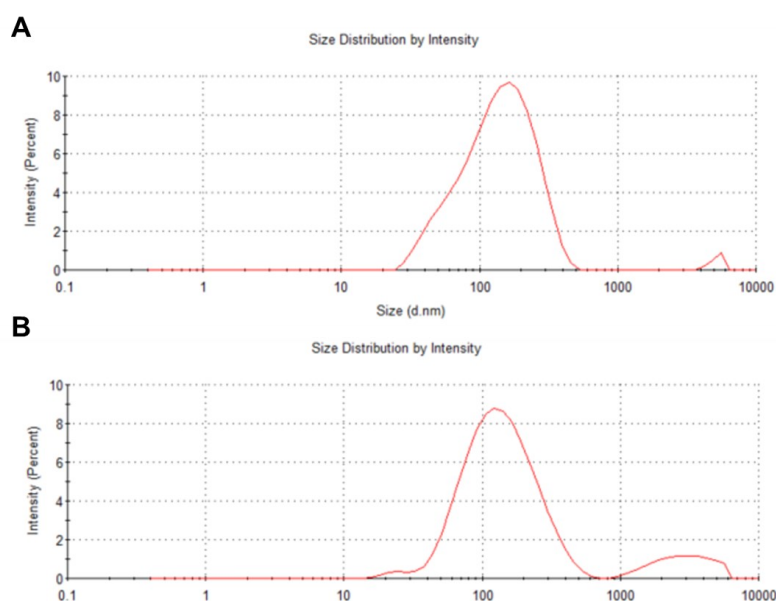


Figure 1. A) Graphical representation of the particle size distribution of liposomes as a function of intensity at day 0 after liposome formation. B) Graphical representation of the particle size distribution of liposomes as a function of intensity at day 166 after liposome formation.

It is noteworthy that the mean particle size remains relatively stable over time, but the PDI does exhibit significant changes, suggesting particle aggregation phenomena [27].

This is supported by **Figure 1**, which indicates an increase in the population of particles

larger than 1000 nm after six months. However, it is observed that the predominant population remains the same and despite the passage of 6 months the average particle size remains without significant differences.

On the other hand, when comparing the PDI of the glycosome at day 0 and day 166 there is no significant difference, which agrees with other investigations in which the addition of glycerol decreases the aggregation phenomenon of the nanovesicles [28].

3.3 Antitumoral activity of olive leaf extract and its nanovesicles forms

The antiproliferative activity of both olive leaf extract and its nanovesicles (i.e., liposome, ethosome and glycosome) was evaluated in MCF7 and MCF10A cell lines. These cell lines were exposed to various concentrations of the nanovesicles (**Figure 2**). When compared with their respective controls, our results reveal a distinct effect between tumoral and non-tumoral cells (i.e., MCF7 and MCF10A, respectively). Specifically, exposure of MCF7 to the different formulations for 24 hours resulted in a drastic reduction (90%) in cell viability (**Figure 2A**). The GI_{50} values are shown in **Table 3**.

Table 3. Growth inhibition values of olive leaf extract, liposomes, ethosomes and glycosomes after 24h incubation in MCF7 cells.

GI_{50} values in MCF7 (mg/mL)	
Extract	0.13 ± 0.02^a
Liposomes	0.14 ± 0.01^a
Ethosomes	0.11 ± 0.01^a
Glycosomes	0.31 ± 0.02^b

Data are presented as mean $\pm$ standard error of the mean (SEM) of three independent experiments conducted in triplicate. Statistically significant differences as determined by Tukey's HSD post-hoc test ($p < 0.05$). Treatments with the same letter within each column are not significantly different from each other.

The results indicate that all nanovesicles are effective as antiproliferative agents against MCF7 tumour cells. These findings are in agreement with those obtained by Han et al. (2009) in which they evaluated the antiproliferative effect of olive leaf extract and two

of its main components, oleuropein and hydroxytyrosol in MCF7 human breast cancer cells, obtaining similar inhibition results at comparable concentrations [29]. Among the nanovesicles tested, ethosomes showed greater effectiveness as evidenced by significantly lower GI_{50} values. The heightened impact of ethanol could be attributed to its ability to enhance lipid fluidity, resulting ethanol-based nanovesicles are softer and less rigid than liposomes, thereby facilitating easier cellular entry and enhancing bioavailability [30]. Despite the effectiveness of all nanovesicles, glycosomes have a higher GI_{50} value compared to other formulations (**Table 2**). We did not detect LDH activity in the culture medium following any of the treatments (data not shown), indicating that the decrease observed in cell viability could not be attributed to any acute cytotoxicity resulting from plasma membrane leakage. Conversely, when analysing the effect of olive leaf extract and various encapsulations on MCF10A, no decrease in cell viability was observed (**Figure 2B**). Not only that, but the results also demonstrated that the tested formulations trend to increase cell proliferation in MCF10A, as observed under an inverted Olympus CK2 microscope (Olympus, Spain) (**Supplementary Figure S1**). Based on these findings, our study shows that olive leaf extract, both soluble and nanovesicles, specifically and solely targets cancer cells. These results are consistent with previous studies on specific phenolic compounds, such as resveratrol and certain methoxylated analogs, which exhibited a similar trend to our extract. They demonstrated an antiproliferative effect in MCF-7 mammary carcinoma cells, while showing no such effect in non-tumorigenic MCF10A mammary epithelial cell lines [31].

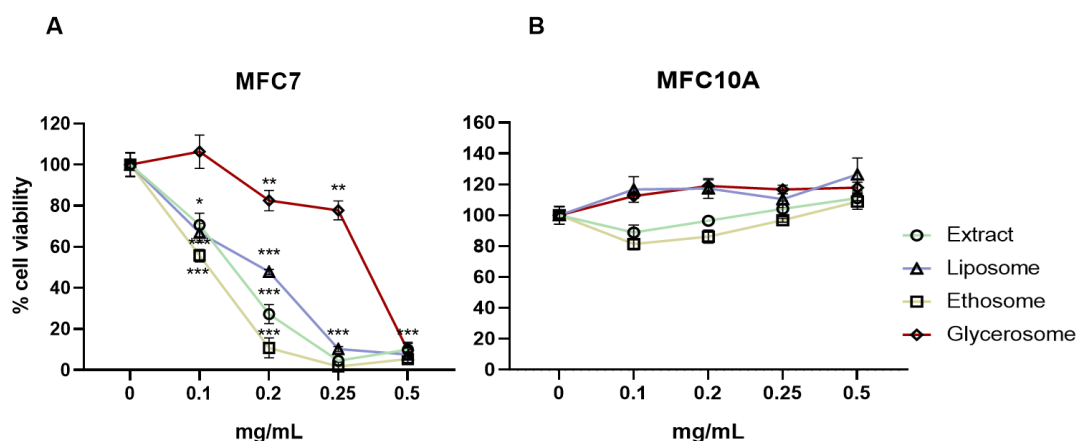


Figure 2. MCF7 (A) and MCF10A (B) cell viability in presence of increasing concentrations (mg/mL) of *Olea europea* extract and the nanovesicles formulations (i.e., liposome, ethosome, glycosome). Results are expressed as the mean $\pm$ SEM of three independent experiments conducted in triplicate. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ compared to the control.

3.3.1 Mechanisms of cell death

To understand the mechanisms triggering a decrease in cell viability following exposure to olive leaf extract and its diverse nanovesicles methods, levels of ROS, mitochondrial membrane potential, and apoptotic stage were assessed. These assays were exclusively performed in the MCF7 cell line, as the MCF10A cells did not exhibit increased cell death in response to the extract and nanovesicles.

In the apoptosis experiments, MCF7 cells were treated with the GI_{50} concentrations previously determined for each formulation. As previously mentioned, this concentration represents the point at which viability is reduced by 50% without observing cell lysis, as indicated by the lack of LDH release into the medium (data not shown). We quantified the percentage of apoptosis in MCF7 cells at basal levels and in the presence of olive leaf extract and its various nanovesicles. Exposure to the different formulations resulted in a higher percentage of late apoptosis (**Figure 3**). As depicted in the same figure, the percentage of cells undergoing apoptosis at basal levels was low,

reflecting the normal cell death in culture. Extract, Liposomes, Ethosomes, and Glycosomes treatments showed significantly different apoptosis and death percentages compared to the control, specifically late apoptosis and cell death. However, there are no statistically significant differences among Extract and the different nanovesicles (**Table 4**).

Table 4. Early apoptosis, late apoptosis, and dead cell percentages on MCF7 cells after treatment with *Olea europea* extract and the nanovesicles formulations (i.e., liposome, ethosome, glycosome).

	Early Apoptosis (mean %, SEM)	Late Apoptosis (mean %, SEM)	Dead (mean %, SEM)
Control	8.20 ± 0.51 ^b	5.67 ± 0.65 ^c	2.80 ± 0.26 ^d
Extract	0.23 ± 0.12 ^a	23.10 ± 0.96 ^a	40.47 ± 0.94 ^a
Liposomes	0.43 ± 0.03 ^a	14.87 ± 0.22 ^b	45.43 ± 2.92 ^a
Ethosomes	0.40 ± 0.06 ^a	14.00 ± 1.71 ^b	47.60 ± 6.32 ^a
Glycosomes	0.17 ± 0.09 ^a	16.37 ± 2.11 ^b	44.73 ± 6.23 ^a
Positive control - Staurosporine	6.43 ± 2.98 ^b	12.10 ± 1.08 ^c	2.23 ± 0.44 ^d

Data are presented as mean ± standard error of the mean (SEM) of three independent experiments conducted in triplicate. Statistically significant differences as determined by Tukey's HSD post-hoc test ($p < 0.05$). Treatments with the same letter within each column are not significantly different from each other.

The level of ROS was also determined and the results are shown in **Figure 4A**. The results indicate that in tumour cells MCF7, both olive leaf extract and its nanovesicles induce higher oxidative stress compared to the positive control (0.5 mM of H₂O₂). This mechanism seems to be coupled with a mitochondrial depolarization indicated by a decrease in the red/green fluorescence ratio, as shown in **Figure 4B**. Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) was used as a positive control for determination of MMP. Our finding suggests that the antitumoral effect of the extract and its nanovesicles may be attributed to an increased in oxidative stress mediated by mitochondrial damage.

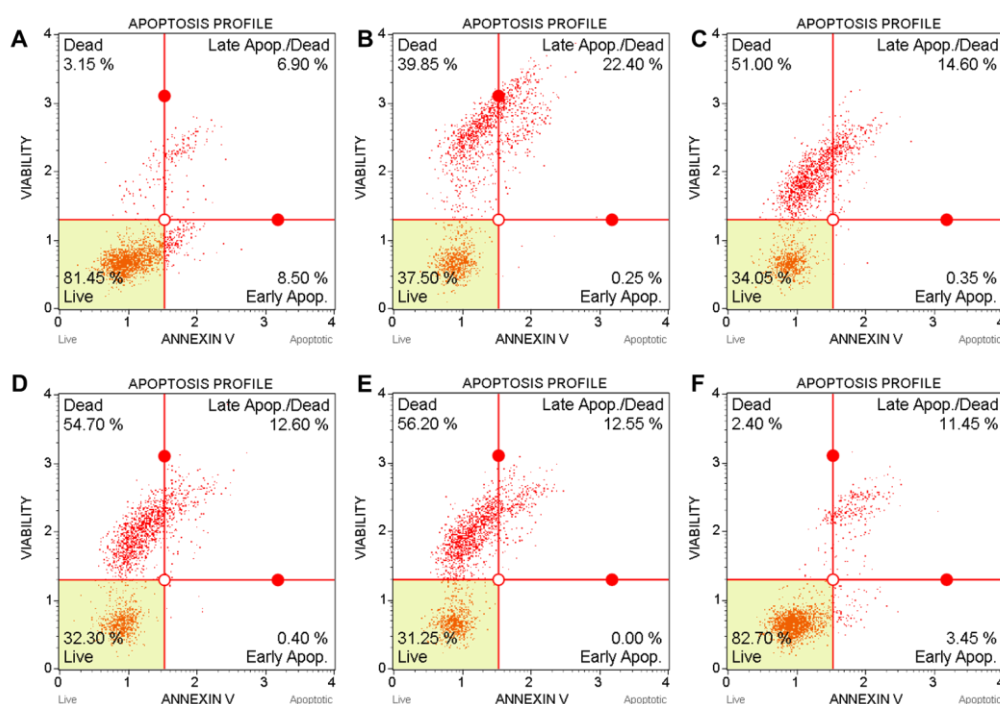


Figure 3. Apoptosis state determined by annexin V-cell death assay of *Olea europaea* leaf extract (B), liposomes (C), ethosomes (D), glycosomes (E) on MCF7 cell line after GI_{50} exposure during 24h. Control MCF7 cells incubated with just media are plotted (A). Staurosporine as well-known apoptotic agent was used as positive control (F). Plots are from a representative experiment out of three replicates.

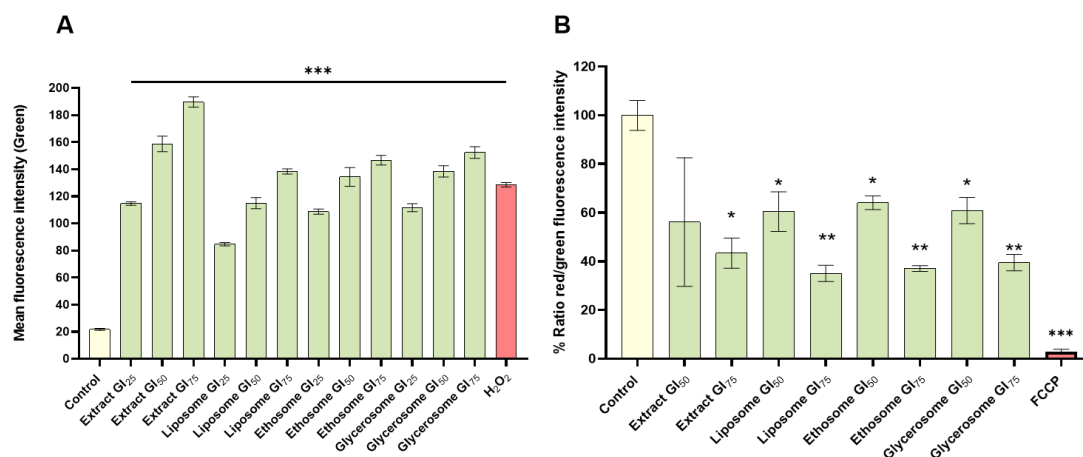


Figure 4. Effects of 24-hour exposure of MCF7 cells to GI_{50} and GI_{75} concentrations of *Olea europaea* leaf extract and nanovesicles on A) reactive oxygen species (ROS) levels and B) mitochondrial membrane potential (MMP). Results are expressed as the mean $\pm$ SEM of three independent experiments conducted in triplicate * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ compared to the control.

In this study, it is demonstrated that the production of ROS, and consequently the presence of oxidative stress, induced by olive leaf extract and its nanovesicles, results in cellular damage ultimately leading to cell death. Excessive ROS production leads to mitochondrial damage, as evidenced by the observed decrease in MMP. Therefore, these findings suggest that the loss of MMP is regarded as the initial stage in mitochondria-mediated apoptosis. Consequently, the study indicates the involvement of a mitochondria-dependent ROS-mediated pathway in the apoptosis induced by the formulations. These results agree with those obtained by other authors with the use of other compounds present in plants such as dryofragin [32] or geraniin [33].

4. Conclusion

Olive leaf extract, whether in its free form or encapsulated in various formulations (liposome, ethosome, and glycerosome), exhibits efficacy as an antiproliferative agent in MCF7 breast tumor cells. However, in the non-tumorigenic MCF10A cell line, it does not exert an antiproliferative effect; instead, it leads to increased cell proliferation. The mechanism through which olive leaf extract exerts its antiproliferative effect on the breast cancer tumor line implies that apoptosis may be induced by the extract via the involvement of a mitochondria-dependent ROS-mediated pathway.

In this context, the encapsulation of the extract does not compromise its anticancer properties. on the contrary, the nanovesicle form has the potential to enhance the bioavailability of the extract for the development of functional foods. These nanovesicles can facilitate a more targeted release along the gastrointestinal tract while also providing protection. Additionally, in the context of breast cancer, there is a compelling interest in developing a topical release formulation. Encapsulation is essential for enhancing the

permeability of the extract through the skin in such applications. Furthermore, the stability of these nanovesicles over time ensures that they can be stored effectively, safeguarding the extract from degradation.

Author contributions

María del Carmen Villegas-Aguilar: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Project administration, Funding acquisition. **María de la Luz Cádiz-Gurrea:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Resources. **Andres Salumets:** Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **David Arráez-Román:** Resources, Supervision, Project administration, Funding acquisition. **Antonio Segura-Carretero:** Resources, Supervision, Project administration, Funding acquisition. **Alberto Sola-Leyva:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Project administration, Funding acquisition. **María Paz Carrasco-Jiménez:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

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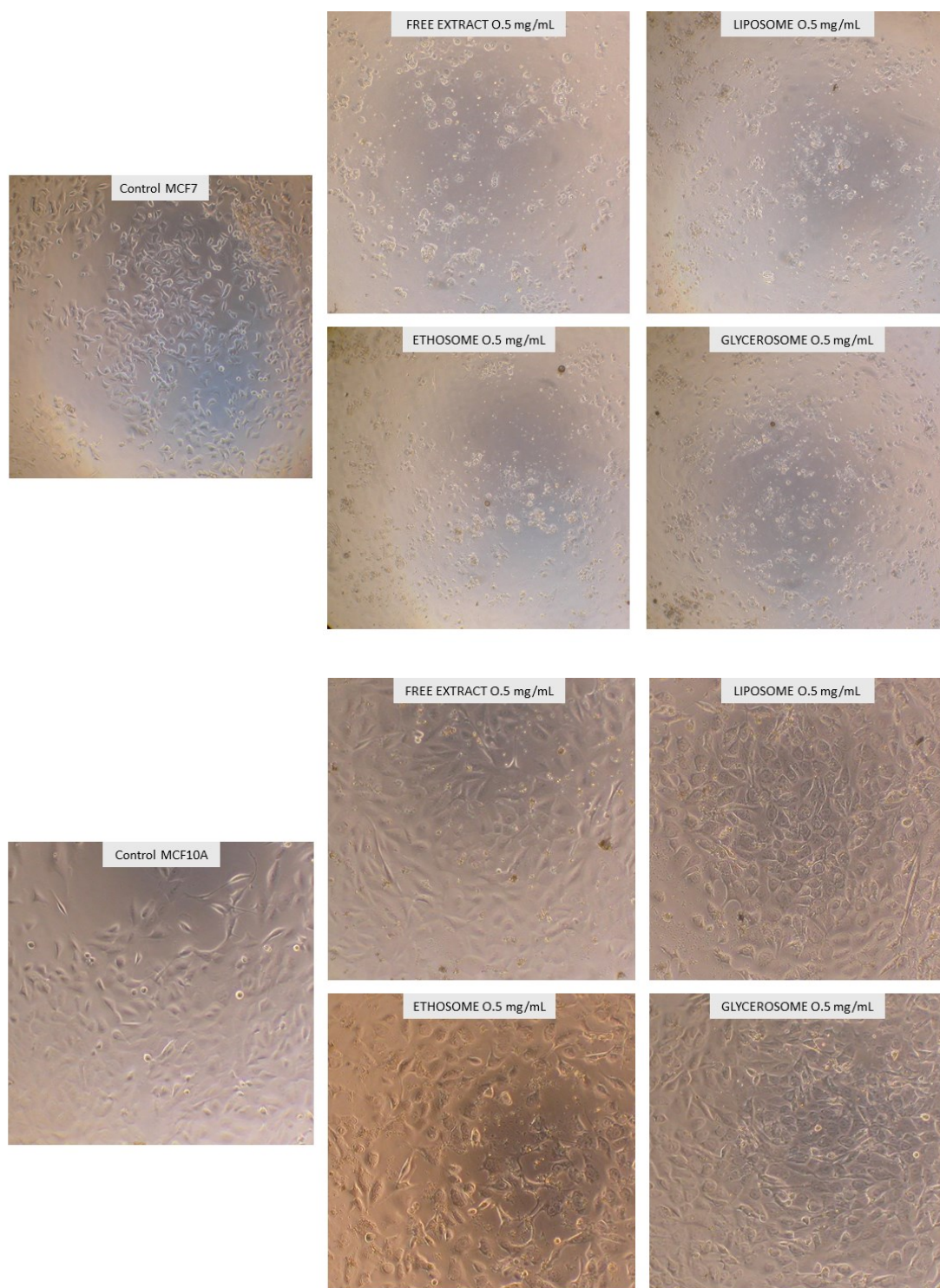
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Supplementary Material

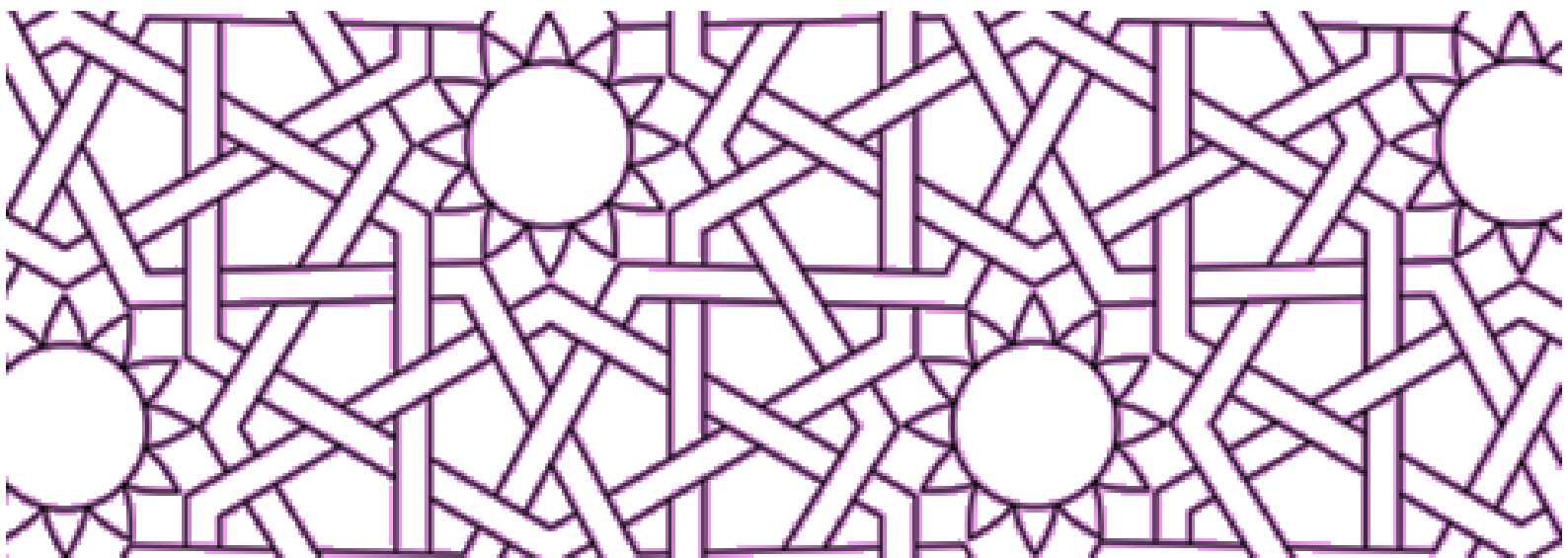
Figure S1. Cell imaging under an inverted microscope. This image displays a field of cultured MCF7 and MCF10A cells treated with 0.5 mg/mL of *Olea europaea* free extract and nanoformulations (i.e., liposomes, ethosomes, and glycosomes), alongside their respective culture media as controls. Magnification: 10x.



***Section B. New methodological
approach for the study of
phenolic compounds***



Chapter 4



*An untargeted metabolomics approach
applied to the study of the bioavailability
and metabolism of three different bioactive
plant extracts in human blood samples*

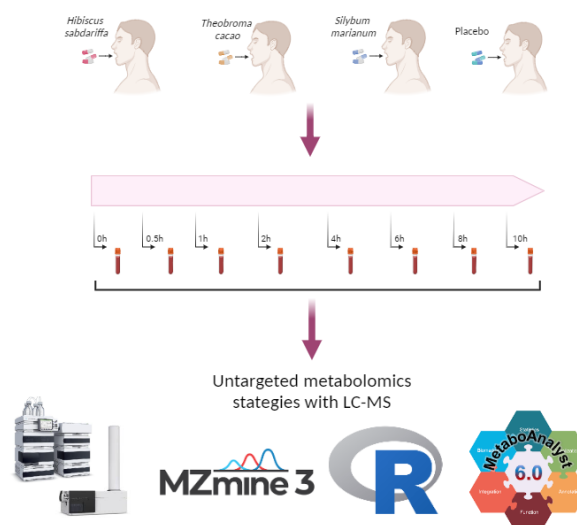
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Abstract

Advances in the understanding of bioavailability and metabolism of bioactive compounds have been achieved primarily through targeted or semi-targeted metabolomics approaches using the hypothesis of potential metabolized compounds. The recent development of untargeted metabolomics approaches can present great advantages in this field, such as in the discovery of new metabolized compounds or to study the metabolism of compounds from multiple matrices simultaneously. Thus, this study proposes the use of an untargeted metabolomics strategy based on HPLC-ESI-QTOF-MS for the study of bioavailability and metabolism of bioactive compounds from different vegetal sources. Specifically, this study has been applied to plasma samples collected in an acute human intervention study using three matrices (*Hibiscus sabdariffa*, *Silybum marianum* and *Theobroma cacao*). This approach allowed the selection of those significant variables associated with exogenous metabolites derived from the consumption of bioactive compounds for their subsequent identification. As a result, 14, 25 and 3 potential metabolites associated with supplement intake were significantly detected in the plasma samples from volunteers who ingested the *H. sabdariffa* (HS), *S. marianum* (SM) and *T. cacao* (TC) extracts. Furthermore, T_{max} values have been computed for each detected compound. The results highlight the potential of untargeted metabolomics for rapid and comprehensive analysis when working with a wide range of exogenous metabolites from different plant sources in biological samples.

1. Introduction

Traditionally, plant-rich diets have been characterised by reports of various health benefits in humans. A significant portion of the beneficial properties of plant sources are associated with bioactive phytochemical compounds such as phenolic compounds¹. When bioactive phenolic compounds are consumed, these phytochemicals can be absorbed or metabolised by host and intestinal microbiota enzymes². The complex mixture of resulting metabolites may contribute additively or synergistically to bioactivities and may explain their beneficial effects ¹.

Given that the biochemical mechanisms of action of phenolic compounds are related to disease prevention, it is essential to fully understand their bioavailability and metabolization reactions to know exactly which chemical structures reach the target organs and tissues. Typical transformations in such phenolic compounds often involve the elimination of sugar moieties, resulting in the generation of aglycones from the initial compounds ³. Furthermore, these compounds can undergo phase I and II metabolic biotransformation in the liver. Primary phase I metabolic reactions involve oxidation and reduction, while phase II reactions include processes such as methylation, sulphation, or glucuronidation ³.

The absorption, bioavailability and metabolism of ingested phenolic compounds in both human and animal is generally explored by applying targeted metabolomics approaches ⁴⁻⁶. This methodological choice revolves around the systematic search in biological samples for specific predetermined metabolites based on the original composition of the bioactive extracts and their hypothesized metabolic transformations ⁴. These targeted strategies allow both the identification and quantification of the main

metabolites predicted for plant matrices in biological samples, as long as commercial standards are available. Therefore, these strategies require a detailed characterization of the original composition of the bioactive extracts. However, these strategies do not have the ability to detect unpredicted or non-preselected compounds, and therefore the existence of completely new metabolites could go undetected ⁷. This limitation becomes particularly obvious in plant matrices where information about the metabolites in plasma or urine is scarce. In such cases, relying solely on targeted strategies might result in substantial loss of valuable information ⁸. To address this limitation, untargeted metabolomics strategies are gaining attention. These approaches involve analysing the entire range of compounds present in the samples without predefining a list of targets. Although the initial hypothesis of an untargeted methods does not pursue quantification, they offer a valuable advantage considering that they can reveal previously unknown or unexpected metabolites ^{6,9,10}.

In this context, by employing untargeted metabolomics strategies, we can acquire a much broader list of exogenous metabolites derived from the ingested extracts. Similarly, it enables the resolution of the issue of identifying metabolites originating from matrices with limited metabolomic studies ^{9,10}. Moreover, these untargeted strategies possess an added advantage in their potential application in multi-matrix studies. They allow rapid detection of circulating metabolites from various extracts without requiring prior knowledge of the original composition of the extract or the hypothesis of possible circulating metabolites. Considering all these aspects, this study proposes the application of an untargeted metabolomics-based study to study the bioavailability and metabolism of three extracts with different phenolic composition (*Hibiscus sabdariffa*, *Silybum marianum*, and *Theobroma cacao*).

H. sabdariffa, *S. marianum*, and *T. cacao* are three plant sources that have traditionally been employed for human consumption because of their reported beneficial properties¹. Traditionally, these three plant sources have been consumed in different formats: *H. sabdariffa* (HS) in the form of infusions ¹¹, *S. marianum* (SM) as pharmaceutical preparations ¹² and *T. cacao* (TC) in the form of chocolate and all its variations ¹³.

The phenolic compounds present in the original extracts of these three plants have been widely studied by the scientific community. However, there are hardly any studies focused on the bioavailability and metabolism of these three plant matrices. Moreover, these three plants present different phenolic composition ¹⁴ allowing to cover the bioavailability and metabolism of different families of compounds through the application of this study.

Considering all the context, this study proposes an untargeted metabolomics methodology for the study of the bioavailability and metabolism of different bioactive extracts simultaneously. For its application, a case study is presented based on an acute intervention in humans where HS, TC and SM extracts were administered, and blood samples were collected at different times after the intake.

2. Experimental

2.1 Chemicals

All solvents employed in the analysis of metabolites were of analytical reagent grade and utilized in their original state. LC-MS grade methanol, water and formic acid, utilized as mobile phase components, were procured from Sigma-Aldrich (Steinheim, Germany). For plasma treatment, ethanol and methanol of LC-MS grade were sourced from Fisher

Scientific (Madrid, Spain). The chemical standards used were (Hibiscus Acid from Phytolab (Vestenbergsgreuth, Germany) and Quercetin-3 glucuronide from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Bioactive plant extracts

The HS, TC and SM extracts selected for the present study were pre-industrial extracts of the three plant matrices provided by NATAC Biotech S.L. (Cáceres, Spain). The extraction parameters were optimised for each plant matrix individually. For HS and TC extracts, a solid-liquid extraction (maceration) was performed using an EtOH:H₂O mixture (80:20; v:v) for two hours with a solvent:plant ratio of 20:1. For the SM extract a maceration was also performed but using an EtOH:H₂O mixture (96:4; v:v) for two hours with a solvent:plant ratio of 25:1. Extraction temperatures were set at 45 °C for HS, and 55 °C for SM and TC. The extracts obtained were dried under vacuum, stored at room temperature and protected from light until encapsulation. HS, SM and TC extracts at 5000 mg/L were characterised by high-performance liquid chromatography (Agilent 1290 HPLC, Agilent Technologies, Palo Alto, CA, USA) coupled to mass spectrometry with a quadrupole time-of-flight analyser (Agilent 6545 QTOF Ultra High Definition, Agilent Technologies, Palo Alto, CA, USA) using the methodology carried out in the previous study by Villegas-Aguilar et al. (2024) ¹⁴.

2.3 Biological Samples

The study protocol adhered to the ethical recommendations of the Declaration of Helsinki and received approval from the Ethics Committee of the Miguel Hernández University of Elche and the University Hospital of Elche (Alicante, Spain), reference PI 57/2019. The study involved 33 healthy subjects with a mean age and body mass index

of 27 ± 9 years and 23 ± 3 kg/m², respectively. Participants were not taking medication or nutritional supplements and did not have any chronic pathology or gastrointestinal disorder. The sample size was estimated based on similar previous bioavailability studies⁴. Volunteers provided informed consent before participating.

The study was conducted at the facilities of Universidad Miguel Hernández. After an overnight fast, volunteers were divided into three groups: 8 consumed an encapsulated (pill) 500 mg *H. sabdariffa* calyxes extract, 8 consumed an encapsulated (pill) 500 mg *S. marianum* fruit extract, 8 consumed an encapsulated (pill) 500 mg *T. cacao* fruit extract and 9 consumed an encapsulated (pill) placebo. A polyphenol-free breakfast was offered 30 minutes after encapsulation consumption, and at 2.5, 4.5, and 9.5 hours after ingestion, a polyphenol-free snack and lunch were provided. Water was available ad libitum.

Before encapsulation ingestion, a nurse inserted a cannula into the ulnar vein of the non-preferred arm of the volunteers. Blood samples were collected in EDTA-coated tubes at baseline ($t = 0$) and subsequently at 0.5, 1, 2, 4, 6, 8, and 10 hours after encapsulation consumption. Plasma was separated by centrifugation (10 min, 3000 rpm, 4 °C) and stored at -80 °C until further analysis.

2.4 Sample treatment

The plasma samples, preserved at -80 °C until analysis, were initially thawed on ice. For sample processing, a 100 µl plasma aliquot was blended with 200 µl of methanol:ethanol (50:50, v/v). Following a 10-second vortexing, the mixture was maintained at -20 °C for 30 minutes to facilitate sufficient protein precipitation. Subsequently, the sample underwent centrifugation for 10 minutes at 14,000 r.p.m. and 4 °C. The resulting

supernatant was subjected to a 2-hour vacuum evaporation in a centrifugal evaporator (Concentrator Plus, Eppendorf, Hamburg, Germany). The reconstituted residue was dissolved in 80 μ l of the initial mobile phase conditions (0.1% aqueous formic acid:methanol, 95:5, v/v). Finally, a 60 μ l aliquot was transferred to an HPLC vial and stored at -80 °C until the day of analysis. A quality control (QC) sample was prepared by combining equal volumes (20 μ l) from each original sample. Multiple QC aliquots were processed following the same methodology as described for the samples.

2.5 HPLC-ESI-QTOF-MS analysis

The analyses were conducted in an Agilent 1260 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate Mass Q-TOF, equipped with a dual Jet Stream ESI interface. A reversed-phase analytical C18 column (Agilent Zorbax Eclipse Plus, 1.8 μ m, 4.6 $\times$ 150 mm) with a protective cartridge of the same packing was employed. The mobile phases included water with 0.1% formic acid (mobile phase A) and methanol (mobile phase B). To ensure effective separation, a gradient of these mobile phases was applied as follows: 0 min [A:B, 95/5], 5 min [A:B, 90/10], 15 min [A:B, 15/85], 30 min [A:B, 5/95], and 35-40 min [A:B, 95/5]. The column and autosampler compartment temperatures were maintained at 25 and 4 °C, respectively. The injection volume and flow rate were set at 2.5 μ l and 0.4 mL/min.

Detection was performed in negative ion mode within the range of 50 to 1700 m/z. All spectra underwent correction via continuous infusion of two reference masses: TFA anion (m/z 112.985587) and HP-921 (m/z 1033.988109). Both reference ions provided accurate mass measurements, typically better than 2 ppm.

Ultrapure nitrogen and a nebulizer served as drying and nebulizing gas at temperatures of 200 and 350 °C, with flow rates of 10 and 12 L/min, respectively. Other optimised parameters were as follows: fragmentor, 130 V; capillary voltage, +4000 V; nebuliser, 20 psi; nozzle voltage, 500 V; skimmer, 45 V and octopole 1 RF Vpp, 750 V.

The sequence design has been carried out according to the guidelines and considerations established in untargeted metabolomics studies ¹⁵. For sample analysis, analytical blanks were analysed at the beginning and at the end of the sequence. Quality control samples were analysed at the beginning of the sequence, after the first blanks, to stabilize instrumental conditions. Additionally, QCs were also injected throughout the entire sequence, every 6 real samples, to ensure analytical reproducibility. Samples from different groups (placebo or different supplements) were randomised in the sequences, but different samples from the same volunteer were analysed consecutively. Finally, targeted MS/MS analyses were performed on the signals of interest to obtain a fragmentation spectrum for metabolite annotation. This experiment was performed using nitrogen as collision gas with the following collision energies: 10 eV, 30 eV and 60 eV.

2.6 Data processing

Firstly, the raw data (.d format) acquired by HPLC-ESI-QTOF-MS were converted to .mzML format using the data processing tool Msconvert (ProteoWizard). MZmine 3.9.0 software was used to run several data preprocessing stages including mass detection, chromatogram builder, deconvolution, alignment, isotopic grouping and duplicate peak filter. The following parameters were optimized and selected to carry out the different data processing stages. For the mass detection stage, a noise level of 3.0E2 was selected.

The construction of chromatograms was based on the ADAP algorithm selecting the following parameters: intensity threshold: 3.0E2; highest minimum intensity: 1.E3; m/z tolerance: 20 ppm. For chromatogram deconvolution, the wavelet algorithm (ADAP) was applied with the following parameters: S/N threshold of 10, minimum peak height of 6.0E2, coefficient/area threshold of 110, peak duration range from 0.00 to 10.00, and RT wavelet width range from 0.00 to 0.10. Chromatogram alignment was conducted using the "Join Aligner" algorithm, with an m/z tolerance of 15 ppm and an RT tolerance of 0.25 min, assigning equal weight to both m/z and RT. Finally, the duplicate peak filter stage was implemented with a selected m/z tolerance of 15 ppm and a retention time tolerance of 0.1 min.

The following data processing steps were conducted using an open-source methodology through a combination of different R packages ¹⁶. For the batch normalization step, missing values (NAs) were imputed by a small value. The batchCorr (v 0.2.5.) package was used to correct the signal intensity drift between and within batches ¹⁷. The imputed NAs were subsequently converted back to NAs after batch normalization. Notame R package was mainly used to filter biologically meaningless signals such as potential contaminants or low detected signals. Thus, the *flag_contaminants* function was used to filter out those molecular features considered as contaminants since they were also detected in blank samples. The *flag_detection* function was also run to exclude the molecular features with low detection rates for the study groups. A flag detection threshold of 0.5 was chosen for filter out those signals with low detection in the analysed samples. 8 groups were established for each matrix according to the different sample collection times (0, 0.5, 1, 2, 4, 6, 8 and 10 hours). Therefore, all those signals that were not detected in 50% of the volunteers in at least one of these subgroups were filtered.

For this filter, all chromatographic areas less than 80 counts were considered NAs since those integrated values are below the noise level.

2.7 Statistical analysis

Statistical analyses were aimed at selecting those variables associated with the intake of bioactive supplements, which are related to bioavailable and/or metabolized compounds. For this objective, a combination of univariate and multivariate statistical analyses was applied to the pre-processed dataset. All statistical analyses were run using MetaboAnalyst 6.0 software. Prior to conducting multivariate analyses, the data were logarithmic transformed, and Pareto scaled. These measures aimed to normalize the data and mitigate the impact of variables with large scales.

Firstly, a Principal Component Analysis (PCA) was performed to assess reproducibility, check data quality and identify potential outliers. A hierarchical clustering analysis was conducted through a heatmap, providing a preliminary visual representation of the data based on the different experimental groups. For those analyses, the variables with a relative standard deviation (RSD) higher than 30% in QC samples were also filtered out.

Finally, the following criteria was used to select the potential molecular features related to exogenous metabolites of the extract for subsequent identification:

- Molecular features do not have to be identified as contaminants based on the flag contaminant function.
- Molecular features have to be detected in at least 50% of one of the subgroups, excluding time 0, of the volunteers who consumed the extracts. On the contrary, molecular features do not have to be detected in the subgroups of the volunteers who consumed the placebo supplement.

- The molecular features do not have to be detected at the time 0 subgroups.

The time to reach maximum concentration (Tmax) was calculated for each significant metabolite was calculated using PKSolver, an add-in program for pharmacokinetic data analysis in Microsoft Excel¹⁸.

2.8 Metabolite identification

The metabolite identification of selected molecular features was conducted by comparing the acquired accurate mass, isotopic distribution, and fragmentation spectra derived from MS/MS analysis with information available in comprehensive metabolomic databases. The metabolite annotation step was facilitated by and the CEU Mass Mediator tool, a versatile resource developed by Gil de la Fuente et al. (2018)¹⁹. This tool enabled simultaneous exploration of multiple databases, including METLIN, LipidMaps, and the Human Metabolome Database.

Furthermore, to enhance the accuracy of our identifications, MS/MS patterns were cross-referenced with in silico MS/MS fragmentation resources, specifically using SciFinder®, Sirius 5.8.5 and MetFrag (<https://ipb-halle.github.io/MetFrag/>).

According to the identification guidelines proposed by Sumner et al. (2007)²⁰, compounds were annotated at level 1 with commercial standards, at level 2 by comparing the MS/MS spectra with those present in the databases, at level 3 based on the molecular formulation and MS1 spectra and at level 4 where the molecules remain as unknowns.

3. Results and discussion

3.1 Phytochemical composition of *Hibiscus sabdariffa*, *Silybum marianum* and *Theobroma cacao* extracts

The detailed phytochemical characterization of HS, SM and TC extracts is reported in **Tables S1, S2 and S3** (Supplementary Material), respectively.

In summary, 40 compounds were annotated in the HS extract, including a high presence of hibiscus acid, hibiscus acid lactone, and its methylated forms and glycosylated flavonoids, such as quercetin 3-O-rutinoside and quercetin 7-glucoside.

In the SM extract, a total of 67 compounds were tentatively identified. Notably, there was a high content of flavolignans, with prominent compounds including silybin and its isomers such as silychrystin, isosilybin B, as well as modified forms like dehydrosilybin, hydrogenated silybin, and acetylsilybin A/B. Additionally, the extract contained other flavonoids such as taxifolin (dihydroquercetin).

Finally, 53 compounds were annotated in the TC extract. Quinic acid and gluconic acid being the main compounds present. Additionally, the presence of vanillic acid was noteworthy in this extract.

Consequently, the phenolic content varies significantly among these three matrices, showcasing a diverse array of phenolic compounds from various families. Hence, these three matrices were chosen for the implementation of untargeted metabolomics methodology in bioavailability and metabolism studies. One of the key advantages of employing untargeted methodology is its versatility and therefore in multi-matrix studies makes it unnecessary to hypothesize beforehand all potential metabolization reactions.

In addition, total phenolic content was measured by the Folin-Ciocalteu method using gallic acid as the reference compound for the standard curve of the extracts of HS, SM and TC used in the acute intervention study in the previous study by Villegas-Aguilar et al. (2024) ¹⁴. The total phenolic content values obtained were 51 ± 1 , 617 ± 8 , and 255 ± 12 mg gallic acid equivalent/g dry extract for HS, SM, and TC, respectively. Therefore, it is evident that SM exhibits a higher total phenolic content compared to the other two extracts.

3.2 Untargeted metabolomic data analysis for the study of bioavailability and metabolism of bioactive compounds of the three bioactive extracts

After data processing with MZmine 3.9.0 software, a data matrix consisting of 22,598 molecular features was obtained. Subsequently, the various filtering steps mentioned in the experimental section (e.g. potential contaminants, low presence signals, etc.), resulting in a refined data matrix containing 6,360 molecular features. These molecular features correspond to potential compounds that are present in at least half of the individuals in the defined subgroups (i.e., one subgroup per matrix and collection time), including the time 0 and placebo subgroups. This dataset based on the 6,360 molecular features was subjected to the different statistical analyses. Additionally, to check data quality, the molecular features with a relative standard deviation (RSD) higher than 30% in QC samples were filtered out. Following this filter, 5,328 molecular features were finally selected for further analysis.

To assess data quality after applying the selected parameters, an initial overview of the performance quality was obtained through PCA of the entire dataset, including all QC samples (**Figure 1**). The PCA reveals well-clustered QC samples, indicating good data

quality. Regarding the remaining samples, it is notable that the SM samples, in particular, exhibit the most distinct separation from the placebo samples. This subset of differentiated SM samples showed lower scores in the PC1 and higher scores in the PC3 with respect to the rest of biological samples. This deviation of a significant percentage of samples from the SM group from the rest of the samples in the PCA score analysis is indicative of the presence of upregulated signals in these SM samples, which may be associated with the intake of the bioactive SM supplement. This highlights that PCA analysis can offer a first overview of the data, revealing the data quality as well as the relevance of potential exogenous metabolites in the datasets. In addition, additional PCA analysis were performed considering data from the placebo group and each of the supplement groups (HS, SM, and TC) but separately (**Figure 2**). Similar trends are observed with respect to the global PCA (**Figure 1**) since there is a subset of SM samples separated from the placebos through high scores values at PC1 and low values at PC2. (**Figure 2B**). However, in the case of all TC and HS samples, a general overlap of almost all studied samples is observed with the placebo samples (**Figure 2A** and **2C**).

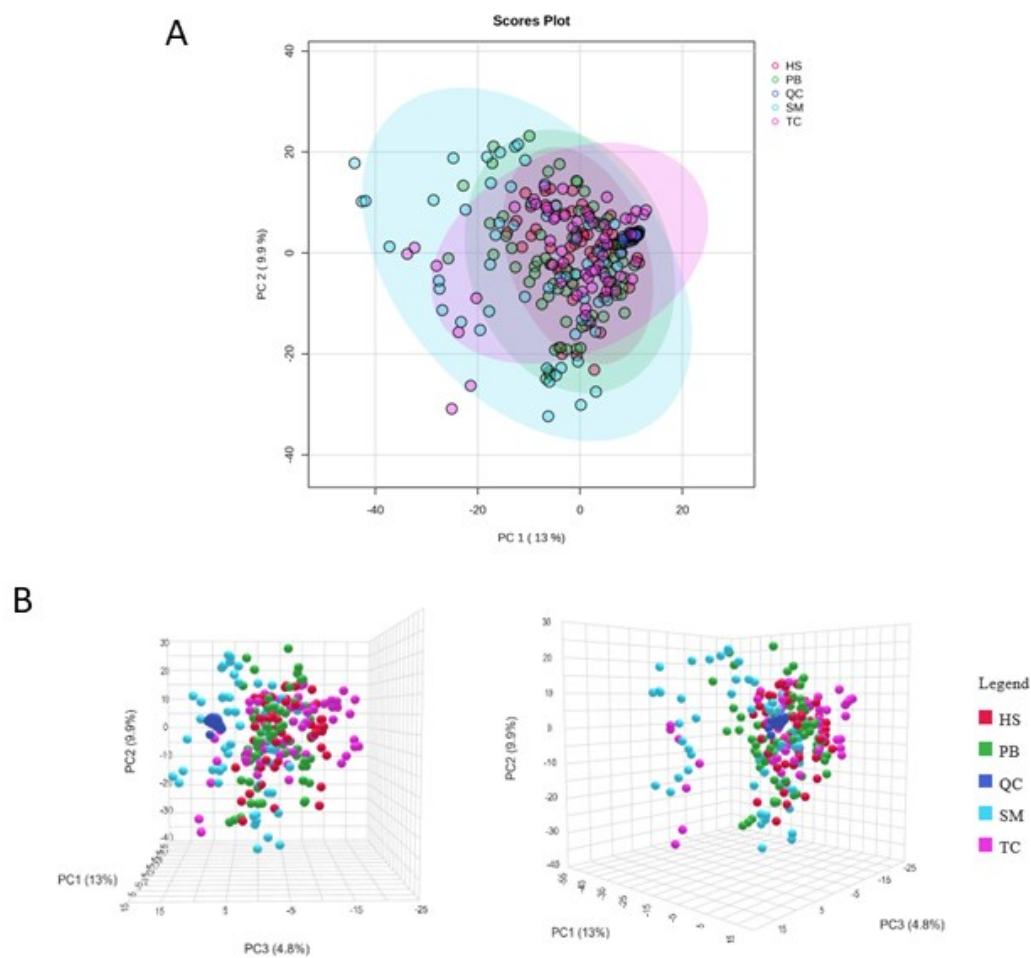


Figure 1. A: 2D PCA scores plots from normalized data for plasma samples. B: 3D PCA scores plots from normalized data for plasma samples.

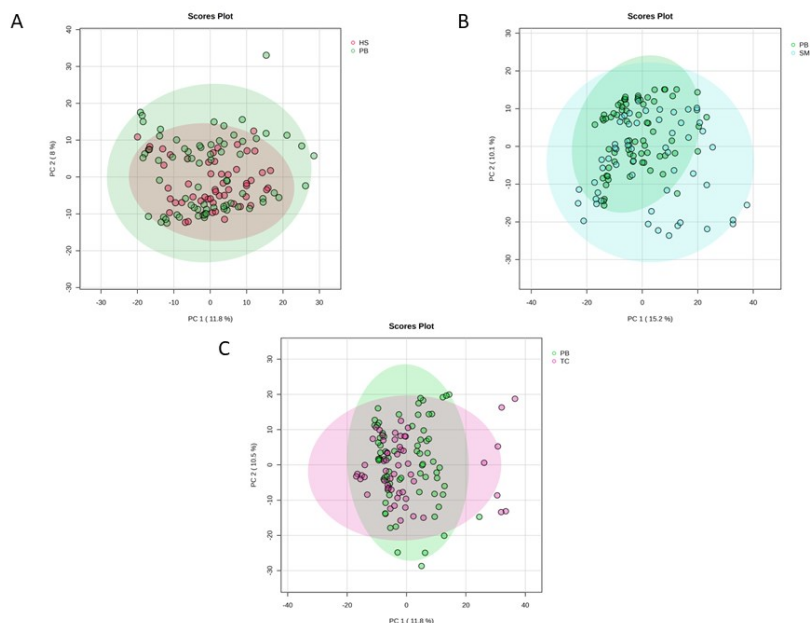


Figure 2. A: 2D PCA scores plots from normalized data for plasma samples from *H. sabdariffa* and placebo. B: 2D PCA scores plots from normalized data for plasma samples from *S. marianum* and placebo. C: 2D PCA scores plots from normalized data for plasma samples from *T. cacao* and placebo.

The subsequent step involves filtering those molecular features detected in the placebo samples as well as in the plasma samples collected at time 0 before the intake of the supplements. After applying the filter, a matrix with 85 molecular features was generated, and a hierarchical clustering analysis via heatmap considering those signals was generated (**Figure 3**). In this heatmap, samples collected at time 0 in the experimental groups were not considered for averaging the signals in those groups. Therefore, the rest of samples collected at 0.5, 1, 2, 4, 6, 8, and 10 hours were used for this analysis. In this heatmap, it is evident the presence of three different clusters that each one of them is associated with a different matrix, highlighting the difference in size in terms of the number of signals in each one of them. The most numerous being the SM cluster, then the HS cluster, and finally the smallest being the TC cluster.

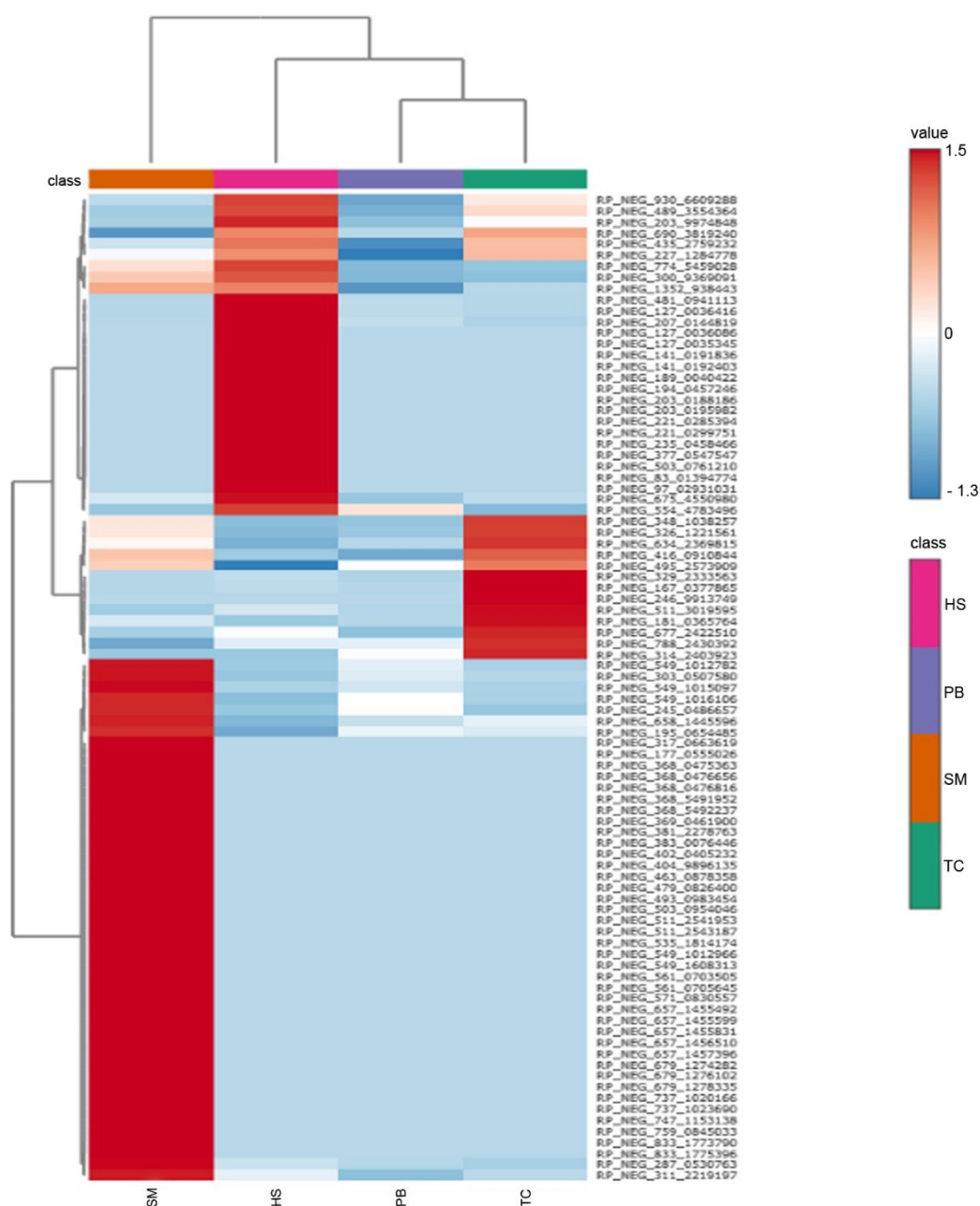


Figure 3. Hierarchical clustering analysis by heat map using the areas under the curve of the 85 characteristic molecules selected for plasma from the three matrices.

The interactive heatmap allows users to visualise broad multidimensional results from untargeted metabolomics and identify significantly altered features by customising the visualisation.²¹ These results illustrate the swift acquisition of such plots once the filtering criteria are established, enabling the prompt identification of signals linked to supplement intake. Moreover, it is worth noting the considerable variances in the

number of biomarkers associated with intake across the different matrices in this study. These distinctions may primarily arise from variances in the richness of the original extract compositions or the bioavailability of phenolic compounds within each matrix. Significantly, certain phenolic compounds exhibit higher bioavailability than others. For instance, based on the literature isoflavones compounds are the most bioavailable followed by phenolic acids, flavanols, flavanones, and flavonols, and the least bioavailable are anthocyanins and proanthocyanidins ²².

The analysis carried out prior to the identification using statistical analysis can be highly beneficial when analysing this type of data involving exogenous metabolites from different plant samples. In this regard, the results of the heatmaps revealed that there are no compounds in common between the three matrices, a finding that has been corroborated by identifying the molecular features. Additionally, the heatmaps predicted that the SM matrix has the widest variety of compounds, a conclusion further supported by the identification process, while highlighting the scarcity of metabolites present in plasma after TC intake.

After analysing these findings, a heatmap analysis was conducted, categorizing the selected metabolites in plasma from the three matrices by group and time (**Figure 4**) to assess whether this graphical representation enables us to discern the times at which the metabolites appear for each type of ingested sample. Indeed, as indicated by the calculated Tmax, it is observed that for the metabolites from HS, there is a prevailing trend for most of them to appear at a time very close to 1 - 2 hours after ingestion of the extract. Conversely, for the metabolites resulting from the ingestion of SM, there is a

subset of compounds with a Tmax of approximately one hour, while others exhibit a Tmax of around 3 hours.

This heatmap offers a wealth of information surpassing that of the previous one depicted (**Figure 3**), as it disaggregates the groups according to their respective time points.

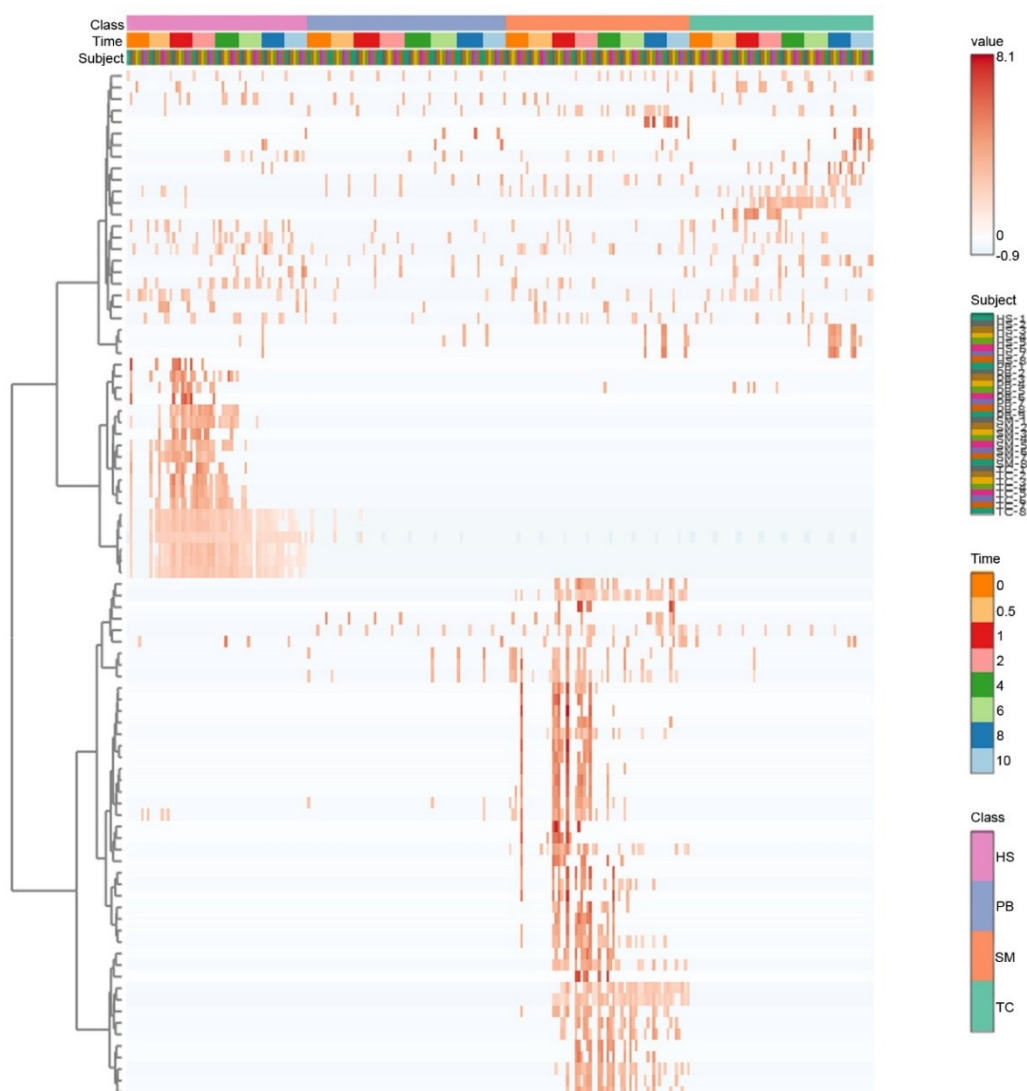


Figure 4. Hierarchical clustering analysis by heat map using the areas under the curve of the selected metabolites in plasma from the three matrices by group and time.

3.3 Identification of significant signals related to supplement intake.

After applying the criteria for selecting significant signals associated with supplement intake using an untargeted metabolomic approach, a total of 85 compounds were proposed as exogenous metabolites present in plasma following extract ingestion. Finally, 25, 14 and 3 compounds were annotated in the volunteers who consumed SM, HS and TC, respectively. 43 molecular features could not be annotated and were kept as unknown compounds (**Table S4**).

In addition, Tmax values have been calculated for each compound detected.

For greater detail of the bioavailable and metabolized compounds detected in plasma samples, the subsections focus on describing these results for each of the studied vegetal matrix.

3.3.1 Circulating metabolites detected in plasma related to the intake of *Hibiscus sabdariffa* extract

Table 1 lists the bioavailable metabolites detected in the plasma samples related to the intake of the *H. sabdariffa* extract supplement. In total, 14 compounds were annotated in volunteers who ingested the HS extract. Among the 14 compounds analysed, hydroxycitric acid lactone was identified by comparison with its commercial standard. **Figure S1** reports the MS/MS spectra of the analysed standard, which along with the retention time matched with the significant feature detected in the HS volunteers. Additionally, 10 compounds were annotated at level 2 by comparison of MS/MS spectra with those reported in databases, and 3 compounds at level 3. Consequently, approximately 79% of the annotated compounds were annotated at levels 1 and 2, indicating high annotation reliability. The remaining 21% of the compounds annotated

at level 3 annotation are considered less accurate, although the supporting bibliographic data suggests a high likelihood of proper annotation.

When comparing the presence of metabolites in plasma with the content of the original extract as reported by Villegas-Aguilar et al. (2024) ¹⁴, it is evident that certain compounds pass into plasma without undergoing modification in their native form. Hence, compounds like hydroxycitric acid, hibiscus acid and two isomers of hibiscus acid monomethyl ester, which are characteristic constituents found in significant quantities in this plant matrix, enter the plasma directly without undergoing any alteration. In this sense, when these compounds enter the bloodstream, they can exhibit in target cells their bioactivity demonstrated *in vivo* in several studies, such as the antimicrobial and anti-diabetic power of hibiscus acid and the antioxidant and anti-inflammatory power demonstrated for hydroxycitric acid ²³.

Other compounds detected in the plasma were not originally present in the extract but are metabolized derivatives of compounds characterised in it. Hence, for instance, hibiscus acid hydroxyethylester may have been generated in plasma upon the addition in hibiscus acid of a methyl group and a hydroxyl group to the methyl ester group. Therefore, these compounds undergo both phase I and phase II metabolic reactions. Methylation of this group can occur via catechol-O-methyltransferases in the liver, small intestine or kidneys, with the liver being the primary site ²⁴.

Also noteworthy is the presence in plasma of two isomers of methoxycitric acid, compounds that were not found in the original extract but may owe their presence in plasma to the methylation of hydroxycitric acid, which was characterized in the extract and in plasma.

It should be noted that most of the compounds detected in plasma after ingestion of the HS extract exhibit a T_{max} between 1 and 2 hours. When compared with the T_{max} values obtained in the study conducted by Arce-Reynoso et al. (2023)²⁵, where a *H. sabdariffa* drink containing compounds such as hibiscus acid and hydroxycitric acid were also detected in plasma but with T_{max} values of 2.7 ± 1.2 and 2.5 ± 2.34 hours, respectively, our study shows earlier T_{max} values of 1.75 ± 0.43 and 1.19 ± 0.50 hours for these same compounds. The earlier T_{max} values observed in our study could be attributed to differences may be related to the format of the product (beverage vs. capsule). However, in accordance with the findings of these authors, the low T_{max} values are indicative of intestinal absorption of the compounds²⁵.

The detection of 5-hydroxy-2-furoic acid in the extract is likely attributable to the presence of vitamin C (ascorbic acid). While our methodology, designed for the identification of phenolic compounds, cannot directly detect vitamin C, existing literature supports a high ascorbic acid content in the source material (HS)²⁶. Ascorbic acid oxidation leads to dehydroascorbic acid, a known precursor to 5-hydroxy-2-furoic acid through subsequent hydroxylation via metabolic pathways²⁷.

In the case of the compounds annotated as D-gluconate and 2-furanmethanol, their presence in plasma is justified by previous reports of these compounds in HS extract^{28,29}.

Table 1. HPLC-ESI-QTOF-MS identification information and relative nutrikinetic parameters of the metabolites detected in plasma samples after intake of an extract of *H. sabdariffa*.

Peak	RT (min)	Observed m/z	Adduct	Theoretical m/z	Mass error (ppm)	Molecular formula	Level of annotation	Proposed compound	MS/MS fragments	Reference*	n	Tmax
1	1.12	127.0035	(M-H) ⁻	127.0037	-1.16	C ₅ H ₄ O ₄	2	5-Hydroxy-2-furoic acid isomer 1	85.0298; 55.0219	HMDB59784	7	1.43 ± 1.05
2	1.13	377.0548	(M-H) ⁻	377.0514	8.84	C ₁₇ H ₁₄ O ₁₀	2	3,4,5-trihydroxy-6-({7-oxo-7H-furo[3,2-g]chromen-4-yl}oxy)oxane-2-carboxylic acid	300.006	HMDB0129424	7	1.43 ± 0.68
3	1.17	207.0157	(M-H) ⁻	207.0146	5.12	C ₆ H ₈ O ₈	2	Hydroxycitric acid	127.0034; 87.0078; 189.0014	HMDB0031159	8	1.19 ± 0.50
4	1.19	481.0941	(M-H) ⁻	481.0988	-9.67	C ₂₁ H ₂₂ O ₁₃	2	Epigallocatechin-7-glucuronide	313.0655; 191.0181	HMDB0041640	8	1.50 ± 0.50
5	1.23	127.0036	(M-H) ⁻	127.0037	-0.32	C ₅ H ₄ O ₄	2	5-Hydroxy-2-furoic acid isomer 2	85.0298; 55.0221	HMDB59784	8	1.50 ± 0.50
6	1.43	221.0285	(M-H) ⁻	221.0303	-7.92	C ₇ H ₁₀ O ₈	2	Methoxycitric acid isomer 1	127.0036; 189.0030	Pubchem: 44149934	8	1.88 ± 0.33
7	1.50	203.0188	(M-H) ⁻	203.0197	-4.47	C ₇ H ₈ O ₇	3	Hibiscus acid monomethyl ester isomer 1	-	²⁹	8	2.13 ± 1.17
8	2.02	203.0196	(M-H) ⁻	203.0197	-0.63	C ₇ H ₈ O ₇	3	Hibiscus acid monomethyl ester isomer 2	-	²⁹	8	1.63 ± 0.48
9	2.21	189.0040	(M-H) ⁻	189.0041	-0.31	C ₆ H ₆ O ₇	1 [#]	Hydroxycitric acid lactone (Hibiscus acid)	127.0039; 87.0072; 111.0066; 57.0347	HMDB0259498	8	1.75 ± 0.43
10	2.22	221.0300	(M-H) ⁻	221.0303	-1.43	C ₇ H ₁₀ O ₈	2	Methoxycitric acid isomer 2	127.0029; 189.0023	Pubchem: 44149934	8	1.75 ± 0.43
11	2.22	127.0036	(M-H) ⁻	127.0037	-0.58	C ₅ H ₄ O ₄	2	5-Hydroxy-2-furoic acid isomer 3	85.0298; 55.0220	HMDB59784	8	1.75 ± 0.43
12	5.45	97.0293	(M-H) ⁻	97.0295	-1.99	C ₅ H ₆ O ₂	3	2-Furanmethanol	-	²⁸		1.00 ± 0.00
13	6.27	235.0458	(M-H) ⁻	235.0459	-0.40	C ₈ H ₁₂ O ₈	2	Hibiscus acid hydroxyethylester	127.0032	³⁰	8	1.50 ± 0.50

14	11.87	194.0457	(M-H) ⁻	194.0432	13.01	C ₆ H ₁₁ O ₇	2	D-Gluconate	151.1002	²⁹	5	0.90 ± 0.20
Average												1.52 ± 0.32

n: number of volunteers in whom the metabolite appears after ingestion of the *H. sabdariffa* encapsulate; RT: retention time; Tmax: time required to reach the relative maximum plasma level (h); Values represent mean ± standard deviation (n = number of volunteers in whom the metabolite appears); * Database or bibliography used for compound annotation. # Mass fragmentation spectrum of the analytical standard in **Figure S1**.

3.3.2 Circulating metabolites detected in plasma related to the intake of *Silybum marianum* extract

Table 2 presents a comprehensive list of 25 metabolites annotated in plasma through an untargeted metabolomics approach in volunteers who consumed *S. marianum* extract. Among the 25 compounds, quercetin glucuronide was identified by comparison with its analytical standard (**Figure S2**). 22 molecular features were annotated at level 2 annotation (comparison of MS/MS spectra), and 2 at level 3 (based on molecular mass and literature search). Consequently, approximately 92% of the annotated compounds achieved at least level 2 of annotation, indicating high annotation reliability.

The main phenolic compounds found in the original SM extract administered to the volunteers include silybin, or any of its isomers such as silychristin, silydianin, isosilybin A, isosilybin B ¹⁴. However, upon observing the presence of metabolites in plasma, it became apparent that these compounds did not appear in their intact form in plasma. Instead, they underwent metabolic transformations. In fact, five isomers of silybin glucuronide, two isomers of silybin sulphate, two isomers of silybin sulphate glucuronide, and three isomers of silybin 3,23-bishemisuccinate were detected. It should be noted that the glucuronidated and sulphated forms of silybin found can be either silybin or any of the isomers silychristin, silydianin, isosilybin A, isosilybin B ¹⁴.

Examining the T_{max} values of the silybin glucuronide isomers, differences in T_{max} values were detected since three isomers exhibit T_{max} values between 1.5 and 2 hours, while the other two showed values around 4.5 and 8.5 h, respectively. These variations in T_{max} values could stem from the specific silybin isomer conjugated to glucuronide. However, the limitation of untargeted metabolomics using HPLC-ESI-QTOF-MS lies in determining

the exact isomer. It is plausible that certain silybin isomers are absorbed more rapidly, allowing for earlier conjugation. This hypothesis finds support in the findings of Marhol et al. (2015), who compared the absorption profiles of different silybin isomers in rats. For instance, they reported a T_{max} of 4.1 hours for silybin A in its free form, whereas for silybin B, it was 1.0 hour³¹. Another explanation could be the site of glucuronidation, as glucuronidation enzymes are expressed in both the liver and small intestine³². Studies conducted on rats indicated that isoflavones exhibited slower intestinal glucuronidation compared to hepatic glucuronidation³³.

In the case of silybin sulphate glucuronide, one of the isomers exhibits a T_{max} of 2.17 ± 0.90 , while the other shows a T_{max} of 4.00 ± 2.62 . This variation could be attributed to the factors discussed previously for the silybin glucuronide results.

Another compound present in the extract and detected in plasma is taxifolin (dihydroquercetin). Besides being found in its native form in plasma, it also appears with various metabolic phase II transformations or conjugations. These include taxifolin sulphate, methyltaxifolin, and methyltaxifolin glucuronide.

These data agree with the study by Lakeev et al. (2023), where they evaluated the metabolism and bioavailability of taxifolin in rats, obtaining T_{max} values of 0.20 ± 0.14 for free taxifolin, 0.12 ± 0.09 for taxifolin sulphate, 0.18 ± 0.12 for methyltaxifolin, and 1.61 ± 1.4 for methyltaxifolin glucuronide, in which the times are shorter due to being rats but appear at early maximum metabolization times, suggesting the rapid metabolization of these compounds³⁹.

Table 2. HPLC-ESI-QTOF-MS identification information and relative nutrikinetic parameters of the metabolites detected in plasma samples after intake of an extract of *S. marianum*.

Peak	RT (min)	Observed m/z	Adduct	Theoretical m/z	Mass error (ppm)	Molecular formula	Level of annotation	Proposed compound	MS/MS fragments	Reference*	n	Tmax
1	11.55	463.0878	(M-H) ⁻	463.0882	-0.79	C ₂₁ H ₂₀ O ₁₂	2	Myricetin rhamnoside	195.8083	³⁴	4	1.00 ± 0.00
2	11.64	493.0983	(M-H) ⁻	493.0988	-0.85	C ₂₂ H ₂₂ O ₁₃	2	Methyl taxifolin glucuronide	317.0665; 195.8111	³⁵	6	1.33 ± 0.47
3	12.02	369.0462	(M-H) ⁻	369.0463	-0.39	C ₁₅ H ₁₄ O ₁₁	2	Caffeoylhydroxycitric acid	191.0232; 135.0456	Pubchem: 24797519	6	1.00 ± 0.00
4	13.02	737.1020	(M-H) ⁻	737.1029	-1.23	C ₃₁ H ₃₀ O ₁₉ S	2	Silybin sulphate glucuronide isomer 1	561.0701; 481.1215; 325.0723	-	6	2.17 ± 0.90
5	13.22	383.0076	(M-H) ⁻	383.0078	-0.51	C ₁₅ H ₁₂ O ₁₀ S	2	Taxifolin sulphate	125.0945	³⁶	8	1.25 ± 0.43
6	13.32	657.1457	(M-H) ⁻	657.1461	-0.70	C ₃₁ H ₃₀ O ₁₆	2	Silybin glucuronide isomer 1	481.1150; 301.0309; 113.0227	³⁷	4	8.50 ± 0.87
7	13.33	477.0649	(M-H) ⁻	477.0675	-5.45	C ₂₁ H ₁₈ O ₁₃	1 [#]	Quercetin glucuronide	301.0348; 151.0044; 178.9993; 113.0248	HMDB0029212	6	1.33 ± 0.47
8	13.46	737.1024	(M-H) ⁻	737.1029	-0.75	C ₃₁ H ₃₀ O ₁₉ S	2	Silybin sulphate glucuronide isomer 2	561.0701; 481.1215; 325.0724	-	7	4.00 ± 2.62
9	13.48	303.0508	(M-H) ⁻	303.0510	-0.88	C ₁₅ H ₁₂ O ₇	2	Taxifolin (Dihydroquercetin)	125.0953	HMDB0303943	6	2.42 ± 2.54
10	13.72	657.1457	(M-H) ⁻	657.1461	-0.56	C ₃₁ H ₃₀ O ₁₆	2	Silybin glucuronide isomer 2	481.1150; 113.0224; 301.0309;	³⁷	8	1.38 ± 0.48
11	13.84	679.1274	(M-H) ⁻	679.1305	-4.46	C ₃₃ H ₂₈ O ₁₆	2	Silybin 3,23-bishemisuccinate isomer 1	551.6985; 116.9318; 262.9476	³⁸	7	2.43 ± 2.32
12	13.98	657.1456	(M-H) ⁻	657.1461	-0.83	C ₃₁ H ₃₀ O ₁₆	2	Silybin glucuronide isomer 3	481.1150; 113.0224; 301.0309;	³⁷	7	4.38 ± 2.34
13	14.16	195.0654	(M-H) ⁻	195.0663	-4.27	C ₁₀ H ₁₂ O ₄	2	Xanthoxylin	160.8413	Pubchem ID: 66654	5	4.60 ± 4.41
14	14.27	657.1455	(M-H) ⁻	657.1461	-0.85	C ₃₁ H ₃₀ O ₁₆	2	Silybin glucuronide isomer 4	481.1150; 113.0224; 301.0309; 113.0224	³⁷	6	1.42 ± 1.17

15	14.34	317.0664	(M-H) ⁻	317.0667	-0.99	C ₁₆ H ₁₄ O ₇	2	Methyltaxifolin	187.0063; 125.0217	³⁵	6	1.17 ± 0.37
16	14.38	287.0531	(M-H) ⁻	287.0561	-10.58	C ₁₅ H ₁₂ O ₆	2	Eriodictyol	251.1362	Pubchem ID: 440735	6	1.25 ± 0.56
17	14.73	679.1276	(M-H) ⁻	679.1305	-4.19	C ₃₃ H ₂₈ O ₁₆	2	Silybin 3,23 bishemisuccinate isomer 2	551.6985; 116.9318; 262.9477	³⁸	5	2.20 ± 0.98
18	14.75	657.1456	(M-H) ⁻	657.1461	-0.80	C ₃₁ H ₃₀ O ₁₆	2	Silybin glucuronide isomer 5	481.1150; 113.0224; 301.0309; 113.0226	³⁷	5	1.80 ± 1.17
19	14.91	561.0704	(M-H) ⁻	561.0708	-0.86	C ₂₅ H ₂₂ O ₁₃ S	2	Silybin sulphate isomer 1	481.1130; 455.1290; 367.1237; 125.0237	³⁷	6	2.17 ± 0.90
20	14.93	679.1278	(M-H) ⁻	679.1305	-3.86	C ₃₃ H ₂₈ O ₁₆	2	Silybin 3,23- bishemisuccinate isomer 3	551.6985; 116.9318; 262.9478	³⁸	4	1.75 ± 0.43
21	15.12	561.0706	(M-H) ⁻	561.0708	-0.48	C ₂₅ H ₂₂ O ₁₃ S	2	Silybin sulphate isomer 2	481.1130; 455.1290; 367.1237; 125.0238	³⁷	7	3.14 ± 2.10
22	15.59	571.0831	(M-H) ⁻	571.0882	-9.01	C ₃₀ H ₂₀ O ₁₂	3	2,3-Dihydro-5',3'''- dihydroxyamentoflavone	-	C00006510	5	1.20 ± 0.40
23	16.73	381.2279	(M-H) ⁻	381.2283	-1.01	C ₂₁ H ₃₄ O ₆	3	Sarcostin	-	Pubchem ID: 46173994	6	1.33 ± 0.47
24	17.27	177.0555	(M-H) ⁻	177.0557	-1.22	C ₁₀ H ₁₀ O ₃	2	3-Methoxycinnamic acid	89.0240; 103.3856; 133.0685	Pubchem ID: 637668	8	4.75 ± 4.09
25	19.96	311.2219	(M-H) ⁻	311.2228	-2.77	C ₁₈ H ₃₂ O ₄	2	8,13-dihydroxy-9,11- octadecadienoic acid	249.2194	Pubchem ID: 5282963	4	7.50 ± 3.28
Average											2.62 ± 1.91	

n: number of volunteers in whom the metabolite appears after ingestion of the *S. marianum* encapsulate; RT: retention time; Tmax: time required to reach the relative maximum plasma level (h); Values represent mean ± standard deviation (n = number of volunteers in whom the metabolite appears); * Database or bibliography used for compound annotation. # Mass fragmentation spectrum of the analytical standard in **Figure S2**.

There are other metabolites present in plasma such as 2-o-caffeoylhydroxycitric acid, quercetin glucuronide or eriodictyol, whose presence is due to different reasons. The presence of 2-o-caffeoylhydroxycitric acid in plasma may be attributed to the chlorogenic acid content in the original extract. Chlorogenic acid consists of an ester of caffeic acid and quinic acid, and 2-o-caffeoylhydroxycitric acid could arise from the degradation of chlorogenic acid. Previous studies have shown that when the original extract contains chlorogenic acid, this compound appears in plasma after ingestion ^{40,41}. The importance of using untargeted metabolomics strategies to search for metabolites that are not easily predictable to appear in this matrix should be emphasised based on these results.

The presence of quercetin glucuronide in plasma can be attributed to the presence of dihydroquercetin (taxifolin) in the extract and in plasma samples. Dihydroquercetin can undergo reduction to yield quercetin, which is subsequently glucuronidated to form quercetin glucuronide. And lastly, the presence of eriodictyol can be attributed to its presence in the ingested extract, allowing it to pass into plasma in its native form.

3.3.3 *Circulating metabolites detected in plasma related to the intake of Theobroma cacao extract*

Table 3 provides a detailed compilation of 3 annotated metabolites detected in plasma via an untargeted metabolomics approach in participants who ingested *T. cacao* extract. Vanillic acid sulphate and trihydroxyoctadecenoic acid were annotated at level 2, while Artonin C was annotated at level 3.

In contrast to the metabolites identified in the ingestion of the two other plant matrices discussed previously, the presence of metabolites following the consumption of *T. cacao* extract is notably lower, with the majority remaining unknown (**Table S1**). Although

these are unknown compounds, this could be considered a future potential advantage of untargeted metabolomics strategies, as they allow us to detect signals related to supplement intake, which would be undetectable by a targeted approach. It is hoped that with the progress of analytical techniques or the expansion of databases these unknowns can be annotated in the future.

The presence of certain metabolites in plasma stands out, such as vanillic acid sulphate, whose appearance can be attributed to the presence of vanillic acid in the original extract consumed by the volunteers. Additionally, the detection of trihydroxyoctadecenoic acid is noteworthy, as it arises from the metabolism of linolenic acid, a fatty acid present in the ingested extract, catalysed by the microbiota ⁴².

The low metabolite content observed in the plasma of volunteers who ingested the TC extract could be attributed to the insufficient richness in phenolic compounds in the extract. The main metabolites that appear in plasma in most previous studies on the bioavailability of cocoa consumption are the flavan-3-ols catechin and epicatechin, or their conjugated forms ^{43–45}, however, in our study we have not been able to identify these compounds in plasma. This may be due to the fact that the original extract used in our study was not sufficiently rich in these flavan-3-ol compounds. In order to verify this hypothesis, these flavan-3-ols present in the TC extract were quantified (see the Supplementary material for the specific details of this quantification). Based on the results of this quantification, it is estimated that each volunteer ingested approximately 0.09 mg and 0.98 mg of catechin and epicatechin, respectively, from the 500 mg capsule of extract.

These compound quantities are significantly lower compared to those consumed in previous reported studies where these bioavailable compounds have been detected in plasma. For example, Ostertag et al. (2012) evaluated the bioavailability of these compounds from flavan-3-ol-enriched dark and standard dark chocolates in a total of 42 healthy subjects ⁴³. They found that both compounds were bioavailable in plasma after the ingestion of 60 g of either flavan-3-ol enriched dark or standard dark chocolate, with levels significantly higher after consuming the enriched one. The amounts ingested of epicatechin and catechin were 257 mg and 53.6 mg for the flavan-3-ol-enriched chocolate, and 84.1 mg and 25.8 mg for the standard dark chocolate, respectively. Thus, it is evident that these values are much higher than those ingested from the extract capsule in our study, which may explain the absence of these metabolites in the plasma samples. Glucuronidated and sulphonated derivatives of these compounds were also detected in plasma in another example reported by Tomas-Barberán et al. (2007), who formulated a flavonoid-enriched cocoa powder and conducted a human trial, administering 196.1 mg of epicatechin and 53.3 mg of catechin. Following the consumption of the flavonoid-enriched cocoa, metabolites such as epicatechin glucuronide and epicatechin sulphate were detected in plasma. In the same study, after the administration of conventional cocoa containing 39.6 mg of epicatechin and 24.2 mg of catechin, no sulphated epicatechin was detected in plasma, and the level of glucuronidated epicatechin was five times lower compared to that after consumption of the enriched cocoa. Therefore, this study illustrates that the quantity of ingested flavan-3-ols determines the presence and concentration of the resulting metabolites ⁴⁴. Another study by Gómez-Juaristi et al. (2019) examined the presence of catechin and epicatechin phase II derivatives in plasma after the consumption of two soluble cocoa

products (15 or 25 g of a conventional or a flavanol-rich product, respectively, dissolved in 200 mL of milk). The presence of these metabolites in plasma was attributed to the consumption of 8.55 mg of epicatechin and 4.8 mg of catechin from the conventional drink, while the enriched drink resulted in a total intake of 28.75 mg of epicatechin and 13.25 mg of catechin. These values show clearly that the amounts ingested in our study are much lower in comparison, suggesting a possible reason for why these derivatives compounds were not detected bioavailable in plasma ⁴⁵.

Furthermore, the format of cocoa consumption in our study (extract) may have influenced flavan-3-ol bioavailability. Schramm et al. (2003) investigated the impact of co-administered foods on catechin and epicatechin absorption from cocoa, demonstrating that concurrent ingestion of bread, sugar, or grapefruit juice affected human bioavailability. Notably, carbohydrate-rich foods significantly increased plasma flavanol levels ⁴⁶. Consequently, most cocoa bioavailability studies in the literature utilise cocoa in the form of chocolate, incorporating it within a matrix that includes carbohydrates such as sugar ⁴⁷.

Therefore, the fact of not having used an extract rich in flavan-3-ols in this intervention study could be the main reason for having detected so few bioavailable compounds for TC. This may be also in line with the fact that untargeted methods have limited sensitivity, meaning that compounds present in very low concentrations cannot be detected. In this context, it is evident that despite the great advantages of untargeted methods such as the detection of unexpected compounds or the simultaneous application to bioavailability studies of multiple matrices, there are also limitations. All this suggests that future studies should be carried out with extracts enriched in phenolic

compounds to increase the probabilities of detecting bioavailable compounds through untargeted methods.

Table 3. HPLC-ESI-QTOF-MS identification information and relative nutrikinetic parameters of the metabolites detected in plasma samples after intake of an extract of *T. cacao*.

Peak	RT (min)	Observed m/z	Adduct	Theoretical m/z	Mass error (ppm)	Molecular formula	Level of annotation	Proposed compound	MS/MS fragments	Reference*	n	Tmax
1	11.66	246.9914	(M-H) ⁻	246.9918	-1.71	C ₈ H ₈ O ₇ S	2	Vanillic acid sulphate	167.0365	HMDB0041788	6	1.00 ± 0.00
2	17.36	329.2334	(M-H) ⁻	329.2333	0.03	C ₁₈ H ₃₄ O ₅	2	Trihydroxyoctadecenoic acid	211.1347; 139.2686	⁴⁸	4	7.63 ± 4.11
3	21.55	677.2423	(M-H) ⁻	677.2392	4.47	C ₄₀ H ₃₈ O ₁₀	3	Artonin C	-	C00008079	4	2.50 ± 2.06
Average												3.71 ± 2.84

n: number of volunteers in whom the metabolite appears after ingestion of the *T. cacao* encapsulate; RT: retention time; Tmax: time required to reach the relative maximum plasma level (h); Values represent mean ± standard deviation (n = number of volunteers in whom the metabolite appears); * Database or bibliography used for compound annotation.

4. Conclusion

This study highlights the potential of untargeted metabolomics for rapid and comprehensive analysis in biological samples of a wide variety of bioavailable or metabolized exogenous compounds derived from the ingestion of different bioactive plant sources.

This untargeted approach enables a visual assessment and comprehensive analysis of biological samples, providing an initial indication of whether further identification is necessary and identifying the samples along the nutrikinetics that are most significant. This approach has been tested with three different matrices (HS, SM and TC) resulting in the annotation of the main metabolites that appear bioavailable after ingestion of the three matrices in an acute assay in humans. It should be noted that some metabolites such as 2-o-caffeoylhydroxycitric acid, quercetin glucuronide or eriodictyol that appear in plasma after SM ingestion could hardly have been obtained by a targeted approach. Thus, this study has been able to annotate the main metabolites without the need to predict the possible routes of metabolism prior to analysis as would have been necessary in a targeted study. Additionally, for a more in-depth analysis, it facilitates the selection of samples for a targeted metabolomics approach, which complements the information obtained.

In this sense, with this research we have proven the potential, but also the limitations, of using this type of untargeted method for bioavailability studies. In fact, we have discovered how the type of bioactive matrix influences the number of metabolites detected by the untargeted method.

Author Contributions

María del Carmen Villegas-Aguilar: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **María de la Luz Cádiz-Gurrea:** Conceptualization, Methodology, Formal analysis, Supervision. **María Herranz-López:** Methodology, Investigation, Data Curation. **Enrique Barraón-Catalán:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **David Arráez-Román:** Resources, Project administration, Funding acquisition. **Álvaro Fernández-Ochoa:** Conceptualization, Methodology, Investigation, Software, Formal analysis, Writing – review & editing, Supervision, **Antonio Segura-Carretero:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

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Supplementary information

Table S1. Identification of phytochemical compounds in *H. sabdariffa* extract by HPLC-ESI-qTOF-MS.

RT (min)	Observed m/z [M-H] ⁻	Theoretical m/z [M-H] ⁻	Mass error (ppm)	Molecular Formula	Level of annotation	Compound name	MS/MS fragments	Relative area	References
0.96	132.0302	132.0302	0.00	C ₄ H ₇ NO ₄	2	Aspartic Acid	88/71	1.7E5	HMDB0000191
0.99	294.0807	294.0831	-8.16	C ₁₀ H ₁₇ NO ₉	2	Fructose-aspartic acid	132/88	1.6E5	¹
1.07	207.0150	207.0146	1.93	C ₆ H ₈ O ₈	2	Hibiscus acid lactone isomer 1	127	1.3E5	²
1.15	207.0159	207.0146	6.28	C ₆ H ₈ O ₈	2	Hibiscus acid lactone isomer 1	127	3.8E4	²
1.32	189.0078	189.0041	19.58	C ₆ H ₆ O ₇	2	Hibiscus acid	127	1.6E6	³
1.66	203.0215	203.0197	8.87	C ₇ H ₈ O ₇	2	Hibiscus acid monomethyl ester isomer 1	142/157/201	1.5E5	⁴
1.82	203.0215	203.0197	8.87	C ₇ H ₈ O ₇	2	Hibiscus acid monomethyl ester isomer 2	142/157/201	3.0E5	⁴
2.63	217.0332	217.0354	-10.14	C ₈ H ₁₀ O ₇	2	Hibiscus acid dimethylester	155/125	2.5E5	⁵
4.02	353.0867	353.0878	-3.12	C ₁₆ H ₁₈ O ₉	2	Chlorogenic acid isomer 1	191/135	7.7E4	HMDB0003164
4.20	297.0266	297.0252	4.71	C ₁₂ H ₁₀ O ₉	2	Methyl gallate derivative isomer 1	125/82	6.2E4	⁶
4.98	297.0266	297.0252	4.71	C ₁₂ H ₁₀ O ₉	2	Methyl gallate derivative isomer 2	125/82	6.9E4	⁶
6.34	353.0867	353.0878	-3.12	C ₁₆ H ₁₈ O ₉	2	Chlorogenic acid isomer 2	191/135	6.3E4	HMDB0003164
6.89	353.0867	353.0878	-3.12	C ₁₆ H ₁₈ O ₉	2	Chlorogenic acid isomer 3	191/135	8.2E4	HMDB0003164
8.56	335.0771	335.0772	-0.30	C ₁₆ H ₁₆ O ₈	2	5-O-Caffeoylshikimic acid	161/191/133	3.4E4	⁵
8.90	367.1030	367.1035	-1.36	C ₁₇ H ₂₀ O ₉	2	3-O-Feruloylquinic acid	135/179	1.4E4	HMDB0030669
9.60	609.1458	609.1461	-0.49	C ₂₇ H ₃₀ O ₁₆	3	Quercetin 3-O-rutinoside	-	4.1E5	HMDB0037934
9.84	463.0884	463.0882	0.43	C ₂₁ H ₂₀ O ₁₂	2	Quercetin 7-glucoside	301/463	1.0E4	HMDB0302151
10.23	539.1751	539.1770	-3.52	C ₂₅ H ₃₂ O ₁₃	3	Oleuropein	-	1.6E4	HMDB0035872
10.96	317.0294	317.0303	-2.84	C ₁₅ H ₁₀ O ₈	2	Myrecetin	151/107	1.2E4	HMDB0002755
11.07	312.1239	312.1241	-0.64	C ₁₈ H ₁₉ NO ₄	2	N-Feruloyltyramine	148/190	1.9E4	Pubchem: 6440659
12.04	522.3276	522.3283	-1.34	C ₂₅ H ₄₉ NO ₁₀	4	Unknown	-	1.1E4	-

12.15	301.0339	301.0354	-4.98	C ₁₅ H ₁₀ O ₇	1	Quercetin	151/121/65	4.3E4	HMDB0005794
12.95	329.0654	329.0667	-3.95	C ₁₇ H ₁₄ O ₇	2	3,7-Dimethylquercetin	285/299/314	2.4E4	HMDB0029263
13.05	299.0538	299.0561	-7.69	C ₁₆ H ₁₂ O ₆	3	Kaempferide	-	2.4E4	Pubchem: 5281666
13.13	394.2945	394.2936	2.28	C ₂₀ H ₄₃ O ₇	4	Unknown	-	2.9E4	-
13.56	343.0821	343.0823	-0.58	C ₁₈ H ₁₆ O ₇	3	Eupatorin isomer 1	-	1.4E4	HMDB0252128
13.61	343.0818	343.0823	-1.46	C ₁₈ H ₁₆ O ₇	3	Eupatorin isomer 2	-	2.8E4	HMDB0252128
13.69	313.0684	313.0718	-10.86	C ₁₇ H ₁₄ O ₆	3	Cirsimaritin	-	2.6E4	Pubchem: 188323
13.92	293.1767	293.1758	3.07	C ₁₇ H ₂₆ O ₄	3	Gingerol	236/221	3.0E4	HMDB0005783
15.74	293.2107	293.2122	-5.12	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 1	-	3.8E4	HMDB0011108
15.81	293.2119	293.2122	-1.02	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 2	-	3.5E4	HMDB0011108
15.85	293.2113	293.2122	-3.07	C ₁₈ H ₃₀ O ₃	3	Hydroxylinolenic acid isomer 3	-	4.7E4	HMDB0011108
16.41	295.2265	295.2279	-4.74	C ₁₈ H ₃₂ O ₃	3	Hydroxylinoleic acid	-	5.5E4	HMDB0247599
18.46	277.2159	277.2173	-5.05	C ₁₈ H ₃₀ O ₂	2	Linolenic acid	205/97	1.2E5	HMDB0001388
18.73	375.2712	375.2752	-10.66	C ₂₀ H ₄₀ O ₆	2	Myristyl glucoside	291/311	3.8E4	Pubchem: 6453025
19.15	279.2328	279.2330	-0.72	C ₁₈ H ₃₂ O ₂	3	Linoleic acid	-	5.8E4	HMDB0000673
19.86	403.3052	403.3065	-3.22	C ₂₂ H ₄₄ O ₆	3	Hexadecyl D-glucoside	-	9.0E4	Pubchem: 171356
19.94	281.2508	281.2486	7.82	C ₁₈ H ₃₄ O ₂	2	Oleic Acid	211/101	2.9E4	HMDB0000207
20.81	443.2490	443.2439	11.51	C ₂₆ H ₃₆ O ₆	3	Bufotalin	-	2.1E4	Pubchem: 12302120
20.97	383.1916	383.1923	-1.83	C ₁₆ H ₃₂ O ₁₀	3	Hexanedioic acid derivative	-	5.6E4	Pubchem: 88032455

RT: Retention Time.

Table S2. Identification of phytochemical compounds in *S. marianum* extract by HPLC-ESI-qTOF-MS.

RT (min)	Observed m/z [M-H] ⁻	Theoretical m/z [M-H] ⁻	Mass error (ppm)	Molecular Formula	Level of annotation	Compound name	MS/MS fragments	Relative area	References
0.80	112.9866	112.9880	-12.39	C ₄ H ₂ O ₄	3	Acetylenedicarboxylic acid	-	1.9E4	HMDB0247933
0.90	134.0479	134.0472	5.22	C ₅ H ₅ N ₅	3	Adenine	-	1.1E4	HMDB0000034
0.99	179.0556	179.0561	-2.79	C ₆ H ₁₂ O ₆	3	Fructose	-	4.4E4	HMDB0000660
1.06	266.0883	266.0894	-4.13	C ₁₀ H ₁₃ N ₅ O ₄	3	Adenosine	-	7.9E2	HMDB0000050
1.09	117.0130	-	-	-	4	Unknown	-	1.5E4	-
1.32	197.8088	-	-	-	4	Unknown	-	2.5E4	-
6.57	303.0492	303.0510	-5.94	C ₁₅ H ₁₂ O ₇	2	Dihydroquercetin	285/125	2.7E4	⁷
6.87	353.0881	353.0878	0.85	C ₁₆ H ₁₈ O ₉	2	Chlorogenic acid	191/161	2.3E4	HMDB0003164
7.75	373.1487	373.1504	-4.56	C ₁₇ H ₂₆ O ₉	2	Deoxyloganin	165/135	6.7E4	Pubchem: 440906
7.93	441.1949	441.1978	-6.57	C ₁₈ H ₃₄ O ₁₂	2	Hexo-Glucose	395/249	4.9E4	Pubchem: 129630443
8.05	505.1915	505.1927	-2.38	C ₂₂ H ₃₄ O ₁₃	2	Oleuropeic acid derivative isomer 1	459/293	3.6E4	⁸
8.41	475.1843	475.1821	4.63	C ₂₁ H ₃₂ O ₁₂	3	Cistanoside E	-	2.5E4	Pubchem: 21632979
8.58	505.1935	505.1927	1.58	C ₂₂ H ₃₄ O ₁₃	2	Oleuropeic acid derivative isomer 2	459/293	3.1E4	⁸
8.92	459.1856	459.1872	-3.48	C ₂₁ H ₃₂ O ₁₁	2	Apiosylepirhododendrin	89/59	8.9E4	Pubchem: 101287073
8.99	163.0389	163.0401	-7.36	C ₉ H ₈ O ₃	2	4-Hydroxycinnamic acid	119	6.3E4	HMDB0002035
9.12	193.0141	193.0142	-0.52	C ₉ H ₆ O ₅	2	Trihydroxycoumarin	137/93	2.7E5	⁹
9.26	481.1161	481.1140	4.36	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 1	125/178	2.6E5	MoNA:VF-NPL-QTOF009680
9.42	455.2130	455.2134	-0.88	C ₁₉ H ₃₆ O ₁₂	3	Alkyl glycoside	-	1.3E5	¹⁰
9.58	303.0512	303.0510	0.66	C ₁₅ H ₁₂ O ₇	2	Dihydroquercetin	285/125	5.1E5	⁷
9.73	607.1081	607.1093	-1.98	C ₃₀ H ₂₄ O ₁₄	2	(Epi)galocatechin-A-(epi)galocatechin	285/303	4.5E5	¹¹
9.82	187.0957	187.0976	-10.16	C ₉ H ₁₆ O ₄	3	Azelaic acid	-	5.3E4	HMDB0000784

9.87	433.1123	433.1140	-3.93	C ₂₁ H ₂₂ O ₁₀	2	Naringenin 4'-O-glucoside	271/151	5.1E4	Pubchem: 42607906
10.12	481.1125	481.1140	-3.12	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 2	125/178	7.0E4	MoNA:VF-NPL-QTOF009680
10.25	483.1293	483.1297	-0.83	C ₂₅ H ₂₄ O ₁₀	2	Silybin hydrogenated	125/151/285	1.4E5	¹²
10.35	813.3162	813.3187	-3.07	C ₃₈ H ₅₄ O ₁₉	2	Tricrocin	767/473	9.1E4	Pubchem: 22833598
10.46	383.1604	383.1612	-2.09	C ₂₁ H ₂₄ N ₂ O ₅	3	Alscomine	-	1.3E5	Pubchem: 11969856
10.54	675.3212	675.3174	5.63	C ₃₉ H ₄₈ O ₁₀	2	Gambogoic acid A	629/293	1.5E5	ChemBK: 1592842-93-7
10.61	481.1120	481.1140	-4.16	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 3	125/178	3.5E5	MoNA:VF-NPL-QTOF009680
10.69	287.0516	287.0561	-15.68	C ₁₅ H ₁₂ O ₆	2	Aromadendrin	125/259	3.8E5	HMDB0030847
10.75	317.0655	317.0667	-3.78	C ₁₆ H ₁₄ O ₇	2	(+)-Dihydroisorhamnetin	245/259	1.7E5	HMDB0037501
10.96	481.1114	481.1140	-5.40	C ₂₅ H ₂₂ O ₁₀	2	Silychrystin isomer 1	151/125	1.8E5	MoNA:RIKENPlaSMA007844
11.00	481.1139	481.1140	-0.21	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 3	125/178	1.7E5	MoNA:VF-NPL-QTOF009680
11.03	481.1150	481.1140	2.08	C ₂₅ H ₂₂ O ₁₀	2	Silychrystin isomer 2	107/125/151/325/463	2.9E5	MoNA:RIKENPlaSMA007844
11.08	481.1146	481.1140	1.25	C ₂₅ H ₂₂ O ₁₀	2	Silychrystin isomer 3	125/151/178/325/355/433	2.4E5	MoNA:MetaboBASE0687
11.54	677.1892	677.1876	2.36	C ₃₅ H ₃₄ O ₁₄	4	Unknown	-	1.5E5	-
11.85	685.3060	685.3077	-2.48	C ₃₃ H ₅₀ O ₁₅	2	Pterocicide B	477/639	6.1E4	Pubchem: 122228272
11.90	659.1765	659.1770	-0.76	C ₃₅ H ₃₂ O ₁₃	2	Phylloflavanine	178/125	1.1E5	¹³
12.06	481.1130	481.1140	-2.08	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 4	125/152/301	1.3E5	MoNA:VF-NPL-QTOF009680
12.12	481.1176	481.1140	7.48	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 5	125/152/301	2.5E5	MoNA:VF-NPL-QTOF009680
12.26	481.1172	481.1140	6.65	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 6	125/152/273	2.6E5	MoNA:VF-NPL-QTOF009680

12.34	481.1158	481.1140	3.74	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 7	125/152/301	3.3E5	MoNA:VF-NPL-QTOF009680
12.44	481.1120	481.1140	-4.16	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 8	125/152/301	2.1E5	MoNA:VF-NPL-QTOF009680
12.48	481.1156	481.1140	3.33	C ₂₅ H ₂₂ O ₁₀	2	Silybin isomer 9	125/152/301	2.2E5	MoNA:VF-NPL-QTOF009680
12.67	381.2269	381.2282	-3.41	C ₂₁ H ₃₄ O ₆	3	Sarcostin	-	6.2E4	Pubchem: 46173994
12.94	477.0837	477.0827	2.10	C ₂₅ H ₁₈ O ₁₀	3	Pradinone I	-	2.0E4	Pubchem: 460846
13.01	477.2489	477.2494	-1.05	C ₂₆ H ₃₈ O ₈	3	Adenanthin B isomer 1	-	3.4E4	¹⁴
13.12	811.1896	811.1938	-5.18	C ₃₅ H ₄₀ O ₂₂	3	Kaempferol 3-[2''-glucosyl-6''-acetylgalactoside] 7-glucoside	-	8.7E4	FDB016214
13.42	989.2502	989.2510	-0.81	C ₅₂ H ₄₆ O ₂₀	4	Unknown	-	2.8E5	-
13.70	523.1249	523.1246	0.57	C ₂₇ H ₂₄ O ₁₁	3	Acetylsilybin A/B	-	1.0E5	Pubchem: 10236891
13.78	477.2522	477.2493	6.08	C ₂₆ H ₃₈ O ₈	3	Adenanthin B isomer 2	-	1.2E5	¹⁴
14.14	479.0975	479.0984	-1.88	C ₂₅ H ₂₀ O ₁₀	2	Dehydrosilybin	125/177/179/283/327/449	6.2E4	HMDB0040513
14.87	627.1569	627.1567	0.32	C ₂₇ H ₃₂ O ₁₇	2	Vitexia-glucoside	152/271/435/463	4.2E4	Pubchem: 56933064
15.02	313.2378	313.2384	-1.92	C ₁₈ H ₃₄ O ₄	3	Octadecanedioic acid	-	4.2E4	Pubchem: 70095
15.61	564.3351	-	-	-	4	Unknown	-	8.3E4	-
15.71	476.2772	476.2780	-1.68	C ₂₇ H ₄₁ O ₇	3	Sinapoyloxypalmitate	-	3.1E4	Pubchem: 90657145
15.83	564.3364	-	-	-	4	Unknown	-	2.4E5	-
16.04	540.3289	540.3304	-2.78	C ₂₉ H ₄₉ O ₉	3	Steroid compound	-	3.7E4	¹⁵
16.30	540.3306	540.3304	0.37	C ₂₉ H ₄₉ O ₉	3	Steroid compound	-	1.6E5	¹⁵
16.37	566.3453	-	-	-	4	Unknown	-	6.0E4	-
16.60	566.3530	-	-	-	4	Unknown	-	2.1E5	-
18.66	591.4117	591.4114	0.51	C ₃₁ H ₆₀ O ₁₀	4	Unknown	-	6.6E4	-
18.73	375.2712	375.2752	-10.66	C ₂₀ H ₄₀ O ₆	2	Myristyl glucoside	291/311	2.9E4	Pubchem: 6453025

19.16	279.2328	279.2330	-0.72	C ₁₈ H ₃₂ O ₂	3	Linoleic acid	-	5.6E4	HMDB0000673
19.74	577.3730	577.3746	-2.77	C ₃₃ H ₅₄ O ₈	3	Timosaponin A	-	4.1E4	¹⁶
19.85	403.3052	403.3065	-3.22	C ₂₂ H ₄₄ O ₆	3	Hexadecyl D-glucoside	-	8.5E4	Pubchem: 171356
19.94	281.2508	281.2486	7.82	C ₁₈ H ₃₄ O ₂	2	Oleic Acid	211/101	6.4E4	HMDB0000207
20.62	605.4040	605.4059	-3.14	C ₃₅ H ₅₈ O ₈	3	Deoxybafilomycin A1	-	3.5E4	CAS: 1883587-79-8
20.98	621.4417	621.4372	7.24	C ₃₆ H ₆₂ O ₈	3	Ginsenoside Rh2	-	2.1E5	Pubchem:119307
21.56	633.4375	633.4371	0.63	C ₃₇ H ₆₂ O ₈	3	Karaviloside III	-	5.4E4	CHEMBL2335924
22.92	371.2592	371.2592	0.00	C ₂₄ H ₃₆ O ₃	3	Pelandjauic acid	-	1.6E5	Pubchem: 178575
24.49	373.2751	373.2748	0.80	C ₂₄ H ₃₈ O ₃	3	Dehydrolithocholic acid	-	1.4E5	Pubchem: 4446994

RT: Retention Time.

Table S3. Identification of phytochemical compounds in *T. cacao* extract by HPLC-ESI-qTOF-MS.

RT (min)	Observed m/z [M-H] ⁻	Theoretical m/z [M-H] ⁻	Mass error (ppm)	Molecular Formula	Level of annotation	Compound name	MS/MS fragments	Relative area	Phenolic content (mg/g dry extract)	References
0.75	272.9512	-	-	-	4	Unknown	-	5.5E4		-
0.91	203.1035	203.1037	-0.98	C ₈ H ₁₆ N ₂ O ₄	2	Valylserine	74/141/173	5.9E4		Pubchem: 18218236
0.92	217.1187	217.1194	-3.22	C ₉ H ₁₈ N ₂ O ₄	2	Leucylserine	74/129/173	7.7E4		Pubchem: 3621685
1.01	195.0519	195.0510	4.61	C ₆ H ₁₂ O ₇	2	Gluconic acid	75/129/177	4.3E5		HMDB0000625
1.13	191.0207	191.0197	5.24	C ₆ H ₈ O ₇	2	Citric acid isomer 1	87/111	3.9E4		HMDB0000094
1.33	191.0228	191.0197	16.23	C ₆ H ₈ O ₇	2	Citric acid isomer 2	87/111	1.8E5		HMDB0000094
1.69	96.9605	96.9601	4.13	H ₂ O ₄ S	3	Sulfate	-	7.1E5		HMDB0001448
2.67	117.0554	117.0557	-2.56	C ₅ H ₁₀ O ₃	2	3-Hydroxyvaleric acid	71/99	6.4E4		HMDB0000531
4.66	294.0606	294.0619	-4.42	C ₁₃ H ₁₃ NO ₇	2	N-caffeoyl-L-aspartate isomer 1	88/132/179	9.7E4		¹⁷
5.05	294.0610	294.0619	-3.06	C ₁₃ H ₁₃ NO ₇	2	N-caffeoyl-L-aspartate isomer 2	88/132/179	1.2E5		¹⁷
5.40	131.0704	131.0714	-7.63	C ₆ H ₁₂ O ₃	2	L-Leucate	85	2.4E5		Pubchem: 69955
5.61	407.1554	407.1559	-1.23	C ₁₇ H ₂₈ O ₁₁	3	Secoiridoid derived	99/263/305	3.1E4		¹⁸
6.01	289.0721	289.0718	1.04	C ₁₅ H ₁₄ O ₆	1	Catechin	161/163/174/192	2.0E4	0.18	HMDB0001871
6.88	278.0670	278.0670	0.00	C ₁₃ H ₁₃ NO ₆	2	L-Aspartic acid. N-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]	71/93/119	1.3E5		¹⁷
7.01	165.0554	165.0557	-1.82	C ₉ H ₁₀ O ₃	2	Dihydro-p-coumaric acid	72/103/147	2.2E5		HMDB0002199
7.60	289.0716	289.0718	-0.69	C ₁₅ H ₁₄ O ₆	2	Epicatechin	161/163/174/192	1.4E5	1.95	HMDB0002780
7.80	275.1006	-	-	-	4	Unknown	-	8.7E4		-
8.01	577.1352	577.1352	0.00	C ₃₀ H ₂₆ O ₁₂	2	Procyanidin B1	-	4.6E4	0.79	¹⁷

8.37	437.2040	437.2028	2.74	C ₁₉ H ₃₄ O ₁₁	2	Ebracteatoside D isomer 1	57/99/161	6.5E4	-
8.61	167.0366	167.0350	9.58	C ₈ H ₈ O ₄	3	Vanillic acid	-	1.0E5	HMDB0000484
8.79	865.1988	865.1985	0.35	C ₄₅ H ₃₈ O ₁₈	2	Procyanidin C	289/577	2.6E4	0.47 ¹⁷
9.18	305.0690	305.0700	-3.28	C ₁₂ H ₁₈ O ₇ S	2	Hydroxy-jasmonic acid sulfate	225/67/59	7.6E5	¹⁹
9.51	437.2057	437.2028	6.63	C ₁₉ H ₃₄ O ₁₁	2	Ebracteatoside D isomer 2	57/99/161	2.2E4	-
9.70	737.1750	737.1723	3.66	C ₃₆ H ₃₄ O ₁₇	2	(Epi)catechin dimer hexose	449/611/53 9/289	1.9E4	¹⁷
9.85	463.0878	463.0882	-0.86	C ₂₁ H ₂₀ O ₁₂	1	Quercetin glucoside	149/242/27 1/285/300/3 35	3.3E4	HMDB0037362
9.93	707.1787	707.1828	-5.80	C ₃₂ H ₃₆ O ₁₈	2	Kalambroside A	449/539/58 1/289	1.9E4	Pubchem: 10349838
9.99	516.2476	516.2450	5.04	C ₂₄ H ₃₉ NO ₁₁	4	Unknown	-	2.8E4	¹⁷
10.18	326.1075	326.1034	12.57	C ₁₈ H ₁₇ NO ₅	2	Deoxyclovamide (N-[(2E)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]-L-tyrosine)	206/282/11 9	1.7E4	¹⁷
10.24	433.0806	433.0776	6.93	C ₂₀ H ₁₈ O ₁₁	2	Quercetin arabinoside	300/271	3.5E4	¹⁷
10.45	238.1091	238.1085	2.52	C ₁₂ H ₁₇ NO ₄	3	Dihydroisoflavipucine	-	1.3E4	Pubchem: 38354263
10.75	272.0935	272.0928	2.57	C ₁₅ H ₁₅ NO ₄	2	L-Thyronine	94/124	1.4E4	HMDB0000667
11.09	421.2064	421.2079	-3.56	C ₁₉ H ₃₄ O ₁₀	2	1-Octen-3-yl primeveroside	57/97/277	1.3E5	HMDB0032960
12.16	301.0339	301.0354	-4.98	C ₁₅ H ₁₀ O ₇	1	Quercetin	151/121/65	1.3E4	HMDB0005794
12.36	391.1196	391.1187	2.30	C ₂₃ H ₂₀ O ₆	2	Dehydrodeguelin	117/62	1.6E4	²⁰
12.46	329.2329	329.2333	-1.21	C ₁₈ H ₃₄ O ₅	2	5,8,12-Trihydroxy-9-octadecenoic acid isomer 1	211	4.1E4	HMDB0030936
12.57	329.2358	329.2333	7.59	C ₁₈ H ₃₄ O ₅	2	5,8,12-Trihydroxy-9-octadecenoic acid isomer 2	211	1.0E4	HMDB0030936
12.79	357.1215	357.1191	6.72	C ₁₆ H ₂₂ O ₉	2	Sweroside	97/198	1.5E5	¹⁷
13.13	394.2954	394.2963	-2.28	C ₂₃ H ₄₁ NO ₄	4	Unknown	-	2.5E4	-
13.47	276.1227	276.1241	-5.07	C ₁₅ H ₁₉ NO ₄	3	Barmumycin	-	4.1E4	²¹

13.92	293.1748	293.1758	-3.41	C ₁₇ H ₂₆ O ₄	2	Gingerol	236/221	2.9E4	HMDB0005783
14.07	333.1815	333.1820	-1.50	C ₁₈ H ₂₆ N ₂ O ₄	3	Hydroxyhuperzine	-	1.9E4	²²
14.20	367.1671	367.1663	2.18	C ₂₁ H ₂₄ N ₂ O ₄	3	3,4-dimethoxy-N-[4-[oxo(1-piperidiny)]methyl]phenyl]benzamide	-	2.3E4	CHEBI:114263
14.77	195.0686	195.0663	11.79	C ₁₀ H ₁₂ O ₄	2	Ethyl vanillate	123	6.9E4	²³
15.61	564.3295	-	-	-	4	Unknown	-	3.1E4	-
15.71	476.2780	476.2780	0.00	C ₂₇ H ₄₁ O ₇	2	Sinapoyloxypalmitate	279	5.1E4	Pubchem: 90657145
15.83	564.3292	-	-	-	4	Unknown	-	1.8E5	-
16.30	540.3288	-	-	-	4	Unknown	-	1.1E5	-
16.60	566.3492	-	-	-	4	Unknown	-	2.3E5	-
17.58	299.2585	299.2592	-2.34	C ₁₈ H ₃₆ O ₃	3	Hydroxyoctadecanoic acid	-	7.3E4	HMDB0112182
17.81	297.2424	297.2435	-3.70	C ₁₈ H ₃₄ O ₃	2	Oxoctadecanoic acid	253/155	6.1E4	HMDB0030981
18.46	277.2159	277.2173	-5.05	C ₁₈ H ₃₀ O ₂	2	Linolenic acid	205/97	6.7E4	HMDB0001388
18.73	375.2712	375.2752	-10.66	C ₂₀ H ₄₀ O ₆	2	Myristyl glucoside	291/311	4.5E4	Pubchem: 6453025
19.16	279.2328	279.2330	-0.72	C ₁₈ H ₃₂ O ₂	3	Linoleic acid	-	1.4E5	HMDB0000673
19.82	255.2325	255.2330	-1.96	C ₁₆ H ₃₂ O ₂	2	Palmitic acid	190/110	1.6E5	HMDB0000220
19.87	403.3052	403.3065	-3.22	C ₂₂ H ₄₄ O ₆	3	Hexadecyl D-glucoside	-	9.9E4	Pubchem: 171356
19.95	281.2482	281.2486	-1.42	C ₁₈ H ₃₄ O ₂	2	Oleic Acid	211/101	1.6E5	HMDB0000207
20.98	383.1934	383.1923	2.87	C ₁₆ H ₃₂ O ₁₀	3	Hexanedioic acid derivative	-	1.2E5	Pubchem: 88032455

RT: Retention Time.

Table S4. Unknown compounds detected in plasma samples obtained after intake of an extract of *H. sabdariffa*, *S. marianum* and *T. cacao*.

RT (min)	<i>H. sabdariffa</i> *	<i>S. marianum</i> *	<i>T. cacao</i> *	Observed m/z	Adduct	Theoretical m/z	Mass error (ppm)	Molecular formula	MS/MS fragments
1.2	8	0	0	83.0139	(M-H) ⁻	83.0139	1.14	C ₄ H ₄ O ₂	-
1.2	6	0	0	503.0761	(M-H) ⁻	503.0772	-2.23	C ₃₀ H ₁₆ O ₈	313.0631; 189.0036
1.4	6	0	0	203.9975	(M-H) ⁻	203.9945	14.46	C ₃ H ₉ O ₈ S	-
2.0	6	0	0	141.0192	(M-H) ⁻	141.0193	-0.65	C ₆ H ₆ O ₄	-
4.1	0	0	7	181.0366	(M-H) ⁻	181.0354	6.63	C ₅ H ₁₀ O ₇	134.0356; 107.0499; 164.0357
5.5	8	0	0	141.0192	(M-H) ⁻	141.0193	-1.05	C ₆ H ₆ O ₄	-
10.4	0	0	8	167.0378	(M-H) ⁻	167.0384	-3.39	C ₅ H ₁₂ O ₄ S	96.9588
11.8	0	5	0	368.0475	(M-H) ⁻	368.0538	-16.93	C ₁₉ H ₁₃ O ₈	175.0267
12.2	0	5	0	833.1774	(M-H) ⁻	-	-	-	-
12.8	0	5	0	833.1775	(M-H) ⁻	-	-	-	-
13.0	0	5	0	245.0487	(M-H) ⁻	245.0489	-1.03	C ₁₀ H ₁₄ O ₅ S	
13.0	0	4	0	368.5492	(M-H) ⁻	-	-	-	280.0324; 113.0260
13.1	0	8	0	368.0477	(M-H) ⁻	368.0538	-16.53	C ₁₉ H ₁₃ O ₈	175.0267
13.2	0	4	0	404.9896	(M-H) ⁻	404.9922	-6.36	C ₁₇ H ₁₀ O ₁₀ S	-
13.4	0	6	0	368.5492	(M-H) ⁻	-	-	-	280.0324; 113.0261
13.4	0	4	0	535.1814	(M-H) ⁻	535.1821	-1.28	C ₂₆ H ₃₂ O ₁₂	175.0222; 113.0299
13.5	0	8	0	368.0477	(M-H) ⁻	368.0538	-16.57	C ₁₉ H ₁₃ O ₈	175.0267
13.5	0	5	0	759.0845	(M-H) ⁻	-	-	-	-
13.5	0	5	0	402.0405	(M-H) ⁻	-	-	-	-
13.5	0	6	0	549.1608	(M-H) ⁻	549.1614	-0.97	C ₂₆ H ₃₀ O ₁₃	459.3905; 174.0624
14.5	0	5	0	549.1016	(M-H) ⁻	-	-	-	371.1065;

14.7	0	5	0	747.1153	(M-H) ⁻	747.1143	1.39	C ₁₉ H ₄₀ O ₂₆ S ₂	-
15.6	0	6	0	549.1015	(M-H) ⁻	-	-	-	371.1065
15.6	0	5	0	503.0954	(M-H) ⁻	503.0984	-5.89	C ₂₇ H ₂₀ O ₁₀	-
15.6	0	4	0	511.2542	(M-H) ⁻	-	-	-	175.0208; 113.0194
15.7	0	6	0	549.1013	(M-H) ⁻	-	-	-	371.1065
15.9	0	7	0	511.2543	(M-H) ⁻	511.2549	-1.08	C ₂₆ H ₄₀ O ₁₀	175.0208; 113.0194
16.0	0	5	0	549.1013	(M-H) ⁻	-	-	-	371.1065
17.1	4	0	0	227.1285	(M-H) ⁻	227.1289	-1.78	C ₁₂ H ₂₀ O ₄	157.1219;182.0693
17.5	0	0	5	326.1222	(M-H) ⁻	-	-	-	-
17.5	0	0	4	416.0911	(M-H) ⁻	-	-	-	240.1475; 295.2614; 159.0587
17.5	0	0	5	348.1038	(M-H) ⁻	348.1037	0.38	C ₁₈ H ₂₁ O ₅ S	-
17.6	0	0	4	511.3020	(M-H) ⁻	-	-	-	-
17.6	0	0	4	788.2430	(M-H) ⁻	-	-	-	-
18.5	0	0	4	314.2404	(M-H) ⁻	-	-	-	-
18.6	0	0	5	435.2759	(M-H) ⁻	-	-	-	-
19.3	0	0	5	495.2574	(M-H) ⁻	-	-	-	287.9892; 147.0447
20.5	0	0	4	634.2370	(M-H) ⁻	-	-	-	-
29.0	5	0	0	489.3554	(M-H) ⁻	489.3585	-6.36	C ₃₀ H ₅₀ O ₅	-
29.3	5	0	0	690.3819	(M-H) ⁻	-	-	-	-
29.4	4	0	0	675.4551	(M-H) ⁻	-	-	-	-
29.5	5	0	0	930.6609	(M-H) ⁻	-	-	-	-
32.7	5	0	0	774.5459	(M-H) ⁻	-	-	-	-

*Number of volunteers in whom the metabolite appears; RT: retention time.

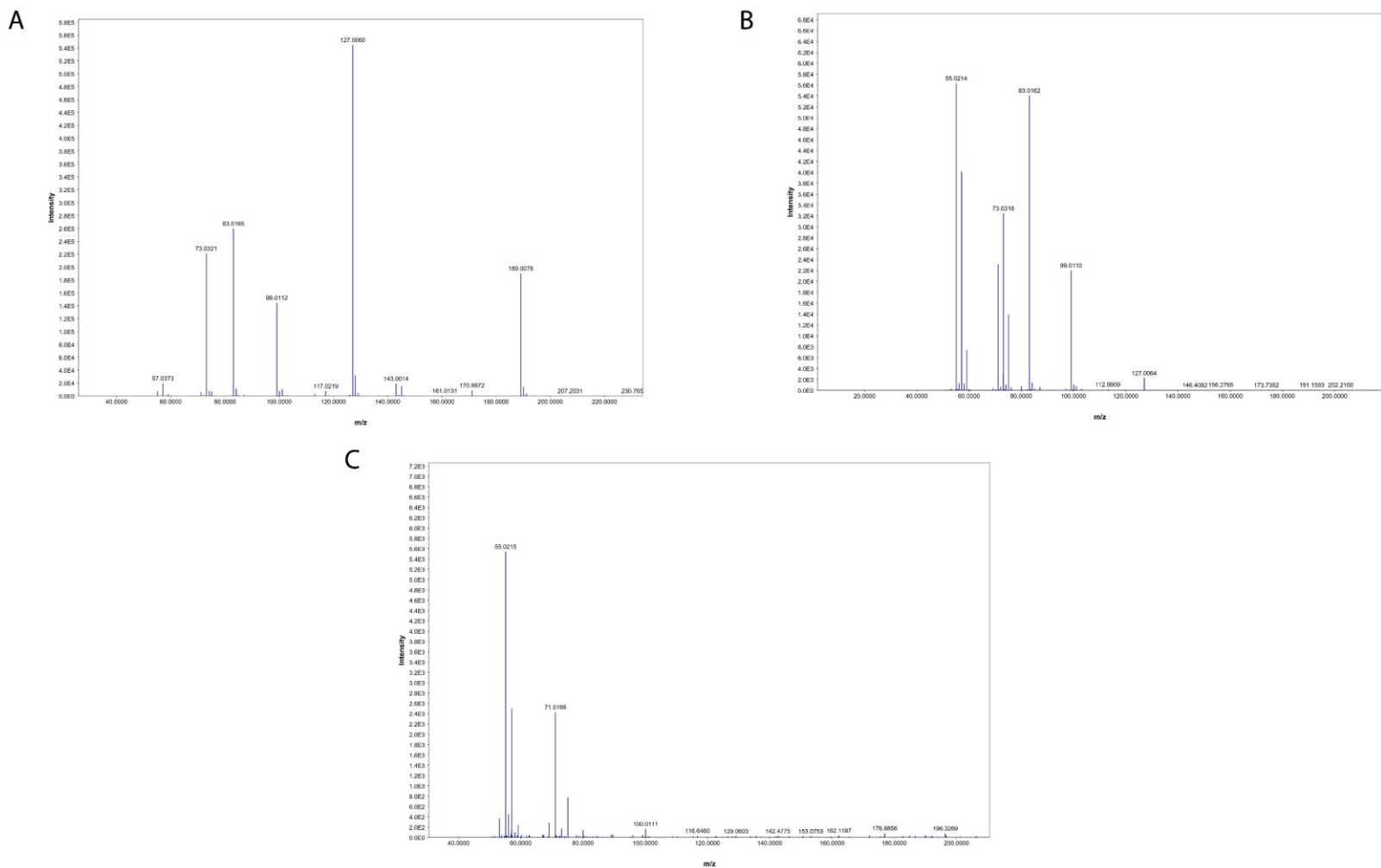


Figure S1. Mass fragmentation spectrum of analytical standard Hydroxycitric acid lactone (Hibiscus acid) (m/z : 189.0078). A: collision energies: 10 eV. B: collision energies: 30 eV. C: collision energies: 60 eV.

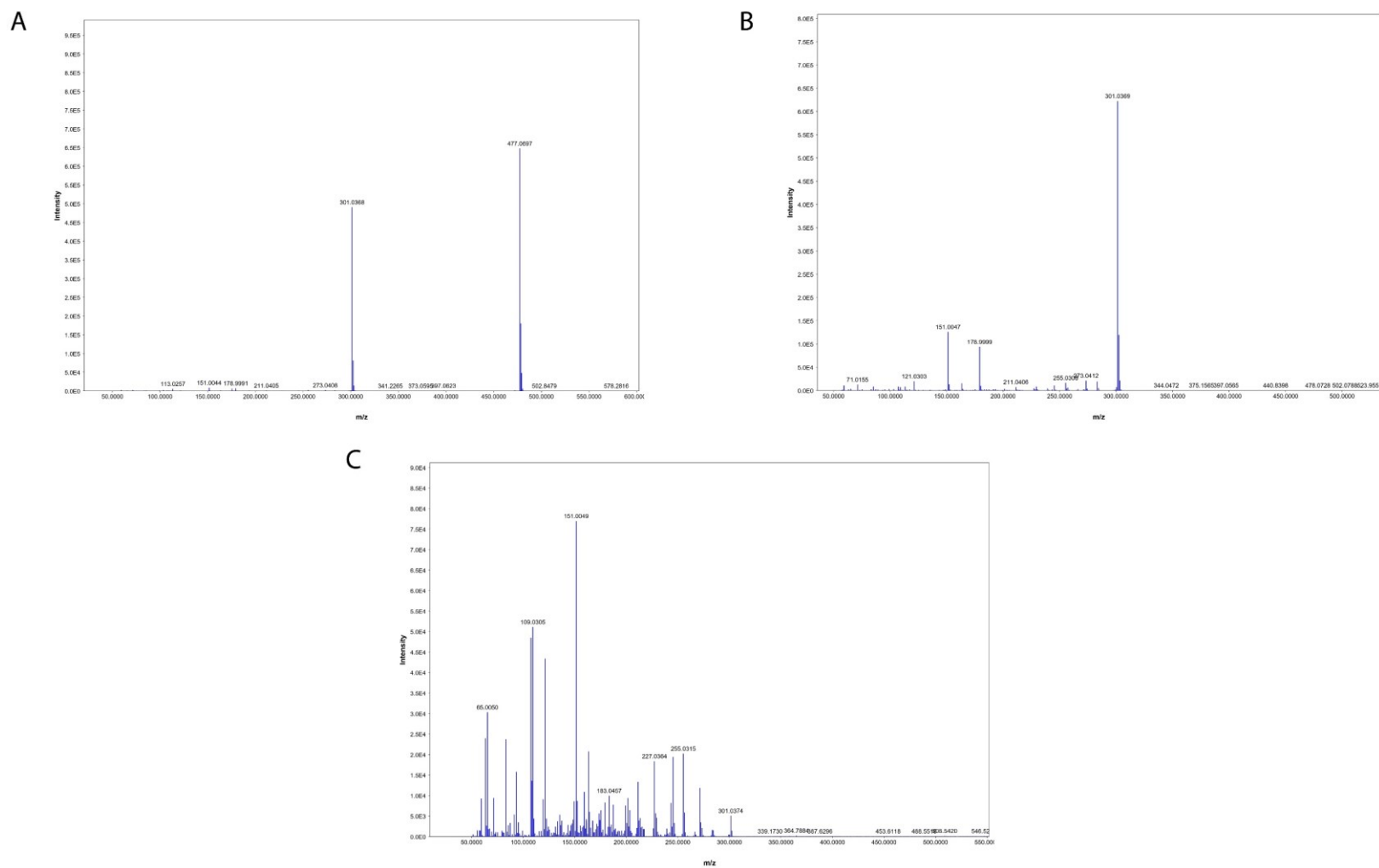


Figure S2. Mass fragmentation spectrum of analytical standard Quercetin glucuronide (m/z : 477.0697). A: collision energies: 10 eV. B: collision energies: 30 eV. C: collision energies: 60 eV.

Quantification of flavan-3-ols and proanthocyanidins by HPLC-ESI-QTOF-MS in *T. cacao* extract

To justify the low number of circulating metabolites detected in plasma samples from the TC extract, the content of catechin and epicatechin in the original extract was quantified, as these compounds have been identified in previous studies as the common bioavailable compounds from the cocoa matrix ^{24–26}. In addition, the quantification of the two proanthocyanidins identified in the cocoa extract has been included, as these compounds are composed of flavan-3-ol monomers. The quantification was performed using the same HPLC-ESI-QTOF-MS method that had been previously used for the qualitative characterization of the extract ²⁷. A calibration curve using the commercial analytical standard of catechin (catechin $\geq 97\%$ from Sigma-Aldrich (Steinheim, Germany)) was used for all compounds. Different standard solution at different concentrations (from 0.39 to 6.25 mg/L) and the TC extract at 1000 mg/L were prepared and analysed by the HPLC-ESI-QTOF-MS. The analytical parameters of the catechin calibration curve are summarised in **Table S5**. Based on the calibration curve and the chromatographic areas for the TC extract, concentrations of 0.18 mg/(g of extract), 1.96 mg/(g of extract), 0.79 mg/(g of extract) and 0.47 mg/(g of extract) were obtained for catechin, epicatechin, procyanidin B1 and procyanidin C, respectively.

Table S5. Standard calibration curve used for the quantification of the compounds of interest in TC extract.

Analytical standard	LOD (mg/L)	LOQ (mg/L)	Calibration Range (mg/L)	Calibration Equation	R ²
Catechin	0.074 $\pm$ 0.004	0.25 $\pm$ 0.01	(LOQ – 6.25)	y = 29638x - 4779.6	0.998

LOD: Limit of Detection; LOQ: Limit of Quantification, R²: Coefficient of variation.

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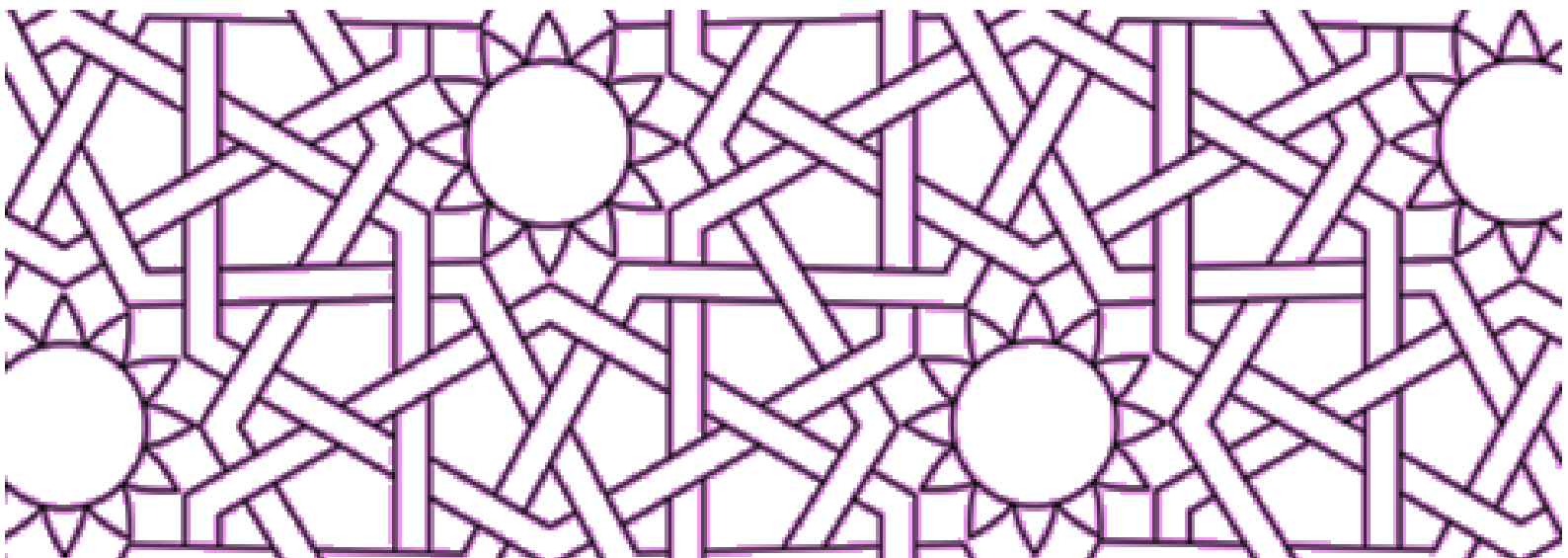
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Chapter 5



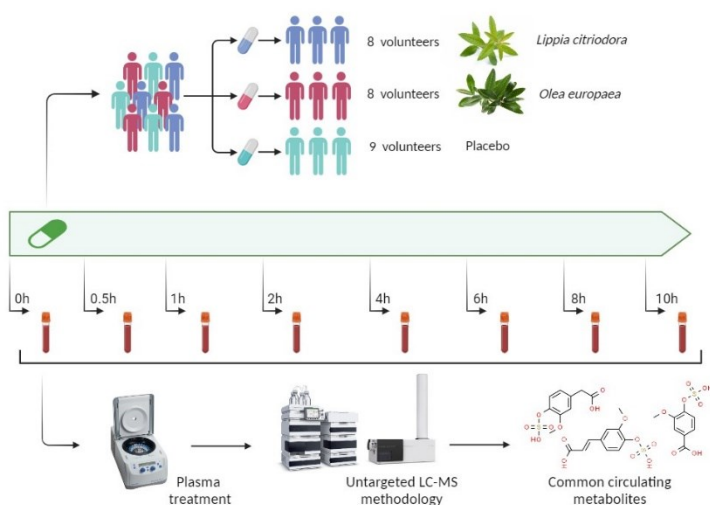
CHAPTER 5

*The application of untargeted metabolomic approaches for the search of common bioavailable metabolites in human plasma samples from *Lippia citriodora* and *Olea europaea* extracts*

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Abstract

Lippia citriodora and *Olea europaea* are known for their shared common bioactivities. Although both matrices are rich in similar families of bioactive compounds, their specific phytochemical compounds are mostly different. Since these compounds can be metabolized in the organism, this study hypothesized that common bioavailable metabolites may contribute to their similar bioactive effects. To test this, an acute double-blind intervention study in humans was conducted, with blood samples collected at multiple time points. Using an untargeted metabolomic approach based on HPLC-ESI-QTOF-MS, 66 circulating metabolites, including 9 common to both extracts, such as homovanillic acid sulphate and glucuronide derivatives, hydroxytyrosol sulphate, etc. These common metabolites displayed significantly different T_{max} values depending on the source, suggesting distinct metabolization pathways for each extract. The study highlights how shared bioavailable metabolites may underlie the similar bioactivities observed between these two plant sources.

Keywords: *Lippia citriodora*; *Olea europaea*; metabolomics; untargeted; bioavailability; metabolism; mass spectrometry; liquid chromatography; bioactive compounds; phenolic compounds.

1. Introduction

Two plant matrices whose phenolic composition has been extensively studied are *Lippia citriodora* (LC) and *Olea europaea* (OE). Both matrices have a common bioactivity, since both species have potent antioxidant ^{1,2}, and anti-inflammatory ^{3,4}, antimicrobial ^{5,6} and anti-tumour properties ^{7,8}. However, although both matrices share similar bioactive properties, their phenolic compositions differ significantly. LC is characterised by a high content of glycosylated phenylpropanoids and iridoids and OE by the presence of secoiridoids ⁹.

The bioactive compounds in both matrices exhibit strong biological activity. The bioactivity of these compounds has traditionally been studied through *in vitro* studies first, and subsequently by *in vivo* assays. However, it is well known that these compounds suffer metabolic reactions before reaching the therapeutic targets ¹⁰. In this regard, ingested phenolic compounds may first be hydrolysed by gastric fluids in the stomach and subsequently metabolised by enzymes in the intestinal cells or by the colonic microbiota ¹¹. Common transformations in such compounds include the removal of sugar moieties, leading to the formation of aglycones from the original compounds¹². In addition, these compounds may undergo phase I and II metabolic biotransformations in the liver. The major phase I metabolic reactions include oxidation and reduction, whereas phase II reactions encompass methylation, sulphation, or glucuronidation ¹². In the literature, there are few studies that have investigated the bioavailability and metabolism of compounds present in LC and OE. These studies have been conducted in rats for LC ^{13,14} and in both rats and humans for OE ^{13,15}. Examples of phase II metabolites derived from compounds detected in LC and OE include hydroxytyrosol-sulphate and hydroxytyrosol-glucuronide ¹⁶. However, there is a lack of research specifically focusing

on the search for bioactive compounds from both plant sources, conducted in either animals or humans.

The methodologies traditionally applied in bioavailability and metabolism studies are based on the use of targeted or semi-targeted metabolomic approaches¹⁷. This type of methodology focuses on the analysis of predefined metabolites from the original composition of the extracts or potential predictions of metabolites derived through known metabolization mechanisms¹⁸. While these approaches allow for the detection and quantification of known metabolites, they are limited in their ability to discover unexpected bioavailable metabolites¹⁹. To overcome this limitation, untargeted metabolomics strategies cover the entire range of detected compounds in the analysis without having a predefined list of candidates. This type of approach has hardly been used in bioavailability and metabolism studies. Although quantification is not possible using untargeted approaches, these methodologies have the great advantage of discovering new or unexpected metabolites^{17,20,21}.

Based on this context, we hypothesize that common bioavailable metabolites from OE and LC may be responsible for their similar bioactive effects. Given that the phytochemical compositions are mostly different, the potential common bioavailable compounds may be unexpected, and therefore, the untargeted metabolomic approaches have great potential to resolve the hypothesis. Identifying these metabolites is crucial, as they can be distributed through the bloodstream to target tissues, where they may exert beneficial biological effects. Then, the present study aimed to detect circulating blood metabolites from LC and OE extracts using an untargeted metabolomics approach applied in an acute nutritional intervention assay in humans.

2. Material and methods

2.1 Chemicals

All solvents used for metabolite analysis were of analytical reagent grade and were used as received. LC-MS grade methanol and formic acid for mobile phases were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany). Water was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Ethanol and methanol (Fisher Scientific Madrid, Spain) for plasma treatment were LC-MS grade. The chemical standards oleuropein ($\geq 98\%$), hydroxytyrosol ($\geq 98\%$) and verbascoside ($\geq 97\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxytyrosol glucuronide ($\geq 98\%$), Homovanillic acid sulphate sodium salt ($\geq 96\%$) and Vanillic Acid 4-Sulphate Sodium Salt ($\geq 97\%$) were purchased from TRC-Canada (Toronto, Ontario, Canada).

2.2 OE and LC extracts

The bioactive extracts were selected based on their composition and bioactive potential, the results of which have been previously published ²². Briefly, pre-industrial extracts of the two plant matrices were provided by NATAC Biotech S.L. (Cáceres, Spain). The extraction parameters were optimised for each plant matrix individually. For the two extracts, a solid-liquid extraction (maceration) was carried out using an EtOH:H₂O mixture (80:20; v:v) for two hours with a solvent:plant ratio of 20:1. Extraction temperatures were set at 45 °C for OE, and 55 °C for LC. The extracts obtained were dried under vacuum, stored at room temperature and protected from light until encapsulation. Considering that most commercial supplements based on these plant matrices range in doses between 250 and 600 mg, the extract was encapsulated in a 500 mg dosage form. The OE and LC extracts were previously qualitatively characterized by high-performance liquid chromatography (Agilent 1290 HPLC, Agilent Technologies, Palo

Alto, CA, USA) coupled to mass spectrometry with a quadrupole time-of-flight analyser (Agilent 6545 QTOF Ultra High Definition, Agilent Technologies, Palo Alto, CA, USA)²². The main compounds of the extracts were quantified in the current study as described in subsection 2.7.

2.3 Subjects and study design

The study protocol was conducted in accordance with the ethical recommendations of the Declaration of Helsinki and was approved by the Ethics Committee of the Miguel Hernández University of Elche and the University Hospital of Elche (Alicante, Spain), reference PI 57/2019. The study was conducted in 25 healthy subjects. The mean age and body mass index of the subjects were 27 ± 9 years and 23 ± 3 kg/m², respectively. The subjects were not taking medication or nutritional supplements, did not suffer from any chronic pathology or gastrointestinal disorder. The sample size was estimated following similar previous bioavailability studies¹⁸. Volunteers gave informed consent prior to participation.

The study was conducted in the facilities of Universidad Miguel Hernández. After an overnight fast, volunteers were divided into the following three groups: a subgroup to evaluate the LC leaf extract (n=8), another for the OE leaf extract (n=8) and the placebo subgroup (n=9). This was a double-blind study in which sample collection and treatment were carried out uniformly across all three subgroups.

A polyphenol-free breakfast was offered 30 minutes after consumption of the encapsulation; 2, 4 and 9 hours later (2.5, 4.5 and 9.5 hours after ingestion of the encapsulated extracts respectively), a polyphenol-free snack and a lunch were also offered to the volunteers. Water was provided ad libitum. Prior to encapsulation

ingestion, a nurse inserted a cannula into the ulnar vein of the non-preferred arm of the volunteers and blood samples were collected in EDTA-coated tubes at baseline ($t = 0$). Subsequently, samples were collected at 0.5, 1, 2, 4, 6, 8 and 10 h after consumption of the 500 mg capsule. Plasma was separated by centrifugation (10 min, 3000 rpm, 4 °C) and stored at -80 °C until further analysis.

2.4 Plasma sample treatment

The plasma samples, which were stored at -80 °C until analysis, were first thawed on ice. For sample treatment, a 100 µl plasma aliquot was mixed with 200 µl methanol:ethanol (50:50, v/v). The mixture was then vortexed for 10 seconds and kept at -20 °C for 30 minutes to achieve adequate protein precipitation. The sample was then centrifuged for 10 minutes at 14 800 r.p.m. and 4 °C. The collected supernatant was evaporated to dryness under vacuum in a centrifugal evaporator (Concentrator Plus, Eppendorf, Hamburg, Germany) for 2 h. The dried residue obtained was reconstituted in 80 µl of the initial mobile phase conditions (0.1% aqueous formic acid:methanol, 95:5, v/v). Finally, a 60 µl aliquot was transferred to an HPLC vial and stored at -80 °C until the day of analysis. A pooled quality control (QC) sample was prepared by mixing equal volumes (20 µl) of each original sample. Several QC aliquots were treated with the same methodology as described for the samples.

2.5 HPLC-ESI-QTOF-MS plasma analysis

Analyses were performed on an Agilent 1260 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate Mass Q-TOF equipped with a dual Jet Stream ESI interface.

A reversed-phase analytical C18 column (Agilent Zorbax Eclipse Plus, 1.8 μm , 4.6 $\times$ 150 mm) with a protective cartridge of the same packing was used. The mobile phases used were water with 0.1 % formic acid (mobile phase A) and methanol (mobile phase B). For efficient separation, the following gradient of these mobile phases was used: 0 min [A:B, 95/5], 5 min (A:B, 90/10), 15 min [A:B, 15/85], 30 min [A:B, 5/95] and 35-40 min [A:B, 95/5]. The column and autosampler compartment temperatures were set at 25 and 4 $^{\circ}\text{C}$, respectively, while the injection volume and flow rate were 2.5 μL and 0.4 mL/min.

Detection was performed in negative ion mode in the range of 50 to 1700 m/z . All spectra were corrected by continuous infusion of two reference masses: TFA anion (m/z 112.985587) and HP-921 (m/z 1033.988109). Both reference ions provided an accurate mass measurement, typically better than 2 ppm.

Ultrapure nitrogen and nebuliser were used as drying gas at temperatures of 200 and 350 $^{\circ}\text{C}$ and flow rates of 10 and 12 L/min, respectively. Other optimised parameters were as follows: fragmenter, 130 V; capillary voltage, +4000 V; nebuliser, 20 psi; nozzle voltage, 500 V; skimmer, 45 V and octopole 1 RF Vpp, 750 V.

For sample analysis, analytical blank samples were analysed at the beginning and at the end of the sequence. Quality control samples were analysed at the beginning of the sequence, after the first blank samples, to equilibrate and stabilise the instrumental conditions. In addition, QCs were also analysed throughout the sequence every 6 biological samples to ensure analytical reproducibility. Samples from different groups (placebo or different supplements) were randomised in the sequences, but different samples from the same volunteer were analysed consecutively. Finally, targeted MS/MS analyses were performed on the signals of interest to acquire fragmentation spectra to

annotate the metabolites. This experiment was performed using nitrogen as collision gas with the following collision energies: 10 eV, 20 eV and 40 eV.

2.6 Data processing and statistical analysis

Firstly, the .d raw data acquired by HPLC-ESI-QTOF-MS were converted to .mzML format using MSConvert (ProteoWizard). For data pre-processing, MZmine 3.9.0 software was used to perform the following stages: mass detection, ADAP Chromatogram Builder, ADAP Chromatogram deconvolution, alignment, isotope grouping and gap filling. The selected noise level was 3.0E2. The following parameters were used for the ADAP chromatogram builder step: intensity threshold 3.0E2; highest minimum intensity 1.0E3; m/z tolerance 20 ppm. For chromatogram deconvolution: S/N threshold 10; minimum peak height 6.0E2; coefficient/area threshold 110; peak duration range 0.00–10.00; RT wavelet width range 0.00–0.10. The chromatograms were aligned using the “Join Aligner” algorithm with an m/z and RT tolerance of 15 ppm and 0.25 min, respectively. After the processing steps, the resulting dataset contained 22598 molecular features, from which the information on the RT, m/z and the corresponding areas of the different samples analysed was obtained. Before applying the selection criteria for significant molecular features, those appearing in the analytical blanks were filtered out from the dataset.

The following data processing steps were conducted using an open-source methodology through a combination of different R packages ²³. For the batch normalization step, missing values (NAs) were imputed by a small value. The batchCorr (v 0.2.5.) package was used to correct the signal intensity drift between and within batches ²⁴. The imputed NAs were subsequently converted back to NAs after batch normalization. Notame R

package was mainly used to filter biologically meaningless signals such as potential contaminants or low detected signals. Thus, the *flag_contaminants* function was used to filter out those molecular features considered as contaminants since they were also detected in blank samples. The *flag_detection* function was also run to exclude the molecular features with low detection rates for the study groups. Subsequently, the applied filters resulted in a refined data matrix containing 6360 molecular features. A principal component analysis (PCA) was performed using MetaboAnalyst 6.0 software to assess reproducibility, check data quality and identify possible outliers. Prior to performing PCA, the data were log-transformed and scaled using Pareto scaling.

Subsequently, to identify statistically significant differences in signals between the experimental groups (placebo vs. olive group; placebo vs. lippia group), linear models with covariate adjustments were applied using the appropriate module in MetaboAnalyst software. The primary metadata included the experimental group, with the placebo group set as the reference. The variable time was fixed as a covariate to account for variations in metabolite levels over time. A p-value cutoff of 0.05 was used, with False Discovery Rate (FDR) correction applied to identify metabolites with significant differences between the groups. Considering mainly the placebo group, and the samples taken for each volunteer at time 0, additional filtering criteria were applied to statistically significant variables to be certain that these signals correspond to exogenous metabolites whose origin is associated with the ingestion of the extract. The criteria associated with these additional filters are detailed below.

1. The molecular features, in which the areas in the samples at baseline (time 0) were higher than the noise level (8 counts), were excluded.

2. Molecular features were selected when the areas of at least one sample from each volunteer's set of 7 plasma samples (0.5, 1, 2, 4, 6, 8 and 10 h), excluding the sample at baseline, were 10 times above the noise level (80 counts).
3. The second condition must be met in a percentage higher than 50% of the volunteers who consumed either the OE and LC extracts. In contrast, this second condition should not be met in any volunteer in the placebo group.

Based on these criteria, a list of molecular features related to the potential circulating metabolites related to the studied extracts was obtained. These selected molecular features were proposed for annotation in the next stage. The annotation was carried out by comparing the accurate mass, isotopic distribution, and MS/MS fragmentation spectra with information available in public databases (METLIN, FoodDB, HMDB, KEGG, Pubchem) and mass banks. CEU Mass Mediator tool was used to search for potential candidates in the different mentioned databases²⁵. MS/MS spectra were also imported into other metabolomics annotation tools, such as Sirius²⁶ or MetFrag (<https://ipb-halle.github.io/MetFrag/>), to search for potential annotated metabolites. Following the identification guidelines proposed by Sumner et al. (2007)²⁷, compounds were annotated as follows: level 1 with commercial standards, level 2 by comparing MS/MS spectra with those in the databases, level 3 based on molecular formulation and MS1 spectra, and level 4 where the molecules remain unidentified.

Metabolite nutrkinetics were studied using PKSolver, an add-in program for pharmacokinetic data analysis in Microsoft Excel²⁸. The time to reach maximum concentration (observed Tmax) was calculated for each significant metabolite. As an untargeted metabolomic approach was used to detect the circulating metabolites, these

were not quantified. Therefore, the Maximum concentration (C_{max}) and area under the curve (AUC) parameters were calculated in a relative way using the deconvoluted chromatographic areas. Statistical analyses to compare these nutrkinetic parameters between the two matrices were performed with GraphPad Prism version 8.01 (GraphPad Software, San Diego, CA, USA). Statistical differences were determined by unpaired t-tests. All *p* values less than 0.05 were considered statistically significant.

2.7 Quantification of potential precursor phenolic compounds of common bioavailable metabolites in OE and LC extracts.

For a better interpretation of the potential common bioavailable metabolites, the phenolic compounds, verbascoside, oleuropein, hydroxytyrosol and their derivatives present in the original extracts were quantified using an analytical method based on a HPLC-ESI-QTOF-MS platform ²². These compounds were quantified using calibration curves prepared with their corresponding analytical standards. Different dilutions of the analytical standards were prepared from a pool mix with a concentration of 100 mM per standard. Log-transformed data were used for the calibration models to adjust the exponential behavior detected between the concentration and peak areas. For each analytical standard, the calibration range, the limits of detection (LOD) and quantification (LOQ) and the coefficient of determination (R^2) were calculated (**Table S1**), showing good linearity ($R^2 > 0.99$). LC and OE extracts at different dilutions (5000, 1000, 100 and 10 mg/L) were analysed by the HPLC-ESI-QTOF-MS method to be able to quantify the phenolic compound present in high and low concentration in the extracts. In the case of hydroxytyrosol- and oleuropein-derived compounds, the quantification of

these compounds was tentatively performed using the calibration models of the hydroxytyrosol and oleuropein standards, respectively.

3. Results and discussion

3.1 Phytochemical composition of *L. citriodora* and *O. europaea* extracts

The bioactive extracts of LC and OE used in the acute intervention study were tentatively characterized by HPLC-ESI-QTOF-MS in a previous study ²². As a summary of the phytochemical composition, 85 and 98 compounds were detected in LC and OE extracts, respectively. In general, the LC extract was characterised by a particularly high content of phenylpropanoids. Within the phenylpropanoid group, verbascoside had the highest content, followed by its isobervascoside isomer. In addition, a high content of iridoids and secoiridoids, such as shanziside and loganic acid, and glycosylated compounds of this type, such as gardoside, were detected. In the OE extract, the content of the glycosylated secoiridoid oleuropein was particularly high, as well as some derivative forms of these compounds such as oleuropein-glucoside, methoxyoleuropein, hydroxyoleuropein and oleuropein aglycone. Other compounds belonging to the flavonoid family, such as quercetin 3-O-rutinoside, were also detected in the OE extract ^{18,29}.

Among the characterised compounds in both extracts, only 10 compounds were annotated in both matrices (**Table 1**). Some of the compounds present in the two matrices were vesbascoside, isoverbascoside, gluconic acid, malic acid and fatty acids such as linolenic acid and linoleic acid. In general, it stands out that these common compounds are more abundant in the extract of LC than in that of OE. The low number of common compounds in the original composition of the plant extracts from both

matrices supports their use to solve the study hypothesis focused on the search for common circulating metabolites originating from different chemical compounds present in the extracts.

Table 1. Common compounds characterized in *L. citriodora* and *O. europaea* extracts.

Peak	Rt (min)	Observed [M-H] ⁻	Mol. Formula	Compounds	Relative area <i>L. citriodora</i> (relative units)	Relative area <i>O. europaea</i> (relative units)
1	1.01	195.0511	C ₆ H ₁₂ O ₇	Gluconic acid	1.3E7	3.1E6
2	1.07	133.0140	C ₄ H ₆ O ₅	Malic acid isomer 1	2.4E6	9.8E6
3	8.19	593.1497	C ₂₇ H ₃₀ O ₁₅	Kaempferol 3-O-rutinoside	7.0E6	4.6E5
4	9.33	623.1978	C ₂₉ H ₃₆ O ₁₅	Verbascoside	2.5E8	4.0E6
5	13.53	307.1920	C ₁₈ H ₂₈ O ₄	Dihydrocapsiate	6.0E6	2.0E5
6	18.46	277.2159	C ₁₈ H ₃₀ O ₂	Linolenic acid	1.5E7	7.0E6
7	18.73	375.2712	C ₂₇ H ₃₆ O	10'-Apo-beta-carotenal	2.4E4	2.2E5
8	19.16	279.2328	C ₁₈ H ₃₂ O ₂	Linoleic acid	6.1E6	3.7E6
9	19.82	255.2325	C ₁₆ H ₃₂ O ₂	Palmitic acid	9.9E6	3.8E6
10	19.95	281.2482	C ₁₈ H ₃₄ O ₂	Oleic Acid	2.7E6	4.7E6

RT: retention time

3.2 Selection of significant signals related to the intake of supplements using an untargeted metabolomic methodology

Blood plasma samples were analysed using an innovative untargeted metabolomics methodology based on HPLC-ESI-QTOF-MS to detect circulating compounds related to the intake of OE and LC extracts. After pre-processing the data with the indicated filters, a total of 6360 molecular features were obtained. To assess data quality, an initial overview of the performance quality was obtained through PCA of the entire dataset, including all QC samples (**Figure S1**). The PCA reveals well-clustered QC samples, indicating good data quality.

Following the application of linear models with covariate adjustments with an FDR of 0.05, a total of 740 signals were found to be statistically significant when comparing the

olive group to the placebo group, while 756 signals were significant in the comparison between the Lippia group and the placebo group. The high number of statistically significant variables is notable; however, it was observed that not all of these were associated with exogenous compounds. This is because the statistical analysis is sensitive to any changes in intensities between the groups, including potential changes in endogenous signals across different groups. Therefore, additional filtering stages were applied to the set of statistically significant signals to ensure that the proposed signals for identification were associated with exogenous metabolites related to the intake of bioactive supplements. Specifically, three additional criteria were applied, based primarily on the samples at t0 and in the placebo groups, as described in the Materials and Methods section. After applying these three filtering criteria, 71 variables associated with exogenous metabolites from the OE extracts and 43 variables from the LC extracts were selected for identification. Nine of these selected molecular features were common between the two matrices. The decrease in the number of signals selected in this last stage stands out, with all those features filtered in the third criterion potentially related to other factors unrelated to the intake of bioactive supplements. However, some molecular features related to the extracts could have been detected in a percentage less than 50% of the volunteers, possibly related to very low concentration compounds, but they were not selected as they were not reproducible in a significant proportion of the volunteers. Taking this criterion into account, it should be noted that the metabolites that appear in the 8 volunteers (100%) among those selected will exhibit greater reproducibility and consequently biological importance, compared to those that appear in a smaller number of volunteers. Then, the selected 106 molecular features were proposed for annotation, of which 66 could be annotated. Among these 66 compounds,

26 and 48 were detected in the volunteers who intake the LC and OE extract (**Table 2** and **Table 3**), respectively. 9 of these 66 were common compounds detected in both LC and OE groups (**Table 4**). Among the 66 annotated compounds, 3 were identified at level 1 through verification with their commercial analytical standards. 46 compounds were annotated at level 2 by comparison of their MS/MS spectra with those reported in public databases or mass banks, and 17 with Level 3 annotation (based on molecular mass and literature search). Consequently, most of the annotated were annotated at least at level 2, indicating high annotation reliability. The remaining 16 compounds were annotated at level 3, indicating less reliability, although the supporting bibliographic data suggests a high likelihood of the indicated compounds due to their relationship with the plant matrices studied. 39 molecular features could not be annotated and were kept as unknown compounds (**Table S2**).

*3.3 Circulating metabolites detected in plasma related to the intake of *Lippia citriodora*.*

Table 2 shows the identification and nutrkinetic parameters of the metabolites detected in the plasma samples of the volunteers who consumed the LC extract. More information is reported in **Table S3** where the means of the chromatographic areas of these compounds for the different times are detailed.

Among these compounds, two isomers of gardoside, a compound that appeared in the original extract, were tentatively identified with MS² fragments m/z 211.0607 ([M-glucose]⁻) and 122.8951 ([M-glucose-88]⁻), suggesting that this compound could be absorbed in its native form. In a study in which LC extract was administered to rats, the presence of gardoside in its native form in plasma was also observed, which is

consistent with our results in humans ¹³. It is noteworthy that verbascoside, despite being one of the major compounds characterized in the LC extract, has not been detected in plasma samples either in its native form or in its possible phase II metabolites. However, compounds resulting from the cleavage of verbascoside and with modifications resulting from phase II metabolic reactions have been detected. These compounds are caffeic acid 4-sulphate (with MS² fragments m/z 179.0352 (C₉H₇O₄) and 135.0447(C₈H₇O₂)), ferulic acid 4-O-glucuronide (with MS² fragments m/z 193.0510 (C₁₀H₉O₄), 235.9254 (C₁₂H₁₁O₅) and 175.0228 (C₁₀H₇O₃)) and vanillic acid 4-O-sulphate (with MS² fragments m/z 123.0425 (C₇H₇O₂), 167.0359 (C₈H₇O₄), 78.9581 (C₅H₃O) and 96.9533 (HO₄S)). Although these three metabolites could potentially derive from their respective acids (caffeic, ferulic, and vanillic acids), these acids are not present in the original composition of the LC extract. This rules out this possible origin and indicates that their presence is likely related to the metabolization of verbascoside. Verbascoside is easily fragmented into caffeic acid ³⁰, and from this by methylation modification or fragmentation can be derived to hydroxytyrosol, ferulic acid and vanillic acid ^{31–33} (**Figure 1**). Some of these compounds, such as caffeic acid, which has been identified in its sulphate form, and the glucuronide forms of ferulic, have been identified in previous bioavailability studies in rats. This consistency aligns with the results obtained in the current study. ¹³.

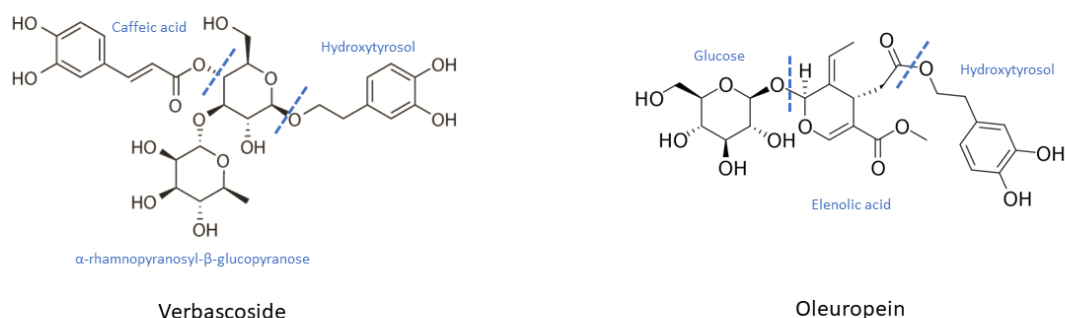


Figure 1. Chemical structure of verbascoside and oleuropein.

Similar observations apply to other compounds, like various isomers of abscisic alcohol 11-glucoside (with MS² fragments m/z 75.0075 (C₂H₃O₃), 55.0206 (C₄H₇) and 307.1387 (C₁₇H₂₃O₅)), which appear in plasma but were not present in the original LC extract. These compounds found in plasma may originate from the fragmentation of other compounds. In the case of abscisic alcohol 11-glucoside, it may also represent a fragment derived from the metabolism of more complex phenolic compounds, a phenomenon observed in other studies involving the consumption of foods rich in phenolic compounds ³⁴.

When the observed Tmax of the different metabolites present in plasma is observed, it can be seen that in the case of the volunteers who ingested LC (**Table 2**), compounds such as gardoside isomers, caffeic acid 4-sulphate isomers and ferulic acid 4-sulphate show a maximum observed Tmax around 2-3 hours after ingestion of the extract. However, other compounds such as hydroxytyrosol sulphate isomer 2, vanillic acid 4-o-sulphate isomer 1, hydroxytyrosol glucuronide isomer 2, homovanillyl alcohol sulphate, 3-methylcatechol, two isomers of homovanillic acid sulphate and homovanillic acid glucuronide have the highest observed Tmax values between 8 and 10 hours after

ingestion of the extract. This may be explained by the fact that the compounds that appear earlier (at 2 hours) are compounds that appear after fewer metabolic reactions. In this sense verbascoside is split into caffeic acid and hydroxytyrosol in phase I metabolism³⁵ and in phase II metabolism these compounds are sulphated. In the case of gardoside, it is found in the original extract and therefore directly undergoes the sulphation reaction. Caffeic acid is easily transformed into ferulic acid by the enzyme caffeate O-methyltransferase and then passed on³⁶. Most of the hydroxytyrosol is transformed into the sulphated form, as the AUCt is about ten times higher at the peak compared to the glucuronidated form, which is also excreted much later. These results are in agreement with those obtained in other study in which they evaluated oral bioavailability and metabolism of hydroxytyrosol from food supplements and found that hydroxytyrosol in its sulphated form was more than 10 times higher than the glucuronidated form³⁷.

The other compounds that show the observed Tmax after 6-8 hours after ingestion undergo more metabolic transformations, so it is justified that they take longer to appear. For example, homovanillyl alcohol sulphate (with MS² fragments m/z 167.0706 (C₉H₁₁O₃) and 137.3427 (C₈H₉O₂)), can appear in plasma from hydroxytyrosol, which by catechol-O-methyl transferase gives rise to homovanillyl alcohol and sulfotransferase gives rise to homovanillyl alcohol sulphate (**Figure 2**). In the case of homovanillic acid sulphate and homovanillic acid glucuronide, hydroxytyrosol via aldehyde dehydrogenase gives rise to 3,4-dihydroxyphenylacetaldehyde (DOPAL) and this compound via alcohol dehydrogenase gives rise to 3,4-dihydroxyphenylacetic acid (DOPAC) which is finally transformed to homovanillic acid by catechol-O-methyl transferase. Finally, this homovanillic acid may undergo sulphation or glucuronidation in phase II metabolism.

Moreover, vanillic acid can be formed from ferulic acid by transformation to vanillin and then to vanillic acid, by oxidation reactions or it can be transformed by bacteria present in the microbiota such as *Paraburkholderia aromaticivora* via vanillin^{38,39}.

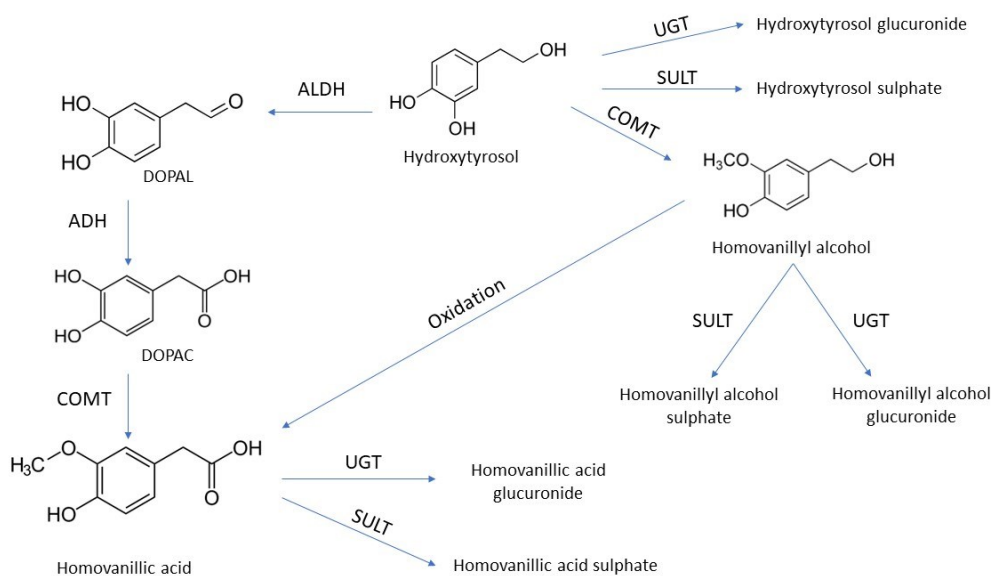


Figure 2. Hydroxytyrosol metabolic pathways. ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; COMT: catechol-O-methyl transferase; DOPAC: 3,4-dihydroxyphenylacetic acid; DOPAL: 3,4-dihydroxyphenylacetaldehyde; SULT: sulfotransferase; UGT: UDP-glucuronosyl transferase.

Table 2. HPLC-ESI-QTOF-MS identification information and relative nutrikinetic parameters of the metabolites detected in plasma samples after intake of an extract of *L. citriodora*.

RT (min)	[M-H] ⁻	Molecular formula	FDR	Proposed compound	Level of annotation	MS/MS fragments	Reference*	n	C _{max}	Observed T _{max}	AUC _t
6.53	233.0122	C ₈ H ₁₀ O ₆ S	6.41E-06	Hydroxytyrosol sulphate isomer 2	2	153.0597, 123.0441, 96.9549	⁴⁰	7	902 ± 428	8 ± 2	3802 ± 2564
7.25	246.9916	C ₈ H ₈ O ₇ S	2.31E-06	Vanillic acid 4-O-sulphate isomer 1	1 ^a	123.0425, 167.0359, 78.9581, 96.9533	HMDB0041788	7	874 ± 644	8 ± 3	3576 ± 3122
8.92	329.0875	C ₁₄ H ₁₈ O ₉	4.90E-02	Hydroxytyrosol glucuronide isomer 2	1 ^b	153.0543, 123.0435, 95.0112	⁴⁰	4	106 ± 17	8 ± 2	350 ± 158
9.24	247.0279	C ₉ H ₁₂ O ₆ S	4.80E-02	Homovanillyl alcohol sulphate	2	167.0706, 137.3427	⁴⁰	4	156 ± 49	7 ± 2	437 ± 278
9.26	123.0450	C ₇ H ₈ O ₂	3.90E-02	3-methylcatechol	2	121.0305	⁴⁰	6	169 ± 59	8 ± 3	565 ± 407
9.69	373.1128	C ₁₆ H ₂₂ O ₁₀	3.07E-08	Gardoside isomer 1	2	211.0607, 122.8951	⁴¹	8	211 ± 72	2 ± 0	608 ± 375
9.81	261.0075	C ₉ H ₁₀ O ₇ S	2.06E-05	Homovanillic acid sulphate isomer 1	1 ^c	181.0494, 79.9585, 137.0609, 217.1051	HMDB0011719	7	341 ± 164	9 ± 2	1391 ± 1144
10.29	261.0071	C ₉ H ₁₀ O ₇ S	1.89E-02	Homovanillic acid sulphate isomer 2	2	181.0495, 79.9587, 137.0619, 217.1055	HMDB0011719	5	476 ± 363	9 ± 2	1771 ± 1599
10.54	357.082	C ₁₅ H ₁₈ O ₁₀	6.99E-04	Homovanillic acid glucuronide	2	181.0501, 313.0923	⁴⁰	6	360 ± 219	8 ± 2	1345 ± 1042
10.85	258.9912	C ₉ H ₈ O ₇ S	1.89E-09	Caffeic acid 4-sulphate isomer 1	2	179.0352, 96.9537, 135.0447	HMDB0041708	7	325 ± 147	3 ± 1	1157 ± 757
11.28	258.9924	C ₉ H ₈ O ₇ S	1.67E-07	Caffeic acid 4-sulphate isomer 2	2	179.0350, 96.953, 135.0452	HMDB0041708	6	357 ± 268	3 ± 2	1656 ± 1922
11.58	273.0049	C ₁₀ H ₁₀ O ₇ S	1.27E-08	Ferulic acid 4-sulphate	2	193.0506, 96.9602	HMDB0240716	7	354 ± 254	3 ± 2	1752 ± 1781
12.37	369.0839	C ₁₆ H ₁₈ O ₁₀	4.70E-04	Ferulic acid 4-O-glucuronide	2	193.0510, 235.9254, 175.0228	HMDB0041733	5	282 ± 358	5 ± 2	1304 ± 1975
12.43	201.1130	C ₁₀ H ₁₈ O ₄	7.34E-03	Sebacic acid Isomer	3	-	HMDB000079	5	119 ± 27	1.2 ± 0.4	141 ± 86
12.55	246.9907	C ₈ H ₈ O ₇ S	7.81E-03	Vanillic acid 4-O-sulphate isomer 2	2	123.0423, 167.0356, 96.9529	HMDB0041788	5	119 ± 41	1.2 ± 0.4	152 ± 118

13.75	373.1132	C ₁₆ H ₂₂ O ₁₀	4.55E-29	Gardoside isomer 2	2	211.0615, 122.8953	⁴¹	6	508 ± 110	2.3 ± 0.7	2015 ± 547
14.16	411.2032	C ₂₁ H ₃₂ O ₈	2.14E-04	Abscisic alcohol 11-glucoside isomer 1	2	75.0075, 55.0206, 307.1387	HMDB0039636	6	202 ± 82	1.2 ± 0.4	259 ± 134
16.12	343.1388	C ₁₆ H ₂₄ O ₈	7.07E-04	Dihydroconiferin isomer 1	2	181.0870	Pubchem: 14427336	6	139 ± 19	0.9 ± 0.2	126 ± 28
16.32	291.0863	C ₁₅ H ₁₆ O ₆	1.94E-04	Picrotoxinin	3	-	Pubchem: 442292	6	136 ± 44	1.3 ± 0.5	231 ± 102
16.43	343.1390	C ₁₆ H ₂₄ O ₈	1.84E-04	Dihydroconiferin isomer 2	2	181.0873	Pubchem: 14427336	5	136 ± 28	1 ± 0	244 ± 102
16.75	411.2010	C ₂₁ H ₃₂ O ₈	3.32E-09	Abscisic alcohol 11-glucoside isomer 2	2	75.0073, 55.0205, 307.1389	HMDB0039636	8	562 ± 251	2 ± 1	757 ± 174
17.44	411.2008	C ₂₁ H ₃₂ O ₈	4.52E-05	Abscisic alcohol 11-glucoside isomer 3	2	75.0069, 307.1393	HMDB0039636	7	258 ± 95	1 ± 0	267 ± 94
17.92	393.1899	C ₂₁ H ₃₀ O ₇	5.48E-03	Pteroside Z isomer 1	2	231.1387, 177.0312	HMDB32587	6	120 ± 22	1.2 ± 0.4	123 ± 53
18.05	393.1909	C ₂₁ H ₃₀ O ₇	4.43E-07	Pteroside Z isomer 2	2	231.1385	HMDB32587	8	181 ± 58	2 ± 1	310 ± 84
18.38	395.2051	C ₂₁ H ₃₂ O ₇	1.47E-08	Isopetasoside	3	-	HMDB29622	8	455 ± 162	2 ± 1	722 ± 166
19.17	293.2116	C ₁₈ H ₃₀ O ₃	1.41E-11	17-Hydroxylinolenic acid	3	-	HMDB00111	8	335 ± 306	6 ± 4	1621 ± 1914
Average									307 ± 210	4 ± 3	997 ± 970

n: number of volunteers in whom the metabolite appears after ingestion of the *L. citriodora* extract; RT: retention time; Cmax: relative maximum plasma level (relative chromatographic area); Observed Tmax: time required to reach Cmax (h); AUCt: area under the zero-moment curve (relative chromatographic area /h); Values represent mean ± standard deviation (n = number of volunteers in whom the metabolite appears);* Database or bibliography used for compound annotation. ^a Mass fragmentation spectrum of the analytical standard in **Figure S2**. ^b Mass fragmentation spectrum of the analytical standard in **Figure S3**. ^c Mass fragmentation spectrum of the analytical standard in **Figure S4**.

3.4 Circulating metabolites detected in plasma related to the intake of *Olea europaea*.

Table 3 shows the identification and nutrkinetic parameters of metabolites detected in plasma samples obtained after ingestion of an extract of OE. In addition, **Table S4** present the mean values of the chromatographic areas for each compound detected in *OE* group.

Although oleuropein is the major compound in the original extract, it was detected as significant in plasma samples. In this case the explanation lies in the fact that the compound was detected in plasma but it was also detected in the volunteers in the placebo group, and therefore it was not selected as significant. This fact could be explained by the fact that olive oil is very consumed in the Mediterranean region, where the study was carried out⁴². Therefore, oleuropein in plasma of all volunteers was found at basal levels, well below the values found by the consumption of the extract. In the case of the compounds presents only in plasma samples from volunteers who ingested OE, the following different isomers of four oleuropein-derived structures were detected: four isomers of hydroxyoleuropein, three isomers of oleuropein aglycone glucuronide, two isomers of 10-hydroxy-7-methoxyoleuropein, and three isomers of methoxyoleuropein. These oleuropein-derived compounds result from enzymatic hydrolysis reactions and metabolic phase I and II reactions. Some of these compounds have also detected in humans due to the consumption of olive oil, such as oleuropein aglycone glucuronide ⁴⁰.

Hydroxytyrosol, one of the most characteristic phenolic compounds in olive leaves, was also detected in plasma samples of the volunteers who consumed the OE extract. The presence of hydroxytyrosol in plasma confirms the bioavailability of this compound. It is intriguing to note that the extensive array of bioactive properties associated with this

compound can be exerted *in vivo* within an organism⁴³. In addition, some metabolised compounds derived from hydroxytyrosol were also detected in plasma, such as hydroxytyrosol glucuronide (with MS² fragments m/z 153.0541 (C₈H₉O₃), 123.0433 (C₇H₇O₂) and 95.0115 (C₆H₇O)) and hydroxytyrosol sulphate (with MS² fragments m/z 153.0599 (C₈H₉O₃), 123.0443 (C₇H₇O₂) and 96.9557 (HO₄S)). This compound may come either from the native form present in the extract or from the oleuropein-derived form, as it is part of its structure and by enzymatic hydrolysis can give rise to the free compound⁴⁴ (**Figure 1**).

Two isomers of homovanillyl alcohol glucuronide (with MS² fragments m/z 167.0614 (C₉H₁₁O₃) and 135.0423 (C₈H₇O₂)) were also detected in plasma. This compound is a metabolite derived from hydroxytyrosol (**Figure 2**). This form without the glucuronide⁴⁵ has been detected in other studies in rats focused on the bioavailability of the metabolites of hydroxytyrosol. Therefore, it is worth noting that in our study in humans, the homovanil alcohol form does not appear but rather its glucuronide form in volunteers who ingested OE. Another metabolite derived from phase II metabolism that appeared in plasma samples was kaempferol sulphate (with MS² fragments m/z 151.0037 (C₇H₃O₄) and 96.9557 (HO₄S)). This metabolite is the result of the metabolic sulfation reaction of kaempferol, which is a compound characterized in the original extract.

When attention is paid to the observed T_{max} of the metabolites that appeared in the volunteers who ingested OE, as opposed to what happens with the metabolites in LC, most of them show their observed T_{max} in the time period of 1 to 2 hours after the ingestion of the extract. This may be because the main metabolites that appear either

were in the original extract as different isomers of hydroxyoleuropein and methoxyoleuropein or compounds that were in the extract and undergo direct phase II metabolic reactions; for example, both oleuropein aglycone and hydroxytyrosol are in the original extract so the sulphated and glucuronidated forms of these that appear in plasma may be the product of these direct reactions.

When examining the mean values for all C_{max} , observed T_{max} , and AUC_t for the metabolites resulting from the ingestion of both extracts (**Table 2** and **Table 3**), it is generally observed that the mean values for these three variables are 307 ± 210 ; 4 ± 3 ; and 997 ± 970 , respectively, for LC, while for OE, they are 392 ± 417 ; 2 ± 1 ; and 1072 ± 1208 , respectively. After conducting a T-Student test, it was noted that only in the case of observed T_{max} were the mean values significantly different. Therefore, similar to the metabolites shared by both matrices, there is an observed trend for metabolites from the LC extract to manifest approximately two hours later on average compared to those from OE.

Table 3. HPLC-ESI-QTOF-MS identification information and relative nutrikinetic parameters of the metabolites detected in plasma samples after intake of an extract of *O.europaea*.

RT (min)	[M-H] ⁻	Molecular formula	FDR	Proposed compound	Level of annotation	MS/MS fragments	Reference*	n	C _{max}	Observed T _{max}	AUC _t
6.41	233.0124	C ₈ H ₁₀ O ₆ S	9.53E-06	Hydroxytyrosol sulphate isomer 1	2	153.0599, 123.0443, 96.9557	⁴⁰	6	593 ± 382	3 ± 3	1755 ± 1392
6.53	233.0122	C ₈ H ₁₀ O ₆ S	2.60E-12	Hydroxytyrosol sulphate isomer 2	2	153.0597, 123.0441, 96.9549	⁴⁰	8	836 ± 359	3 ± 3	2914 ± 1792
7.25	246.9916	C ₈ H ₈ O ₇ S	2.81E-18	Vanillic acid 4-O-sulphate isomer 1	1 ^a	123.0425, 167.0359, 78.9581, 96.9533	HMDB0041788	8	489 ± 147	1.0 ± 0.0	2361 ± 729
8.65	329.0875	C ₁₄ H ₁₈ O ₉	1.19E-23	Hydroxytyrosol glucuronide isomer 1	2	153.0541, 123.0433, 95.0115	⁴⁰	8	1002 ± 411	0.9 ± 0.2	2266 ± 993
8.92	329.0875	C ₁₄ H ₁₈ O ₉	1.74E-34	Hydroxytyrosol glucuronide isomer 2	1 ^b	153.0543, 123.0435, 95.0112	⁴⁰	8	2627 ± 1167	0.9 ± 0.2	6846 ± 2440
8.93	351.0697	C ₁₆ H ₁₆ O ₉	3.48E-12	Chlorogenoquinone	2	175.0318	HMDB0029383	8	324 ± 7	0.9 ± 0.2	605 ± 254
9.24	247.0279	C ₉ H ₁₂ O ₆ S	4.59E-05	Homovanillyl alcohol sulphate	2	167.0706, 137.3427	⁴⁰	8	135 ± 32	2.0 ± 0.9	360 ± 155
9.26	123.0450	C ₇ H ₈ O ₂	1.09E-05	3-methylcatechol	2	121.0305	⁴⁰	7	307 ± 160	0.6 ± 0.2	585 ± 562
9.75	343.0686	C ₁₄ H ₁₆ O ₁₀	2.76E-04	Vanillic acid glucuronide	2	255.0046	HMDB0060024	7	111 ± 48	4 ± 3	351 ± 232
9.81	261.0075	C ₉ H ₁₀ O ₇ S	5.94E-16	Homovanillic acid sulphate isomer 1	1 ^c	181.0494, 79.9585, 137.0609, 217.1051	HMDB0011719	8	233 ± 69	3 ± 2	1338 ± 674
10.10	343.1033	C ₁₅ H ₂₀ O ₉	4.27E-23	Homovanillyl alcohol glucuronide isomer 1	2	167.0614, 135.0423	HMDB0240527	8	504 ± 154	1.1 ± 0.3	1623 ± 620
10.28	259.0819	C ₁₁ H ₁₆ O ₇	1.98E-14	3-Furanmethanol glucoside isomer 1	2	165.0562, 121.0648, 241.0689, 95.0823	HMDB0032924	8	236 ± 104	1.6 ± 0.5	688 ± 337
10.29	261.0071	C ₉ H ₁₀ O ₇ S	3.73E-02	Homovanillic acid sulphate isomer 2	2	181.0495, 79.9587, 137.0619, 217.1055	HMDB0011719	8	215 ± 68	4 ± 2	1484 ± 597
10.52	181.0867	C ₁₀ H ₁₄ O ₃	2.57E-10	1,2,3-trimethoxy-5-methyl benzene	3	-	⁴⁶	8	221 ± 72	1 ± 0	384 ± 150
10.53	149.0604	C ₉ H ₁₀ O ₂	4.26E-08	p-Vinylguaiaicol	3	-	⁴⁷	8	184 ± 45	1 ± 0	320 ± 138
10.54	225.0753	C ₁₁ H ₁₄ O ₅	1.23E-04	Desoxy elenolic acid	3	-	⁴⁸	6	124 ± 24	1.1 ± 0.4	174 ± 85

10.54	357.0820	C ₁₅ H ₁₈ O ₁₀	6.65E-08	Homovanillic acid glucuronide	2	181.0501; 313.0923	⁴⁰	7	199 ± 54	4 ± 1	1091 ± 562
10.55	243.0870	C ₁₁ H ₁₆ O ₆	2.33E-16	threo-Syringoylglycerol isomer 1	2	123.0818, 211.0603,167.0707	HMDB0031237	8	442 ± 138	1 ± 0	1001 ± 389
10.60	211.0606	C ₁₀ H ₁₂ O ₅	8.44E-05	Eudesmic acid	2	167.0708	FDB012013	6	120 ± 27	1 ± 0	150 ± 93
11.06	411.0904	C ₂₂ H ₂₀ O ₆ S	1.25E-07	4β-Benzylthioepicatechin	2	242.8023	⁴⁹	7	158 ± 62	1.7 ± 0.5	432 ± 270
11.09	343.1032	C ₁₅ H ₂₀ O ₉	1.64E-31	Homovanillyl alcohol glucuronide isomer 2	2	167.0616, 135.0425	HMDB0240527	8	735 ± 257	1.6 ± 0.5	2867 ± 1078
11.24	259.0819	C ₁₁ H ₁₆ O ₇	5.54E-38	3-Furanmethanol glucoside isomer 2	2	165.0563, 121.0649, 241.0692, 95.0827	HMDB0032924	8	1234 ± 489	1 ± 0	4372 ± 1633
11.24	327.0695	C ₁₄ H ₁₆ O ₉	5.70E-11	Vanillin glucuronide	3	-	HMDB0240573	8	180 ± 66	1 ± 0	448 ± 297
11.58	273.0049	C ₁₀ H ₁₀ O ₇ S	4.25E-02	Ferulic acid 4-sulphate	2	193.0506, 96.9602	HMDB0240716	5	142 ± 20	1.6 ± 0.5	267 ± 218
12.13	229.0714	C ₁₂ H ₂₂ O ₄	1.48E-16	Decanedioic acid isomer 1	3	-	0975-5071	8	232 ± 48	2 ± 0	967 ± 366
12.40	229.0712	C ₁₂ H ₂₂ O ₄	1.03E-04	Decanedioic acid isomer 2	3	-	0975-5071	5	112 ± 11	6 ± 3	438 ± 201
12.75	275.0229	C ₁₀ H ₁₂ O ₇ S	3.62E-52	Dihydroferulic acid 4-sulfate	3	-	HMDB0041724	8	190 ± 20	1.3 ± 0.4	297 ± 238
13.09	371.0979	C ₁₆ H ₂₀ O ₁₀	3.43E-18	Dihydroferulic acid 4-O-glucuronide	3	-	HMDB0041723	8	171 ± 110	2 ± 2	896 ± 861
13.64	243.0869	C ₁₁ H ₁₆ O ₆	1.22E-21	threo-Syringoylglycerol isomer 2	2	123.0821, 211.0603,167.0705	HMDB0031237	8	568 ± 141	0.8 ± 0.2	1393 ± 460
13.90	243.0868	C ₁₁ H ₁₆ O ₆	1.09E-16	threo-Syringoylglycerol isomer 3	2	123.0819, 211.0601,167.0709	HMDB0031237	8	414 ± 105	0.8 ± 0.2	939 ± 302
13.91	555.1712	C ₂₅ H ₃₂ O ₁₄	5.31E-07	Hydroxyoleuropein isomer 1	2	393.0907,323.0458	⁴⁴	8	524 ± 281	0.9 ± 0.5	690 ± 401
13.95	553.1556	C ₂₅ H ₃₀ O ₁₄	1.36E-07	Oleuropein aglycone glucuronide isomer 1	2	377.1260, 275.1608, 165.0559	⁴⁰	8	244 ± 128	0.8 ± 0.2	289 ± 205
13.97	457.0805	C ₂₂ H ₁₈ O ₁₁	5.27E-04	Epigallocatechin 7-O-gallate	2	305.0661	HMDB0003153	7	126 ± 30	1 ± 0	120 ± 53
13.97	593.1486	C ₂₇ H ₃₀ O ₁₅	1.41E-03	Vicenin-2	3	-	HMDB0030708	5	137 ± 59	1 ± 0	190 ± 135
14.05	555.1709	C ₂₅ H ₃₂ O ₁₄	1.66E-17	Hydroxyoleuropein isomer 2	2	393.0903, 323.0456	⁴⁴	8	865 ± 264	0.9 ± 0.2	1754 ± 511
14.05	623.1584	C ₂₈ H ₃₂ O ₁₆	7.96E-04	Isorhamnetin 3-O-glucoside7-O-rhamnoside	3	-	Pubchem: 72188972	6	133 ± 18	0.9 ± 0.2	137 ± 65

14.07	585.1815	C ₂₆ H ₃₄ O ₁₅	1.10E-03	10-Hydroxy-7 - methoxyyleuropein isomer 1	2	409.1498, 113.0227, 176.0274	⁵⁰	5	107 ± 22	2 ± 0	240 ± 113
14.11	541.1562	C ₂₇ H ₄₂ O ₁₁	1.55E-03	Cortolone-3-glucuronide	2	175.0223	HMDB0010320	5	163 ± 88	4.4 ± 0.8	610 ± 515
14.16	553.1558	C ₂₅ H ₃₀ O ₁₄	4.25E-13	Oleuropein aglycone glucuronide isomer 2	2	377.1262, 275.1612, 165.0560	⁴⁰	8	549 ± 230	0.9 ± 0.2	905 ± 398
14.26	585.1817	C ₂₆ H ₃₄ O ₁₅	1.56E-14	10-Hydroxy-7 - methoxyyleuropein isomer 2	2	409.1499, 113.0230, 176.0275	⁵⁰	8	241 ± 89	1.9 ± 0.3	837 ± 406
14.27	569.1868	C ₂₆ H ₃₄ O ₁₄	2.81E-03	Methoxyyleuropein isomer 1	2	529.1235; 551.657	HMDB0035445	6	147 ± 31	1.5 ± 0.5	209 ± 82
14.95	555.1712	C ₂₅ H ₃₂ O ₁₄	7.32E-09	Hydroxyyleuropein isomer 3	2	393.0906, 323.0457	⁴⁴	8	471 ± 143	0.7 ± 0.2	601 ± 318
15.07	569.1870	C ₂₆ H ₃₄ O ₁₄	1.23E-07	Methoxyyleuropein isomer 2	2	529.1237, 551.653	HMDB0035445	6	181 ± 49	0.7 ± 0.2	553 ± 433
15.28	555.1714	C ₂₅ H ₃₂ O ₁₄	1.48E-03	Hydroxyyleuropein isomer 4	2	393.0917, 329.0459	⁴⁴	5	359 ± 121	0.8 ± 0.2	339 ± 196
15.49	569.1867	C ₂₆ H ₃₄ O ₁₄	1.09E-16	Methoxyyleuropein isomer 3	2	529.1229, 551.661	HMDB0035445	8	794 ± 449	0.9 ± 0.2	1745 ± 1341
15.56	364.9974	C ₁₅ H ₁₀ O ₉ S	1.10E-04	Kaempferol-sulphate	2	151.0037, 96.9557	⁵¹	7	172 ± 102	1.1 ± 0.3	226 ± 186
15.77	201.1130	C ₁₀ H ₁₈ O ₄	8.10E-07	Sebacic acid isomer 3	3	-	HMDB000079	7	330 ± 109	6 ± 2	1628 ± 518
15.90	393.1184	C ₂₀ H ₂₆ O ₈	3.83E-07	10-hydroxyyleuropein aglycone	3	-	⁵²	8	194 ± 76	0.7 ± 0.2	214 ± 126
22.37	535.3086	C ₃₃ H ₄₄ O ₆	5.90E-05	Dihydrocelastryl diacetate	3	-	⁵³	5	239 ± 78	3 ± 1	837 ± 571
Average									392 ± 417	2 ± 1	1072 ± 1208

n: number of volunteers in whom the metabolite appears after ingestion of the *O. europaea* extract; RT: retention time; Cmax: relative maximum plasma level (relative chromatographic area); Observed Tmax: time required to reach Cmax (h); AUCt: area under the zero-moment curve (relative chromatographic area /h); Values represent mean ± standard deviation (n = number of volunteers in whom the metabolite appears);* Database or bibliography used for compound annotation. ^a Mass fragmentation spectrum of the analytical standard in **Figure S2**. ^b Mass fragmentation spectrum of the analytical standard in **Figure S3**. ^c Mass fragmentation spectrum of the analytical standard in **Figure S4**.

Table 4. Nutrikinetic parameters and statistical analysis of common metabolites after ingestion of an extract of *L. citriodora* and *O. europaea*.

Proposed compound	n		Cmax			Observed Tmax			AUCt		
	LC	OE	LC	OE	Stat	LC	OE	Stat	LC	OE	Stat
Hydroxytyrosol sulphate isomer 2	7	8	902 ± 428	836 ± 359	-	8 ± 2	3 ± 3	****	3802 ± 2564	2914 ± 1792	-
Vanillic acid 4-O-sulphate isomer 1	7	8	874 ± 644	489 ± 147	-	8 ± 3	1.0 ± 0.0	****	3576 ± 3122	2361 ± 729	-
Hydroxytyrosol glucuronide isomer 2	4	8	106 ± 17	2627 ± 1167	****	8 ± 2	0.9 ± 0.2	****	350 ± 158	6846 ± 2440	****
Homovanillyl alcohol sulphate	4	8	156 ± 49	135 ± 32	-	7 ± 2	2.0 ± 0.9	****	437 ± 278	360 ± 155	-
3-methylcatechol	6	7	169 ± 59	307 ± 160	-	8 ± 3	0.6 ± 0.2	****	565 ± 407	585 ± 562	-
Homovanillic acid sulphate isomer 1	7	8	341 ± 164	233 ± 69	-	9 ± 2	3 ± 2	****	1391 ± 1144	1338 ± 674	-
Homovanillic acid sulphate isomer 2	5	8	476 ± 363	215 ± 68	-	9 ± 2	4 ± 2	****	1771 ± 1599	1484 ± 597	-
Homovanillic acid glucuronide	6	7	360 ± 219	199 ± 54	-	8 ± 2	4 ± 1	**	1345 ± 1042	1091 ± 562	-
Ferulic acid 4-sulphate	7	5	354 ± 254	142 ± 20	-	3 ± 2	1.6 ± 0.5	-	1752 ± 1781	267 ± 218	-

n: number of volunteers in whom the metabolite appears after ingestion of the *L. citriodora* or *O.europaea* extracts; Cmax: relative maximum plasma level (relative chromatographic area); Observed Tmax: time required to reach Cmax (h); AUCt: area under the zero-moment curve (relative chromatographic area /h); Values represent mean ± standard deviation (n = number of volunteers in whom the metabolite appears); LC: *L. citriodora*; OE: *O. europaea*; Stat: statistically significant differences at **p<0.01, ****p<0.0001 between the parameters for metabolites from *L. citriodora* and *O. europaea*.

3.5 Circulating metabolites related to the intake of both matrices.

The 9 common bioavailable metabolites (hydroxytyrosol sulphate, vanillic acid sulphate, hydroxytyrosol glucuronide, homovanillyl alcohol sulphate, 3-methylcatechol, homovanillic acid glucuronide, ferulic acid 4-sulphate and two isomers of homovanillic acid sulphate) detected in plasma for both extracts are detailed in **Table 4** along with their calculated nutrkinetic parameters.

Based on these results, the hypothesis of the origin of most common bioavailable metabolites lies on the common origin related to the compound hydroxytyrosol (**Figure 2**). This compound is present only in the OE extract. However, the compounds verbascoside, present in LC and OE extracts, and oleuropein and its derivatives (e.g. hydroxyoleuropein, methoxyoleuropein isomer, oleuropein aglycone, etc.), present in OE, contain a hydroxytyrosol unit in their structure (**Figure 1**). For this reason, all these sources of hydroxytyrosol in both OE and LC extracts were quantified for better interpretation and discussion of the achieved results of the common metabolites. The quantification results of the potential precursor compounds of common circulating metabolites are shown in **Table 5**. These quantification results shows that the OE extract, apart from having many more precursors than the LC extract, also has a higher total concentration of hydroxytyrosol equivalents. Although verbascoside is present in the composition of both extracts, the concentration level of abundance in the OE extract is much lower than that present in the LC extract. Therefore, it is demonstrated that the origin of the bioavailable common compounds is not solely due to this common phenolic compound present in the original extracts.

Interestingly, despite the OE extract exhibiting greater precursor abundance for metabolites derived from hydroxytyrosol, verbascoside, and oleuropein (**Table 5**), the average AUCt values between the two matrices were not statistically significant. This global trend based on the means of all compounds detected in both matrices is also in line with the lack of significant differences in the Cmax and AUC parameters for the 9 common metabolites, except for hydroxytyrosol glucuronide (**Table 4**).

In addition to the reported 9 common metabolites, three other common compounds were detected but were significant in less than 50% of volunteers in LC. Specifically, these metabolites were another isomer of hydroxytyrosol sulphate, vanillic acid glucuronide, and sebacic acid, which were only detected in 3, 2 and 3 volunteers, respectively, who took the LC extract of the total of 8 volunteers. However, these compounds were considered in the analysis of the volunteers who consumed OE, as they were present in more than 50% of these volunteers. These differences can be attributed to the lower concentration of hydroxytyrosol precursors in the LC extract compared to the OE extract, as reported in **Table 5**.

Table 5. Quantification of potential precursor compounds of common circulating metabolites in *L. citriodora* and *O. europaea* extracts.

Compound	m/z [M-H] ⁻	LC Content (μmol/g dry extract)	LC content (% g/g dry extract)	OE Content (μmol/g dry extract)	OE content (% g/g dry extract)
Hydroxytyrosol isomer 1	153.0553	ND	ND	10 ± 1	0.15 ± 0.02
Hydroxytyrosol isomer 2	153.0556	0.14 ± 0.03	0.0021 ± 0.0005	16.3 ± 0.7	0.25 ± 0.01
Hydroxyoleuropein isomer 1	555.1715	ND	ND	0.52 ± 0.08	0.029 ± 0.004
Hydroxytyrosol acetate	195.0660	ND	ND	10 ± 1	0.19 ± 0.02
Demethyleuropein	525.1608	ND	ND	0.091 ± 0.003	0.0047 ± 0.0002
Hydroxyoleuropein isomer 2	555.1729	ND	ND	<LOQ	<LOQ
Verbascoside isomer 1	623.1990	195 ± 36	12 ± 2	8.4 ± 0.3	0.52 ± 0.02
Oleuropein-glucoside isomer 1	701.2302	ND	ND	4.1 ± 0.4	0.29 ± 0.03
Verbascoside isomer 2	623.1988	2.84 ± 0.02	0.177 ± 0.001	0.039 ± 0.006	0.0024 ± 0.0004
Oleuropein-glucoside isomer 2	701.2309	ND	ND	0.24 ± 0.06	0.017 ± 0.004
Verbascoside isomer 3	623.1986	21.1 ± 0.9	1.32 ± 0.06	0.99 ± 0.05	0.062 ± 0.003
Oleuropein-glucoside isomer 3	701.2294	ND	ND	0.169 ± 0.002	0.0118 ± 0.0001
Methoxyoleuropein isomer 1	569.1883	ND	ND	1.04 ± 0.07	0.059 ± 0.004
Oleuropein isomer 1	539.1783	ND	ND	258 ± 37	14 ± 2
Methoxyoleuropein isomer 2	569.1895	ND	ND	0.0090 ± 0.0001	0.00051 ± 0.00005
Oleuropein isomer 2	539.1782	ND	ND	8.4 ± 0.7	0.45 ± 0.04
4''-Methyloleuropein	553.1935	ND	ND	0.014 ± 0.003	0.0008 ± 0.0001
Oleuropein aglycone isomer 1	377.1244	ND	ND	0.018 ± 0.007	0.0007 ± 0.0003
10-Hydroxyoleuropein aglycone isomer 1	393.1197	ND	ND	0.16 ± 0.01	0.0063 ± 0.0004
10-Hydroxyoleuropein aglycone isomer 2	393.1189	ND	ND	0.594 ± 0.008	0.0234 ± 0.0003
Oleuropein aglycone isomer 2	377.1244	ND	ND	9 ± 1	0.34 ± 0.04
Total (μmol hydroxytyrosol equivalent/g dry extract)		219 ± 37		328 ± 42	
% Total			14 ± 2		16 ± 2

LC: *L. citriodora*; OE: *O. europaea*; ND: Not detectable; LOQ: Limit of Quantification

The immediate metabolites resulting from phase II reactions involving hydroxytyrosol were identified as hydroxytyrosol sulphate and a hydroxytyrosol glucuronide isomer. It

was observed that hydroxytyrosol sulphated may be derived from verbascoside, as in the case of LC, or from the free or oleuropein-derived hydroxytyrosol form, related to the OE extract. A relevant result is that a single isomer of the hydroxytyrosol glucuronide was detected common between the two matrices. However, another isomer of hydroxytyrosol glucuronide was detected exclusively in the plasma of volunteers who consumed OE. Furthermore, the common hydroxytyrosol glucuronide isomer exhibited substantially higher C_{max} and AUC_t in OE compared to LC extracts. These observations are likely attributable to the appreciably higher levels of precursors for this compound in OE extracts, as reported in **Table 5**. The presence of two isomers of hydroxytyrosol in plasma of OE volunteers may also be because the free form of hydroxytyrosol in this matrix makes it more likely to be metabolised by different OH groups, resulting in both isomers being found in plasma ⁵⁴. An alternative hypothesis to explain this difference could be attributed to the steric hindrance posed by hydroxytyrosol in the verbascoside structure that prevents it from being metabolized by different OH groups. Nevertheless, there is limited information in the current literature to substantiate this hypothesis.

The presence of vanillic acid 4-O-sulphate, homovanillyl alcohol sulphate, homovanillic acid sulphate, homovanillic acid glucuronide, ferulic acid 4-sulphate and 3-methylcatechol in plasma samples of both groups may be due to the action of enzymatic hydrolysis and metabolic phase I and II reactions. In this context, the hydroxytyrosol derived from both compounds, through the action of catechol-O-methyltransferase, gives rise to homovanillic acid and alcohol derivatives. Subsequently, by the actions of UDP-glucuronosyltransferases and sulfotransferases, the compounds homovanillic acid glucuronide, homovanillyl alcohol sulphate, and homovanillic acid sulphate can be produced ⁵⁵. 3-methylcatechol is also a derivative of hydroxytyrosol which has been

detected in other bioavailability studies focused on olive oil, a producer derived from the fruit of OE ⁴⁰.

It should be noted that ferulic acid 4-sulphate appears in the plasma of volunteers who consumed LC and OE, while ferulic acid 4-O-glucuronide only appears in volunteers who ingested LC. This result may be due to a higher content of ferulic acid coming from verbascoside compared to the parent compounds present in the OE, as caffeic acid is easily transformed into ferulic acid by the enzyme caffeate O-methyltransferase ³⁶. This is consistent with a study in which it was found that when the concentration of a metabolite is lower, sulphation is more effective, while glucuronidation is much more effective than sulphation at higher concentrations ⁵⁶.

The comparison of the observed Tmax values of common reveals that there were statistical differences for this parameter, confirming different metabolization mechanisms between the two matrices. (**Table 4** and **Figure 3**)

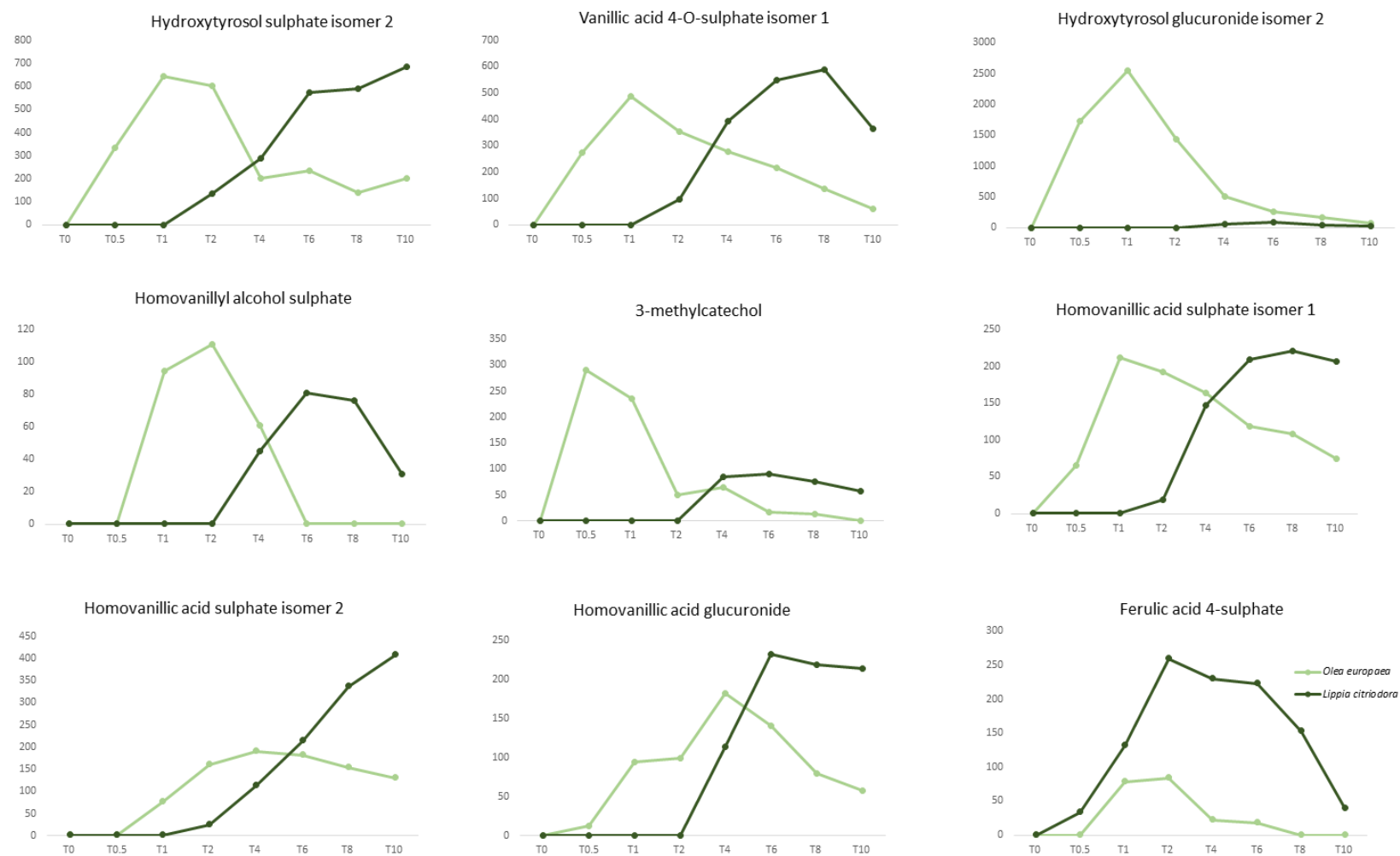


Figure 3. Mean relative abundances of the common annotated metabolites present in plasma between *L. citriodora* and *O. europaea* at different sample collection times. Ordinate axis: relative maximum plasma level (chromatographic area); Abscissa axis: hours.

When examining the AUC graphics of the two common hydroxytyrosol metabolites, hydroxytyrosol sulphate and hydroxytyrosol glucuronide (**Figure 3**), it becomes apparent that, for OE, the maximum observed T_{max} occurs one hour after the extract ingestion. In contrast, for LC, the AUC values are statistically significantly lower, especially for hydroxytyrosol glucuronide. Furthermore, in the case of the sulphate form, the observed T_{max} appears at 1.1 ± 0.3 hours in OE and at 8 ± 2 hours in LC, while for the glucuronide form, it occurs at 0.9 ± 0.2 and 7 ± 2 hours, for OE and LC, respectively. These significant differences might be attributed to the fact that OE extract contains hydroxytyrosol, whereas this compound is not present in LC composition. Therefore, all hydroxytyrosol in LC must first be cleaved from verbascoside. As for hydroxytyrosol glucuronide, it is clear that the disparity in AUC values and the different times of appearance suggest that hydroxytyrosol, both the free form present in the OE extract and that resulting from oleuropein cleavage, may undergo phase II metabolic transformations in the liver, as the expression of liver UDP-glucuronosyltransferases is high. In contrast, the hydroxytyrosol present in the LC extract, derived from verbascoside, may be metabolised in the small intestine ⁵⁷, where glucuronidase expression is also present but to a lesser extent which would explain the lower AUC, since the highest expression occurs in the liver. These findings align with the results obtained, which indicate that the presence of hydroxytyrosol glucuronide in rat plasma results from both phase II metabolism in the liver and small intestine. Furthermore, the concentration of this metabolite differs based on the source of hydroxytyrosol ⁵⁸.

When we focus on the metabolites hydroxytyrosol sulphate, homovanillyl alcohol sulphate, vanillic acid 4-O-sulphate isomer 1 and homovanillic acid sulphate isomer 1 (**Figure 3**), they exhibit a similar pattern. For the volunteers who ingested LC, there is a

observed T_{max} at 7 ± 2 hours after the compound intake, while those consuming OE have a peak at 2.0 ± 0.9 hours, followed by a small resurgence at 8 hours. One hypothesis that could explain this is the difference in the location where sulfation of compounds derived from different matrices occurs. There are studies that indicate a high expression of sulfotransferases in the small intestine ⁵⁹, suggesting that metabolites derived from the OE extract could bind to the sulphate group in the small intestine, explaining their earlier appearance, and later in the liver, as evidenced by a second maximum around 8 hours. Meanwhile, LC metabolites could undergo the sulfation reaction both in the small intestine or in the liver, and their later appearance could be related to the previous breakdown of verbascoside, undergoing several metabolic reactions before reaching these compounds. These hypotheses are supported by previous studies in which homovanillic acid sulphate has been shown to be produced in both the liver and the small intestine. ^{60,61}. The differences in observed T_{max} between the two isomers of homovanillic acid sulphate are also notable, suggesting different metabolization mechanisms ⁶².

The observed difference in observed T_{max} values for homovanillic acid glucuronide between volunteers who consumed LC and those who ingested OE could be attributed to the metabolic pathway of hydroxytyrosol, from which homovanillic acid is derived. Volunteers who ingested LC may undergo more metabolic reactions before hydroxytyrosol is converted into homovanillic acid glucuronide, leading to a later observed T_{max} (9 ± 2 hours). In contrast, volunteers consuming OE may experience a more direct metabolic route, resulting in an earlier observed T_{max} (4 ± 2 hours).

Finally, in the case of the compound ferulic acid 4-sulphate is the only compound that, being in common in plasma in both matrices, follows the same trend, but with much higher AUC values for LC than for OE, which may be explained by the fact that verbascoside fragments into caffeic acid and is easily transformed into ferulic acid by the enzyme caffeate O-methyltransferase ³⁶. If the origin of this metabolite comes from verbascoside, the observed differences in AUC may be related to the fact that the concentration of this compound is much higher concentration in the LC extract compared to that of OE (**Table 1**).

The results obtained in this study seem to highlight a clear distinction. Depending on the original source of the same compound, in this case, hydroxytyrosol, which can either be derived freely in the extract or from oleuropein or verbascoside, the resulting circulating metabolites appear in plasma at very different times after the intake. This suggests that, depending on their source, these compounds may be subject to the action of endogenous enzymes located in different organs such as the small intestine or liver. With these findings, we can consider that when formulating nutraceuticals or functional foods based on bioactive extracts, it may be interesting to create combinations of different types of extracts or compounds. This approach could potentially increase the time that circulating bioactive compounds are in the bloodstream, increasing the chances of reaching target tissues. However, numerous studies in the literature attribute the bioactive potential to compounds present in the extracts of OE and LC. However, this study has shown that in general the compounds from the original extract do not reach the bloodstream but rather their metabolized compounds. Despite the wealth of information on the bioactive potential of the original compounds, there is limited literature on the bioactive effects of these specific bioavailable metabolites. Hence, one

of the future perspectives is the need to study the potential bioactive effects of these metabolites given that they are the ones most likely to exert effects on target tissues.

In conclusion, the study of bioavailable metabolites in plasma from OE and LC extracts using an untargeted approach has shown significant and novel results. This approach has allowed the annotation of 64 circulating metabolites from the OE and LC bioactive extracts, 9 of them being common between both. The significant differences in observed Tmax for common metabolites suggest the existence of different metabolization mechanisms, which depend on the plant matrix and, consequently, on the original compounds. This highlights the potential of combining both extracts in the development of nutraceuticals to allow circulating metabolites to reach the bloodstream for a longer period, and therefore increase the chances of reaching target tissues.

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CRediT authorship contribution statement

María del Carmen Villegas-Aguilar: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **María de la Luz Cádiz-Gurrea:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Noelia Sánchez-Marzo:** Investigation, Data Curation. **Enrique Barrajón-Catalán:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **David Arráez-Román:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Álvaro Fernández-Ochoa:** Conceptualization, Methodology, Investigation, Software, Formal analysis, Writing – review & editing, Supervision. **Antonio Segura-Carretero:** Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supporting Information description

Table S1. Quantification data of the reference standards. **Table S2.** Unknown compounds detected in plasma samples obtained after intake of an extract of *L. citriodora* and *O. europaea*. **Table S3.** Mean values of the chromatographic area for each of the times for each compound present in plasma after ingestion of *L. citriodora* extract. **Table S4.** Mean values of the chromatographic area for each of the times for each compound present in plasma after ingestion of *O. europaea* extract. **Figure S1.** PCA scores plots from normalized data for plasma samples. **Figure S2.** Mass fragmentation spectrum of analytical standard Vanillic acid sulphate. **Figure S3.** Mass fragmentation spectrum of analytical standard Hydroxytyrosol glucuronide. **Figure S4.** Mass fragmentation spectrum of analytical standard Homovanillic acid sulphate.

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Supporting information

Table S1. Standard calibration curves used for the quantification of the compounds of interest in the OE and LC extracts.

Analytical standard	LOD (μM)	LOQ (μM)	Calibration Range (μM)	Calibration Equations	R ²
Verbascoside	0.011 $\pm$ 0.001	0.033 $\pm$ 0.006	(LOQ – 185)	log y = 0.84 log x + 4.48	0.99
Oleuropein	0.004 $\pm$ 0.002	0.013 $\pm$ 0.007	(LOQ – 185)	log y = 0.52 log x + 4.86	0.99
Hydroxytyrosol	0.022 $\pm$ 0.002	0.052 $\pm$ 0.006	(LOQ – 125)	log y = 0.93 log x + 4.13	1

LOD: Limit of Detection; LOQ: Limit of Quantification, R²: Coefficient of variation.

Table S2. Unknown compounds detected in plasma samples obtained after intake of an extract of *L. citriodora* and *O. europaea*

Peak	<i>L.</i> <i>citriodora</i> *	<i>O.</i> <i>europaea</i> *	RT (min)	[M-H] ⁻	Molecular formula	Proposed compound	MS/MS fragments
1	5	2	1.46	304.9350		Unknown	-
2	0	5	8.59	414.0648		Unknown	351.0680, 369.0382
3	0	6	8.91	414.0650		Unknown	351.0680, 369.0382
4	0	5	8.91	331.0929		Unknown	-
5	6	0	9.73	436.1082		Unknown	-
6	5	0	9.73	405.1037	C ₁₆ H ₂₂ O ₁₂	Unknown	-
7	0	8	10.53	311.0743	C ₁₄ H ₁₆ O ₈	Unknown	159.8886, 99.9255, 100.9341
8	0	6	10.55	328.0644	C ₁₀ H ₁₇ O ₁₂	Unknown	-
9	0	5	11.23	281.0644	C ₂₀ H ₁₀ O ₂	Unknown	-
10	0	6	11.25	344.0596		Unknown	-
11	6	0	11.8	429.2117		Unknown	-
12	0	6	12.58	276.0695		Unknown	-
13	0	5	13.75	328.0648		Unknown	-
14	8	0	13.76	395.0946	C ₂₂ H ₂₀ O ₅ S	Unknown	-
15	0	8	13.81	311.0741	C ₁₄ H ₁₆ O ₈	Unknown	-
16	0	7	13.91	311.0741	C ₁₄ H ₁₆ O ₈	Unknown	-
17	0	8	13.96	571.1668	C ₂₉ H ₃₂ O ₁₀ S	Unknown	-
18	7	0	14.05	429.2110		Unknown	-
19	0	5	14.10	567.1715		Unknown	197.0811, 175.0202, 113.0237
20	0	6	14.25	543.1714	C ₂₄ H ₃₂ O ₁₄	Unknown	-
21	0	5	14.49	459.0596		Unknown	-
22	0	7	14.62	514.1370	C ₁₅ H ₃₁ O ₁₉	Unknown	-
23	7	4	14.95	197.0816		Unknown	-
24	5	0	14.95	287.0510		Unknown	-
25	5	0	15.02	427.1969		Unknown	-

26	8	0	15.15	465.1976		Unknown	-
27	0	7	15.15	459.0954		Unknown	-
28	0	6	15.45	591.1681	C ₃₂ H ₃₂ O ₉ S	Unknown	-
29	0	8	15.54	580.2024		Unknown	-
30	0	8	15.59	484.1276		Unknown	-
31	8	0	15.70	429.2120		Unknown	-
32	8	0	15.77	467.2134	C ₂₀ H ₃₆ O ₁₂	Unknown	113.0232, 144.9160, 222.9614
33	6	0	16.28	253.1059		Unknown	-
34	8	0	16.59	413.2163		Unknown	-
35	7	0	16.79	479.1899		Unknown	-
36	1	7	17.89	487.3420	C ₃₀ H ₄₈ O ₁₅	Unknown	-
37	6	0	18.16	679.3471		Unknown	-
38	5	0	19.01	465.2094		Unknown	-
39	8	0	19.12	361.1999		Unknown	315.1962, 293.2109, 96.9677
40	0	6	23.72	795.3551		Unknown	-

*Number of volunteers in whom the metabolite appears; RT: retention time.

Table S3. Mean values of the chromatographic area for each of the times for each compound present in plasma after ingestion of *L. citriodora* extract.

<i>L. citriodora</i> *	RT (min)	[M-H] ⁺	Formula	Compound	T0	T0.5	T1	T2	T4	T6	T8	T10
5	1.46	304.9350		Unknown	0	0	0	38	35	0	56	41
7	6.53	233.0122	C ₈ H ₁₀ O ₆ S	Hydroxytyrosol sulphate isomer 2	0	0	0	138	289	575	591	686
7	7.25	246.9916	C ₈ H ₈ O ₇ S	Vanillic acid 4-O-sulphate isomer 1	0	0	0	97	395	548	588	365
4	8.92	329.0875	C ₁₄ H ₁₈ O ₉	Hydroxytyrosol glucuronide isomer 2	0	0	0	0	48	81	34	23
4	9.24	247.0279	C ₉ H ₁₂ O ₆ S	Homovanillyl alcohol sulphate	0	0	0	0	45	81	76	31
6	9.26	123.045	C ₇ H ₈ O ₂	3-methylcatechol	0	0	0	0	86	91	76	58
8	9.69	373.1128	C ₁₆ H ₂₂ O ₁₀	Gardoside isomer 1	0	0	85	211	86	27	0	0
5	9.73	436.1082		Unknown	0	0	86	238	130	26	0	0
7	9.81	261.0075	C ₉ H ₁₀ O ₇ S	Homovanillic acid sulphate isomer 1	0	0	0	19	147	209	221	207
5	10.29	261.0071	C ₉ H ₁₀ O ₇ S	Homovanillic acid sulphate isomer 2	0	0	0	25	112	215	336	408
6	10.54	357.082	C ₁₅ H ₁₈ O ₁₀	Homovanillic acid glucuronide	0	0	0	0	114	232	219	214
7	10.85	258.9912	C ₉ H ₈ O ₇ S	Caffeic acid 4-sulphate isomer 1	0	21	89	280	177	86	67	0
6	11.28	258.9924	C ₉ H ₈ O ₇ S	Caffeic acid 4-sulphate isomer 2	0	0	67	277	266	189	129	22
7	11.58	273.0049	C ₁₀ H ₁₀ O ₇ S	Ferulic acid 4-sulphate	0	33	131	259	230	222	152	39
6	11.80	429.2117		Unknown	0	0	18	116	98	16	0	0
5	12.37	369.0839	C ₁₆ H ₁₈ O ₁₀	Ferulic acid 4-O-glucuronide	0	0	0	66	163	217	177	89
5	12.43	201.113	C ₁₀ H ₁₈ O ₄	Sebacic acid Isomer 1	0	22	102	35	0	0	0	0
5	12.55	246.9907	C ₈ H ₈ O ₇ S	Vanillic acid 4-O-sulphate isomer 2	0	18	102	43	0	0	0	0
6	13.45	201.113	C ₁₀ H ₁₈ O ₄	Sebacic acid Isomer 2	0	15	86	86	15	0	0	0
6	13.75	373.1132	C ₁₆ H ₂₂ O ₁₀	Gardoside isomer 2	0	123	328	480	284	151	51	15
8	13.76	395.0946	C ₂₂ H ₂₀ O ₅ S	Unknown	0	11	86	133	46	12	0	0
7	14.05	429.211		Unknown	0	12	142	25	0	0	0	0
6	14.16	411.2032	C ₂₁ H ₃₂ O ₈	Abscisic alcohol 11-glucoside isomer 1	0	50	188	61	0	0	0	0

7	14.95	197.0816		Unknown	0	88	236	235	133	72	70	24
5	14.95	287.0510		Unknown	0	22	102	84	0	0	0	0
5	15.02	427.1969		Unknown	0	21	119	17	0	0	0	0
8	15.15	465.1976		Unknown	0	56	192	100	0	0	0	0
8	15.70	429.2120		Unknown	0	63	254	206	35	0	0	0
8	15.77	467.2134		Unknown	0	112	253	64	0	0	0	0
6	16.12	343.1388	C ₁₆ H ₂₄ O ₈	Dihydroconiferin isomer 1	0	44	137	0	0	0	0	0
6	16.28	253.1059		Unknown	0	48	141	165	68	47	0	0
6	16.32	291.0863	C ₁₅ H ₁₆ O ₆	Picrotoxinin	0	32	102	91	0	0	0	0
5	16.43	343.139	C ₁₆ H ₂₄ O ₈	Dihydroconiferin isomer 2	0	34	136	83	0	0	0	0
8	16.59	413.2163		Unknown	0	66	170	37	0	0	0	0
8	16.75	411.201	C ₂₁ H ₃₂ O ₈	Abscisic alcohol 11-glucoside isomer 2	0	111	519	176	23	0	0	0
7	16.79	479.1899		Unknown	0	24	181	12	0	0	0	0
7	17.44	411.2008	C ₂₁ H ₃₂ O ₈	Abscisic alcohol 11-glucoside isomer 3	0	56	258	13	13	0	0	0
6	17.92	393.1899	C ₂₁ H ₃₀ O ₇	Pteroside Z isomer 1	0	0	105	28	0	0	0	0
8	18.05	393.1909	C ₂₁ H ₃₀ O ₇	Pteroside Z isomer 2	0	28	152	97	18	0	0	0
6	18.16	679.3471		Unknown	0	0	0	0	0	41	75	66
8	18.38	395.2051	C ₂₁ H ₃₂ O ₇	Isopetasoside	0	95	394	200	25	14	0	0
5	19.01	465.2094		Unknown	0	0	103	95	48	18	0	17
8	19.12	361.1999		Unknown	0	14	154	150	25	0	0	0
8	19.17	293.2116	C ₁₈ H ₃₀ O ₃	17-Hydroxylinolenic acid	0	81	428	445	184	31	12	20

* Number of volunteers in whom the metabolite appears; RT: retention time.

Table S4. Mean values of the chromatographic area for each of the times for each compound present in plasma after ingestion of *O. europaea* extract.

<i>O. europaea</i> *	RT (min)	[M-H] ⁺	Formula	Compound	T0	T0.5	T1	T2	T4	T6	T8	T10
6	6.41	233.0124	C ₈ H ₁₀ O ₆ S	Hydroxytyrosol sulphate isomer 1	0	280	323	178	328	118	202	45
8	6.53	233.0122	C ₈ H ₁₀ O ₆ S	Hydroxytyrosol sulphate isomer 2	0	336	646	605	202	235	140	202
8	7.25	246.9916	C ₈ H ₈ O ₇ S	Vanillic acid 4-O-sulphate isomer 1	0	275	489	354	279	216	137	60
5	8.59	414.0648		Unknown	0	75	137	21	0	0	0	0
8	8.65	329.0875	C ₁₄ H ₁₈ O ₉	Hydroxytyrosol glucuronide isomer 1	0	724	955	463	158	61	28	0
6	8.91	414.065		Unknown	0	211	367	193	0	0	0	0
5	8.91	331.0929		Unknown	0	38	101	0	0	0	0	0
8	8.92	329.0875	C ₁₄ H ₁₈ O ₉	Hydroxytyrosol glucuronide isomer 2	0	1726	2553	1437	507	251	161	75
8	8.93	351.0697	C ₁₆ H ₁₆ O ₉	Chlorogenoquinone	0	181	315	154	12	11	0	0
8	9.24	247.0279	C ₉ H ₁₂ O ₆ S	Homovanillyl alcohol sulphate	0	0	94	111	61	0	0	0
7	9.26	123.045	C ₇ H ₈ O ₂	3-methylcatechol	0	290	235	50	64	16	13	0
5	9.73	405.1037	C ₁₆ H ₂₂ O ₁₂	Unknown	0	0	0	0	99	67	30	22
7	9.75	343.0686	C ₁₄ H ₁₆ O ₁₀	Vanillic acid glucuronide	0	0	37	77	63	44	22	0
8	9.81	261.0075	C ₉ H ₁₀ O ₇ S	Homovanillic acid sulphate isomer 1	0	65	212	192	165	119	108	75
8	10.10	343.1033	C ₁₅ H ₂₀ O ₉	Homovanillic alcohol glucuronide isomer 1	0	274	503	372	183	66	26	0
8	10.28	259.0819	C ₁₁ H ₁₆ O ₇	3-Furanmethanol glucoside isomer 1	0	27	200	184	107	17	0	0
8	10.29	261.0071	C ₉ H ₁₀ O ₇ S	Homovanillic acid sulphate isomer 2	0	0	76	161	191	182	154	130
8	10.52	181.0867	C ₁₀ H ₁₄ O ₃	1,2,3-trimethoxy-5-methyl benzene	0	117	221	105	0	0	0	0
8	10.53	149.0604	C ₉ H ₁₀ O ₂	p-Vinylguaiacol	0	90	184	83	0	0	0	0
8	10.53	311.0743	C ₁₄ H ₁₆ O ₈	Unknown	0	129	240	126	13	0	0	0
6	10.54	225.0753	C ₁₁ H ₁₄ O ₅	Desoxy elenolic acid	0	56	123	35	0	0	0	0

7	10.54	357.082	C ₁₅ H ₁₈ O ₁₀	Homovanillic acid glucuronide	0	13	94	99	182	141	80	58
6	10.55	328.0644	C ₁₀ H ₁₇ O ₁₂	Unknown	0	91	161	44	0	0	0	0
8	10.55	243.0870	C ₁₁ H ₁₆ O ₆	threo-Syringoylglycerol isomer 1	0	267	442	253	78	0	0	0
6	10.60	211.0606	C ₁₀ H ₁₂ O ₅	Eudesmic acid	0	34	120	28	0	0	0	0
7	11.06	411.0904	C ₂₂ H ₂₀ O ₆ S	4β-Benzylthioepicatechin	0	25	123	151	50	0	0	0
8	11.09	343.1032	C ₁₅ H ₂₀ O ₉	Homovanillic alcohol glucuronide isomer 2	0	242	677	704	365	165	61	0
5	11.23	281.0644	C ₂₀ H ₁₀ O ₂	Unknown	0	18	94	60	0	0	0	0
8	11.24	259.0819	C ₁₁ H ₁₆ O ₇	3-Furanmethanol glucoside isomer 2	0	625	1234	961	483	250	113	0
8	11.24	327.0695	C ₁₄ H ₁₆ O ₉	Vanillin glucuronide	0	60	180	121	40	11	0	0
6	11.25	344.0596		Unknown	0	96	209	140	35	0	0	0
5	11.58	273.0049	C ₁₀ H ₁₀ O ₇ S	Ferulic acid 4-sulphate	0	0	78	84	22	18	0	0
8	12.13	229.0714	C ₁₂ H ₂₂ O ₄	Decanedioic acid isomer 1	0	0	169	232	167	68	11	0
5	12.40	229.0712	C ₁₂ H ₂₂ O ₄	Decanedioic acid isomer 2	0	0	0	26	47	84	38	60
6	12.58	276.0695		Unknown	0	111	120	77	32	16	8	0
8	13.09	371.0979	C ₁₆ H ₂₀ O ₁₀	Dihydroferulic acid 4-O-glucuronide	0	74	114	94	113	109	74	41
8	13.64	243.0869	C ₁₁ H ₁₆ O ₆	threo-Syringoylglycerol isomer 2	0	411	542	309	115	23	14	13
5	13.75	328.0648		Unknown	0	56	75	0	0	0	0	0
8	13.81	311.0741	C ₁₄ H ₁₆ O ₈	Unknown	0	121	159	67	0	0	0	0
8	13.90	243.0868	C ₁₁ H ₁₆ O ₆	threo-Syringoylglycerol isomer 3	0	330	401	233	61	0	0	0
7	13.91	311.0741	C ₁₄ H ₁₆ O ₈	Unknown	0	97	132	57	0	0	0	0
8	13.91	555.1712	C ₂₅ H ₃₂ O ₁₄	Hydroxyoleuropein isomer 1	0	262	382	181	0	0	0	0
8	13.95	553.1556	C ₂₅ H ₃₀ O ₁₄	Oleuropein aglycone glucuronide isomer 1	0	186	202	29	0	0	0	0
8	13.96	571.1668	C ₂₉ H ₃₂ O ₁₀ S	Unknown	0	151	359	217	23	0	0	0
7	13.97	457.0805	C ₂₂ H ₁₈ O ₁₁	Epigallocatechin 7-O-gallate	0	14	126	12	0	0	0	0
5	13.97	593.1486	C ₂₇ H ₃₀ O ₁₅	Vicenin-2	0	34	137	46	0	0	0	0

6	14.05	623.1584	C ₂₈ H ₃₂ O ₁₆	Isorhamnetin 3-O-glucoside7-O-rhamnoside	0	34	130	14	0	0	0	0
8	14.05	555.1709	C ₂₅ H ₃₂ O ₁₄	Hydroxyyleuropein isomer 2	0	530	841	455	88	0	0	0
5	14.07	585.1815	C ₂₆ H ₃₄ O ₁₅	10-Hydroxy-7 -methoxyyleuropein isomer 1	0	0	61	107	16	0	0	0
5	14.10	567.1715		Unknown	0	38	94	42	0	0	0	0
5	14.11	541.1562	C ₂₇ H ₄₂ O ₁₁	Cortolone-3-glucuronide	0	0	0	0	160	82	63	0
8	14.16	553.1558	C ₂₅ H ₃₀ O ₁₄	Oleuropein aglycone glucuronide isomer 2	0	401	482	197	24	0	0	0
6	14.25	543.1714	C ₂₄ H ₃₂ O ₁₄	Unknown	0	0	0	0	232	161	154	83
8	14.26	585.1817	C ₂₆ H ₃₄ O ₁₅	10-Hydroxy-7 -methoxyyleuropein isomer 2	0	11	164	229	148	34	0	0
6	14.27	569.1868	C ₂₆ H ₃₄ O ₁₄	Methoxyyleuropein isomer 1	0	0	92	92	0	0	0	0
5	14.49	459.0596		Unknown	0	0	0	18	95	60	60	42
8	14.57	555.1710	C ₂₅ H ₃₂ O ₁₄	Hydroxyyleuropein isomer 3	0	0	0	19	113	78	48	0
7	14.62	514.1370	C ₁₅ H ₃₁ O ₁₉	Unknown	0	0	147	101	0	0	0	0
8	14.95	555.1712	C ₂₅ H ₃₂ O ₁₄	Hydroxyyleuropein isomer 4	0	405	350	71	14	0	0	0
6	15.07	569.187	C ₂₆ H ₃₄ O ₁₄	Methoxyyleuropein isomer 2	0	159	123	103	47	25	41	0
7	15.15	459.0954		Unknown	0	115	160	17	0	0	0	0
5	15.28	555.1714	C ₂₅ H ₃₂ O ₁₄	Hydroxyyleuropein isomer 5	0	227	265	17	0	0	0	0
6	15.45	591.1681	C ₃₂ H ₃₂ O ₉ S	Unknown	0	23	143	0	0	23	0	0
8	15.49	569.1867	C ₂₆ H ₃₄ O ₁₄	Methoxyyleuropein isomer 3	0	457	783	450	81	46	0	0
8	15.54	580.2024		Unknown	0	192	340	74	42	0	0	0
7	15.56	364.9974	C ₁₅ H ₁₀ O ₉ S	Kaempferol-sulphate	0	25	165	59	0	0	0	0
8	15.59	484.1276		Unknown	0	98	447	141	20	0	0	0
7	15.77	201.1130	C ₁₀ H ₁₈ O ₄	Sebacic acid isomer 3	0	13	0	33	209	226	178	141
8	15.90	393.1184	C ₂₀ H ₂₆ O ₈	10-hydroxyyleuropein aglycone	0	165	142	15	0	0	0	0
7	17.89	487.3420	C ₃₀ H ₄₈ O ₁₅	Unknown	0	30	40	141	153	102	38	18

5	22.37	535.3086	C ₃₃ H ₄₄ O ₆	Dihydrocelastryl diacetate	0	0	38	150	163	81	38	20
6	23.72	795.3551		Unknown	0	74	123	0	20	52	0	0

* Number of volunteers in whom the metabolite appears; RT: retention time.

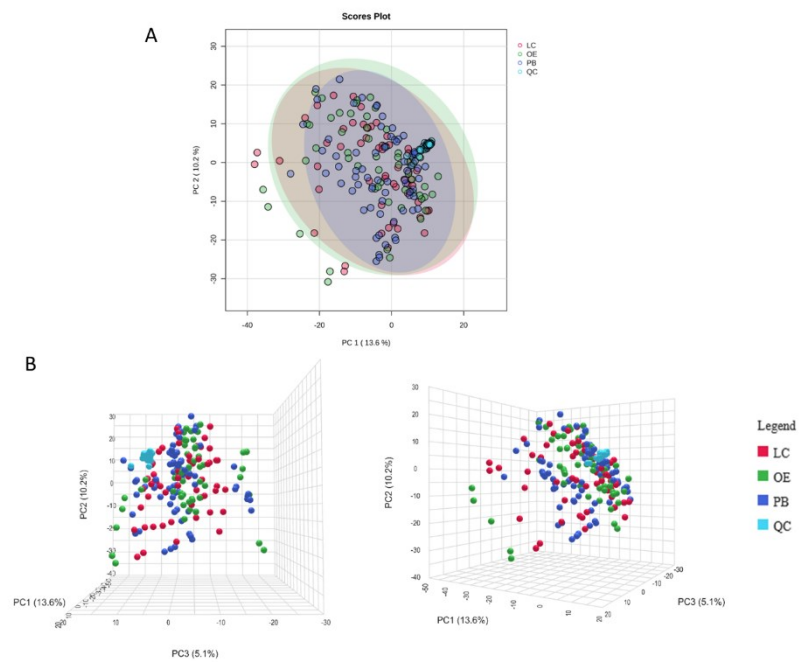


Figure S1. A: 2D PCA scores plots from normalized data for plasma samples. B: 3D PCA scores plots from normalized data for plasma samples.

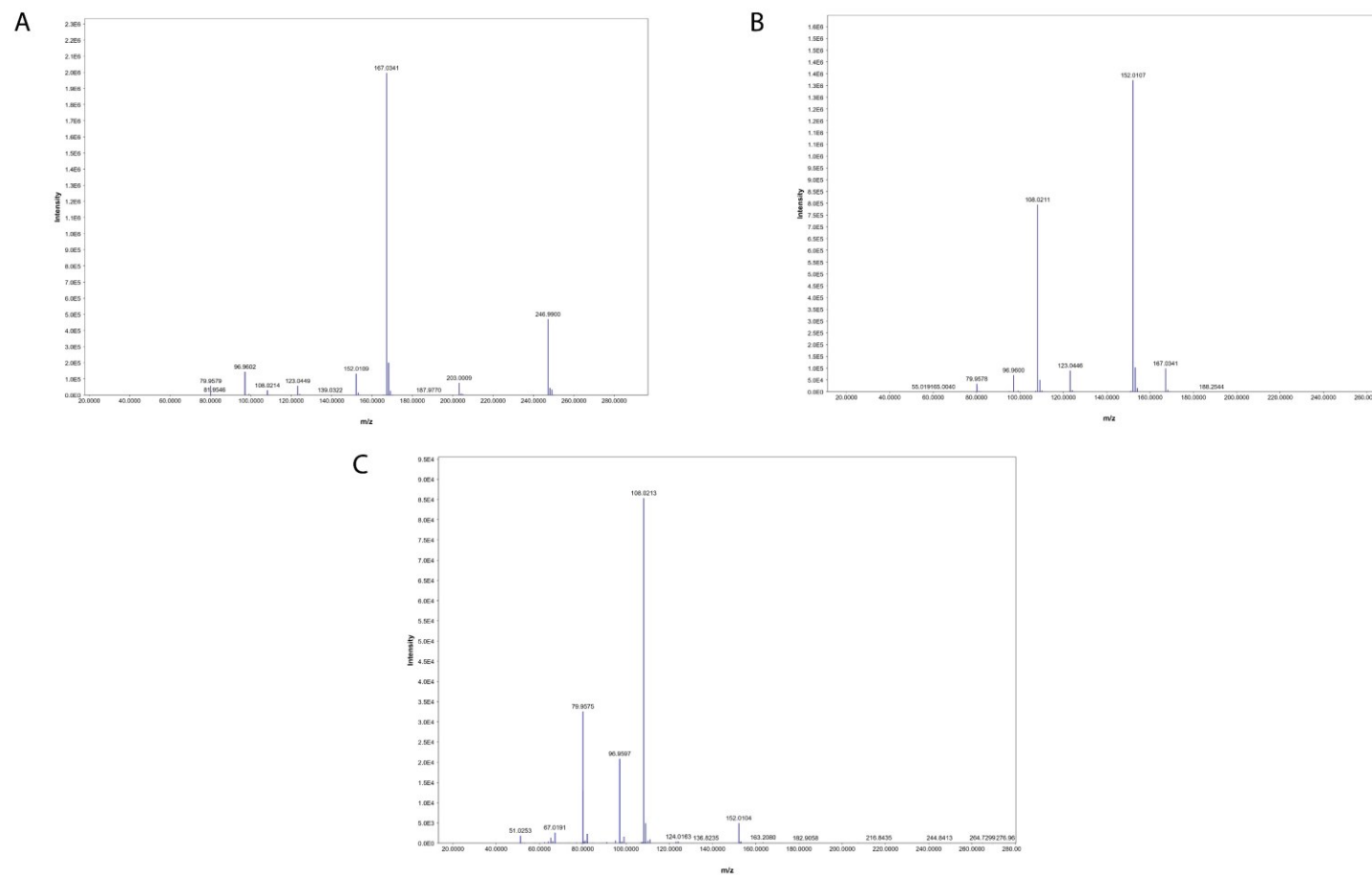


Figure S2. Mass fragmentation spectrum of analytical standard Vanillic acid sulphate (m/z : 246.9914). A: collision energies: 10 eV. B: collision energies: 30 eV. C: collision energies: 60 eV.

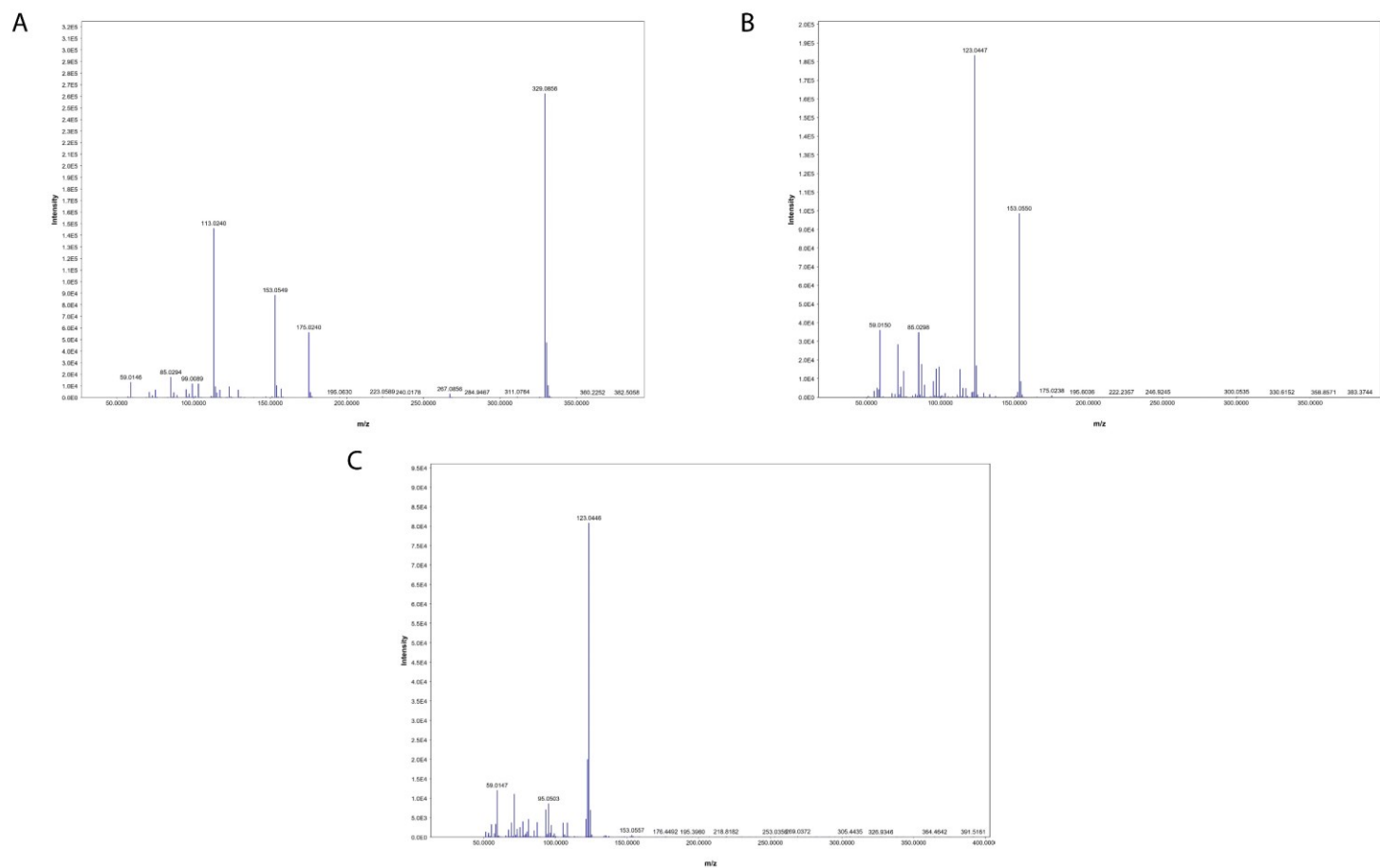


Figure S3. Mass fragmentation spectrum of analytical standard Hydroxytyrosol glucuronide (m/z: 329.0856). A: collision energies: 10 eV. B: collision energies: 30 eV. C: collision energies: 60 eV.

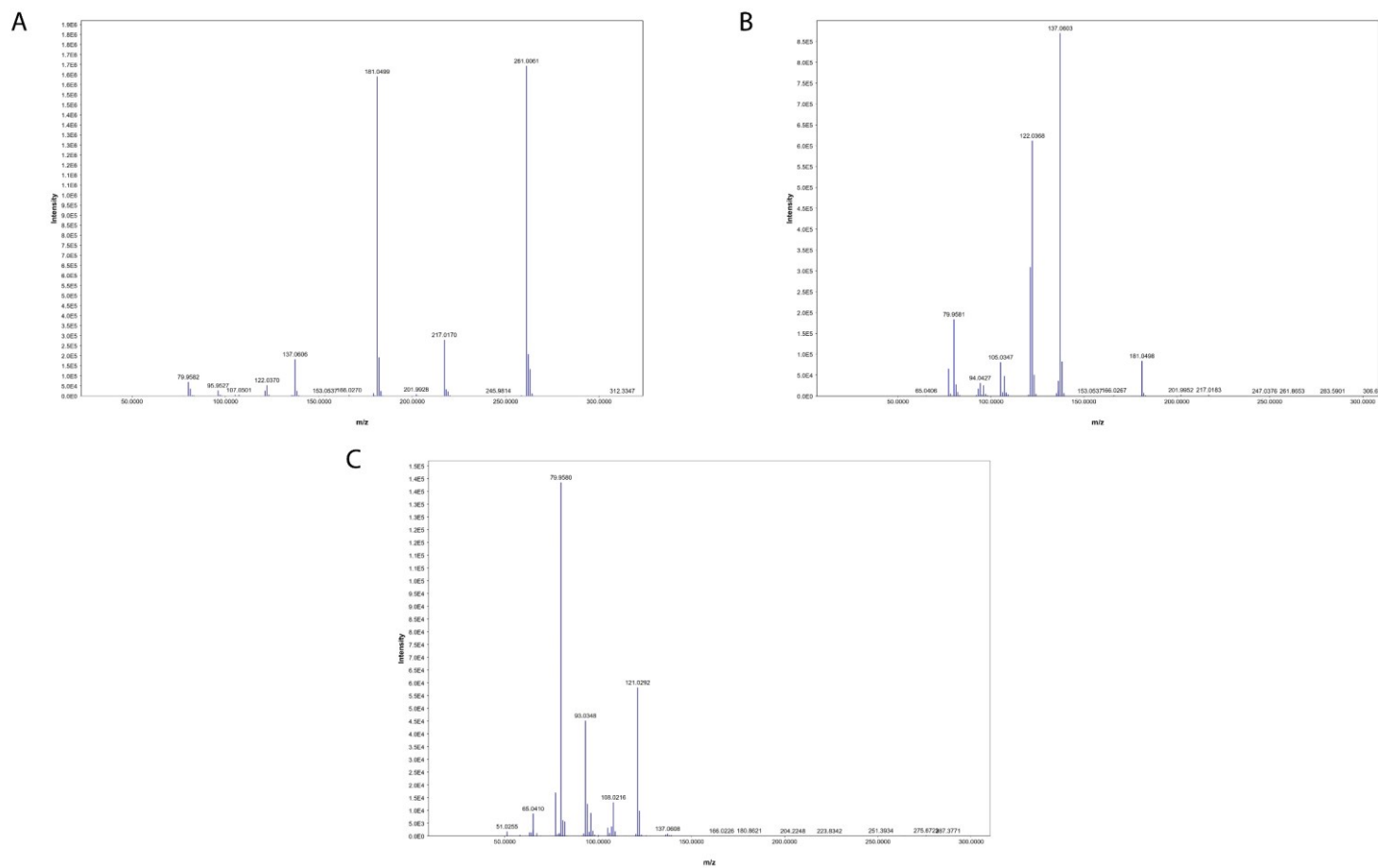
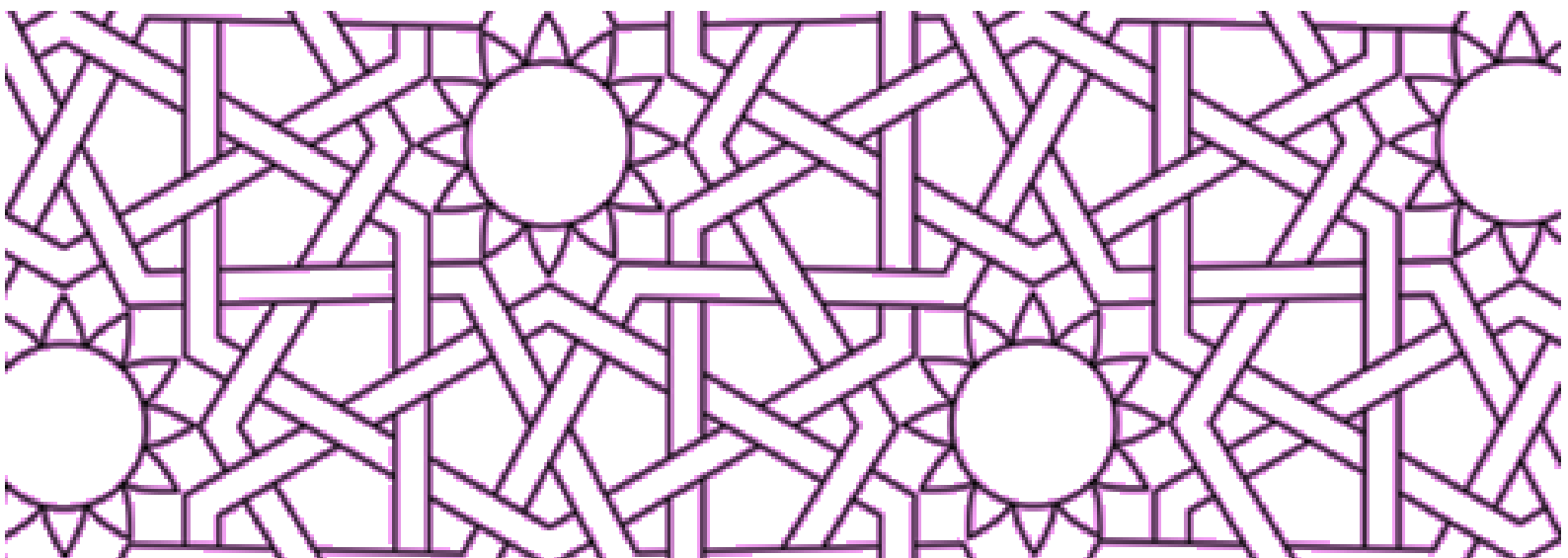


Figure S4. Mass fragmentation spectrum of analytical standard Homovanillic acid sulphate (m/z: 261.0061). A: collision energies: 10 eV. B: collision energies: 30 eV. C: collision energies: 60 eV.

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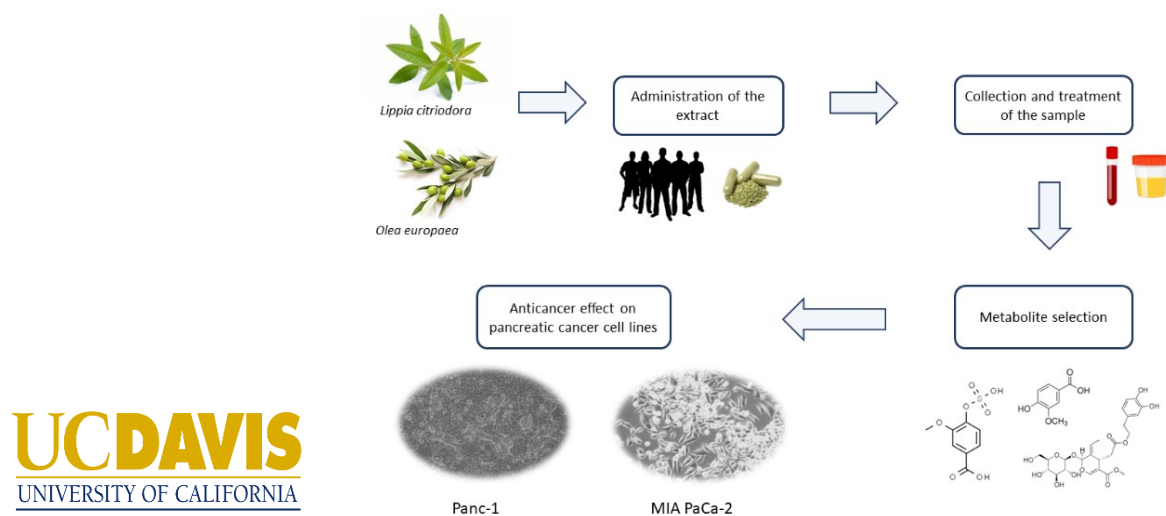
CHAPTER 6

Potential anticancer effects of bioavailable metabolites from Lippia citriodora and Olea europaea extracts on pancreatic cancer cell lines

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Abstract

Pancreatic cancer is a highly aggressive and diverse disease that shows significant resistance to standard treatments. The toxicity of current chemotherapeutic options underscores the critical need for novel therapeutic approaches. In this sense, phenolic compounds with antiproliferative effects offer a promising alternative. *Lippia citriodora* (LC) and *Olea europaea* (OE) are known for their antitumor properties, despite having different phytochemical compositions, there are nine common derived metabolites bioavailable in plasma after ingestion, which has been demonstrated by an intervention study in humans. These common compounds are generally phase II metabolites, such as (vanillic acid sulfate (VA-Sul), homovanillic acid sulfate (HVA-Sul), and hydroxytyrosol glucuronide (HT-G)), from larger chemical structures such as verbascoside or oleuropein. In this sense, the aim of this study is to evaluate the anticancer effects of common plasma and urine metabolites derived from LC and OE extracts, administered during an acute nutritional intervention trial in humans, on pancreatic cancer cells *in vitro*. The study results indicate that consuming OE and LC extracts leads to elevated plasma levels of metabolites derived from the metabolism of verbascoside and oleuropein, the main phenolic compounds in LC and OE extracts, respectively. Oleuropein has shown anticancer effects against pancreatic cancer cells by inducing apoptosis independently of the caspase pathway. Additionally, other metabolites such as VA-Sul and HVA-Sul from both extracts have demonstrated inhibitory effects on pancreatic cancer cell growth, likely through a non-apoptotic mechanism. In conclusion, LC and OE extracts are rich sources of bioavailable metabolites with potential anticancer properties.

1. Introduction

Pancreatic cancer represents a devastating and heterogeneous disease characterized by substantial resistance to conventional treatment options. Current chemotherapeutic agents are often highly toxic (Goldsmith et al., 2015; Siegel et al., 2012), underscoring the urgent need to develop new therapeutic strategies to combat this disease. An alternative avenue in the pursuit of potential treatments for this form of cancer lies in investigating phenolic compounds with antiproliferative properties. Studies have evaluated the anti-proliferative power of olive leaf extract against pancreatic cancer (Goldsmith et al., 2018, 2015).

Lippia citriodora (LC) and *Olea europaea* (OE) are two plant matrices whose phenolic compositions have common bioactivity, including their antitumor capacity (Ohno et al., 2002; Tahara et al., 2009). However, despite having similar bioactivity, their phenolic compositions are mostly different. *L. citriodora* is characterized by a high content of glycosylated phenylpropanoids and iridoids, with verbascoside as a major component (Leyva-Jiménez et al., 2018) and *O. europaea* by the presence of secoiridoids, where oleuropein is the major compound (Guo et al., 2018).

In this context, the discrepancy between different phenolic composition and similar bioactivity led to an acute nutritional intervention trial in humans in which nutraceuticals developed with extracts from both matrices were administered in order to evaluate the bioavailability and the main metabolic reactions of the phenolic compounds contained in the extracts of both matrices. The results obtained from an untargeted metabolomic approach, which facilitates the identification of a wide array of non-preselected metabolites, revealed the presence of nine common metabolites following the ingestion

of both extracts (Villegas-Aguilar et al., 2024). The aforementioned nine metabolites are the products of the metabolic processing of the original phenolic compounds present in the extracts. Notable examples include vanillic acid sulfate, homovanillic acid sulfate, and hydroxytyrosol glucuronide, among others (Villegas-Aguilar et al., 2024). These metabolites are not present in the original extracts; however, they are generated by hydrolysis of gastric fluids within the stomach (Karayaka, 2004). This process converts oleuropein and verbascoside into hydroxytyrosol, as it is part of the structure of both compounds (Huertas-Alonso et al., 2022; López-Munguía et al., 2011). Subsequent metabolic transformations are facilitated by enzymes in intestinal cells, by the colonic microbiota, or by the oxidation-reduction reactions of phase I metabolism occurring in organs such as the liver (Velderrain-Rodríguez et al., 2014). These lead to the formation of additional compounds, including vanillic acid and homovanillic acid. Ultimately, phase II metabolic reactions occurring in organs such as the liver, through processes including methylation, sulfation or glucuronidation, result in the formation of the aforementioned common metabolites (Velderrain-Rodríguez et al., 2014). Therefore, the common metabolites that appear in plasma after ingestion of the extracts separately may be responsible for the common bioactivity of both extracts in *in vivo* models where these metabolic transformations occur (Rashid et al., 2022; Sepporta et al., 2014). In this scenario, phase II metabolites such as resveratrol glucuronide and resveratrol sulfate or quercetin sulfate and quercetin glucuronide have been evaluated for their potential to inhibit carcinogenesis, specifically in breast cancer (Ávila-Gálvez et al., 2021; Wu et al., 2018). However, the existing literature lacks findings on the anticarcinogenic properties of certain compounds, such as vanillic acid sulfate, homovanillic acid sulfate, and hydroxytyrosol glucuronide, along with other metabolites common to both matrices.

This highlights the need to further evaluate compounds that are known to be bioavailable and capable of crossing the systemic circulation to reach target organs. Once at these sites, these compounds may modulate various metabolic pathways and exert their mechanisms of action on gene expression (Heleno et al., 2015).

Therefore, the objective of this study is to assess the anticancer effect of common plasma and urine metabolites of LC and OE extracts, following administered during an acute nutritional intervention trial in humans, on pancreatic cancer cells *in vitro*.

2. Material and methods

2.1 Chemicals and reagents

LC-MS grade methanol and formic acid for mobile phases were procured from Fluka, Sigma-Aldrich (Steinheim, Germany). Water was purified utilizing a Milli-Q system from Millipore (Bedford, MA, USA). Ethanol and methanol (Fisher Scientific Madrid, Spain) for plasma treatment were of LC-MS grade. Oleuropein ($\geq 98\%$), hydroxytyrosol ($\geq 98\%$) and homovanillic acid ($\geq 97\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and a stock solution (100 mM) were prepared in sterile DMSO. Vanillic acid ($\geq 97\%$) was purchased from Merck KGaA (Darmstadt, Germany) and a stock solution (100 mM) was prepared in sterile DMSO. Hydroxytyrosol glucuronide ($\geq 98\%$) was purchased from TRC-Canada (Toronto, Ontario, Canada) and a stock solution (100 mM) was prepared in sterile DMSO. Homovanillic acid sulfate sodium salt ($\geq 96\%$) and Vanillic Acid 4-Sulphate Sodium Salt ($\geq 97\%$) were purchased from TRC-Canada (Toronto, Ontario, Canada) and a stock solution (100 mM) were prepared in sterile water. RIPA lysis buffer, Halt Protease Inhibitor Cocktail, and Phosphatase Inhibitor Cocktail were purchased from MilliporeSigma (St. Louis, MO). SuperSignal™ West Dura Extended Duration Substrate

were purchased from ThermoFisher Scientific (Waltham, MA). Bradford protein assay reagent, 30% (w/v) Acrylamide/Bis Solution, 4 × Laemmli sample buffer, Immun-Blot Polyvinylidene difluoride (PVDF) Membranes and were purchased from Bio-Rad (Hercules, CA).

2.2 Acute nutritional human intervention study

The human acute nutritional intervention study in which nutraceuticals developed with LC leaf extract and OE leaf extract were administered allowed the identification of common metabolites using an untargeted metabolomics approach. Details of volunteer selection and study design have been published previously (Villegas-Aguilar et al., 2024). In general volunteers were divided into three groups: 8 consumed 500 mg of encapsulated LC leaf extract, 8 consumed 500 mg of encapsulated OE leaf extract and 9 consumed encapsulated placebo. In relation to the results obtained by the previous work (Villegas-Aguilar et al., 2024), the common metabolites that appeared in plasma with the ingestion of the two plant matrices were selected to quantify the selected compounds. The selection of the metabolites to be tested in the cellular assays was made on the basis of their commercial availability standards. Thus, among the new common metabolites identified in the previous work, hydroxytyrosol glucuronide, vanillic acid sulfate and homovanillic acid sulfate were selected. In addition to these three, the metabolites oleuropein, hydroxytyrosol, vanillic acid and homovanillic acid were also selected and analyzed, since these metabolites were not selected on the basis of the selection criteria established in the work (Villegas-Aguilar et al., 2024), but rather to enable comparison with their phase II metabolized forms, which were selected according to the criteria outlined in the aforementioned study.

In addition, the presence of these compounds in the urine samples collected in this study has been analyzed. Urine samples were collected in plastic containers at baseline (prior to capsule ingestion, $t=0$) and at two subsequent time intervals following supplement intake: interval 1 (0-6 h) and interval 2 (6-12 h). Additionally, volunteers provided a 24-hour urine sample, marking the conclusion of the urine sample collection. Urine samples were normalized to a standardized osmolality value of 100 mOsm/kg (OSMOMAT 3000 (Gonotec, Berlin, Germany)) using Milli-Q water. Samples were centrifuged for 10 min at 14,800 rpm and 4°C and 60 μ l of supernatant was taken in an HPLC vial and stored at -80°C until the day of analysis.

2.3 Quantification of target metabolites in plasma and urine samples

The metabolites oleuropein, hydroxytyrosol, hydroxytyrosol glucuronide, vanillic acid, vanillic acid sulfate, homovanillic acid and homovanillic acid sulfate present in plasma and urine samples were quantified by an analytical method based on an HPLC-ESI-QTOF-MS platform. Briefly, high performance liquid chromatography (Agilent 1290 HPLC, Agilent Technologies, Palo Alto, CA, USA) coupled to mass spectrometry with a quadrupole time-of-flight analyzer (Agilent 6545 QTOF Ultra High Definition, Agilent Technologies, Palo Alto, CA, USA) was employed. Chromatographic analysis was performed in reversed phase with a C18 ACQUITY UPLC BEH column (1.7 μ m, 2.1 mm, 150 mm, 130 Å, Waters Corporation, Milford, MA, USA). The mobile phases were (A) water acidified with 0.1% formic acid (v/v) and (B) acetonitrile. THE mobile phase gradient for optimal separation selected was: 0.00 min [A:B 100/0], 5 min [A:B 90/10], 18 min [A:B 15/85], 24 min [A:B 0/100], 25.50 min [A:B 0/100], 26.50 min [A:B 95/5] and 32.50 min [A:B 95/5]. Finally, the acquired data were processed using MassHunter workstation software version B.06.00 (Agilent Technologies) and MZmine 3.9.0.

The compounds were quantified using calibration curves prepared with their corresponding analytical standards. Different dilutions of the analytical standards were prepared from a pool mix with a concentration of 100 mM per standard. For each analytical standard, the calibration range, the limits of detection (LOD) and quantification (LOQ) and the coefficient of determination (R^2) were calculated (**Table S1**), showing good linearity ($R^2 > 0.99$).

To assess the bioavailability and determine the ranges of plasma and urine concentrations of the metabolites under investigation in the present study, the volunteer with the lowest and the volunteer with the highest levels of bioavailable metabolites in plasma were selected. Additionally, the metabolites were classified based on their presence in plasma. The same procedure was employed in urine, with the selection of the volunteer exhibiting the lowest excretion levels and the volunteer exhibiting the highest excretion levels over a 24-hour period.

Metabolite nutrkinetics (C_{max} and AUC) were studied using PKSolver, an add-in program for pharmacokinetic data analysis in Microsoft Excel (Zhang et al., 2010).

2.4 Cell culture

Human pancreatic cancer cell lines (Panc-1 and MIA PaCa-2) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 5% (v/v) penicillin–streptomycin. Cells were maintained in a 5% (v/v) CO_2 , humidified atmosphere at 37°C. These cells were grown as monolayers under conditions suggested by ATCC. All the cell lines were characterized by cell morphology and growth rate and passaged in our laboratory less than 6 months after being received.

2.5 Cell viability

After treating cells with the various compounds for 24, 48 and 72 h, the reduction of MTT dye was determined according to the manufacture's protocol (MilliporeSigma, St. Louis, MO, USA).

2.6 Western blot

Following treatment with the various compounds for 24 hours, cells were lysed, and total cell fractions were obtained according to previously described methods (Wei et al., 2020). Aliquots of total fractions containing 10–30 µg protein were separated using 10% (w/v) polyacrylamide gel electrophoresis and electroblotted onto PVDF membranes. After blocking with the “EveryBlot” Blocking Buffer (Bio-Rad, Hercules, CA) for 5 minutes, membranes were probed overnight with the following primary antibodies (1:1000 dilution) from Cell Signaling Technology (Danvers, MA): Cleaved Caspase-3 (Cat #9664), Caspase-3 (Cat #14220), Cleaved PARP (Cat #9541), PARP (Cat #9542), Cyclin E1 (Cat #20808), Bcl-xL (Cat #2802), XIAP (Cat #14334), Survivin (Cat #2808), p-ERK1/2 (Cat #4370), and ERK1/2 (Cat #9102). β -Actin (Cat #8457) was used at the same time as a loading control. After incubation for 60 min at room temperature in the presence of the secondary antibody (HRP-conjugated; 1:2500 dilution), the conjugates were developed and visualized using a Molecular Imager FX™ System (BioRad; Hercules, CA) and analyzed using ImageJ software (V1.54, NIH, Bethesda, MD, USA).

2.7 Statistical analysis

The data, derived from a minimum of three independent experiments, were presented as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using unpaired t-test or two-factor analysis of variance followed by Tukey's test for multiple

comparisons with GraphPad Prism version 8.01 (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Identification and quantification of metabolites in plasma and urine

Table 1 shows the results of the nutritional values of the metabolites present in plasma after ingestion of LC and OE extracts in a nutritional intervention study.

In the case of LC, verbascoside, which is the main phenolic compound present in the extract and whose metabolization can give rise to some of the selected metabolites, was not detected in plasma after ingestion of the extract. Thus, in the volunteers who consumed LC, the metabolite detected at the highest plasma concentration was of vanillic acid sulfate isomer 1, followed by homovanillic acid isomer 2, although it should be noted that the latter was present in only two volunteers.

On the other hand, in the case of the volunteers who ingested OE, oleuropein, which is the main phenolic compound present in the extract, proved to be the most bioavailable metabolite with the highest concentration detected in plasma, but in only 3 and 2 volunteers who ingested it for isomers 1 and 2, respectively. After oleuropein, the two isomers of hydroxytyrosol glucuronide showed the highest levels quantified in plasma, and each of them was found in all 8 volunteers who ingested the OE extract, so the presence of elevated plasma concentrations of this metabolite can be associated with all volunteers who ingested the extract.

Thus, the main difference between OE and LC ingestion is that in the case of the former it gives rise to a higher concentration of metabolites that have undergone fewer metabolic reactions, since it gives rise to more oleuropein, hydroxytyrosol and its glucuronidated derivatives.

Table 1. Nutrikinetic parameters of selected metabolites detected in human plasma after consumption of *Lippia citriodora* and *Olea europaea* extracts.

RT (min)	[M-H] ⁻	Metabolite	n	LC		n	OE	
				Cmax (μM) range	AUC (μM/h) range		Cmax (μM) range	AUC (μM/h) range
2.92	329.0055	Hydroxytyrosol glucuronide isomer 1	-	N.D.	N.D.	8	0.37 – 1.17	0.65 – 2.61
2.96	153.0553	Hydroxytyrosol isomer 1 *	5	<LOQ – 0.17	<LOQ – 0.81	2	<LOQ – 0.16	<LOQ – 1.07
3.29	329.0050	Hydroxytyrosol glucuronide isomer 2 *	4	<LOQ – 0.07	<LOQ – 0.34	8	0.87 – 2.23	2.30 – 5.06
6.25	181.0508	Homovanillic acid isomer 2	2	<LOQ – 0.45	<LOQ – 1.41	3	<LOQ – 0.35	<LOQ – 0.67
6.32	246.9921	Vanillic acid sulfate isomer 1 *	7	<LOQ – 0.74	<LOQ – 3.37	8	0.22 – 0.31	1.69 – 1.69
6.70	246.9910	Vanillic acid sulfate isomer 2	5	<LOQ – 0.07	<LOQ – 0.63	-	N.D.	N.D.
8.37	261.0071	Homovanillic acid sulfate isomer 1 *	7	<LOQ – 0.15	<LOQ – 0.84	8	<LOQ – 0.10	0.10 – 0.76
9.10	261.0075	Homovanillic acid sulfate isomer 2	5	<LOQ – 0.15	<LOQ – 0.81	8	<LOQ – 0.08	0.13 – 0.72
10.34	539.1795	Oleuropein isomer 1 *	-	<LOQ	N.D.	3	<LOQ – 6.90	<LOQ – 28.39
10.53	539.1787	Oleuropein isomer 2	-	<LOQ	N.D.	2	<LOQ – 1.40	<LOQ – 4.09

RT: retention time; n: number of volunteers in whom the metabolite appears after ingestion of the *L. citriodora* or *O. europaea* extracts; Cmax: relative maximum plasma level; AUC: area under the zero-moment curve; LC: *L. citriodora*; OE: *O. europaea*; N.D.: Not detectable; LOQ: Limit of Quantification; * Analytical standard.

Table 2 shows the range of concentrations between the subject with the highest and lowest content of the selected metabolites to be quantified in urine collected for a total of 24 h after ingestion of the LC and OE extracts.

First, it should be noted that the metabolite with the highest excretion was homovanillic acid isomer 2 for both the LC and OE extracts.

In the case of the two isomers of hydroxytyrosol and the two isomers of its glucuronidated form, the excreted content was higher after consumption of the OE extract compared to the consumption of the LC extract.

Furthermore, it was observed that vanillic acid is predominantly excreted in its sulfated form in both the LC and OE extracts, with particularly high levels associated with the consumption of the LC extract. Conversely, homovanillic acid was excreted in larger quantities in its non-sulfated form.

Notably, oleuropein showed the highest plasma bioavailability in subjects consuming OE extract, but its urinary excretion was very low, suggesting that oleuropein undergoes significant metabolism before excretion.

Table 2. Selected metabolites excreted in urine (from 0 to 24 h) by healthy volunteers after consumption of *Lippia citriodora* and *Olea europaea* extracts.

RT (min)	[M-H] ⁻	Metabolite	LC	OE
			Total 24h (μmol) range	Total 24h (μmol) range
2.92	329.0055	Hydroxytyrosol glucuronide isomer 1	2.02 – 3.96	15.57 – 25.31
2.96	153.0553	Hydroxytyrosol isomer 1 *	0.09 – 0.28	1.71– 1.93
3.61	153.0541	Hydroxytyrosol isomer 2	1.34 – 1.38	0.47 – 2.02
3.29	329.0050	Hydroxytyrosol glucuronide isomer 2 *	4.67 – 10.50	27.20 – 63.87
5.41	181.0508	Homovanillic acid isomer 1*	8.04 – 16.21	0.81 – 6.44
6.25	181.0508	Homovanillic acid isomer 2	114.50 – 151.18	71.59 – 163.90
6.32	246.9921	Vanillic acid sulfate isomer 1 *	7.86 – 80.97	1.44 – 59. 86
6.70	246.9910	Vanillic acid sulfate isomer 2	75.60 – 133.15	0.43 – 41.48
6.92	167.0345	Vanillic acid *	3.96 – 5.33	2.58 – 8.97
8.37	261.0071	Homovanillic acid sulfate isomer 1 *	16.22 – 24.55	11.07 – 26.62
9.10	261.0075	Homovanillic acid sulfate isomer 2	2.57 – 5.74	2.71 – 6.65
10.34	539.1795	Oleuropein isomer 1 *	<LOQ	0.06 – 0.44

RT: retention time; LC: *L. citriodora*; OE: *O. europaea*; LOQ: Limit of Quantification; * Analytical standard.

3.2 Effect of LC and OE metabolites on cell growth of pancreatic cancer lines

To test the growth-inhibitory effect of the metabolites compounds Oleuropein (Oleu), Hydroxytyrosol (HT), Hydroxytyrosol glucuronide (HT-G), Vanillic acid (VA), Vanillic acid sulfate (VA-Sul), Homovanillic acid (HVA) and Homovanillic acid sulfate (HVA-Sul) on cancer cell growth, we treated Panc-1 and MIA PaCa-2 pancreatic cancer cell lines, with three concentrations of each of the metabolites (5, 10 and 20 μM) for 24, 48 and 72 h (Figure 1).

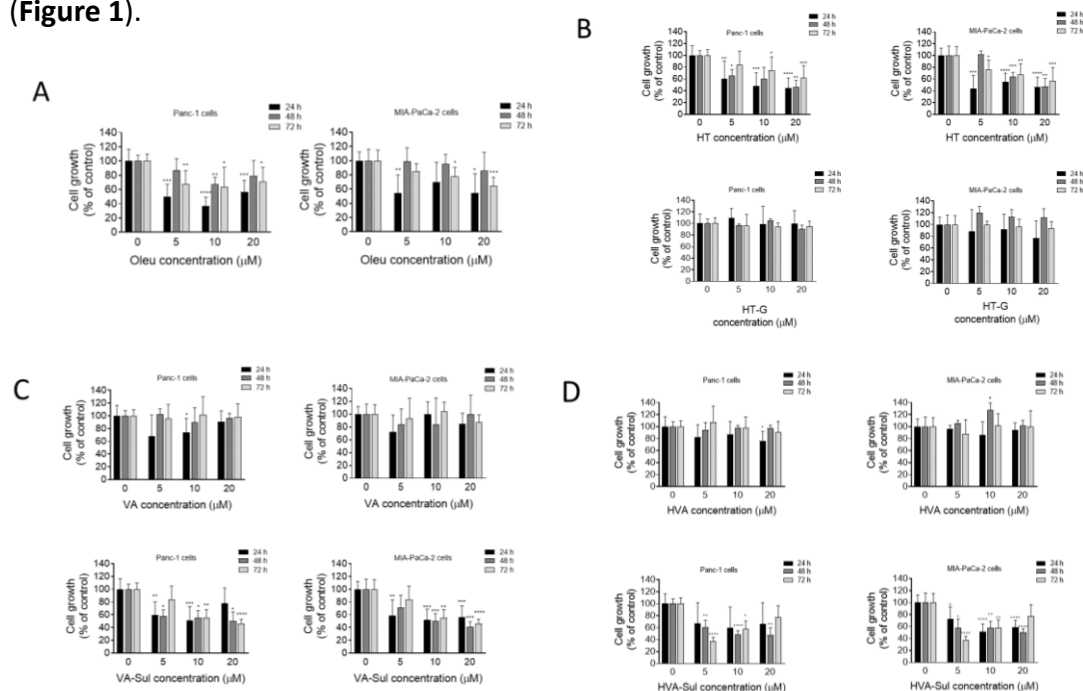


Figure 1. Effect of the compounds Oleuropein (Oleu), Hydroxytyrosol (HT), Hydroxytyrosol glucuronide (HT-G), Vanillic acid (VA), Vanillic acid sulfate (VA-Sul), Homovanillic acid (HVA) and Homovanillic acid sulfate (HVA-Sul) on cell growth in Panc-1 and MIA PaCa-2 cells. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

In the case of the Oleuropein metabolite, the maximum cell growth inhibition was observed for the Panc-1 line at a concentration of 10 μM following 24h treatment resulting in 50% cell growth compared to the control. In addition, for this metabolite it was observed that for both cell lines cell growth was lower for all concentrations when treated with Oleu for 24 h, compared to 48 and 72h (Figure 1A).

When comparing the effect on cell growth of the metabolites HT and HT-Glu, it is observed that for the latter, there were no significant differences in cell growth compared to the vehicle-treated controls for any of the concentrations or incubation times in either cell line. In contrast, HT exerted a significant reduction in cell growth in both cell lines for all three concentrations, especially showing a greater reduction at the 24-hour time point across the concentrations (**Figure 1B**).

For the VA and VA-Sul metabolites, the former exerted no significant effect on cell growth compared to the control for any of the concentrations or incubation times in either cell line, except for the concentration of 10 μ M at 24h. In contrast, VA-Sul exerted a significant effect decreasing cell growth compared to vehicle-treated controls, being the most significant for 20 μ M at 72h for both Panc-1 and MIA Paca-2 (**Figure 1C**).

Finally, when evaluating the effect of HVA, we did not observe any significant cell growth inhibitory effect compared to the control for both cell lines at the concentrations tested and incubation times, except for 20 μ M at 24 hours in Panc-1 and 10 μ M at 48 hours in MIA Paca-2. In contrast, its sulfated form, HVA-Sul, exhibited a significant overall effect decreasing cell growth compared to the control for both cell lines, with the most notable effect observed at 5 μ M at 24 hours for both cell lines. (**Figure 1D**).

3.3 Effect of LC and OE common metabolites on apoptosis-related protein expression

To explore the mechanism of metabolites-induced apoptosis, we determined, in Panc-1 and MIA PaCa-2 cells, the expression levels of multiple proteins that regulate apoptosis, including proteins of the inhibitor of apoptosis protein (XIAP) and Bcl-2 family. Given the cell growth inhibitory results, we chose a metabolite concentration of 10 μ M for 24 hours, for the apoptosis studies.

As shown in **Figure 2**, Oleu was the metabolite with the highest proapoptotic activity as it reduced Bcl-xl and survivin levels in both cell lines, however it did not exert a significant effect on XIAP levels. VA also exerted a proapoptotic effect by reducing Bcl-xl and survivin levels without affecting XIAP expression. However, unlike Oleu, this effect was observed only in Panc-1 cells. In the case of HT, it only exerted a significant effect on the reduction of Bcl-xl for the MIA PaCa-2 cell line, while its glucuronidated form exerted a reduction on Bcl-xl for the Panc-1 cell line and on XIAP for the MIA PaCa-2 cell line.

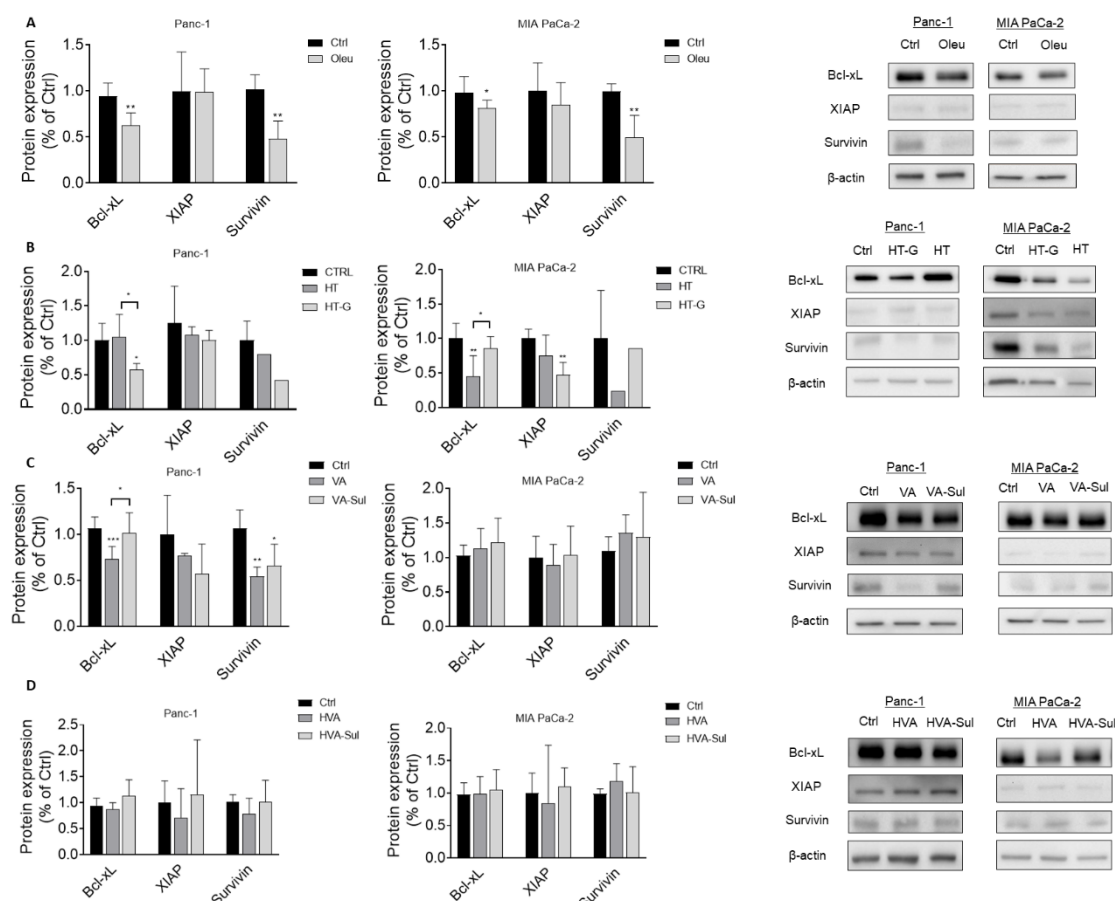


Figure 2. Effect of the compounds Oleuropein (Oleu), Hydroxytyrosol (HT), Hydroxytyrosol glucuronide (HT-G), Vanillic acid (VA), Vanillic acid sulfate (VA-Sul), Homovanillic acid (HVA) and Homovanillic acid sulfate (HVA-Sul) on apoptosis-related protein expression. **A:** Immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Oleu for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * p <0.05, ** p <0.01 vs. control. **B:** Immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of HT and HT-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * p <0.05, ** p <0.01 vs. control. **C:** Immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of VA and VA-Sul for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * p <0.05, ** p <0.01, *** p <0.001 vs. control. **D:** Immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of HVA and HVA-Sul for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control.

In order to test whether Oleu and VA mediated activation of apoptosis in pancreatic cancer cell lines was exerted through activation of caspases pathways, the level of apoptosis-related caspase 3 was assessed by Western blot. As shown in **Figure 3**, there was no activation of caspase 3 for both cell lines by either Oleu or VA. Similar results

were obtained when assessing the levels of full and cleaved poly(ADP-ribose) polymerase (PARP) levels, which followed a similar pattern as for caspase 3.

Consequently, these results suggest that the Oleu-mediated decrease in cell growth in both cell lines and the VA-mediated decrease in cell growth in Panc-1 occur through activation of cell apoptosis, but that apoptosis is not mediated by activation of the caspases pathway.

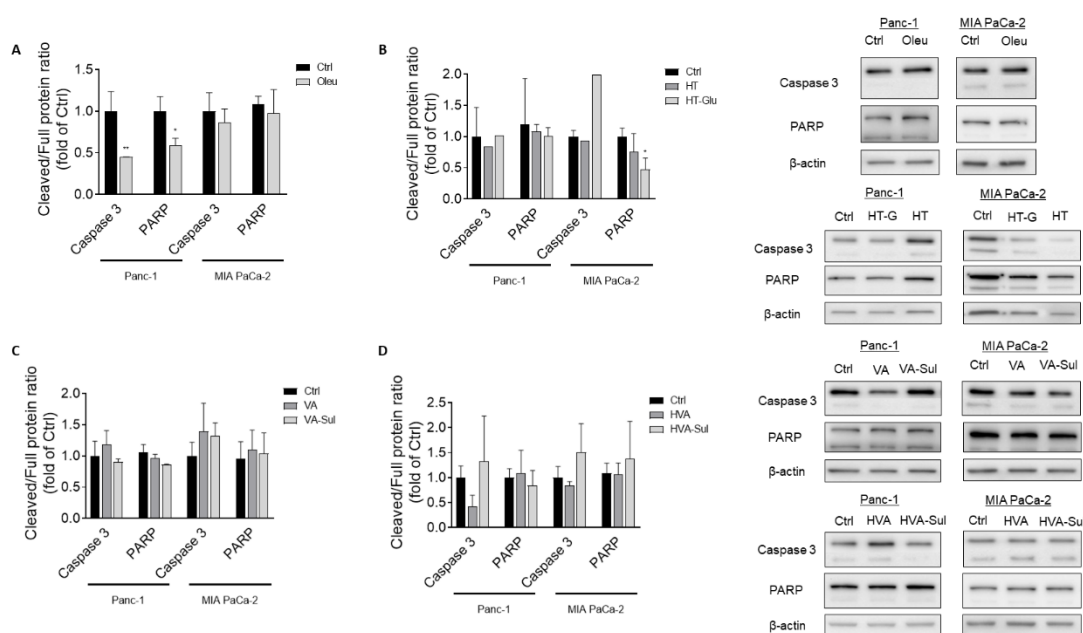


Figure 3. Effect of the compounds Oleuropein (Oleu), Hydroxytyrosol (HT), Hydroxytyrosol glucuronide (HT-G), Vanillic acid (VA), Vanillic acid sulfate (VA-Sul), Homovanillic acid (HVA) and Homovanillic acid sulfate (HVA-Sul) on apoptosis-related protein expression. **A:** Immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Oleu for 24 h. Loading control: β -Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein. * p <0.05, ** p <0.01 vs. control. **B:** Immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of HT and HT-G for 24 h. Loading control: β -Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein. * p <0.05 vs. control. **C:** Immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of VA and VA-Sul for 24 h. Loading control: β -Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein. **D:** Immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of HVA and HVA-Sul for 24 h. Loading control: β -Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein.

3.4 Effect of LC and OE common metabolites on cell cycle progression

The subsequent step involved evaluating whether metabolites could influence cell cycle progression by examining the expression of the cell cycle regulator Cyclin E1 via Western blot analysis. **Figure 4** illustrates that Oleu treatment led to a significant reduction in Cyclin E1 levels in both Panc-1 and MIA PaCa-2 cell lines, while VA treatment significantly decreased Cyclin E1 levels only in Panc-1 cells.

It is noteworthy that both VA-Sul and HVA-Sul despite having an overall negative regulatory effect on cell growth for both cell lines do not affect cell growth through activation of apoptosis or cell cycle regulation.

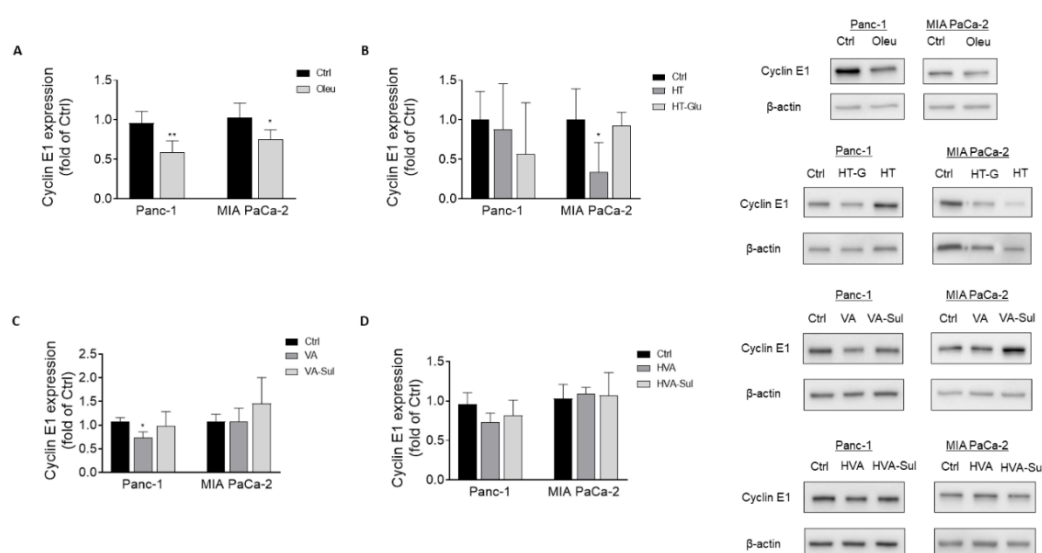


Figure 4. Effect of the compounds Oleuropein (Oleu), Hydroxytyrosol (HT), Hydroxytyrosol glucuronide (HT-G), Vanillic acid (VA), Vanillic acid sulfate (VA-Sul), Homovanillic acid (HVA) and Homovanillic acid sulfate (HVA-Sul) in cell cycle arrest in pancreatic cancer cells. **A:** Immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Oleu for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * p <0.05, ** p <0.01 vs. control. **B:** Immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of HT and HT-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * p <0.05 vs. control. **C:** Immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of VA and VA-Sul for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * p <0.05 vs. control. **D:** Immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of HVA and HVA-Sul for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control.

4. Discussion

Overall, it has been observed that the common metabolites associated with the consumption of LC and OE extracts exhibit different proportions of bioavailable concentrations in plasma, depending on the extract consumed. Specifically, OE extract consumption results in higher levels of oleuropein and hydroxytyrosol-derived metabolites, while LC extract consumption leads to greater production of homovanillic acid, its sulfated isomers, and the sulfated form of vanillic acid.

In the case of metabolites present in plasma following the ingestion of LC extract, it is notable that verbascoside, the primary metabolite in this extract, is absent. This suggests that all ingested verbascoside undergoes metabolism, leading to the production of homovanillic acid, which originates from the hydroxytyrosol component of verbascoside, and vanillic acid sulfate, which is derived from the caffeic acid moiety within verbascoside (Villegas-Aguilar et al., 2024). It should also be noted that the concentration of all the possible metabolites that may result, since the glucuronidated forms of both vanillic acid and homovanillic acid and the sulfated form of hydroxytyrosol have not been quantified. Conversely, upon examining the metabolites in plasma after ingestion of OE extract, oleuropein, hydroxytyrosol, and its glucuronidated derivative are present. This can be attributed to the fact that the OE extract contains both hydroxytyrosol and oleuropein, as well as verbascoside (Villegas-Aguilar et al., 2024), thus providing a greater source of hydroxytyrosol, making it more bioavailable in plasma. Therefore, based on the results obtained, OE extract consumption leads to higher bioavailable levels of hydroxytyrosol and glucuronidated hydroxytyrosol in plasma, whereas LC extract consumption results in hydroxytyrosol predominantly converting to homovanillic acid.

It should be noted that oleuropein was found to be bioavailable in large amounts, especially isomer 1, but only in 3 volunteers. Likewise, in the case of homovanillic acid isomer 2, it only appeared in 2 and 3 volunteers with the consumption of LC and OE extracts, respectively. This indicates that the presence of metabolites varies between people and some people are producers of more metabolites and others of others. These variations in the absorption, distribution, metabolism and excretion of metabolites of phenolic compounds have been related mainly to the composition and activity of the intestinal microbiota, but also to genetic polymorphisms, age, sex, ethnicity, BMI, (patho)physiological state and physical activity, depending on the subclass of (poly)phenols considered (Favari et al., 2024). In the specific case of oleuropein metabolism, sex has been identified as an influencing factor. In a study conducted by Beck et al. (2013), it was observed that women exhibited higher concentrations of bioavailable oleuropein in plasma compared to men. However, when comparing the presence of conjugated metabolites of hydroxytyrosol, men were found to have higher amounts than women (de Bock et al., 2013).

Focusing on the concentration of excreted metabolites, it is notable that oleuropein isomer 1, despite being highly bioavailable in plasma after OE extract consumption in three volunteers, is excreted in very low concentrations compared to the values of other excreted metabolites, and oleuropein isomer 2 is not detected at all. These findings align with the results reported by Kendall et al. (2012), where the excretion of metabolites following chronic ingestion of olive leaf supplements was assessed, and oleuropein was not detected in urine samples. However, glucuronic acid conjugates derived from oleuropein aglycone were present in urine samples (Kendall et al., 2012).

Furthermore, it was observed that vanillic acid is predominantly excreted in its sulfated form in both the LC and OE extracts, with particularly high levels associated with the consumption of the LC extract. Conversely, homovanillic acid was excreted in larger quantities in its non-sulfated form.

According to the results obtained, vanillic acid is excreted in higher concentration in its sulfated form, while homovanillic acid is excreted in its unconjugated form. This is in agreement with literature data according to which vanillic acid is excreted in the urine mainly in its conjugated form, but also as a free metabolite (Sayavongsa et al., 2007), whereas homovanillic acid is excreted mainly in its free unconjugated form (Amin et al., 1992).

In this regard, the daily consumption of encapsulated products containing a combination of LC and OE extracts can sustain the presence of these metabolites in the body over time. This sustained presence may enable a continuous bioactive effect over an extended period. Among all the metabolites investigated from both matrices, oleuropein exhibited the most potent anticancer activity by significantly reducing the growth of the two tumor cells tested. This strong anticancer effect of Oleu stems from its robust cytokinetic properties, including the inhibition of proliferation, induction of apoptosis, and blockade at the G1/S cell cycle transition (Wei et al., 2020). Additionally, this metabolite reached the highest plasma levels after a single dose, suggesting that prolonged consumption of this olive leaf extract could maintain high plasma concentrations of oleuropein. In this context, given that oleuropein has the highest anticancer potential among the metabolites, it would be valuable to develop encapsulation strategies to protect this compound from gastrointestinal degradation. For instance, microencapsulation with

sodium alginate via spray drying has been employed to investigate the evolution of oleuropein during *in vitro* gastrointestinal digestion, as well as its bioaccessibility and bioavailability. The results demonstrated that encapsulating oleuropein provided protection under gastric conditions and facilitated controlled release in the intestines, leading to increased bioaccessibility (58%) and potential bioavailability (20%) (González et al., 2019).

However, it should be noted that Oleu activation appears to occur through activation of cell apoptosis, but that apoptosis is not mediated by activation of the caspase pathway. Research indicates that apoptosis cannot be solely activated by caspases; indeed, cell death can be induced even in the absence of caspase involvement, facilitated by the expression of Bax or Bak. The caspase-independent pathway of apoptosis operates without the involvement of caspases, relying instead on mitochondrial pro-apoptosis proteins like AIF (Apoptosis-Inducing Factor) and endonuclease G. AIF translocates from mitochondria to the nucleus, causing nuclear DNA fragmentation. Unlike the caspase-dependent pathway, apoptosis in this pathway can be induced without caspase activation, involving mitochondrial factors such as AIF, leading to mitochondrial swelling, chromatin condensation, and cytochrome C release (Surya Negara et al., 2020). These findings are groundbreaking because previous literature studies examining the effects of oleuropein on various cancer types, such as liver (YAN et al., 2015) or breast cancer (Chimento et al., 2014), have shown activation of apoptosis through caspase-dependent pathways. However, in the case of pancreatic cancer, there is a lack of studies demonstrating the activation of apoptosis by oleuropein's caspase-dependent action. Therefore, this study provides novel insights into the mechanism of action of oleuropein specifically in pancreatic cancer cells.

Concerning Oleu's capacity to modulate the cell cycle, it exerts an inhibitory effect on the G1/S transition by specifically targeting Cyclin E1. These observations are consistent with findings involving other phenolic compounds, such as gallic acid, which similarly influence the G1/S transition in lung cancer cells (Ko et al., 2022). Notably, the dysregulation of the G1/S transition represents a hallmark mutation in cancer, underscoring the potential utility of inducing cell cycle arrest as a strategy for eradicating cancer cells (Evan and Vousden, 2001). The impact on Cyclin E1 has also been evidenced for HT, albeit solely in the MIA Paca-2 cell lines, and for VA, but exclusively in the Panc-1 line.

It is noteworthy to consider the case of VA-Sul and HVA-Sul in the cell growth assays, which exhibited a significant reduction in the growth of both tumor cell types across the tested concentrations and durations. However, these metabolites did not impact the expression and activation of the apoptosis process, nor did they affect the G1/S transition of the cell cycle. This suggests that while these metabolites may have a growth-suppressive effect on tumor cells, the mechanism of cell death is likely to occur through a non-apoptotic pathway, such as autophagy (Kiruthiga et al., 2020). Phenolic compounds like curcumin and resveratrol have demonstrated anticancer effects by inducing autophagy in malignant glioma cells and ovarian cancer cells, respectively (Kueck et al., 2007; Shinojima et al., 2007).

5. Conclusions

OE and LC extracts are an excellent source of bioavailable metabolites that show promise as anticancer agents. The consumption of both extracts leads to high plasma levels of metabolites derived from the metabolization of verbascoside, the main phenolic compound present in LC, and oleuropein, the main phenolic compound of OE.

Oleuropein, a metabolite in olive leaf extract, has high bioavailability and has shown anticancer properties against pancreatic cancer cells by inducing cell apoptosis, although this process does not involve the caspase pathway.

Additionally, other bioavailable metabolites derived from consuming both extracts, such as VA-Sul and HVA-Sul, have also demonstrated inhibitory effects on the growth of pancreatic cancer cells, likely through a non-apoptotic mechanism.

In this context, it is essential to underscore the importance of conducting assays that assess the bioactive potential of metabolites that are both bioavailable and metabolized from the phenolic compounds originally present in the extracts. These studies are key to understanding which metabolites can effectively reach target organs and exert their biological effects.

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Supplementary material

Table S1. Standard calibration curve used for the quantification of the metabolites.

Analytical standard	LOD (μM)	LOQ (μM)	Calibration Range (μM)	Calibration Equations	R ²
Oleuropein	0.006 $\pm$ 0.004	0.02 $\pm$ 0.01	(LOQ – 10)	$y = 19857.38x + 10067.55$	0.991
Hydroxytyrosol	0.010 $\pm$ 0.007	0.03 $\pm$ 0.02	(LOQ – 10)	$y = 9794.47x - 215.81$	0.999
Hydroxytyrosol glucuronide	0.014 $\pm$ 0.009	0.05 $\pm$ 0.03	(LOQ – 10)	$y = 12914.47x + 153.49$	0.999
Vanillic acid	0.01 $\pm$ 0.01	0.05 $\pm$ 0.03	(LOQ – 100)	$y = 1416.14x - 541.21$	0.999
Vanillic acid sulfate	0.03 $\pm$ 0.01	0.10 $\pm$ 0.04	(LOQ – 50)	$y = 28157.54x - 2173.54$	0.999
Homovanillic acid	0.02 $\pm$ 0.01	0.07 $\pm$ 0.04	(LOQ – 50)	$y = 2870.01x + 1156.43$	0.999
Homovanillic acid sulfate	0.02 $\pm$ 0.01	0.07 $\pm$ 0.05	(LOQ – 10)	$y = 29971.79x - 1303.42$	0.999

LOD: Limit of Detection; LOQ: Limit of Quantification, R²: Coefficient of variation.

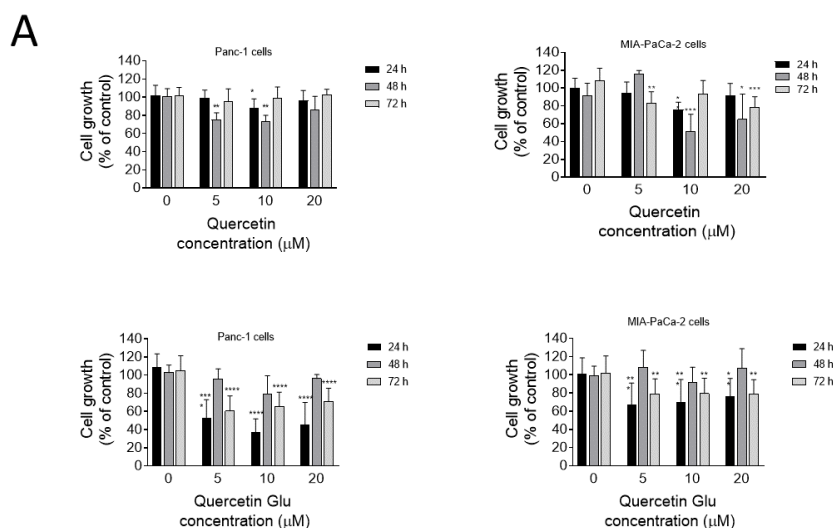
Appendix Chapter 6

During the pre-doctoral research stay, which culminated in the development of Chapter 6, the anticancer potential of additional selected compounds found in plasma after administration of target extracts in a nutritional intervention trial in pancreatic cancer cell lines (Panc-1 and MIA PaCa-2 cells) was also evaluated, together with the compounds analyzed in Chapter 6. Compound selection was carried out based on the results obtained in Chapters 4 and 5, with a total number of 108 compounds identified as candidates. Of the total of 108 bioavailable candidates detected, 24 were selected. The selection of the metabolites to be tested was based on their commercial availability at the time of the search for commercial standards. The metabolites selected, in addition to the seven evaluated in Chapter 6, were Hydroxycitric acid lactone (Phytolab, Vestenbergsgreuth, Germany), Epigallocatechin gallate (Sigma-Aldrich, St. Louis, MO, USA), Catechin (Sigma-Aldrich, St. Louis, MO, USA), Epicatechin (Sigma-Aldrich, St. Louis, MO, USA), Palmitoleic acid (Sigma-Aldrich, St. Louis, MO, USA), Dihydrocaffeic acid (Merck KGaA, Darmstadt, Germany), Dihydrocaffeic acid 3-O-glucuronide diamonium salt (TRC-Canada, Toronto, Ontario, Canada), Caffeic acid (Sigma-Aldrich, St. Louis, MO, USA), Trans ferulic acid (Phytolab, Vestenbergsgreuth, Germany), Quercetin (Sigma-Aldrich, St. Louis, MO, USA), Quercetin-3-O-glucuronide (HWI, Frankfurt am Main, Germany), Dihydroferulic acid (Cymit, Barcelona, Spain), Dihydroferulic acid 4-O-sulfate sodium salt (TRC-Canada, Toronto, Ontario, Canada), Dihydroferulic acid 4-O-glucuronide (TRC-Canada, Toronto, Ontario, Canada), Silybin (HWI, Frankfurt am Main, Germany), Luteolin (Sigma-Aldrich, St. Louis, MO, USA), and Luteolin-7-O-glucuronide (HWI, Frankfurt am Main, Germany).

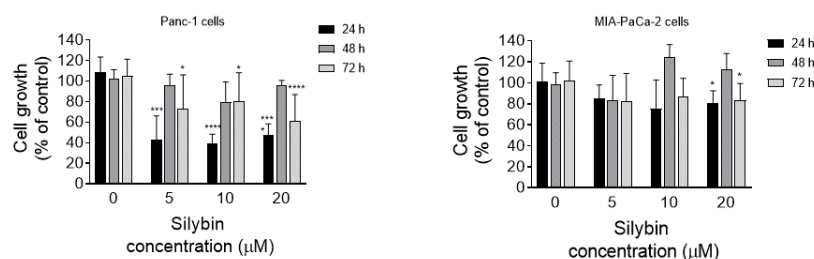
Cell viability and Western blot were carried out using the same methodology used in Chapter 6 for the evaluation of metabolites.

The metabolites Hydroxycitric acid lactone, Epigallocatechin gallate, Catechin, Epicatechin, Palmitoleic acid, Dihydrocaffeic acid, Dihydrocaffeic acid 3-O-glucuronide, Caffeic acid, and Trans ferulic acid not exert a significant effect on decreasing cell viability in the Panc-1 and MIA PaCa-2 cell lines (data not shown), so no further assays were performed with these.

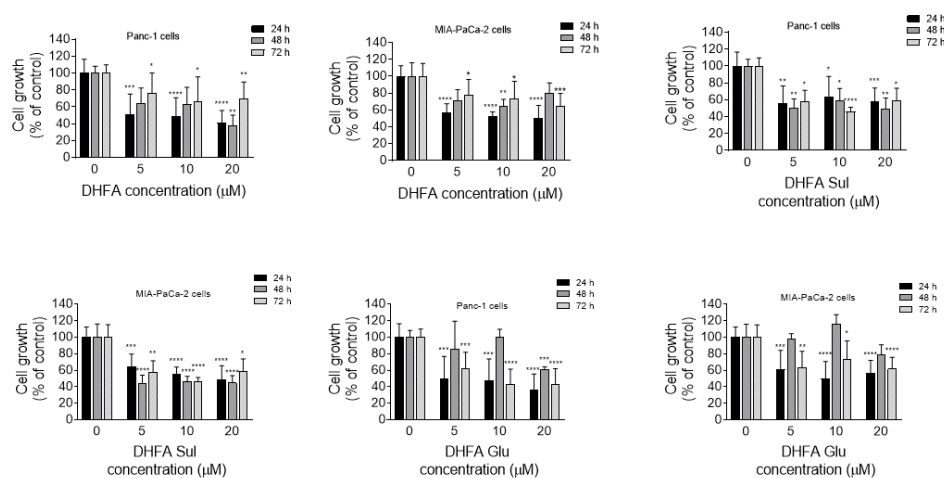
The effect shown by the metabolites, Quercetin, Quercetin-3-O-glucuronide, Dihydroferulic acid, Dihydroferulic acid 4-O-sulfate, Dihydroferulic acid 4-O-glucuronide, Silybin, Luteolin, and Luteolin-7-O-glucuronide on cell viability in the pancreatic cancer cell lines Panc-1 and MIA PaCa-2, with three concentrations of each of the metabolites (5, 10 and 20 μM) for 24, 48 and 72 h is shown in **Figure 1**.



B



C



D

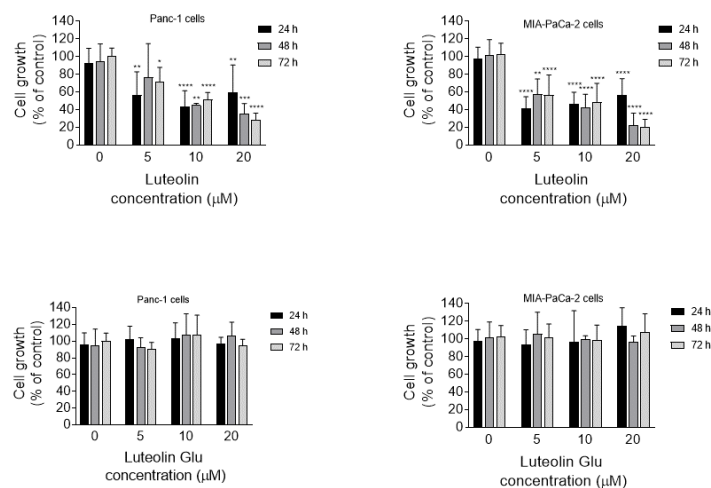


Figure 1. Effect of the compounds Quercetin and Quercetin glucuronide (Quercetin Glu) (A); Silybin (B); Dihydroferulic acid (DHFA), Dihydroferulic acid sulfate (DHFA Sul) and Dihydroferulic acid glucuronide (DHFA Glu) (C) and Luteolin and Luteolin glucuronide (Luteolin Glu) on cell growth in Panc-1 and MIA PaCa-2 cells. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

To explore the mechanism of Quercetin, Quercetin-3-O-glucuronide, Dihydroferulic acid, Dihydroferulic acid 4-O-sulfate, Dihydroferulic acid 4-O-glucuronide, Silybin, Luteolin, and Luteolin-7-O-glucuronide -induced apoptosis, we determined, in Panc-1 and MIA PaCa-2 cells, the expression levels of multiple proteins that regulate apoptosis (**Figures 2 and 3**).

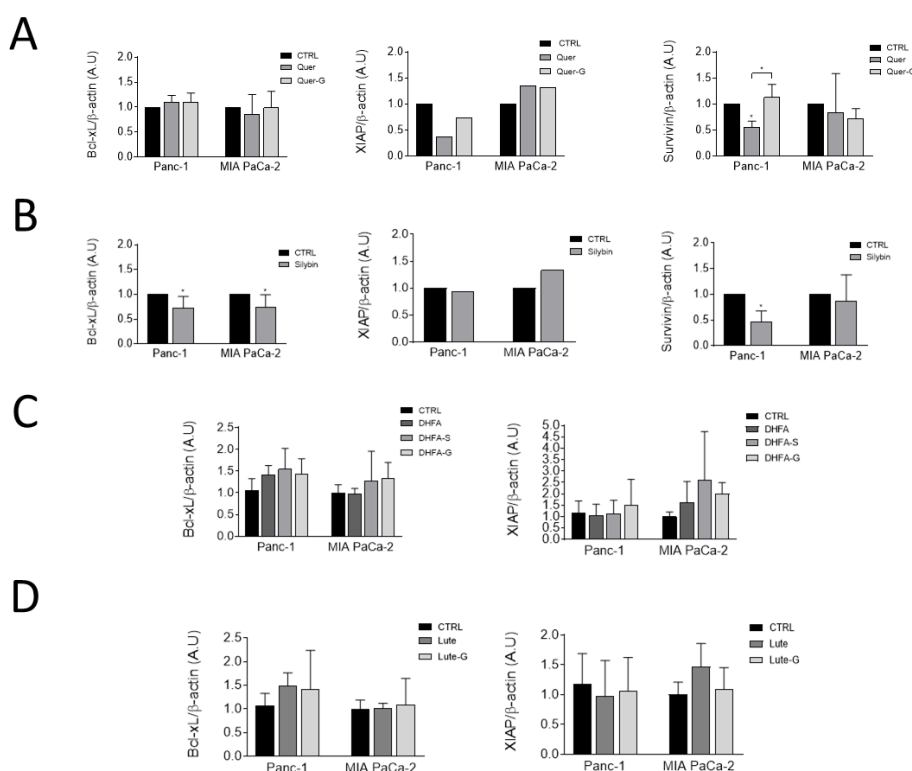


Figure 2. Effect of the compounds Quercetin (Quer), Quercetin glucuronide (Quer-G), Silybin, Dihydroferulic acid (DHFA), Dihydroferulic acid sulfate (DHFA-S), Dihydroferulic acid glucuronide (DHFA-G), Luteolin (Lute) and Luteolin glucuronide (Lute-G) on apoptosis-related protein expression. **A:** Graphical representation of immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Quer and Quer-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * $p < 0.05$ vs. control. **B:** Graphical representation of immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Silybin for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * $p < 0.05$ vs. control. **C:** Graphical representation of immunoblots for Bcl-xL, and XIAP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of DHFA, DHFA-S, and DHFA-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. **D:** Graphical representation of immunoblots for Bcl-xL, and XIAP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Lute and Lute-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control.

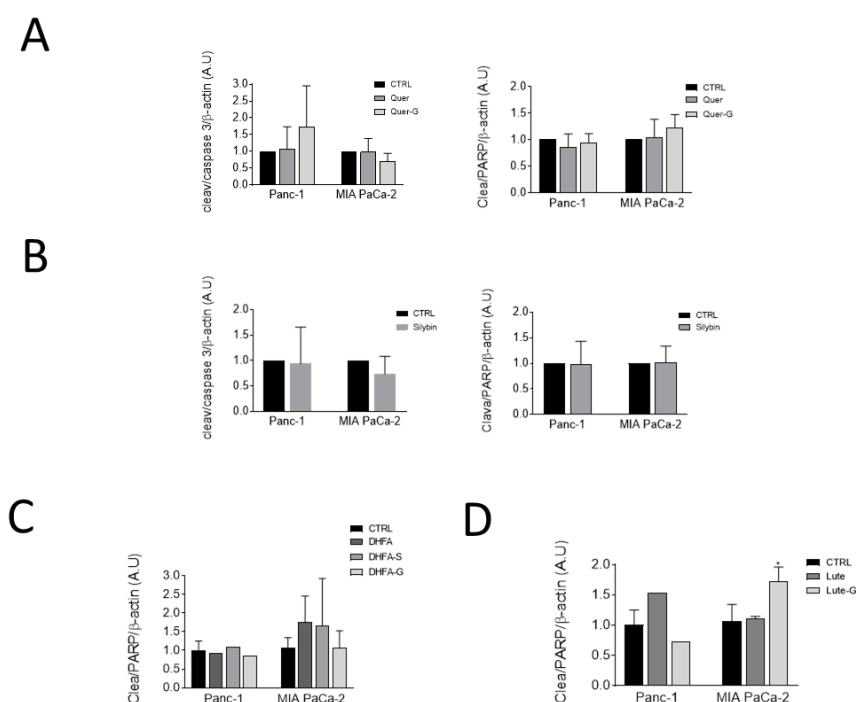


Figure 3. Effect of the compounds Quercetin (Quer), Quercetin glucuronide (Quer-G), Silybin, Dihydroferulic acid (DHFA), Dihydroferulic acid sulfate (DHFA-S), Dihydroferulic acid glucuronide (DHFA-G), Luteolin (Lute) and Luteolin glucuronide (Lute-G) on apoptosis-related protein expression. **A:** Graphical representation of immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Quer and Quer-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. **B:** Graphical representation of immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Silybin for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. **C:** Graphical representation of immunoblots for full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of DHFA, DHFA-S, and DHFA-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. **D:** Graphical representation of immunoblots for full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Lute and Lute-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * $p < 0.05$ vs. control.

Finally, the role of Quercetin, Quercetin-3-O-glucuronide, Dihydroferulic acid, Dihydroferulic acid 4-O-sulfate, Dihydroferulic acid 4-O-glucuronide, Silybin, Luteolin, and Luteolin-7-O-glucuronide in cell cycle progression was then assessed by examining the expression of the cell cycle regulator Cyclin E1 by Western blot analysis (**Figure 3**).

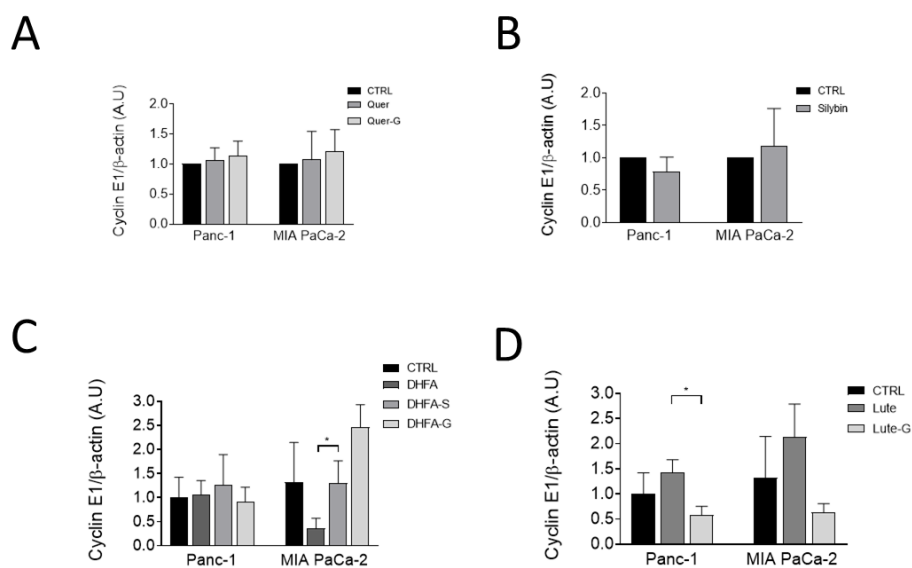
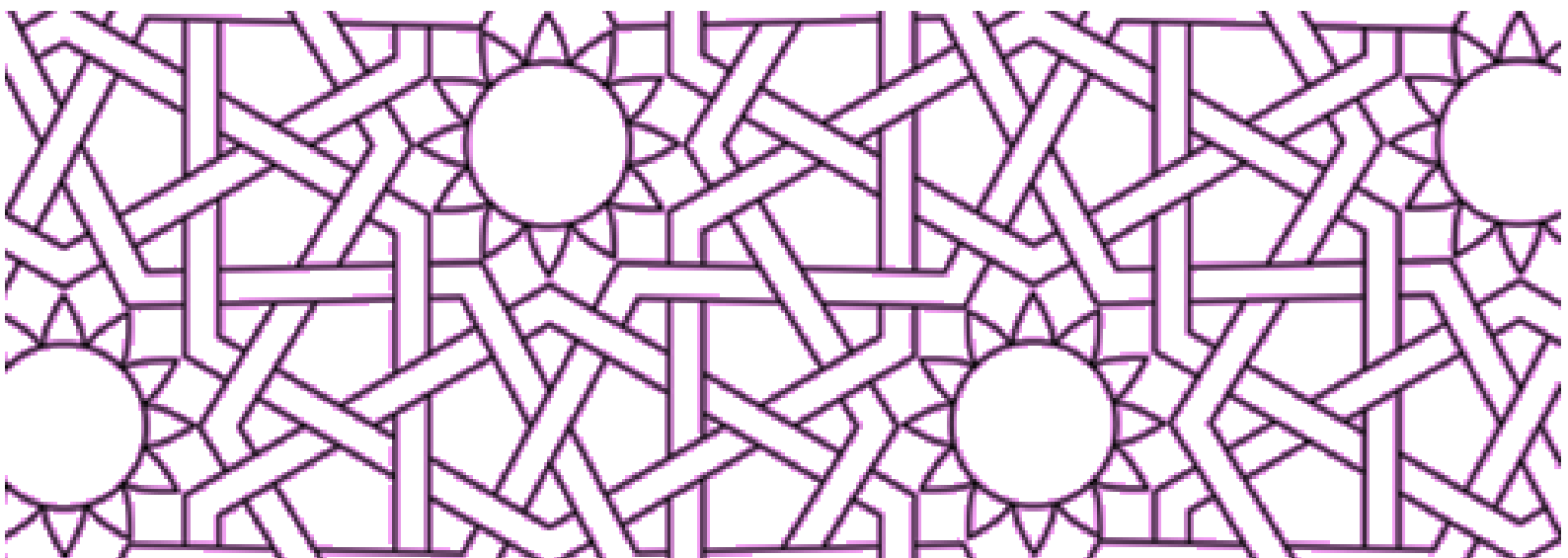


Figure 4. Effect of the compounds Quercetin (Quer), Quercetin glucuronide (Quer-G), Silybin, Dihydroferulic acid (DHFA), Dihydroferulic acid sulfate (DHFA-S), Dihydroferulic acid glucuronide (DHFA-G), Luteolin (Lute) and Luteolin glucuronide (Lute-G) in cell cycle arrest in pancreatic cancer cells. **A:** Graphical representation of immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Quer and Quer-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. **B:** Graphical representation of immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Silybin for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. **C:** Graphical representation of immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of DHFA, DHFA-S, and DHFA-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * $p < 0.05$ vs. control. **D:** Graphical representation of immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μ M of Lute and Lute-G for 24 h. Loading control: β -Actin. Bands were quantified and results are expressed as percentage of control. * $p < 0.05$ vs. control.

In conclusion, the metabolites Quer, Quer-G, Silybin, DHFA, DHFA-S, DHFA-G, Lute, and Lute-G demonstrated a significant reduction in the cell viability of the two pancreatic cancer cell types tested. However, the specific mechanisms through which they decrease cell viability could not be elucidated based on the assays conducted. Therefore, it is crucial to continue investigating these findings to better understand the mechanisms by which these potential anticancer agents exert their bioactive effects in pancreatic cancer cells.

Conclusions

Conclusiones



Conclusions

In conclusion, this doctoral thesis presents a study of phenolic compounds employing two methodological approaches: a traditional one and a novel one in which the sequence of phases of the traditional approach has been modified. The two approaches have generated new insights into the role of phenolic compounds present in different plant matrices in relation to various chronic diseases. It is noteworthy that the novel approach underscores the necessity of initially identifying bioavailable compounds and subsequently examining their bioactivity, a methodology that contrasts with the conventional approach of examining bioactivity prior to identifying bioavailable compounds. The specific conclusions derived from the results obtained are detailed below.

1. Phenolic compounds exert effects on multiple cellular and molecular pathways involved in various chronic disorders, thereby exemplifying their pleiotropic nature. Metabolic pathways associated with energy metabolism, inflammation and ageing are interlinked and rely on central molecules for their regulation. Notwithstanding the aforementioned interconnections, specific pivotal molecules within each pathway are subject to the regulatory influence of phenolic compounds. The AMPK and mTOR regulated pathways play a pivotal role in energy metabolism. *In vitro* and *in vivo* studies have demonstrated that natural extracts, including *L. citriodora* and its principal compound, verbascoside, as well as *S. marianum* extract and secoiridoids derived from extra virgin olive oil, exert a beneficial impact on AMPK and a detrimental effect on mTOR. With respect to inflammatory metabolic pathways, phenolic compounds exert their influence primarily on Nrf2 and NF- κ B. These

compounds, including silybin and isosilybin from *S. marianum* extract, *L. citriodora* extract and (-)-epicatechin from *T. cocoa*, have demonstrated regulatory effects in both *in vitro* and *in vivo* studies. Furthermore, in metabolic pathways related to ageing, numerous intermediates are shared with those already mentioned, indicating an interrelationship between energy metabolism and inflammatory pathways, all of which are subject to the regulatory action of phenolic compounds.

2. The antioxidant, anti-inflammatory and neuroprotective effects of five plant extracts with different phenolic compositions (*T. cacao*, *H. sabdariffa*, *S. marianum*, *L. citriodora* and *O. europaea*) were evaluated in order to analyse the relationship between the different types of phenolic compounds and their bioactivity. The findings demonstrated that, despite variations in their phenolic profiles, all extracts exhibited favourable bioactive outcomes in the majority of tests. In particular, extracts with markedly disparate phenolic compositions, such as those derived from *T. cacao* and *S. marianum*, exhibited comparable inhibitory efficacy on enzymes implicated in inflammatory processes and skin ageing. Furthermore, extracts of *H. sabdariffa* and *T. cacao*, despite exhibiting low phenolic content, demonstrated noteworthy antioxidant and antiradical capacity. The findings of this research demonstrate that phenolic compounds present in a variety of plant matrices possess shared bioactive properties. The diversity of phenolic compound families is indicative of their high bioactivity against a range of biological targets.
3. The antiproliferative efficacy of olive leaf extract, both in its free form and encapsulated in formulations such as liposomes, ethosomes and glycosomes, has been demonstrated in MCF7 breast cancer cells. However, in non-tumorigenic MCF10A cells, the extract has been observed to increase cell proliferation. The

mechanism by which olive leaf extract exerts its antiproliferative effect on the breast cancer tumour line suggests that the extract may induce apoptosis through a mitochondria-dependent ROS-mediated pathway. The encapsulation process does not impair the anticancer properties of the extract; rather, it enhances the bioavailability of the extract, facilitating its use in functional foods and allowing a more targeted release in the gastrointestinal tract. Furthermore, the encapsulation process offers protection to the extract. Additionally, there is a growing interest in the development of topical formulations for the treatment of breast cancer, where the use of encapsulation technology has been shown to enhance the permeation of the extract through the skin. Moreover, the stability of these nanovesicles over time ensures that they can be effectively stored, thereby protecting the extract from degradation.

4. The potential of untargeted metabolomics for rapid and comprehensive analysis of a wide range of exogenous bioavailable or metabolised compounds in biological samples derived from the ingestion of various bioactive plant sources was demonstrated. The methodology was evaluated in three different matrices (*H. sabdariffa*, *S. marianum* and *T. cacao*), resulting in the identification of the main metabolites that appear to be bioavailable after ingestion of the three matrices in an acute study in humans. It is noteworthy that certain metabolites, including 2-o-caffeoylhydroxycitric acid, quercetin glucuronide, and eriodictyol, which are present in plasma following *S. marianum* ingestion, would have been challenging to detect using a targeted approach. Consequently, this study has enabled the identification of the principal metabolites without the necessity of predicting metabolic pathways prior to analysis, which would be required in a targeted study. Moreover, for a more

comprehensive analysis, it allows the selection of samples for a targeted metabolomic approach, thereby complementing the information obtained. In this regard, these results have demonstrated both the potential and limitations of using this untargeted method for bioavailability studies. Indeed, it was observed that the type of bioactive matrix influences the number of metabolites detected by the untargeted method.

5. The study of plasma bioavailable metabolites from the consumption of *O. europaea* and *L. citriodora* extracts in an acute nutritional intervention study in humans, using an untargeted approach, has yielded significant and novel results. This methodology enabled the identification of 64 circulating metabolites derived from bioactive extracts of *O. europaea* and *L. citriodora*, of which nine were common to both. The notable disparities in T_{max} for the shared metabolites indicate the presence of disparate metabolic pathways that are contingent on the plant matrix and, subsequently, on the parent compounds. This emphasises the potential of combining both extracts in nutraceutical development to ensure that circulating metabolites remain in the bloodstream for a longer period, thereby increasing the likelihood of reaching target tissues.
6. The extracts of *O. europaea* and *L. citriodora* represent an excellent source of bioavailable metabolites with significant potential as anticancer agents. The ingestion of these extracts results in elevated plasma levels of metabolites derived from the metabolism of verbascoside, the principal phenolic compound in *L. citriodora*, and oleuropein, the principal phenolic compound in *O. europaea*. Oleuropein has been demonstrated to exhibit high bioavailability and has demonstrated anticancer properties against pancreatic cancer cells by inducing cell apoptosis without involvement of the caspase pathway. Furthermore, other bioavailable metabolites

derived from consumption of these extracts, such as sulphated vanillic acid and sulphated homovanillic acid, have also demonstrated inhibitory effects on pancreatic cancer cell growth, presumably via a non-apoptotic mechanism. In this context, it is crucial to emphasise the significance of conducting assays that evaluate the bioactive potential of bioavailable and metabolised metabolites derived from the initial phenolic compounds present in the extracts. It is imperative that these studies be conducted in order to ascertain which metabolites are capable of effectively reaching target organs and exerting their biological effects.

Conclusiones

En conclusión, en esta tesis doctoral se ha llevado a cabo el estudio de los compuestos fenólicos mediante dos aproximaciones metodológicas, una tradicional y una nueva aproximación en el que se han alterado el orden de las fases del enfoque tradicional. A través de estas dos aproximaciones, se ha generado nuevo conocimiento sobre el papel de los compuestos fenólicos presentes en diversas matrices vegetales en relación con distintas enfermedades crónicas. Cabe destacar, la importancia de la nueva aproximación que destaca la importancia de conocer primero los compuestos biodisponibles y luego examinar la bioactividad de ellos, y no al revés, como se ha hecho tradicionalmente. A continuación, se detallan las conclusiones específicas derivadas de los resultados obtenidos.

1. Los compuestos fenólicos actúan sobre múltiples vías celulares y moleculares, implicadas en diversos trastornos crónicos, mostrando así su naturaleza pleiotrópica. Las vías metabólicas relacionadas con el metabolismo energético, la inflamación y el envejecimiento están interconectadas y comparten moléculas centrales en su regulación. Pese a estas conexiones, ciertas moléculas clave en cada vía están bajo la influencia reguladora de los compuestos fenólicos. En el metabolismo energético, las vías reguladas por AMPK y mTOR son cruciales. Estudios *in vitro* e *in vivo* han demostrado que extractos naturales como el de *L. citriodora* y su principal compuesto verbascósido, el extracto de *S. marianum* y los secoiridoides del aceite de oliva virgen extra, tienen un efecto positivo sobre AMPK y negativo sobre mTOR. Respecto a las vías metabólicas relacionadas con la inflamación, los compuestos fenólicos influyen principalmente en Nrf2 y NF- κ B. Estos compuestos, como la silibina y la isosilibina del

extracto de *S. marianum*, el extracto de *L. citriodora* y la (-)-epicatequina presente en *T. cocoa*, han demostrado efectos reguladores tanto en estudios *in vitro* como *in vivo*. Finalmente, en las vías metabólicas relacionadas con el envejecimiento, muchos intermediarios son comunes a los ya mencionados, indicando una interrelación entre las vías del metabolismo energético y la inflamación, todas sujetas a la acción reguladora de los compuestos fenólicos.

2. Se evaluaron los efectos antioxidantes, antiinflamatorios y neuroprotectores de cinco extractos vegetales con diferentes composiciones fenólicas (*T. cacao*, *H. sabdariffa*, *S. marianum*, *L. citriodora* y *O. europaea*) para analizar la relación entre los distintos tipos de compuestos fenólicos y su bioactividad. Los resultados indicaron que, a pesar de las diferencias en sus composiciones fenólicas, todos los extractos mostraron efectos bioactivos positivos en la mayoría de los ensayos. En particular, extractos con composiciones fenólicas muy distintas, como *T. cacao* y *S. marianum*, demostraron un poder inhibitor similar sobre enzimas involucradas en procesos inflamatorios y envejecimiento cutáneo. Además, los extractos de *H. sabdariffa* y *T. cacao*, aunque con bajo contenido fenólico, destacaron por su notable capacidad antioxidante y antirradical. Esta investigación resalta las propiedades bioactivas compartidas de los compuestos fenólicos presentes en diversas matrices vegetales. La diversidad de familias de compuestos fenólicos demuestra su alta bioactividad frente a diferentes objetivos biológicos.

3. El extracto de hoja de olivo, tanto libre como encapsulado en formulaciones como liposomas, etosomas y glicerosomas, demostró ser eficaz como agente antiproliferativo en células de cáncer de mama MCF7, pero no en células no tumorigénicas MCF10A, donde aumenta la proliferación celular. El mecanismo a

través del cual el extracto de hoja de olivo ejerce su efecto antiproliferativo en la línea tumoral del cáncer de mama implica que el extracto puede inducir la apoptosis a través de una vía mediada por ROS dependientes de las mitocondrias. La encapsulación no compromete sus propiedades anticancerígenas, sino que mejora la biodisponibilidad del extracto, facilitando su uso en alimentos funcionales y permitiendo una liberación más específica en el tracto gastrointestinal, además de ofrecer protección. Además, existe un interés en desarrollar formulaciones tópicas para el cáncer de mama, donde la encapsulación mejora la permeabilidad cutánea del extracto. Asimismo, la estabilidad de estas nanovesículas a lo largo del tiempo asegura que puedan almacenarse eficazmente, protegiendo el extracto de la degradación.

4. Se demostró el potencial de la metabolómica no dirigida para un análisis rápido y exhaustivo de una amplia gama de compuestos exógenos biodisponibles o metabolizados en muestras biológicas derivadas de la ingestión de diversas fuentes vegetales bioactivas. Esta metodología se evaluó en tres matrices diferentes (*H. sabdariffa*, *S. marianum* y *T. cacao*), resultando en la identificación de los principales metabolitos que parecen biodisponibles tras la ingestión de las tres matrices en un estudio agudo en humanos. Es importante destacar que ciertos metabolitos, como el ácido 2-o-cafeoilhidroxycítrico, el glucurónido de quercetina o el eriodictiol presentes en plasma tras la ingestión de *S. marianum*, difícilmente se habrían detectado con un enfoque dirigido. Así, este estudio ha permitido identificar los principales metabolitos sin necesidad de predecir las rutas metabólicas antes del análisis, como sería necesario en un estudio dirigido. Además, para un análisis más detallado, facilita la selección de muestras para una aproximación metabolómica dirigida,

complementando la información obtenida. En este sentido, estos resultados han demostrado tanto el potencial como las limitaciones de usar este método no dirigido para estudios de biodisponibilidad. De hecho, se observó cómo el tipo de matriz bioactiva influye en el número de metabolitos detectados mediante el método no dirigido.

5. El estudio de los metabolitos biodisponibles en plasma a partir del consumo de extractos de *O. europaea* y *L. citriodora* en un estudio de intervención nutricional agudo en humanos, utilizando un enfoque no dirigido, ha mostrado resultados significativos y novedosos. Esta metodología permitió la identificación de 64 metabolitos circulantes provenientes de los extractos bioactivos de *O. europaea* y *L. citriodora*, de los cuales 9 eran comunes entre ambos. Las diferencias significativas en el Tmax para los metabolitos comunes sugieren la existencia de distintos mecanismos de metabolización que dependen de la matriz vegetal y, por consiguiente, de los compuestos originales. Esto destaca el potencial de combinar ambos extractos en el desarrollo de nutracéuticos para lograr que los metabolitos circulantes permanezcan en el torrente sanguíneo durante un período más prolongado, aumentando así las posibilidades de alcanzar los tejidos diana.
6. Los extractos de *O. europaea* y *L. citriodora* son una excelente fuente de metabolitos biodisponibles con gran potencial como agentes anticancerígenos. El consumo de estos extractos resulta en altos niveles plasmáticos de metabolitos derivados de la metabolización del verbascósido, el principal compuesto fenólico en *L. citriodora*, y de la oleuropeína, el principal compuesto fenólico en *O. europaea*. La oleuropeína, ha demostrado poseer una alta biodisponibilidad y ha mostrado propiedades anticancerígenas contra células de cáncer de páncreas al inducir apoptosis celular sin

involucrar la vía de las caspasas. Además, otros metabolitos biodisponibles derivados del consumo de estos extractos, como el ácido vanílico sulfatado y el ácido homovanílico sulfatado, también han demostrado efectos inhibidores sobre el crecimiento de células de cáncer de páncreas, probablemente mediante un mecanismo no apoptótico. En este contexto, es esencial subrayar la importancia de realizar ensayos que evalúen el potencial bioactivo de los metabolitos biodisponibles y metabolizados a partir de los compuestos fenólicos presentes originalmente en los extractos. Estos estudios son fundamentales para comprender qué metabolitos pueden alcanzar eficazmente los órganos diana y ejercer sus efectos biológicos.

