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NEW ANALYTICAL APPROACHES TO ASSESS THE PHENOLIC COMPOSITION OF GUAVA (*Psidium guajava* L.) LEAVES WITH ANTI-DIABETIC RELATED RISK FACTORS BIOACTIVITY

Memoria presentada por

Elixabet Díaz de Cerio Alonso de Mezquía

Para optar al grado de

Doctor Internacional en Química por la Universidad de Granada

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LOS DIRECTORES DE LA TESIS

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POR

Elixabet Díaz de Cerio Alonso de Mezquía

Granada, Junio de 2017

Fdo. Dr. Antonio Segura Carretero

Fdo. Dr. Vito Verardo

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

Fdo. Elixabet Díaz de Cerio Alonso de Mezquía

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

CERTIFICA:

Que el trabajo realizado en la presente tesis doctoral titulada: "**NEW ANALYTICAL APPROACHES TO ASSESS THE PHENOLIC COMPOSITION OF GUAVA (*Psidium guajava* L.) LEAVES WITH ANTI-DIABETIC RELATED RISK FACTORS BIOACTIVITY",** se ha realizado bajo su dirección y la del Dr. Vito Verardo, en los laboratorios del Departamento de Química Analítica de la Universidad de Granada, del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (Parque Tecnológico de la Salud), así como también de manera parcial en las instalaciones del CIRI Agrifood de la Universidad de Bolonia (Cesena, Italia), el Centro de Investigación Biomédica (CIBM) y el Departamento de Farmacología de la UGR, y la Universidad de Almería, reuniendo todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dña. Elixabet Díaz de Cerio Alonso de Mezquía pueda optar al grado de Doctor Internacional en Química por la Universidad de Granada.

Y para que así conste, expido y firmo el presente certificado en Granada a 9 de Junio de 2017.

La doctoranda Elixabet Díaz de Cerio Alonso de Mezquía y los directores de la tesis Dr. Antonio Segura Carretero y Dr. Vito Verardo, garantizamos al firmar esta tesis doctoral que el trabajo ha sido realizado por la doctoranda bajo nuestra dirección y hasta donde nuestro conocimiento alcanza, durante la realización del trabajo, se han respetado los derechos de otros autores al ser citados cuando se han utilizado sus resultados o publicaciones.

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Quiero dar gracias, en primer lugar, a Alberto Fernández, por abrirme las puertas del grupo y darme la oportunidad de comenzar mi carrera investigadora. En segundo lugar, a mis directores, por guiarme en este camino. Antonio, gracias por tomar, entre otras, una de las mejores decisiones y por resolver los "problemillas"que he tenido durante estos años. ¡Veraaardoooo!, que decirte que no sepas ya, siempre ¡POR LA PRIMERA! A mis compañeros y amigos, por "aguantarme" durante estos años, ¡Y lo que os queda bonitos! Y por último, a mi familia, y en especial, a mi madre, que sin niguna duda, sin ella, no habría sido posible, y a Fernando, quien habiendo llegado en uno de los años más "complicados", ha conseguido que no pierda la sonrisa y la ilusión. ¡AHORA COMIENZA LO MEJOR!

“Todo aquello que puedes hacer, o sueñes poder hacer, comiénzalo.

La audacia contiene en si misma genio, poder y magia.”

-Johann Wolfgang von Goethe

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LIST OF ABBREVIATIONS

$ABTS^{\cdot+}$	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation
APCI	Atmospheric pressure chemical ionization
BBD	Box-Behnken design
CLC	Column liquid chromatography
CPC	Centrifugal partition chromatography
CTs	Condensed tannins
DAD	Diode array detector
DCCC	Droplet counter current chromatography
DM	Diabetes mellitus
DPPH	2,2'-diphenyl-1-picrylhydrazyl radical
d.w.	Dry weight
ESI	Electrospray ionization
FAB	Fast atom bombardment ionization
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GPC	Gel permeation chromatography
HHDP	Hexa-hydroxy-di-phenoyl moiety
HPLC	High-performance liquid chromatography
HSCCC	High-speed counter current chromatography
HTs	Hydrolysable tannins
IR	Infrared spectroscopy
IT	Ion trap
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCD	Non-communicable disease
NMR	Nuclear magnetic resonance
PAs	Proanthocyanidins
PC	Paper chromatography
Q	Quadrupole
RSM	Response surface methodology
TEAC	Trolox equivalent antioxidant capacity
TOF	Time-of-flight
TLC	Thin layer chromatography
TPC	Total phenolic content
TPTZ	2,4,6-tripyridyl-s-triazine
UAE	Ultrasound-assisted extraction
UPLC	Ultra performance liquid chromatography
UV-Vis	Ultraviolet-visible

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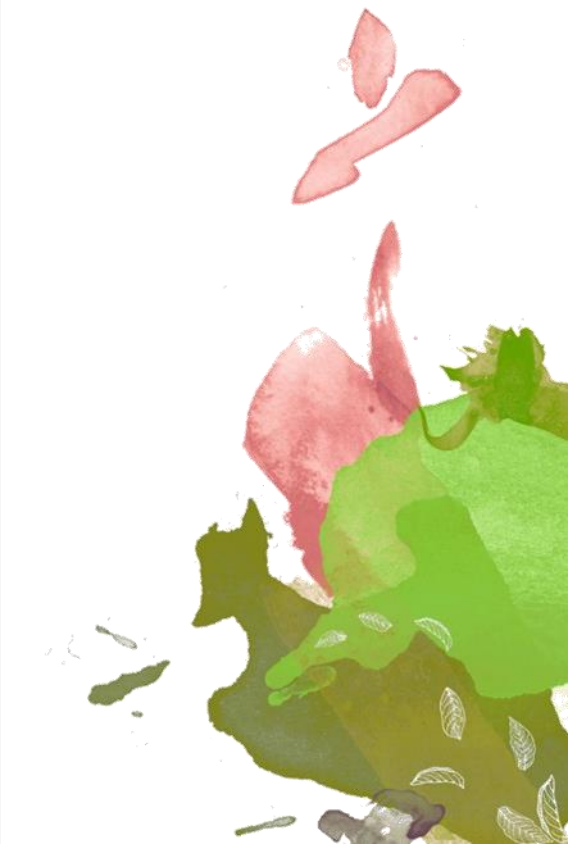
OBJETIVES/OBJETIVOS



Non-communicable diseases are one of the most concerning health problems worldwide, due to the mortality and morbidity rates, and the high cost for the public health system. In the last years, diabetes mellitus (DM) and its risk factors, which include cardiovascular complications, have become one of the illnesses under study. Prior to drug treatment, a change in lifestyle is recommended, physical activity and a plant-based diet, due to the beneficial effects beyond its nutritional composition.

Psidium guajava L., popularly known as guava, is a small tree belonging to the myrtle family (*Myrtaceae*). It is native from America, although its ability to adapt itself to several edapho-climatic conditions, allows its cultivation in areas such as Andalusia region. In particular, preparations of the leaves have traditionally been used in folk medicine for managing several diseases. Currently, there is clear evidence that these beneficial effects are promoted by secondary metabolites present in guava leaves. In this regard, there is increasing interest in studying their bioactive composition, mainly of phenolic compounds, and their effect on DM and some of its risk factors.

Consequently, the overall **aims** of this dissertation are:

To assess different advanced analytical tools in phenolic-enriched extracts of *Psidium guajava* L. leaves and to study their *in vivo* bioactivity to consider the possibility for further nutraceutical and/or functional food development.

For this goal, the following **specific objectives** were established:

- ✓ To optimize, by different experimental methods (one-variable-at-a-time and response surface methodology), the extraction of the target compounds in guava leaves common varieties (*pyrifera* and *pomifera*) and at different oxidative states.
- ✓ To comprehensive characterize, qualitatively and quantitatively, the phenolic fraction present in the leaves by the use of high-performance liquid

chromatography coupled to an appropriate detection technique based on the target compounds.

- ✓ To study the *in vivo* bioactivity of the best guava leaf-enriched extract against one of the most concerning illness, DM and some of its risk factors.

Las enfermedades no transmisibles son uno de los problemas de salud más preocupantes a nivel mundial, debido a sus índices de mortalidad y morbilidad, así como a su alto coste producido en el sistema de sanidad pública. En los últimos años, la diabetes mellitus (DM) y sus factores de riesgos, en los que se incluye las complicaciones cardiovasculares, se han convertido en unas de las enfermedades objeto de estudio. Previo a un tratamiento con medicamentos, se recomienda un cambio en el estido de vida, actividad física y una dieta rica en vegetales, debido a los efectos beneficiosos más allá de su composición nutricional.

La especie *Psidium guajava* L., comúnmente conocida como guayabo, es un árbol pequeño perteneciente a la familia de las Mirtáceas. Es oriundo de América, pero debido a su capacidad de adaptación a diversas condiciones edafoclimátimas, puede ser cultivado en diferentes zonas, como en Andalucía. La importancia de dicho árbol radica tanto en la fruta, por su alto valor nutricional, como en las hojas, las cuales han sido empleadas en medicina tradicional como tratamiento alternativo frente a diversas enfermedades, debido a sus propiedades terapéuticas. Estas propiedades beneficiosas para salud han sido atribuidas a los metabolitos secundarios presentes en las hojas. De ello deriva un creciente interés en el estudio de la composición bioactiva de la hoja, y en particular, de su composición fenólica, así como el efecto que ella provoca en la DM y en algunos de sus factores riesgo.

Teniendo en cuenta estas premisas, los **objetivos principales** que se pretenden conseguir con la presente Tesis Doctoral es:

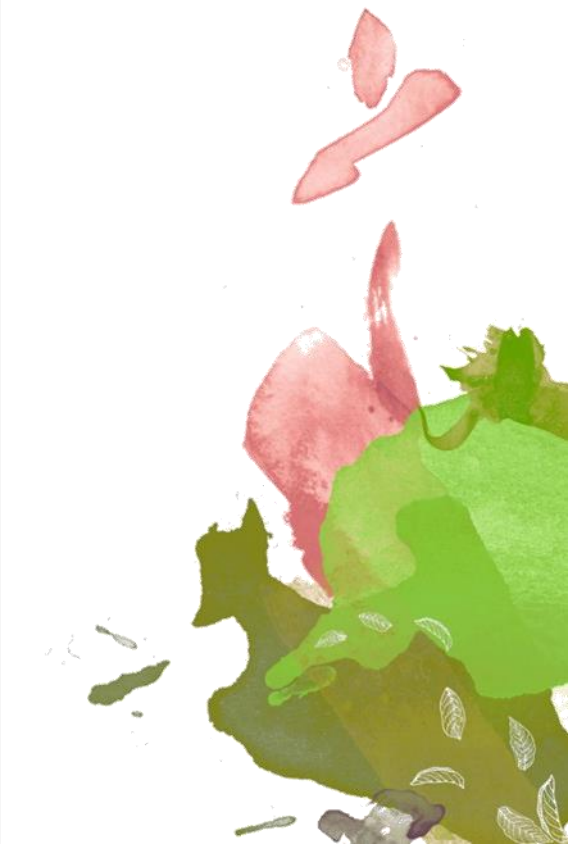
Evaluar la capacidad de diferentes herramientas analíticas avanzadas en extractos de hojas de guayabo enriquecidos en compuestos fenólicos y estudiar su

bioactividad en un modelo *in vivo*, con el fin de analizar su posible desarrollo como nutracéutico y/o alimento funcional.

Para la consecución de los objetivos principales, se establecieron una serie de **objetivos específicos** que se presentan a continuación:

- ✓ Optimizar, mediante diferentes métodos experimentales (variando una única variable y mediante la metodología de superficie de respuesta), la extracción de compuestos de interés a partir de las hojas de guayabo de las variedades más comunes (*pyrifera* y *pomífera*) y a diferentes estados oxidativos.
- ✓ Caracterizar exhaustivamente, es decir, tanto cualitativa como cuantitativamente, la fracción fenólica presente en las hojas de guayabo mediante el uso de cromatografía líquida de alta resolución acoplada a uno o varios detectores en función del tipo de analito.
- ✓ Estudiar la bioactividad del mejor extracto enriquecido de las hojas de guayabo en un modelo *in vivo*, frente a uno de los mayores problemas de salud pública: la DM y algunos de sus factores de riesgo asociados.

SUMMARY/RESUMEN



The present doctoral dissertation encompasses new analytical approaches to assess the phenolic composition of ***Psidium guajava* L. leaves** as a possible source as nutraceutical against diabetes mellitus (DM), compiling the identification, quantification, and also the evaluation of *in vivo* bioactivity of the **phenolic** composition of the leaves towards DM and some of its risk factors. It is divided into two main parts; the first one is the INTRODUCTION, which encompasses a brief explanation of DM disease, and an overview of the description and importance of guava tree, the nutritional value of the fruit, and the composition and the methods most used for the analysis of the **phenolic compounds** of the **leaves**, useful to know the analytical research that had been previously done with the leaves and to properly focus on further studies.

The second part includes the "EXPERIMENTAL PART. RESULTS AND DISCUSSION" carried out in the current PhD thesis and is sub-divided into two sections. ***Section I*** provides an extend characterization of the phenolic composition of guava leaves by using several advanced analytical techniques. **Chapter 1** concerns the optimization of the most common ancient remedy, the infusion, and it is compared with the bath-ultrasound assisted extraction, by one-variable-at-a-time method. It is a first attempt to characterize extracts rich in phenolic compounds from *P. guajava* L. leaves by means of identification and quantification of the compounds by high-performance liquid chromatography coupled to diode array detection and quadrupole mas spectrometer (HPLC-DAD-ESI-Q-MS), as well as the evaluation of their antioxidant capacity by FRAP and ABTS assays.

Chapter 2 deals with the optimization of the extraction of phenolic compounds by bath-ultrasound assisted extraction and one-variable-at-a-time method, as well as the accurate identification of several compounds (some of them for the first time) by high-performance liquid chromatography coupled to diode array detection and quadrupole time of flight spectrometry (HPLC-DAD-ESI-QTOF-MS). Besides, it includes the comparison between the main varieties of guava leaves (*pyrifera* and

pomifera vars.) according to the phenolic composition and the antioxidant capacity by FRAP and ABTS assays.

Chapter 3 studies the influence that the different oxidative states have on the phenolic composition of guava leaves var *pyrifera* by high-performance liquid chromatography coupled to diode array detection and quadrupole time of flight spectrometry (HPLC-DAD-ESI-QTOF-MS), and on antioxidant capacity (FRAP and ABTS), following the extraction method optimized in the previous chapter. Moreover, cyaniding-3-O-glucoside has been identified for the first time.

Chapters 4 and **5** were prepared in collaboration with the Inter-Departmental Centre for Agri-Food Industrial Research (CIRI Agrifood) and the Department of Agro-Food Sciences and Technologies from the University of Bologna (Italy) during a pre-doctoral stay founded by CEI-Biotic mobility fellowship (3 months). In **Chapter 4**, the optimization, by means of response surface methodology (Box-Behnken design (BBD)), of proanthocyanidins extraction from guava leaves var. *pyrifera* is carried out assisted by bath-ultrasound. The influence of the factors (time, temperature, solvent ratio and acid ratio) is evaluated in the response of the sum of the target compounds. Identification and quantification of this family of bioactive compounds is done by normal phase high-performance liquid chromatography coupled to fluorescence detection and quadrupole mass spectrometer (NP-HPLC-FLD-ESI-Q-MS). Also, the composition of guava leaves at different oxidative states is evaluated under the optimal conditions.

In **Chapter 5**, the sonotrode-ultrasound assisted extraction efficiency is evaluated at laboratory scale. Based on response surface methodology (BBD), the response of the factors (time, solvent ratio, and ultrasound power) is studied in the antioxidant capacity (DPPH and TEAC) and the main families of bioactive compounds present in guava leaves. Identification and quantification is carried out by high-performance liquid chromatography coupled to triple quadrupole mass spectrometer

(HPLC-ESI-QqQ-MS). Besides, the best process conditions are applied to *pyrifera* and *pomifera* vars.

The ***Section II*** focuses on the bioactivity of guava leaves. **Chapter 6** provides a view over the last decade of the health benefits, *in vitro* and *in vivo*, as well as some food applications reported with guava leaves. **Chapter 7** was prepared in collaboration with the Center for Biomedical Research (CIBM) and the Department of Pharmacology (CIBER-EHD and CIBER-Enfermedades Cardiovasculares, respectively) from the University of Granada. Following the same analytical scheme as chapter 2, here, is assessed the bioactivity of guava leaves var. *pyrifera* in mice with diet-induced obesity towards the DM related risk factors.

Lastly, the **annex** section was prepared in collaboration with "Advanced NMR methods and metal-based catalysts" research group from the University of Almeria. This appendix includes the fingerprint of guava leaf var. *pyrifera* by nuclear magnetic resonance (NMR) spectroscopy. The structure of this work has not the same appearance as the chapters, since it has not been concluded yet. Nevertheless, it has been included because it has been achieved during this period.

Esta memoria recoge nuevas herramientas analíticas para evaluar la composición fenólica de las hojas de guayabo (*Psidium guajava* L.) como una posible fuente como nutraceutico frente a la diabetes mellitus (DM), incluyendo la identificación, la cuantificación y la evaluación de la bioactividad, *in vivo*, de los compuestos fenólicos presentes en dichas hojas frente a la DM y alguno de los factores de riesgos asociados a la misma. La memoria está dividida en dos apartados principales; la primera de ellas es la INTRODUCCIÓN, la cual hace acopio de una breve explicación de la DM y de todo lo relacionado con la descripción y la importancia del guayabo, así como el valor nutricional del fruto y la composición y los métodos empleados para el análisis de los compuestos fenólicos en la hoja, útil para conocer el estado actual de la investigación analítica y focalizar estudios futuros.

La segunda parte incluye la PARTE EXPERIMENTAL, RESULTADOS Y DISCUSIÓN llevada a cabo durante el periodo predoctoral, y está subdividida a su vez en dos secciones. La ***Sección I*** contiene una extensa caracterización de los compuestos fenólicos presentes en las hojas de guayabo, empleando para ello diversas técnicas analíticas avanzadas. El **Capítulo 1** recoge la optimización del remedio antiguo más común, la infusión, mediante la alteración de una variable, y es comparada con la extracción asistida por baño de ultrasonidos, en un primer intento de caracterizar los extractos de hoja de guayabo ricos en compuestos fenólicos mediante la identificación y cuantificación a través de la plataforma HPLC-DAD-ESI-Q-MS, así como la evaluación de su capacidad antioxidante a través de los ensayos con FRAP y ABTS.

El **Capítulo 2** trata de la optimización de la extracción de los compuestos fenólicos de la hoja de guayabo asistida por baño de ultrasonidos, alterando una variable, así como la identificación de un gran número de compuestos, alguno de ellos identificados por primera vez en las hojas, mediante la plataforma HPLC-DAD-ESI-QTOF-MS. Además, incluye la comparación entre las variedades más comunes de hoja (*pyrifera* y *pomífera*), en función de su composición fenólica y de su capacidad antioxidante a través de los ensayos FRAP y ABTS.

El **Capítulo 3** estudia la influencia de los diferentes estados oxidativos, que pueden presentar las hojas, en la composición fenólica mediante la plataforma HPLC-DAD-ESI-QTOF-MS y en su capacidad antioxidante, siguiendo el método de extracción optimizado en el capítulo previo. En este caso se estudian los estados oxidativos en la variedad *pyrifera*. Además, la cianidina-3-O-glucósido ha sido identificada por primera vez.

Los **Capítulos 4** y **5** han sido realizados en colaboración con el "Inter-departamental Center for Agri-Food Industrial Research" (CIRI Agrifood) y el Departamento "Agro-Food Sciences and Technologies" de la Universidad de Bolonia gracias a una beca de movilidad pre-doctoral del CEI-Biotic de 3 meses de duración. En el **Capítulo 4** se lleva a cabo la optimización, mediante la metodología de la

superficie respuesta con un diseño Box-Behnken, de la extracción asistida por baño de ultrasonidos de proantocianidinas de las hojas de guayabo variedad *pyrifera*. Se evalua la influencia de los factores tiempo, temperatura, porcentaje de disolvente y de ácido, en la respuesta de la suma de los compuestos de interés. La identificación y cuantificación de esta familia de compuestos bioactivos es llevada a cabo mediante NP-HPLC-FLD-ESI-Q-MS. Además, se evalua el efecto de los factores en la composición de las hojas en diferentes estados oxidativos, bajo las condiciones óptimas.

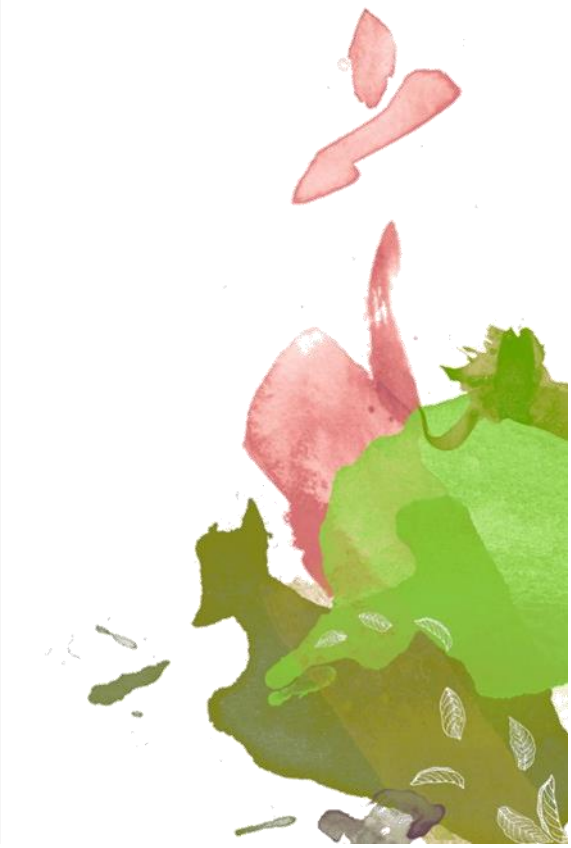
En el **Capítulo 5** se evalúa la eficiencia de la extracción asistida por sonda de ultrasonidos, basándose en la metodología de la superficie de respuesta (diseño Box-Behnken), mediante el estudio de la respuesta de los factores (tiempo, porcentaje de disolvente y potencia del aparato) en la capacidad antioxidante (DPPH y ABTS) y las principales familias de compuestos bioactivos presentes en la hoja. La identificación y cuantificación de los compuestos es llevada a cabo mediante HPLC-ESI-QqQ-MS. Además, las condiciones optimizadas se emplean para comparaar las variedades *pyrifera* y *pomífera*.

La ***Sección II*** se centra en la bioactividad de las hojas de guayabo. En primer lugar, el **Capítulo 6** ofrece una visión, a lo largo de la última década, de los efectos beneficiosos para la salud que ofrecen las hojas de guayabo, en el que se incluyen los estudios *in vitro* e *in vivo*, así como varias aplicaciones alimentarias. En segundo lugar, el **Capítulo 7** ha sido realizado en colaboración con el Centro de Investigaciones Biomédicas (CIBM) y el Departamento de Farmacología (CIBER-EHD y CIBER-Enfermedades Cardiovasculares, respectivamente) pertenecientes a la Universidad de Granada (UGR). Siguiendo el mismo esquema analítico que el capítulo 2, se evalúa la bioactividad de un extracto de la variedad *pyrifera* en un modelo de obesidad inducida en ratones frente a factores de riesgos asociados a la diabetes mellitus.

En último lugar, el **anexo** ha sido realizado en colaboración con el grupo de investigación “Advanced NMR methods and metal-based catalysis” pertenenciente a

la Universidad de Almería (UAL). Este apartado incluye la huella dactilar de la hoja de guayabo (variedad *pyrifera*) realizada mediante la técnica espectroscópica de Resonancia Magnética Nuclear. La estructura de este trabajo no tiene la misma apariencia que el resto de capítulos ya que no se ha concluido el trabajo. No obstante, ha sido incluído en la tesis ya que se ha realizado durante dicho periodo.

INTRODUCTION



1. Diseases of high social impact

Non-communicable diseases (NCDs) are referred to chronic diseases, which are characterized by a large duration and slow progression. These are one of major alarming public health problems worldwide, especially cardiovascular diseases, cancer, respiratory diseases and diabetes mellitus (DM) which represent a great social and economic impact. This incidence is related to a high mortality and morbidity of these ailments, accordingly, in the last report of the World Health Organization (WHO), they accounted for a 68% (38 millions) over the global deaths and it is estimated to rise to 52 million by 2030. Briefly, in 2012, cardiovascular diseases supposed a 46.2% of NCD deaths, followed by cancer (21.7% of NCD deaths), respiratory diseases (10.7% of NCD deaths), and diabetes (4% of NCD deaths)[1]. In spite of being the fourth illness, DM has gained notable attention since the number of people who live with this condition is growing, as well as the number of deaths, for example, in agreement with the International Diabetes Federation, by the end of 2015, DM would have caused 5.1 million deaths[2]. Tackling this emerging global epidemic, in 2015, there were estimated 318 million adults with impaired glucose tolerance and 415 million aged 20-79 with DM, of which 193 million correspond to undiagnosed. Besides, if the prevalence is steadily rising, for 2040 there will be diagnosed 642 million patients spread out by region[2] as is shown in Figure 1.

[1] World Health Organization. *Global status report on noncommunicable diseases*; **2014**.
[2] International Diabetes Federation. *IDF Diabetes Atlas*; 7th edn.; **2015**

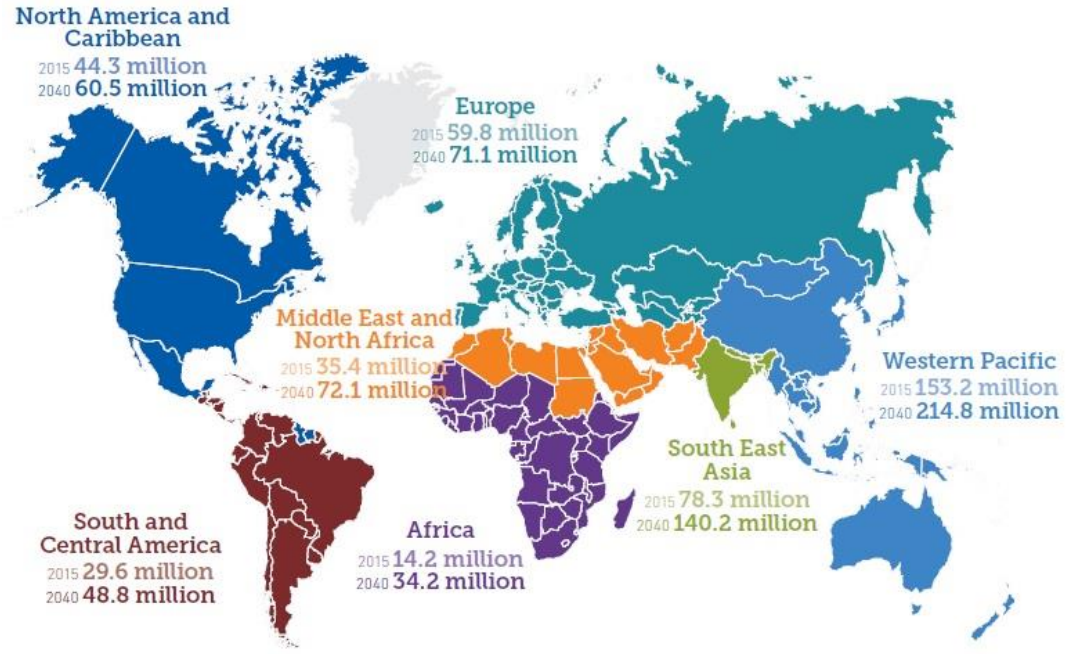


Figure 1. Estimated cases of diabetes (20-79 years) by region. *Adapted from IDF Diabetes Atlas*[2]

Classified as a metabolic disease by the WHO[3], based on its etiology, DM is divided into three main types where type 1 (T1DM), type 2 (T2DM) and gestational diabetes mellitus (GDM) are the most relevant[2,4].

GDM takes place in about 23% of all pregnancies. This condition occurs when pregnant women without a previous diagnosis of diabetes, develop impaired glucose tolerance and hyperglycemia. Generally, is a temporary situation that recovers after birth, nevertheless, it increases the risk of women and their child developing T2DM through their life[2,4].

T1DM affects to a 7-12% of people with diabetes and increases a 3% annually. It comes from an autoimmune response which induces insulin deficiency by

[3] World Health Organization. *International statistical classification of diseases and related health problems 10th revision*. **2016**.Available from: http://apps.who.int/classifications/icd10/browse/2016/en

[4] American Diabetes Association Diagnosis and classification of diabetes mellitus. *Diabetes Care* **2014**, *37*, 81–90.

the destruction of beta cells in the pancreas. This syndrome needs the daily administration of insulin to patients and is common during the childhood[2,4].

T2DM concerns most cases of DM (88-93%). Usually, it is diagnosed in adults (about 40) and is associated with insulin resistance and insulin deficiency. Its symptoms are less severe than in T1DM, so this condition may be unrecognised[2,4].

DM encompassed a combination of illnesses or metabolic syndromes characterized by an increase of blood glucose levels (hyperglycemia) due to a body's failure to produce insulin and/or to a resistance to insulin. The exact cause for developing DM remains unknown, although is related to several risk factors (Figure 2).

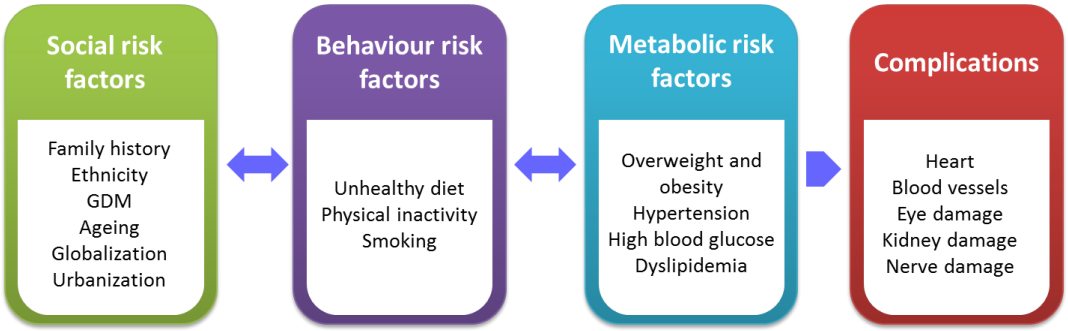


Figure 2. Risk factors and complications related to DM**.** *Adapted from WHO*[2]

The main influence that promotes this disease is the interplay among genetic and metabolic factors coupled to unhealthy lifestyles. Moreover, high blood glucose levels may lead the manifestation of several complications causing a significant deterioration of the quality of life of patients as well as the disability and premature death. These complications are associated with micro and macro-vascular complications, where cardiovascular diseases caused the major rates of mortality[2]. In this sense, endothelial dysfunction precedes the beginning of these complications. This alteration is caused by an imbalance between the vasodilators, particularly nitric oxide,

and the constrictors which relax the blood vessel, resulting in an impairment of endothelium-dependent vasodilation[5].

Taking into account that behavior risk factors play an important role in suffering these affections, correction of lifestyle, with physical activity and intake of a healthy diet, is becoming the most effective preventive therapy against the prevalence of DM and related risk factors and complications[6]. Throughout history, traditional medicine has employed natural remedies for health care. Therefore, it is easy to believe that the intake of plant-based foods can aid in the co-treatment of this disease[7]. This health promoting benefits caused by plants have aroused curiosity about their composition and biological activities.

In this regard, the beneficial effects of ***Psidium guajava* L. (guava)** have been widely studied in the last years, giving rise to a vast number of works. Therefore, the pharmacological research of **guava leaves**, *in vitro* as well as *in vivo*, over the last decade has been reviewed on **Chapter 6**. A fact worthy of comment is that several *in vivo* studies, concerning DM, have been made with **guava leaves**, while few of them have been focused on the vascular reactivity caused by this disease. On the contrary, there is a lack of information concerning the use of advanced analytical techniques to assess the **phenolic composition** of the **leaves**.

[5] Hadi, H. A. R.; Carr, C. S.; Al Suwaidi, J. Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vasc. Health Risk Manag.* **2005**, *1*, 183–98.

[6] Sharma, H.; Chandola, H. M. Prameha in Ayurveda: correlation with obesity, metabolic syndrome, and diabetes mellitus. Part 1–etiology, classification, and pathogenesis. *J. Altern. Complement. Med.* **2011**, *17*, 491–496

[7] Gurib-Fakim, A. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Mol. Aspects Med.* **2006**, *27*, 1–93.

2. *Psidium guajava* L.: The matrix under study

The Myrtle family (Myrtaceae), which is considered of great economic importance worldwide, comprises about 133 genera and more than 3,800 species[8]. Among them, it should be pointed those that produce edible fruit. In this sense, one of the 150 species of the genus *Psidium* L. is universally known as guava in English or "guayabo" in Spanish.

2.1. Origin and description

Psidium guajava L. (Figure 3) is considered such a gregarious fruit tree native from Mexico. In fact, it was extended from southern Mexico to northern South America. Despite of its origin, guava tree was early adopted as a crop in Asia and some parts of Africa[9]. This fact is possible due to the ability of the tree to adapt itself to a wide variety of edapho-climatic conditions[10,11]. As a result of seasonal changes, the tree clearly showed phenological stages[11] varying the development of it.

Figure 3. *Psidium guajava* L. tree

[8] Shruthi, S. D.; Roshan, A.; Timilsina, S. S.; Sajjekhan, S. A review on the medicinal plant Psidium Guajava Linn. (Myrtaceae). *J. Drug Deliv. Ther.* **2013**, *3*, 162–168.
[9] Morton, J. F. Guava. In *Fruits of warm climates*; **1987**; pp. 356–363.
[10] Hao, W. Freezing tolerance and cold acclimation in guava (Psidium guajava L.), Iowa State University, **2008**.
[11] Salazar, D. M.; Melgarejo, P.; Martínez, R.; Martínez, J. J.; Hernández, F.; Burguera, M. Phenological stages of the guava tree (Psidium guajava L.). *Sci. Hortic.* **2006**, *108*, 157–161.

Guava is an evergreen plant up to 3-10 m high and regular-bearing. The branches grow widespread and the trunk could attain a diameter of 25 cm. The simple and oval **leaves**, aromatic when are crushed, are 5-18 cm in length and 3-7 cm in width. The flowers with 4-5 petals and 2-4 cm in length come out singly or in clusters of 2 or 3 at the leaf buds (Figure 3). The ripe fruit could measure between 5 and 12 cm long and between 5 and 7 cm wide. Its pulp varies from white to dark pink and it usually has a yellow peel, while that of the unripe fruit is greenish[12]. In addition, their common varieties are distinguished by the color of the pulp. In fact, as is shown in Figure 4, *pyrifera* variety is the fruit with white pulp, while *pomifera* var. is the one with the red pulp[13]. Besides, the cultivation around the world has led to the emergence of several cultivars such as 'Supreme', 'Ruby', among others[9].

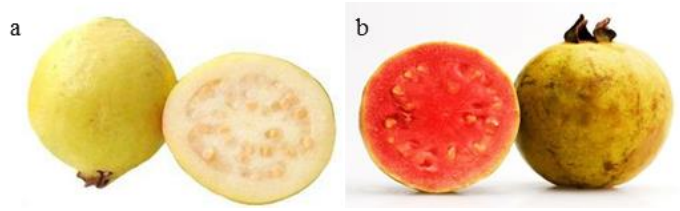


Figure 4. *P. guajava* fruit vars. *pyrifera* (a) and *pomifera* (b)

Formerly, the plant was known for its medicinal properties, mainly for its anti-diarrheal effect. The **leaves** were widely used in countries with a long history of traditional medicine for the treatment of several diseases such as anti-cough, anti-diabetic, anti-inflamatory and anti-nociceptive activities, among others[13,14]. At present, the plant is still been used as medicinal plant and it is also known as an important food crop[14].

[12] Hernández Gomez, E. Análisis de la principal problemática de producción y comercialización del cultivo de guayaba (Psidium guajava L.) en el Estado de Michoacán, Universidad Autónoma Agraria Antonio Narro, **2006**.

[13] Barbalho, S. M.; Farinazzi-Machado, F. M. V.; de Alvares Goulart, R.; Saad Brunnati, A. C.; Machado Bueno Ottoboni, A. M.; Teixeira Nicolau, C. C. Psidium Guajava (Guava): A Plant of Multipurpose Medicinal Applications. *Med. Aromat. Plants* **2012**, *1*, 1–6.

[14] Pérez Gutiérrez, R. M.; Mitchell, S.; Vargas Solis, R. Psidium guajava: A review of its traditional uses, phytochemistry and pharmacology. *J. Ethnopharmacol.* **2008**, *117*, 1–27.

2.2. Worldwide production of guava

Guava crops, both wild and commercial, are distributed through tropical and subtropical countries (Figure 5). The fruit is one of the most widely accepted tropical fruits worldwide and its cultivation presented an increasing tendency in the last decade. Although several countries published production data, there is not specific statistical information; it is contained in the same group as mango and mangosteens. Despite this fact, in 2013, global production covered a total of 5.5 million harvested hectares (ha) which meant a production of 43.9 million tonnes (t) of fruit[15].

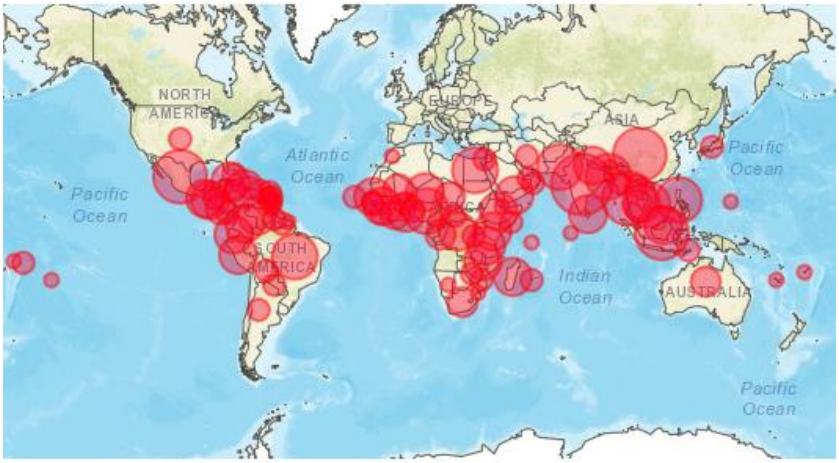


Figure 5. Guava global production. *Adapted from FAOSTAT*[15].

The top five producing countries were India (40.98%), China (10.13%), Thailand (7.15%), Indonesia (4.69%), and Mexico (4.33%). Considering these numbers, Asia conquers the guava production with a 76%, being also the continent with the highest amount of harvested areas, followed by America (12.50%), Africa (11.3%) and Oceania (0.10%)[15]. Instead, the crop yield does not agree with production, in fact, America had the highest value (104.24 t/ha). This agricultural parameter refers to the cultivation efficiency, thus a slight increase in the crop yields would generates a considerable impact on the guava trade.

[15] Statistics Division of the Food and Agricultural Organization of the United Nations, FAOSTAT data. **2013**. Available from http://www.fao.org/faostat/en/

Besides, in the last years, guava is also cultivated in Spain, Italy, Greece and other Mediterranean countries[11]. The climatic conditions of certain parts of Spain facilitate the cultivation of this type of tropical-subtropical tree. Therefore, it can be easily cultivated in the East and South of Spain, as well as in the Canary Island. However, Spain does not represent a big percentage of the total world production; there is a lack of information about guava crops since most of them have been carried out on experimental lands or are located on private plantations. For example, in Valencia region does not play such a relevant role although there are evidences of small cultivated areas both in Valencia and Alicante[11]. In Andalusia, data are from 1974 and 1994 in which the crops were located in Malaga and extended in the province of Granada[16], principally in Almuñecar (Granada)[17]. Moreover, the actual presence of private plantations is supported by the statistics reported by the "Junta de Andalucía"[18] and the existence of several companies such as Frescoral in Huelva, Exoticfruitbox in Málaga[19], and Motriltropicalfruit in Granada[20]. Only in the Canary Islands data of guava crops are gathered together. According to the "Instituto Canario de Estadística", in 2014, the growing area had an extension of 69.9 ha distributed between the different islands[21].

2.3. Guava trade

In the last decade, the demand of this fruit has considerably grown in several markets, causing an important increase of the volume of guava that is intended to export. Guava is mainly grow for fresh consumption, but with the development of the agroindustry, many guava products like nectars, jams, jellies, frozen, sherbet, and

[16] Jose V. Torrellas Cardenas, Cultivo de La Guayaba, **1974**.
[17] Consejería de Agricultura y Pesca, Junta de Andalucía, V Jornadas Andaluzas de Frutos Tropicales, **1998**. Available from: http://www.juntadeandalucia.es/servicios/publicaciones/detalle/44147.html
[18] Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía. Anuario de estadísticas agrarias y pesqueras. **2013**. Available from http://www.juntadeandalucia.es/organismos/agriculturapescaydesarrollorural/consejeria/sobre-consejeria/estadisticas/paginas/agrarias-anuario.html
[19] Available from http://exoticfruitbox.com/es/
[20] Available from http://motriltropicalfruit.com/
[21] Instituto Canario de Estadística. Available from http://www.gobiernodecanarias.org/agricultura/otros/estadistica/agricultura.html

gelatin are also found. In spite of being widespread produced and exploited in many countries of America, Asia, and Africa, commercial guava is of little importance and its market still very restricted. In 2013, the volume exported worldwide is estimated in an export of 1.65 million tonnes while the volume imported account for 1.19 million t[15].

In general, the principal producers' countries are also the main exporters (Figure 6). Mexico stands out exports with a 21% followed by India (16%), Thailand (15%), Peru (8%) and Brazil (7%). On the other hand, the greatest importers of this fruit are the USA (36%), Netherlands (12%), Saudi Arabia and United Kingdom (both 5%) and Germany (4%).

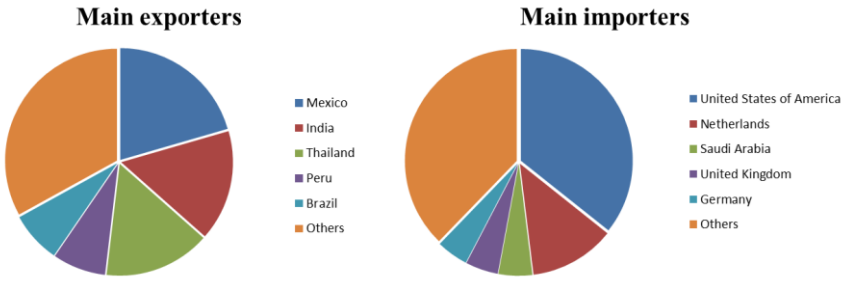


Figure 6. Guava worldwide trade

Regarding the available literature, in Mexico, the most cultivated and commercialized cultivars are 'guayaba china', 'guayaba media' and 'criolla'[12]. In India, the most popular cultivars are Allahabad Safeda and L-49 among others. Furthermore, in Thailand, the ones suitable for fresh consumption are Pansithong and Glomsalee, while the processed cultivars are Samsi, Beaumount and Kahuakula[22]. By last, in Brazil, the cultivars most planted for the production are Pedro Sato, Sassoka and Paluma. Along with the variety Kamugai, these are the basis of the Brazilian export[23].

[22] Thuaytong, W.; Anprung, P. Bioactive compounds and prebiotic activity in Thailand-grown red and white guava fruit (Psidium guajava L.). *Food Sci. Technol. Int.* **2011**, *17*, 205–12.
[23] Parra-Coronado, A. Maduración y comportamiento poscosecha de la guayaba (Psidium guajava L.). Una revisión. *Rev. Colomb. Ciencias Hortícola* **2014**, *8*, 314–327.

Moreover, in Spain, commercial guavas belongs to cultivars 'Guayaba Cas', 'Puerto Rico', 'Guayaba Coronilla', 'Rojo Africano' and 'Guayaba Roja'[24]. Specifically, in the Canary Islands, the varieties cultivated come from different parts of the world, including the main commercial ones 'White x Supreme', 'Stone Ruby', 'Enano Rojo Cubano EEA 18-40', among others[25]

The agricultural works like pruning and harvesting of fruit in commercial crops produce residues over 70% of the tree giving the possibility of using them for further applications[26]. For example, the most common way to consume herbs is in tea. Moreover, in the last years, **guava leaf** tea is promoted for its content on polyphenols that has been claimed as food for specified health use (FOSHU)[27]. Because of these reasons, the tea of the **leaves** is marketed in several shops in Japan as well as on the internet, and is also easy to find the **leaves** as complementary products[28].

2.4. Nutritional value of the fruit and bioactive composition of the leaf

The production and socioeconomic importance of this crop is due to the nutritional value of the fruit, mainly to the content of Vitamin C and dietary fiber, which provided several health benefits for the consumer. Figure 7 compiles the data about the macro- and micronutrients found in 100 g of edible portion from pink and white guava fruit in agreement with the National Nutrient Database for Standard Reference of the United States[29] and with the McCance and Widdowson's composition

[24] Empresa Nacional Mercasa. Distribución y consume. **2007**. Available from http://www.mercasa.es/files/multimedios/1288280121_DYC_2007_95_33_85.pdf
[25] Instituto Canario de Investigaciones Agrarias ICIA. Available from http://www.icia.es/icia/
[26] Vargas-Álvarez, D.; Soto-Hernández, M.; González-Hernández, V. A.; Engleman, E. M.; Martínez-Garza, Á. Kinetics of accumulation and distribution of flavonoids in guava (Psidium guajava L.). *Agrociencia* **2006**, *40*, 109–115.
[27] Arai, S.; Yasuoka, A.; Abe, K. Functional food science and food for specified health use policy in Japan: state of the art. *Curr. Opin. Lipidol.* **2008**, *19*, 69–73.
[28] Matsuda, K.; Nishimura, Y.; Kurata, N.; Iwase, M.; Yasuhara, H. Effects of continuous ingestion of herbal teas on intestinal CYP3A in the rat. *J. Pharmacol. Sci.* **2007**, *103*, 214–221.
[29] Nutrient data laboratory, food and nutrition information center (FNIC); information systems division of the national agricultural library, United states department of agriculture (USDA) national nutrient database for standard reference. Available from http://ndb.nal.usda.gov/

of foods integrated dataset[30], respectively. Besides, micronutrients from the fruit were compared with those reviewed from different parts of guava tree; the flowers[31], the bark, shoots and the **leaves**, reported to have greater amounts of minerals than the fruit[32].

General nutritional composition		
Energy (kcal/kJ):	68/285	26/112
Water:	80.80 g	84.7 g
Protein:	2.55 g	0.8 g
Total lipids:	0.95 g	0.5 g
Carbohydrates:	14.32 g	8.7 g
Total dietary fiber:	5.4 g	3.8 g
Sugars:	8.92 g	4.9 g
Total Aminoacids:	0.971 g	-
Vitamins		
Vitamin C:	228.3 mg	230 mg
Thiamin (B1):	0.067 mg	0.04 mg
Riboflavin (B2):	0.04 mg	0.04 mg
Niacin (B3):	1.084 mg	1.0 mg
Pantothenic acid:	0.451 mg	0.15 mg
Pyridoxine (B6):	0.110 mg	0.14 mg
Folate (DFE):	49 µg	-
Vitamin A (RAE):	31 mg	73 mg
Vitamin E (α-tocopherol):	0.73 mg	-
Vitamin K (phylloquinone):	2.6 µg	-
Minerals		
Calcium (Ca):	18 mg	13 mg
Iron (Fe):	0.26 mg	0.40 mg
Magnesium (Mg):	22 mg	12 mg
Phosphorous (P):	40mg	25 mg
Potassium (K):	417 mg	230 mg
Sodium (Na):	2 mg	5 mg
Zinc (Zn):	0.23 mg	0.2 mg
Manganese (Mn):	0.15 mg	0.10 mg
Selenium (Se):	0.6 µg	-

Figure 7. Nutritional composition of 100 g of *pomifera* and *pyrifera* guava fruits

[30] McCance and Widdowson's composition of foods integrated dataset (CoFID), Public Health England. Available from https://www.gov.uk/government/publications/composition-of-foods-integrated-dataset-cofid

[31] de Souza, H. A.; Rozane, D. E.; Romualdo, L. M.; Natale, W. Differences among nutrients in flowers and fruits of guava trees under different types of pruning. *IDESIA (Chile)* **2012**, *30*.

[32] Adrian, J. A. L.; Arancon, N. Q.; Mathews, B. W.; Carpenter, J. R. Proximate analysis, in vitro organic matter digestibility, and energy content of common guava (*Psidium guajava* L.) and yellow, strawberry guava (*Psidium cattleianum* var. *lucidum*) tree parts and fruits as potential forage. *J. Agric. Food Chem.* **2012**, *60*, 10398–10405.

In fact, the health effects that these nutrients confer to the human body are a consequence of considering them as **bioactive compounds**. In these sense, this term has been defined as "*essential and non-essential compounds that occur in nature, that are part of the food chain, and can be shown to have an effect on human health"* by consensus[33]. In contrast, the classical meaning only includes non-essential components. However, essential compounds, are represented by the primary metabolites (amino-acids, carbohydrates, lipids, proteins, vitamins and minerals) and i.e. it has been demonstrated that the intake of minerals play a role in diabetes mellitus (DM)[34].

Non-essential compounds, known as phytochemicals in plants, are referred to the secondary metabolites like alkaloids, terpenoids and phenolic compounds, among others. In the last years, the search of these metabolites in different food and plant matrices has drawn the attention of the researchers since the bioactive compounds can exhibit greater health benefits beyond the basic nutritional value of the food.

Regarding the literature available of the secondary metabolites present in the different parts of guava, several phytochemical analyses revealed that guava tree is rich in terpenoids, phenolic acids, flavonoids and tannins[13,14], of which the **leaves** contain mainly tannins, flavonoids and less amounts of terpenoids[35]. Particularly, the research has been more focused on the terpenoid content of the **leaves** and the fruit rather than on the phenolic composition. In fact, the essential oil has been widely studied; until 204 volatile components have been identified in the fruit[36] while in the **leaves**, the presence of 86 compounds has been found[37]. Briefly, white **guava leaves**

[33] Biesalski, H.-K.; Dragsted, L. O.; Elmadfa, I.; Grossklaus, R.; Müller, M.; Schrenk, D.; Walter, P.; Weber, P. Bioactive compounds: definition and assessment of activity. *Nutrition* **2009**, *25*, 1202–1205.

[34] Granados-Silvestre, M. de los Á.; Ortíz-López, M. G.; Montúfar-Robles, I.; Menjívar-Iraheta, M. Micronutrientes y diabetes, el caso de los minerales. *Cir. Cir.* **2014**, *82*, 119–125.

[35] Metwally, A. M.; Omar, A. A.; Ghazy, N. M.; Harraz, F. M.; El Sohafy, S. M. Monograph of *Psidium guajava* L. leaves. *Pharmacogn. J.* **2011**, *3*, 89–104.

[36] Joseph, B.; Priya, R. M. Phytochemical and biopharmaceutical aspectcs of *Psidium guajava* L. essential oil: a review. *Res. J. Med. Plant* **2011**, *5*, 432–442.

[37] García, M.; Quijano, C. E.; Pino, J. A. Free and glycosidically bound volatiles in guava leaves (*Psidium guajava* L.) Palmira ICA-I cultivar. *J. Essent. Oil Res.* **2009**, *21*, 131–134.

and fruit from South Korea, were both rich in α-pinene, β-caryophyllene, α-humulene, and β-(Z)-ocimene, although this composition varies in other countries[38]. For example, the essential oil from white guava fruit grown in Reunion Island was found to be rich in β-caryophyllene, nerolidol, and caryophyllene oxide, while for the **leaves**, limonene, menthol and β-bisabolene have been found between the main constituents[14]. Among white and pink fruit forms, variations in the composition have also been noticed in the essential oil of the fruit[22]and of the **leaves**[39].

Despite of the importance given to phenolic compounds few works have been published concerning the **phenolic composition** of the **leaves** and even of the fruit. Until relatively recently, the phenolic composition of the tree was still not comprehensively defined. However, as the phenolic composition from ***P. guajava*** **leaves** has been the subject under study in the present Thesis, they will be described in more depth below.

2.4.1.Phenolic compounds in guava leaves

"Phenolics" are more present in the human diet since plant foods and beverages have proven to possess great amounts of them. In fact, these compounds are developed during the growing of the plant and also in response to stress and ultraviolet radiation. They contribute to the colors of plants and organoleptic properties. Moreover, the intake of these compounds has been associated with several health-promoting benefits which have been attributed to their antioxidant and chelating capacity. Thus, there is a burgeoning interest on the discovery of "phenolics"-rich sources as well as the search of individual target to use them in pharmaceutical and industrial applications, as nutraceuticals, colorants and preservatives.

[38] Lee, S.; Km, Y.-S.; Choi, H.-K.; Cho, S. K. Determination of the volatile components in the fruits and leaves of guava plants (*Psidium guajava* L.) grown on Jeju Island, South Korea. *J. Essent. Oil Res.* **2011**, *23*, 52–56.

[39] Chalannavar, R. K.; Venugopala, K. N.; Baijnath, H.; Odhav, B. The chemical composition of leaf essential oils of *Psidium guajava* L. (white and pink fruit forms) from South Africa. *J. Essent. Oil Bear. Plants* **2014**, *17*, 1293–1302.

The term "phenolics" cover a huge range of families of substances with more than 8,000 compounds. Their particular structural features arise from the pathway they are built up in nature (biosynthesis). These compounds derived from shikimic acid or malonic acid pathway, thus, they possess an aromatic ring bearing one or more hydroxyl groups in their structure, ranging from simple to highly polymerized compounds. Classification of phenolic compounds in different categories is done regarding to their origin or their structure, so there are several classifications available. In fact, it rather depends upon the intention. For the present thesis, a straightforward classification has been attempted dividing phenolic compounds by its flavonoid or non-flavonoid nature[40]. Within them, only those of particular interest due to its presence in **guava leaves** are described, as are highlighted in darker color in Figure 8.

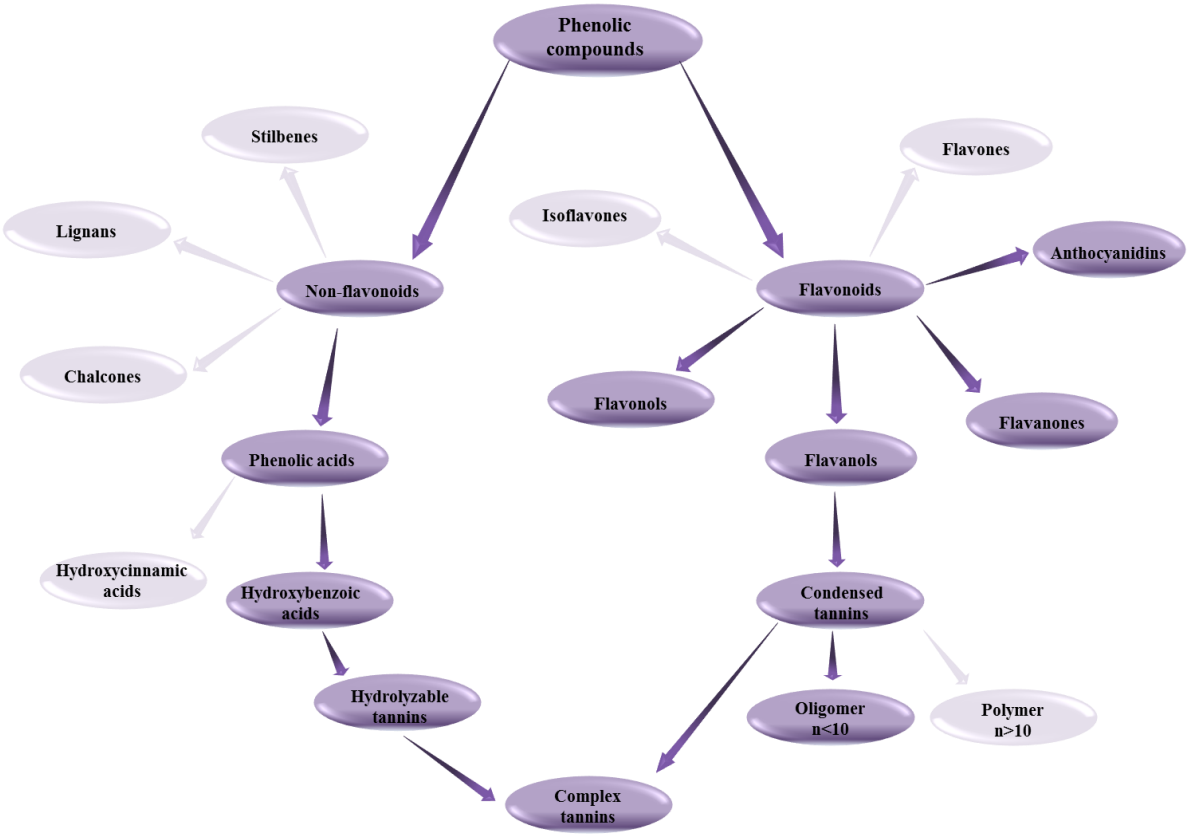


Figure 8. Classification of phenolic compounds highlighting those present in guava leaves

[40] Crozier, A.; Jaganath, I. B.; Clifford, M. N. Phenols, Polyphenols and Tannins: An Overview. In *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*; **2007**; pp. 1–24.

As the main use of **guava leaves** was as anti-diarrheal remedy, tannins have been unavoidably studied as a result of their astringency properties. In fact, this subclass represents 8.5% of the leaf composition[14]. This property has been attributed only to condensed tannins (CTs)[41], although substantially less attention has been aimed at the CTs composition. In contrast to hydrolysable tannins (HTs), which have been elucidated since early on. Table 1 summarizes the phenolic compounds identified in **guava leaves**. Moreover, a brief explanation of the different families to which these compounds belong is given below.

Table 1. An overview of the phenolic compounds identified in *P. guajava* L. leaves

Origin	Phenolic family	Compounds	Phenolic content	Ref.
India	flavonols and HTs	quercetin, guaijaverin, leucocyanidin, ellagic acid amritoside	-	42
Japan	HTs	pedunculagin, casuarinin, stachyurin, tellimagrandin I, strictin, casuariin, casuarictin, HHDP-glucose, isostrictin	-	43
Japan	HTs	guavin B	-	44
Japan	complex tannins	guavin A, C, D	-	45
Japan	flavonols	quercetin, morin and/or their glycosides derivatives	-	46
Sudan	flavanols and CT oligomers	catechin, gallocatechin, procyanidins B1, B2, B3, prodelphinidins	-	47
Mexico	flavonols	quercetin, myricetin, kaempferol	3.10 - 2883.08 mg/kg dry weight (d.w.)	26

[41] Haminiuk, C. W. I.; Maciel, G. M.; Plata-Oviedo, M. S. V.; Peralta, R. M. Phenolic compounds in fruits-an overview. *Int. J. Food Sci. Technol.* **2012**, *47*, 2023–2044.

[42] Seshadri, T. R.; Vasishta, K. Polyphenols of the leaves of *Psidium guava*- quercetin, guaijaverin, leucocyanidin and amritoside. *Phytochemistry* **1965**, *4*, 989–992.

[43] Okuda, T.; Yoshida, T.; Hatano, T.; Yazaki, K.; Ashida, M. Ellagitannins of the Casuarinaceae, Stachyuraceae and Myrtaceae. *Phytochemistry* **1982**, *21*, 2871–2874

[44] Okuda, T.; Hatano, T.; Yazaki, K. Guavin B, an ellagitannin of novel type. *Chem. Pharm. Bull. (Tokyo).* **1984**, *32*, 3787–3788.

[45] Okuda, T.; Yoshida, T.; Hatano, T.; Yazaki, K.; Ikegami, Y.; Shingu, T. Guavins A, C and D, complex tannins from *Psidium guajava*. *Chem. Pharm. Bull. (Tokyo).* **1987**, *35*, 443–446.

[46] Arima, H.; Danno, G. Isolation of antimicrobial compounds from guava (*Psidium guajava* L.) and their structural elucidation. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 1727–1730.

[47] Qa'dan, F.; Petereit, F.; Nahrstedt, A. Polymeric proanthocyanidins from *Psidium guajava*. *Sci. Pharm.* **2005**, *73*, 113–125.

Origin	Phenolic family	Compounds	Phenolic content	Ref.
Taiwan	phenolic acids, flavonols, flavanols, and flavonols	gallic acid, (epi)-catechin, naringenin, quercetin, kaempferol, rutin	56.00 - 132.00 mg/g	48
Taiwan	phenolic acids	gallic and ferulic	TPC: 267.00 - 313.00 mg/g catechin, 414.00 - 483.00 mg GAE/g. Individual compounds: 266.00 - 1621.00 and 108.00 - 672.00 µg/g d.w.	49
Japan	PAs, HTs, complex tannin, flavonols, and phenolic acids	procyanidin B1, pedunculagin, castalagin, casuarinin, Stenophyllanin A, hyperin, isoquercitrin, catechin, ellagic and gallic acid	-	50
USA	anthocyanidins	-	-	10
Taiwan	phenolic acids, flavonols, and favanols	gallic and ferulic acid, quercetin, catechin	1.30 - 21.80 mg/g extract	51
Thailand	phenolic acids, HTs, and flavonols	gallic and ellagic acid, quercetin	TPC: 80.28 - 136.02 mg GAE/g. Individual compounds: 0.93 - 6.27, 2.61 - 36.68 and 10.85 - 26.12 mg/g extract	52
Thailand	flavonols	quercetin, morin and quercetin glycosides	57.20 - 179.90 µg/g d.w.	53
Japan	flavonols	flavonol galloyl glycosides, benzophenone	-	54

[48] Hsieh, C.-L.; Huang, C.-N.; Lin, Y.-C.; Peng, R. Y. Molecular action mechanism against apoptosis by aqueous extract from guava budding leaves elucidated with human umbilical vein endothelial cell (HUVEC) model. *J. Agric. Food Chem.* **2007**, *55*, 8523–8533.

[49] Chen, H. Y.; Yen, G. C. Antioxidant activity and free radical-scavenging capacity of extracts from guava (*Psidium guajava* L.) leaves. *Food Chem.* **2007**, *101*, 686–694.

[50] Yamanaka, F.; Hatano, T.; Ito, H.; Taniguch, S.; Takahashi, E.; Okamoto, K. Antibacterial effects of guava tannins and related polyphenols on Vibrio and Aeromonas species. *Nat. Prod. Commun.* **2008**, *3*, 711–720.

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[52] Nantitanon, W.; Yotsawimonwat, S.; Okonogi, S. Factors influencing antioxidant activities and total phenolic content of guava leaf extract. *LWT - Food Sci. Technol.* **2010**, *43*, 1095–1103.

[53] Rattanachaikunsopon, P.; Phumkhachorn, P. Contents and antibacterial activity of flavonoids extracted from leaves of Psidium guajava. *J. Medinical Plants Res.* **2010**, *4*, 393–396.

[54] Matsuzaki, K.; Ishii, R.; Kobiyama, K.; Kitanaka, S. New benzophenone and quercetin galloyl glycosides from *Psidium guajava* L. *J. Nat. Med.* **2010**, *64*, 252–256.

Origin	Phenolic family	Compounds	Phenolic content	Ref.
Japan	HTs	casuarictin, casuarinin, benzophenones	-	55
China	flavonols	kaempferol, quercetin, quercitrin, isoquercitrin, guaijaverin, avicularin, hyperoside, reynoutrin, benzophenones	-	56
Japan	flavonols	quercetin glycosides	-	57
China	flavonols	hyperoside, isoquercitrin, reynoutrin, quercetin glucosides, benzophenone	-	58
Indonesia	tannins	-	1.68 - 2.35 mg EAT/g	59
Taiwan	phenolic acid; flavanols; flavanons; flavonols; HTs	gallic acid, (epi)-catechin, gallocatechin, galloyl derivatives, quercetin and some derivatives, myricetin and some derivatives, rutin, ellagic acid	TPC: 30.50 - 417.90 mg GAE/g d.w. TFC: 10.50 - 452.50 mg quercetin/g d.w. Individual compounds: 20 compounds tentatively identified (12 quantified 0.2 - 111.3 mg/g)	60

Flavonoids are the widest family of compounds found in the plant kingdom, although their distribution in plants depends on several factors related to the specie[40]. Thus, they comprise roughly two-thirds of the dietary phenols due to their abundant presence in fruits, vegetables, and grains, spreading out all over the plant, like on the

[55] Park, B. J.; Matsuta, T.; Kanazawa, T.; Chang, K. J.; Park, C. H.; Onjo, M. Phenolic compounds from the leaves of *Psidium guajava*. I. Hydrolysable tannins and benzophenone glycosides. *Chem. Nat. Compd.* **2011**, *47*, 632–635.

[56] Shu, J.-C.; Chou, G.-X.; Wang, Z.-T. One new diphenylmethane glycoside from the leaves of *Psidium guajava* L. *Nat. Prodruct Res.* **2012**, *26*, 1971–1975.

[57] Park, B. J.; Matsuta, T.; Kanazawa, T.; Park, C. H.; Chang, K. J.; Onjo, M. Phenolic compounds from the leaves of *Psidium guajava* II. Quercetin and its glycosides. *Chem. Nat. Compd.* **2012**, *48*, 477–479.

[58] Zhu, Y.; Liu, Y.; Zhan, Y.; Liu, L.; Xu, Y.; Xu, T.; Liu, T. Preparative isolation and purification of five flavonoid glycosides and one benzophenone galloyl glycoside from *Psidium guajava* by high-speed counter-current chromatography (HSCCC). *Molecules* **2013**, *18*, 15648–15661.

[59] Mailoa, M. N.; Mahendradatta, M.; Laga, A.; Djide, N. Tannin extract of guava leaves (*Psidium guajava* L.) variation with concentration organic solvents. *Int. J. Sci. Technol. Res.* **2013**, *2*, 106–110.

[60] Chang, C.-H.; Hsieh, C.-L.; Wang, H.-E.; Peng, C.-C.; Chyau, C.-C.; Peng, R. Y. Unique bioactive polyphenolic profile of guava (*Psidium guajava*) budding leaf tea is related to plant biochemistry of budding leaves in early dawn. *J. Sci. Food Agric.* **2013**, *93*, 944–54.

seeds, **leaves**, roots, and stems[61]. The flavan nucleus, which contains 15 carbon atoms distributed in two aromatics rings (A and B) and a heterocyclic ring (C), usually in a C_6-C_3-C_6 configuration, is the basic carbon flavonoid skeleton. The basic flavonoid structure is referred as the aglycone form, although generally, flavonoids occur as glycoside form (with sugar moiety). Further substitutions of the basic structure and the oxidation degree of the C-ring make themselves be distributed into six subclasses[40] (Figure 8). However, only four of them (flavanones, anthocyanidins, flavonols, and flavanols) have been previously identified in **guava leaves** (Figure 9).

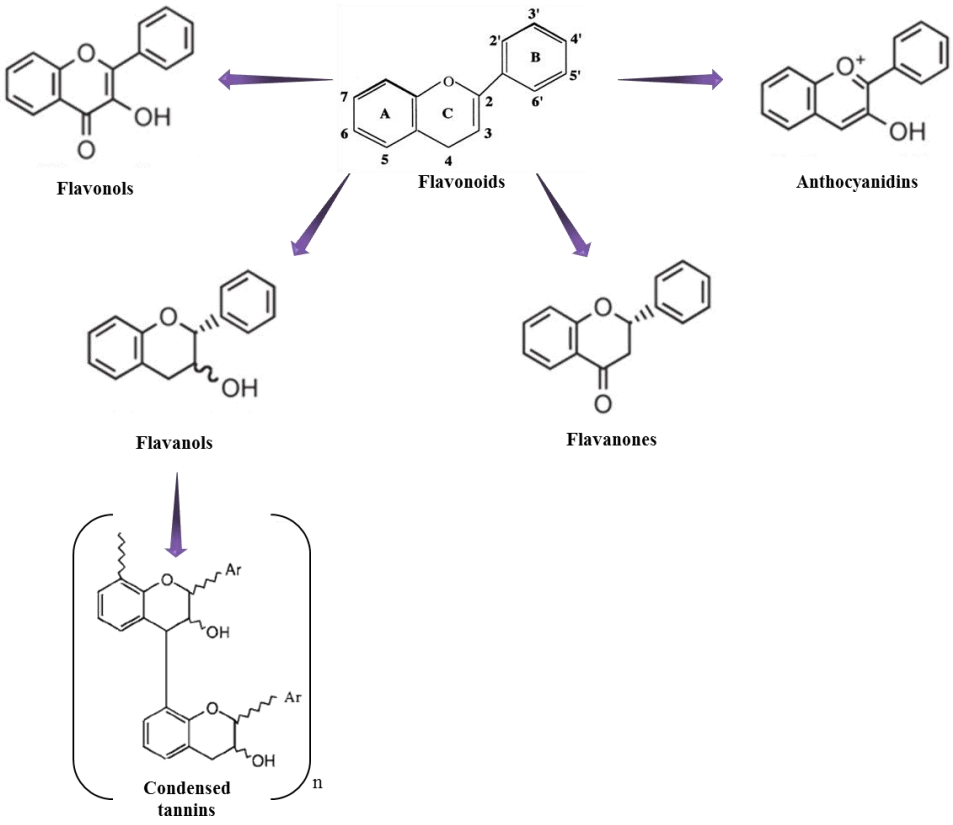

Figure 9. Structures of main flavonoids identified in guava leaves

Structurally, ***flavanones*** are non-planar molecules due to the absence of unsaturation and the presence of a chiral center at C2, which is usually attached in the α-configuration to the B-ring. They also contain a carbonyl group at the 4 position of

[61] Robbins, R. J. Phenolic acids in foods : an overview of analytical methodology. *J. Agric. Food Chem.* **2003**, *51*, 2866–2887.

the C-ring[40]. These compounds are the less common dietary components[40] (Table 2); in the case of **guava leaves**, only naringenin has been quantified in trace amounts[48].

Position Compound	5	7	3'	4'
Naringenin	OH	OH	-	OH
Naringin	OH	O-Rha-Glu	-	OH
Hesperetin	OH	OH	OH	OCH_3
Hesperidin	OH	O-Rha-Glu	OH	OCH_3

Table 2. Flavanones

Anthocyanidins are normally found in every part of plant foods as its glycoside form, named anthocyanins. Their structures are characterized by the flavylium cation which can be conjugated in 3, 5, 7, 3', 4', and 5'[40] (Table 3). Its presence is evident since their synthesis resulted in a wide range of colors such as red, blue and purple. As example, in **guava leaves**, the accumulation of this subclass of compounds caused a dramatic red coloration in response to harmful growing conditions, such as low temperatures and high light intensities[10,62]. Nevertheless, only the anthocyanin content has been published (data not reported) and none of them have been individually identified yet.

Position Compound	3	5	7	3'	4'	5'
Cyanidin	OH	OH	OH	OH	OH	-
Cyanin	O-Glu	OH	OH	OH	OH	-
Peonidin	OH	OH	OH	OCH_3	OH	-
Delphinidin	-	OH	OH	OH	-	OH
Pelargonidin	OH	OH	OH	-	OH	-
Malvidin	OH	OH	OH	OCH_3	OH	OCH_3

Table 3. Anthocyanidins

Flavonol structure is based on a carbonyl group in 4 position and conjugation at C3, although substitutions also take place at the 5, 7, 4', 3', and 5' positions (Table 4). Flavonols are one of the widest subclass of the flavonoids that could be found in almost every plant[40]. The structural variation is widespread although in leafy fruits is

[62] Coley, P. D.; Barone, J. A. Herbivory and plant defenses in tropical forests. *Annu. Rev. Ecol. Syst.* **1996**, *27*, 305–335.

common the presence of glycoside forms, particularly of quercetin derivatives, which have been identified as the main flavonoids in **guava leaves**[56-58,60] with concentrations of 138.60 µg/g and 0.60 mg/g d.w.[53,60]. Concerning the amounts of aglycone form, quercetin has been found in a wide range (0.18 – 56.00 mg/g)[26,48,51-53] and myricetin from 50.97 to 208.44 mg/kg[26]. Kaempferol, indeed, has only been detected in trace amounts[48] and in mature leaves between 35.07 and 97.25 mg/kg d.w.[26]. In addition, morin, myricetin and some of their glycosides could also be found in **guava leaves**.[46,53,60] accounting for concentrations between 122.60 µg/g[53] and 0.40 mg/g d.w.[60]

Position Compound	5	7	3'	4'	5'
Quercetin	OH	OH	OH	OH	-
Kaempferol	OH	OH	-	OH	-
Galagin	OH	OH	-	-	-
Fisetin	-	OH	OH	OH	-
Myricetin	OH	OH	OH	OH	OH

Table 4. Flavonols

Flavanols or flavan-3-ols are arguably the most structurally complex subclass of flavonoids, covering from the simple monomers to the oligomers (from dimers to decamers) and polymers (up to decamer)[40]. Ubiquitously found in nature, flavanols monomers are non-planar molecules characterized by the presence of two chiral centers (C2 and C3). Commonly, catechin and epicatechin are found in several fruits while (epi)-gallocatechin is less abundant[40] (Table 5). Nonetheless, gallocatechin has been identified in the case of **guava leaves**, as well as (epi)-catechin[47,60]. These compounds has been quantified by the sum of every derivative identified (189 mg/g)[60].

Compound/Position	3	5	7	3'	4'	5'
(+)-Catechin	βOH	OH	OH	OH	OH	-
(-)-Epicatechin	αOH	OH	OH	OH	OH	-
(-)-Epigallocatechin	αOH	OH	OH	OH	OH	OH

Table 5. Flavanols

Condensed tannins (CTs) make reference to oligo and polymer molecules formed due to the linkage between several units of flavanols. Traditionally, CTs were divided based on the different linkages. B-type is referred to C4-C8 or C4-C6 linkage, whereas A-type is due to an ether linkage (C2-O-C7 or C2-O-C5)[40]. Nowadays, these compounds are also known as ***proanthocyanidins* (PAs)** since upon a heating treatment in acidic media yield anthocyanidins[63]. However, they could be distinguished as procyanidins, propelargonidins, and prodelphinidins depending on the flavanol subunit. Moreover, gallic acid is the most frequent substitution in both, flavanols and CTs[63]. Only procyanidins B1, B2, B3 and two prodelphinidins have been isolated in **guava leaves**[47,50].

Non-flavonoids cover a variety of structures, although in Figure 10 only are schematized the main families found in **guava leaves**.

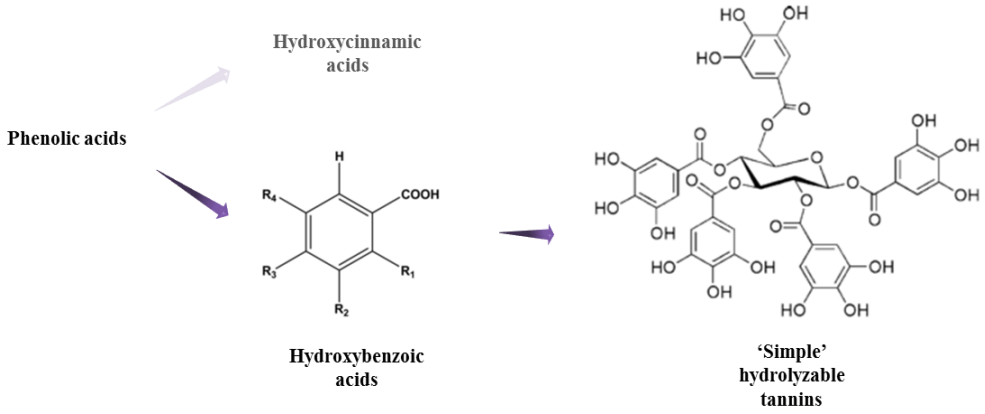

Figure 10. Phenolic acid derivatives structures

Among them, ***phenolic acids*** are one the most important phenolic class and are divided into hydroxycinnamic and hydroxybenzoic acid derivatives. Though the basic structure remains the same, the position and the length of the side substituent make the difference. They account for one-third of the dietary phenols[61]. In the case of

[63] Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds-nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094–1117.

guava leaves, gallic acid together with ferulic acid are almost the unique phenolic acids quantified[49-51].

Hydrolysable tannins (HTs) are derived from gallic acid base unit with a polyol (typically β-D-glucose) which may be further esterified or oxidative cross-linked to form more complex tannins[64]. Based on Okuda's classification[65], HTs could be divided into four groups, where only two of them have been identified in **guava leaves**[42-44,50]:

- Type I: gallotannins resulted from further substitution of several gallic acid units at the pentagalloylglucose core ('simple' tannin) and are rather distributed in nature.

- Type II: ellagitannins are a consequence of oxidation reactions that yield hexa-hydroxy-di-phenoyl (HHDP) units by intra-molecular bonding of C–C linkage between the galloyl subunits. Besides, HHDP units can be subject of lactonization giving rise to ellagic acid.

- Type III: dehydroellagitannin arises from the dehydrogenation of HHDP moieties and differs from ellagitanins in the position of the glucopyranose ring.

- Type IV: are derived from the oxidation of dehydroellagitannin, yielding dehydroellagitannin acids.

Other kind of tannins is further classified as ***complex tannins*** which are formed by a HT linked to flavanol units[65]. Most of these compounds isolated in **guava leaves** were found to be derivatives of catechin type II-based tannins, such as some guavins[45], and based on type III HTs[50].

[64] Ongphimai, N.; Lilitchan, S.; Aryusuk, K.; Bumrungpert, A.; Krisnangkura, K. Phenolic acids content and antioxidant capacity of fruit extracts from Thailand. *Chiang Mai J. Sci.* **2013**, *40*, 636–642.

[65] Okuda, T.; Ito, H. Tannins of constant structure in medicinal and food plants-hydrolyzable tannins and polyphenols related to tannins. *Molecules* **2011**, *16*, 2191–2217.

2.4.2. Factors affecting guava phenolic composition

Phenolic compounds are synthesized during the development of the plant and its composition varies over the full course of growing. In addition to this, its presence or absence is also dependent on the geographic location, season changes, cultivar, varieties and age of the plant[41]. For example, HTs type I emerge favorably in spring and HTs type II in summer[66], while PAs exhibited the opposite seasonal pattern. Between individual compounds different seasonal pattern also arises[67].

In guava tree, vegetative growth takes place from mid spring to autumn. Like most fruit trees, it displays different phenological stages due to environmental conditions[11]. However, few works reported the variation of the phenolic compounds in the different stages of the **leaves**. On the one hand, Vargas-Álvarez et al.[26] found that **mature leaves** provided better flavonoid content than young or senescence leaves, whereas Nantitanon et al.[52] concluded that flavonoid content was highest in **young leaves**, as well as total phenolic content (TPC). On the other hand, certain flavonoids vary substantially during seasonal changes. Although its variation did not follow any trend, in July was found the highest accumulation[26]. In contrast to the anthocyanin accumulation, it was maxima after cold temperatures[10]. Besides, Table 1 shows how geographical position affects the phenolic composition of the **leaves**. Differences between varieties[68] and cultivars[49] have also been published.

3. Analytical approaches for phenolic compounds in guava leaves

Beneficial effects of phenolic compounds have been ignored for long. Nevertheless, their role as protective dietary constituents has attracted attention of the scientific community[40]. Over the past two decades, a huge number of works have

[66] Grundhöfer, P.; Niemetz, R.; Schilling, G.; Gross, G. G. Biosynthesis and subcellular distribution of hydrolyzable tannins. *Phytochemistry* **2001**, *57*, 915–927.

[67] Salminen, J.-P.; Roslin, T.; Karonen, M.; Sinkkonen, J.; Pihlaja, K.; Pulkkinen, P. Seasonal variation in the content of hydrolizable tannins, flavonoid glycosides and proanthocyanidins in oak leaves. *J. Chem. Ecol.* **2004**, *30*, 1693–1711..

[68] Haida, K. S.; Baron, Â.; Haida, K. S.; de Faci, D.; Haas, J.; da Silva, F. J. Phenolic compounds and antioxidant activity of two varieties of guava and rue. *Rev. Bras. Ciências da Saúde* **2011**, *9*, 11–19.

highlighted the significance of developing analytical methods and the effort to elucidate phenolic compounds structures. The diversity of the sample matrix nature and/or analytes makes difficult to find a global analytical strategy for the determination of phenolic compounds. However, general steps such as the recovery of the compounds, their separation, identification and measurement will suffice the aim[69]. Hence, in **guava leaves**, a strategy which involves experimental features, recovery of the compounds followed by biological screening, separation, and characterization has been followed (Figure 11).

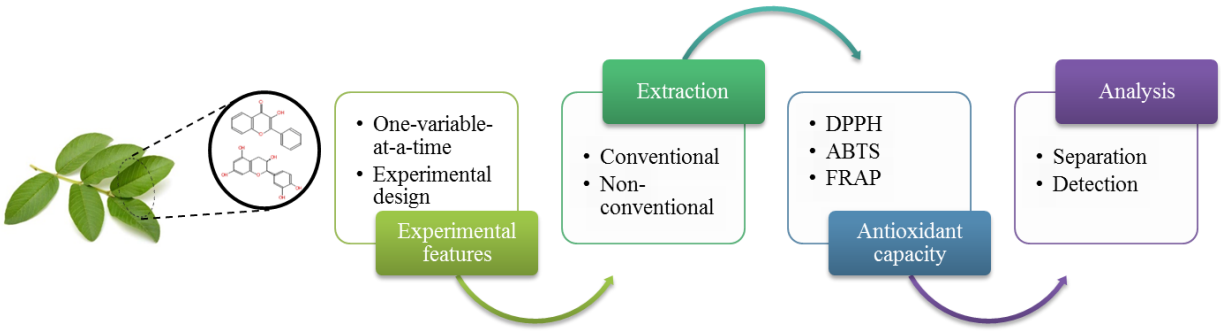

Figure 11. Analytical strategy used for guava leaves

3.1. Experimental features

As it has aforementioned, elucidation of phenolic compounds include, mainly, three steps: compound extraction, separation and quantification. Of these, extraction and separation have been commonly optimized. Traditionally, the method most used for optimization with **guava leaves** is one-variable-at-a-time technique, which consisted on evaluating the effect on the response of one changing parameter while others are kept constant. The major drawback of this approach is that it does not consider the interaction effect between the independent parameters[70]. Hence, it is

[69] Robards, K. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *J. Chromatogr. A* **2003**, *1000*, 657–691.

[70] Almeida Bezerra, M.; Erthal Santelli, R.; Padua Oliveira, E.; Silveira Villar, L.; Escaleira, L. A. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* **2008**, *76*, 965–977.

improbable that the optimum will be discovered. In addition to this, changing one-variable-at-a-time also presents other disadvantages (Table 6)[70,71].

In recent years, the necessity to increase the efficiency of extraction and separation optimization methods has boosted the use of multivariate statistic techniques. Among the chemometric tools more used for this goal, experimental design or design of experiments employing response surface methodology (RSM) is the most relevant ones[70] due to its advantages (Table 6)[71,72]. This approach combined statistical and mathematical theories to fit the experimental data into a function, usually linear or quadratic and their interactions, with the objective of describing the behavior of the system. In fact, is an estimate of the change in response when a factor changes.

Table 6. One-variable-at-a-time vs. RSM

Optimization method	Advantages	Disadvantages	Ref.
One-variable-at-a-time	Simple statistical analysis	• Does not include interaction effects • Large number of experiments • Time consuming	70,71
RSM	•Include interaction effects •Reduction of time and number of experiments •Information about the process	Fitting the data	71, 72

The achievement of the optimal situation of a process by multivariate analysis, usually involves two steps. First, a screening design (full or fractional factorial design) should be carried out as a preliminary test to find out the main effects. However, if a factor has a known effect on the system, it is 'not' necessary this design. The factor

[71] Baş, D.; Boyacı, İ. H. Modeling and optimization I: usability of response surface methodology. *J. Food Eng.* **2007**, *78*, 836–845.

[72] Ferreira, S. L. C.; Bruns, R. E.; Ferreira, H. S.; Matos, G. D.; David, J. M.; Brandão, G. C.; da Silva, E. G. P.; Portugal, L. A.; dos Reis, P. S.; Souza, A. S.; dos Santos, W. N. L. Box-Behnken design: an alternative for the optimization of analytical methods. *Anal. Chim. Acta* **2007**, *597*, 179–186.

could directly be part of an optimization design[73]. Second step consisted on a more complex design (central composite design, Box-Behnken design (BBD) and Doehlert designs) which provided the variation of the system. Comparing them, BBD and Doehlert design are more efficient and economical designs than central composite design, although it is still been the method most used for optimization. BBD is considered as the best option since it is rotatable or nearly rotatable second-order design, allow avoiding extreme situations, and it could be arranged in orthogonal blocks, properties that Doehlert design does not present[72,74]. A brief explanation of BBD is given in the ***appendix*** section.

3.2. Extraction techniques

As mentioned above, extraction is one of the first steps of any analytical process and is crucial for later stages. The complexity of most natural plant matrices and the structural diversity of the phenolic compounds, which affect to its physicochemical behavior, makes difficult to establish a single extraction methodology. In this regard, the choice of extraction method, solvent, extraction time, and temperature is the most critical for recovery the target compounds[69]. In addition, plant matrices also have non-phenolic compounds, such as sugars, so previous extraction steps may be required to remove them[75].

Extraction of phenolic compounds could be done employing different techniques which are classified as conventional or non-conventional methods. Typically, conventional extraction has been used for this purpose, since it has been shown to be effective, and is still dominating this area[69]. The existing classical techniques consisted on the transfer of the analytes from a liquid or solid matrix to different solvents, due to their extracting power, applying heat and/or mixing.

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[73] Hibbert, D. B. Experimental design in chromatography: a tutorial review. *J. Chromatogr. B* **2012**, *910*, 2–13.

[74] Ferreira, S. L. C.; Bruns, R. E.; da Silva, E. G. P.; dos Santos, W. N. L.; Quintella, C. M.; David, J. M.; Bittencourt de Andrade, J.; Breitkreitz, M. C.; Sales Fontes Jardim, I. C.; Barros Neto, B. Statistical designs and response surface techniques for the optimization of chromatographic systems. *J. Chromatogr. A* **2007**, *1158*, 2–14.

[75] Ignat, I.; Volf, I.; Popa, V. I. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chem.* **2011**, *126*, 1821–1835.

Considering that the effectiveness of these methods depends on the solvent, its polarity, the use of co-solvent, environmental and human safety, are factors that should be considered. The major problems of conventional extraction are that it requires long extraction times and large amount of solvent. Hence, in recent years, non-conventional extraction techniques, such as ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), and microwave-assisted extraction (MAE) have been emerged to overcome these issues[76].

Between the most common conventional extraction techniques (soxhlet, maceration, and hydrodistillation), maceration during long and short extraction times (infusion/decoction) have been extensively applied in **guava leaves** (Table 7). Moreover, conventional liquid-liquid extraction has performed to isolate individual phenolic compounds in **guava leaves**. Taking into account the extracting solvent, acetone, methanol, ethanol and their aqueous mixtures have been the most widely employed with **guava leaves** (Table 7), as are generally employed in phenolic compounds[76].

Table 7. Analytical approaches used for guava leaves

Extraction	Separation	Detection	Ref
Solid-liquid: maceration with ethanol (room temperature, 48 h x 3 times). Liquid-liquid: partition with solvents of different polarities	PC	UV-Vis (λ=255, 360, 545 nm) IR	42
Solid-liquid: maceration with Me_2CO:H_2O (70% (v/v) x 3 times). Liquid-liquid: partition with solvents of different polarities	CLC TLC DCCC HPLC	UV (λ=280 nm) NMR	43
Solid-liquid: maceration with Me_2CO:H_2O (70% (v/v) x 3 times). Liquid-liquid: partition with solvents of different polarities	CLC	UV (λ=208, 221, 283 nm) NMR	44
Solid-liquid: maceration with Me_2CO:H_2O (70% (v/v) x 3 times). Liquid-liquid: partition with solvents of different polarities	CPC CLC DCCC	NMR	45

[76] Azmir, J.; Zaidul, I. S. .; Rahman, M. M.; Sharif, K. M.; Mohamed, A.; Sahena, F.; Jahurul, M. H. A.; Ghafoor, K.; Norulaini, N. A. N.; Omar, A. K. M. Techniques for extraction of bioactive compounds from plant materials: A review. *J. Food Eng.* **2013**, *117*, 426–436.

Extraction	Separation	Detection	Ref
Solid-liquid: maceration with MeOH:H_2O (90% (v/v) x 3 times; room temperature). Liquid-liquid: partition with solvents of different polarities	CLC TLC HPLC	UV-Vis (λ=250, 360 nm) APCI-Q-MS NMR IR	46
Solid-liquid: maceration with Me_2CO:H_2O (70% (v/v)) and ethyl acetate	CLC GPC TLC	NMR MALDI-TOF-MS	47
Solid-liquid: reflux with acidified MeOH:H_2O (90% (v/v); 98 °C, 2h)	HPLC	DAD (λ=365 nm)	26
Solid-liquid: reflux with H_2O (100 °C, 30 min)	HPLC	DAD (λ=220, 400 nm)-ESI-IT-MS	48
Solid-liquid: infusion/decoction 5 minutes	HPLC	UV (λ=285 nm)	49
Solid-liquid: maceration with Me_2CO:H_2O (70% (v/v)). Liquid-liquid: partition with solvents of different polarities.	CLC analytical and preparative HPLC	DAD (λ=285 nm) NMR	50
Solid-liquid: maceration with acidified MeOH	-	Vis (λ=530, 657 nm)	10
Solid-liquid: infusion/decoction 5 minutes	HPLC	DAD (λ=280 nm)	51
Solid-liquid: maceration with/without stirring (room temperature, 24h x 3 times), UAE (10 min x 3 times), soxhlet (4 h) with EtOH Solid-liquid: UAE with EtOH, H_2O and ethyl acetate (10 min x 3 times)	HPLC	UV (λ=280 nm)	52
Solid-liquid: maceration with MeOH	CLC	-	53
Solid-liquid: maceration with MeOH:H_2O (80% (v/v); room temperature, 7 days)	CLC TLC HPLC	UV (λ=200 - 400 nm) IR FAB-MS NMR	54
Solid-liquid: infusion with H_2O and EtOH	-	Vis (λ=740 nm)	68
Solid-liquid: maceration with MeOH:H_2O (70% (v/v) x 2 times) Me_2CO:H_2O (80% (v/v) x 2 times). Liquid-liquid: partition with solvents of different polarities.	CLC GPC HPLC	NMR ESI-TOF-MS	55

Extraction	Separation	Detection	Ref
Solid-liquid: maceration with EtOH:H_2O (70% (v/v) x 3 times; room temperature). Liquid-liquid: partition with solvents of different polarities	CLC UPLC	NMR ESI-IT-MS ESI-QTOF-MS IR	56
Solid-liquid: maceration with MeOH:H_2O (70% (v/v) x 2 times) Me_2CO:H_2O (80% (v/v) x 2 times). Liquid-liquid: partition with solvents of different polarities	CLC HPLC	NMR ESI-TOF-MS	57
Solid-liquid: UAE with ethyl acetate (1 h x 7 times)	HSCCC preparative and analytical HPLC	UV (λ=254 nm) DAD (λ=200 - 400 nm) ESI-Q-MS NMR	58
Solid-liquid: maceration with MeOH:H_2O (70, 50 and 30% (v/v), 3 days) or Me_2CO:H_2O (70, 50 and 30% (v/v), 3 days)	-	Vis (λ=724.5 nm)	59
Solid-liquid: UAE with MeOH (20 min). Liquid-liquid: partition with solvents of different polarities	HPLC	DAD (λ=278, 350, 520 nm)-ESI-IT-MS	60

Centrifugal partition chromatography (CPC); column liquid chromatography (CLC); droplet counter current chromatography (DCCC); gel permeation chromatography (GPC); high speed counter current chromatography (HSCCC); high performance liquid chromatography (HPLC); paper chromatography (PC); thin layer chromatography (TLC); ultra performance liquid chromatography (UPLC)

Among the novel techniques, UAE has been almost the unique technique used for the extraction of phenolic compounds in **guava leaves** (Table 7). SFE has been applied to remove non-polar compounds prior to the extraction[56]. In addition, quercetin, gallic, and ellagic acid were extracted by MAE with ionic liquids, although no significant differences were found comparing with ionic liquids conventional extraction[77].

In the present thesis, infusion and UAE have been done for the extraction of phenolic compounds in **guava leaves**. Thus, they are described in the following subsection.

[77] Du, F.-Y.; Xiao, X.-H.; Luo, X.-J.; Li, G.-K. Application of ionic liquids in the microwave-assisted extraction of polyphenolic compounds from medicinal plants. *Talanta* **2009**, *78*, 1177–1184.

3.2.1. Infusion

Infusion and decoction of **guava leaves** have been employed in traditional medicine for the treatment of several diseases[14]. This extraction method is one of the simplest procedures and is suitable because it offers data of daily dietary intakes[69]. It consisted on the maceration of the leaves, during a short period of time, in cold or boiling water. In addition, 25% of alcohol could be added during or after the process[78]. Factors affecting the extraction are liquid-solid ratio, particle size and infusion time. Commonly, **guava leaves** are cut into small pieces, powdered or crushed and infusion during 5 minutes[49,51,78].

3.2.2. Ultrasound assisted extraction (UAE)

Nowadays, much attention has been given to the application of ultrasound to food processing with the purpose of replacing or accelerating the traditional processing techniques, such as degassing or extraction. In the meantime, the possibility to scale up of the ultrasonic extraction is interesting for food industry[79]. Comparing with novel extraction techniques (MAE, PLE, and SFE), UAE represents the advantages of being simpler, faster, and economical[80].

As is shown in Figure 12, two types of ultrasound apparatus are usually employed in laboratory. The most common one is the ultrasonic bath (a), where the samples are submerged, while the ultrasonic probe or horn (b) is immersed into the sample, thus, the delivered intensity is less attenuated. This difference makes the probe a powerful system that could be range to industrial scale. In this case, the fast rise of temperature should be controlled by circulating cooling water into a double mantle[80].

[78] Singh, J. Maceration, percolation and infusion techniques for the extraction of medicinal and aromatic plants. In *Extraction technologies for medicinal and aromatic plants*; **2008**; pp. 67–82.
[79] Tao, Y.; Sun, D.-W. Enhancement of food processes by ultrasound: a review. *Crit. Rev. Food Sci. Nutr.* **2015**, *55*, 570–594.
[80] Chemat, F.; Zill-E-Huma; Khan, M. K. Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrason. Sonochem.* **2011**, *18*, 813–835.

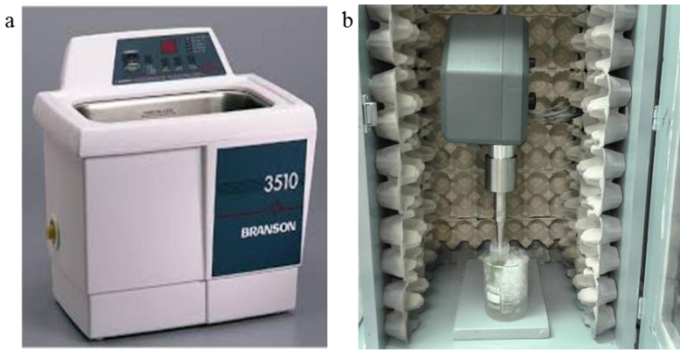

Figure 12. Laboratory ultrasound systems: a) bath, and b) probe

The ultrasound system, which is composed by a generator, a transducer, and a delivery system, generates a mechanical wave with frequencies from 20 kHz to 10 MHz. When the wave passes through the medium, the cavitation bubbles are formed creating in this way the ultrasonic effect. Under this effect, the surface area of the raw material increases, improving the mass transfer rate and the release of the compounds; consequently, it enhances the extraction process. Factors that affected the UAE could be divided into ultrasonic or non-ultrasonic factors. Non-ultrasonic factors include solvent, solvent/solid ratio, particle size, type of container used, temperature, and time. Briefly, the enhancement of extraction could be reached at relatively low temperatures, avoiding degradation of the compounds and keeping their stability and bioactivity. Moreover, small particle size and high solvent/solid ratios facilitate the extraction. Ultrasonic factors involve the ultrasonic frequency, the ultrasonic energy transmitted, the duration, and the treatment mode. In this sense, extraction is favorable at middle-low frequencies and power, short extraction times, and a pulse mode[79].

The extraction of phenolic compounds from **guava leaves** has been carried out employing an ultrasonic bath with different solvents such as ethanol, methanol, water, ethyl acetate[52,60]. Within them, ethanol and methanol provided better recovery of target compounds[52,60] (Table 7). However, it is not possible to make any comparison due to the lack of information about ultrasonic (frequency and power) and non-ultrasonic factors (solvent/solid ratio, particle size, and temperature) and because the extraction time differs from one to another (10 min x 3 times and 20 min, respectively) [52,60].

3.3. Antioxidant capacity

Phenolic compounds, and their dietary intake, have gained especial attention due to their potent antioxidant properties, which could modify the activity of several enzymes and cell receptors[81]. Besides, these properties confer to them the ability to protect us against a vast number of pathological conditions such as DM, and cardiovascular diseases. Their reliable effect in the prevention of some oxidative stress associated diseases lies in the power of the antioxidants to counteract the overproduction of reactive oxygen/nitrogen species (ROS/RNS)[82].

The antioxidant activity or capacity derived from the high redox potential, which allow to these compounds to donate hydrogen or an electron[81,82], acts via different mechanisms, such as reducing activity, ROS/RNS radical-scavenging, chelating of pro-oxidant metals and quenching of singlet oxygen[83]. These properties are largely influenced by the chemical structure, hydroxylation, and conjugation of phenolic compounds. For example, in flavonoids, the position of the hydroxyl groups on the B-ring contributes more than the flavan backbone. However, the lack of a double bond in C-ring, conjugated with a 4-oxo function, decreases the activity of some flavonoids[84].

Antioxidant activity/capacity can be measured by a broad varieties of assays, which are classified as hydrogen atom transfer (HAT), electron transfer (ET), and mixed mode (HAT/ET) based assays[82]. Despite the fact that there are several regards against these methods, they are still useful to evaluate the power of an extract. As there is not a standardized method for this purpose, it is desirable to carry out more than one

[81] Dai, J.; Mumper, R. J. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* **2010**, *15*, 7313–52.
[82] Apak, R.; Özyürek, M.; Güçlü, K.; Çapanoğlu, E. Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *J. Agric. Food Chem.* **2016**, *64*, 997–1027.
[83] Tachakittirungrod, S.; Okonogi, S.; Chowwanapoonpohn, S. Study on antioxidant activity of certain plants in Thailand: Mechanism of antioxidant action of guava leaf extract. *Food Chem.* **2007**, *103*, 381–388.
[84] Heim, K. E.; Tagliaferro, A. R.; Bobilya, D. J. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.* **2002**, *13*, 572–584.

in vitro chemical-based assay. In this regard, the 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), trolox equivalent antioxidant capacity (TEAC), and ferric reducing antioxidant power (FRAP) have been the main assays applied with **guava leaves** because they act by different mechanism of action [52,83,85].

3.3.1. 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) assay

DPPH reactions are supposed to occur by HAT mechanism[86,87]. However, they are mostly attributed to mixed mode method[86]. The assay is based on the reduction of DPPH radical due to the presence of antioxidant compounds, what causes an absorbance reduction (at 515 nm), and a color change from purple to yellow[85] (Figure 13).

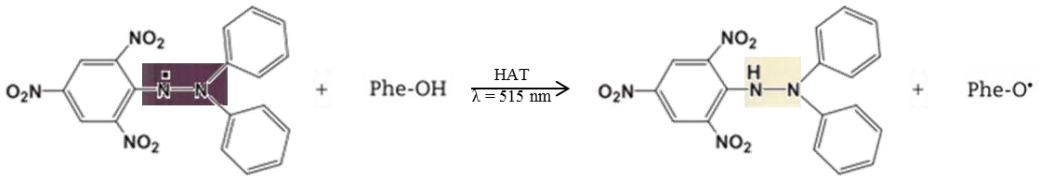

Figure 13. Reaction of DPPH radical. *Adapted from Apak et al.*[87]

Advantages of this assay are that is an economical and simple method. On the contrary, it exhibited several disadvantages: the assay can provide erroneous conclusions since it does not evaluate reaction rates, it does not work well with hydrophilic antioxidants, effects of steric hindrance, and it is solvent and pH dependent[86,87]. Moreover, data obtained by DPPH assay are usually expressed as % of reduction of radical DPPH or as Trolox equivalent antioxidant capacity (TEAC).

[85] Nantitanon, W.; Okonogi, S. Comparison of antioxidant activity of compounds isolated from guava leaves and a stability study of the most active compound. *Drug Discov. Ther.* **2012**, *6*, 38–43.
[86] Schaich, K. M.; Tian, X.; Xie, J. Hurdles and pitfalls in measuring antioxidant efficacy: A critical evaluation of ABTS, DPPH, and ORAC assays. *J. Funct. Foods* **2015**, *18*, 782–796.
[87] Apak, R.; Özyürek, M.; Güçlü, K.; Çapanoğlu, E. Antioxidant Activity/Capacity Measurement. 2. Hydrogen Atom Transfer (HAT)-Based, Mixed-Mode (Electron Transfer (ET)/HAT), and Lipid Peroxidation Assays. *J. Agric. Food Chem.* **2016**, *64*, 1028–1045.

3.3.2. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The mechanism of ABTS assay consisted on the de-colorization of the radical cation ($ABTS^{\cdot+}$) produced by an H atom transfer converting it into a more stable product (Figure 14). It occurs through a mixed of ET and HAT mechanisms, depending on the kinetic reaction.

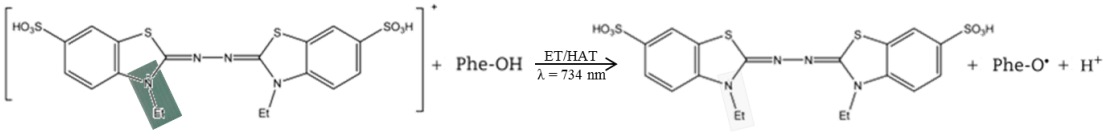

Figure 14. Reaction of ABTS radical cation. *Adapted from Apak et al.*[87]

The ABTS method is operationally simple, reproducible, cost effective, and is useful to determine both hydrophilic and hydrophobic antioxidants. Its major limitations are that results only take into account stoichiometry, molecular size of the antioxidants, and steric hindrance[86,87].

3.3.3. Ferric reducing antioxidant power (FRAP) assay

FRAP method is conducted via the reduction of a colorless ferric complex, with 2,4,6-tripyridyl-s-triazine (TPTZ), to its ferrous colored form (Figure 15). The reducing power indicates that the antioxidants are capable to donate an electron[82,85]. Despite the mechanism of the reaction differs in FRAP and ABTS assays, similar compounds react in them.

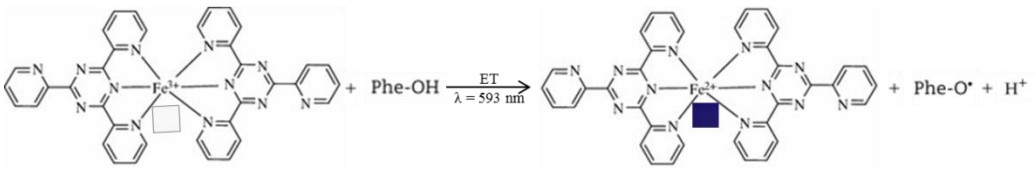

Figure 15. Reduction of Fe(III)-TPTZ complex

The benefits of this assay are the cost (low), its simplicity, its wide linear range, and that it provides a putative value of antioxidant capacity. Major criticisms of FRAP assay are that is not adequate for testing thiols and carotenoids, only measures the reducing capability, which is not much relevant to antioxidant capacity, and also because it generates Fe(II) as the reduction product, which could generate of reactive species[82].

3.4. Application of analytical techniques

Generally, the analysis of the phenolic composition in plants is a challenging task due to the complexity and variability of the matrices. In this sense, analytical techniques both, spectrophotometric and chromatographic were developed for separation and quantification of phenolic compounds, due to the necessity for highly sensitive and selective techniques, especially for the simultaneous determination of the target compounds.

Over the last years, several works have been published concerning the isolation and measurement of several compounds from **guava leaves**. Most of the works have been aimed at the structural elucidation of several compounds after their purification, whilst few works have been focused on the screening and quantification of single and total amount of phenolic compounds (Table 1 and 7).

From the point of view of separation, paper chromatography (PC) was the first technique used to isolate quercetin, guaijaverin, leucocyanidin, ellagic acid, amritoside from **guava leaves**[42]. Subsequent separations of HTs, CTs and some flavonoids were carried out combining column liquid chromatography (CLC) along with thin layer chromatography (TLC) or less common techniques[43-47]. In addition, high performance liquid chromatography (HPLC) started to be used by comparing the data obtained with TLC[43], and gained importance in the last years. This later one is further described because it has been the separation method chosen for the screening of phenolic compounds in **Spanish guava leaves**.

3.4.1. High performance liquid chromatography (HPLC)

Currently, HPLC has become the analytical method of choice for separation and quantification of natural products as it is highly efficient in the analysis of phenolic compounds. Basically, HPLC consisted on the use of high pressure to impulse a mobile phase solution through a stationary phase (column), permitting the separation of compounds in a complex mixture. The system is generally constituted by a pump, injector, column, detector, and data acquisition system (Figure 16).

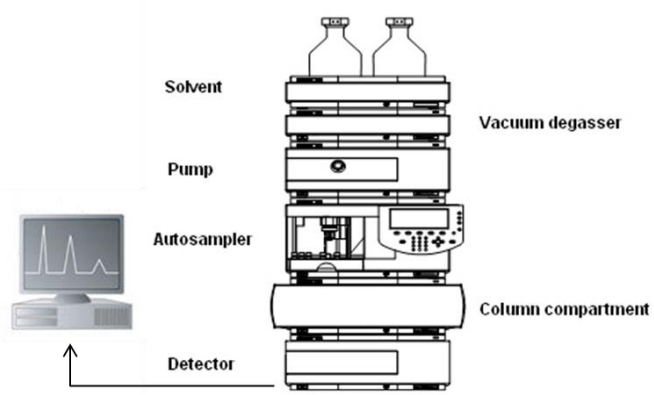

Figure 16. HPLC system

Concerning the nature of the column, different alternatives are available such as partition, adsorption, ionic exchange, affinity, exclusion, and quiral. However, adsorption chromatography is the most used for the analytes of interest. The principle of the technique consisted on the interaction (adsorption/desorption) of sample components, transported by a liquid mobile phase, with a stationary phase, which is a solid material. The separation is due to the different distribution coefficients of the analytes, which results in different elution rates. Depending upon the affinity of the solute for the stationary phase, their retention will provide shorter or longer times.

Based on the type of adsorbent of the stationary phase, HPLC could be achieved by normal-phase (NP) or reversed-phase (RP). On one side, in NP high polar compounds interact with a polar stationary phase while a less polar mobile phase elute the analytes in order of increasing polarity. This HPLC mode is usually employed with

tannins, especially with CTs[88]. On the other side, in RP medium-high polarity compounds are retained in non-polar stationary phase, and eluted by decreasing polarity. This option is commonly chosen for phenolic compounds[69]. The separation is performed in the **column**, although it can reach 40°C, is usually achieved at room temperature because the separation is more reproducible[89]. The dimensions of the most common columns varied from 50 to 300 mm in length with an internal diameter from 2 to 4.6 mm, and packaging between 1.8 and 10 μm. Indeed, shorter and narrower columns with smaller packing would be preferred to obtain better resolution and reduce the analysis time. The normal packed are made up of C_{30}, C_8 and C_{18}-bonded silica for RP, whilst bare silica based is used for NP columns[69,88]. However, the elution of PAs in these last columns is incomplete. Therefore, is recommendable to use a diol stationary phase or even work in hydrophilic interaction chromatography (HILIC) mode[88].

The separation is also highly influenced by the type and the composition of the **mobile phase**, and the choice is based on the column, the compounds of interest, and the detection system. Generally, it consisted on a solvent or a solvent system formed by water (RP) or an organic solvent with low proportions of water (NP), and a less polar organic solvent (methanol, acetonitrile). The mobile phase is pumped at high pressure by isocratic or gradient elution, so solvent systems can be both acidified, usually with acetic, formic and perchloric acid, in order to maintain the concentration during the analysis, and also to avoid ionization, enhancing partly the separation[69].

Parameters such as **flow rate** and **time of analysis** are also important in HPLC to provide greater resolution of the whole analysis. Increasing the flow involves a reduction of the time of analysis and improves the resolution, although it directly

[88] Ikegami, T.; Tomomatsu, K.; Takubo, H.; Horie, K.; Tanaka, N. Separation efficiencies in hydrophilic interaction chromatography. *J. Chromatogr. A* **2008**, *1184*, 474–503.

[89] de Rijke, E.; Out, P.; Niessen, W. M. A.; Ariese, F.; Gooijer, C.; Brinkman, U. A. T. Analytical separation and detection methods for flavonoids. *J. Chromatogr. A* **2006**, *1112*, 31–63.

increases the pump pressure. Normally, time should range from 10 to 150 min at a flow rates between 0.2 to 2 mL/min[69,88].

As is shown in Table 8, there is no consensus for chromatographic separation of phenolic compounds in **guava leaves**. The conditions employed for this goal, are a mix of the most common values summarized in many instances.

Table 8. Chromatographic conditions used in guava leaves

Mode	Column	Elution	Mobile phase	Flow rate	Time	Temp	Ref
RP	LiChrosorb C18 (300 x 4 mm), 10 µm	Isocratic	0.1 M H_3P0_4/0.1 M KH_2P0_4/EtOH/EtOAc (50:50:2:5 (v/v/v/v))	-	-	-	43
NP	Develosil 60 (150 x 4 mm), 5 µm	Isocratic	Acidified (oxalic acid) n-hexane/MeOH/THF/HCOOH (55:33:11:1 (v/v/v/v))	-	-	-	43
RP	Cosmosil C18 (250 × 10 mm), 5 µm	Isocratic	0.1% (v/v) TFA in MeOH/H_2O (50% (v/v))	2 mL/min	-	-	46
RP	ODS hypersil C18 (125 x 4 mm), 5 µm	Isocratic	TFA (pH 2.5) in MeOH/H_2O (35% (v/v))	1.5 mL/min	-	-	26
RP	Luna 3 µm C18 (150 x 2 mm), 3 µm	Gradient	A: H_2O; B: CH_3CN. Both acidified (0.1% HCOOH)	0.2 mL/min	65 min	-	48
RP	LiChrospher C18 (250 mm x 4mm), 5 µm	Gradient	A: 0.5 M NaH_2PO_4; B: 0.5 M NaH_2PO_4/MeOH/ CH_3CN (30:20:50 (v/v/v))	1 mL/min	100 min	-	49
RP	ODS A-320 (150 x 4.6 mm)	Gradient	A: 0.1 M H_3PO_4/0.1 M KH_2PO_4/CH_3CN (45:45:10 (v/v/v)), B: 0.1 M H_3PO_4/0.1 M KH2PO_4 /CH_3CN (35:35:30 (v/v/v))	2 mL/min	30 min	40 ºC	50
NP	SIL A-003 (250 x 4.6 mm)	Isocratic	n-hexane/MeOH/THF/HCOOH (55:33:11:1 (v/v/v/v))	1.5 mL/min	-	-	50

Mode	Column	Elution	Mobile phase	Flow rate	Time	Temp	Ref
RP	LiChrospher C18 (250 x 4.6 mm), 5 µm	Gradient	A: 2% (v/v) CH_3COOH in H_2O, B: 0.5% CH_3COOH in H_2O/CH_3CN (50:50 (v/v))	1 mL/min	80 min	Room	51
RP	Zorbax SB-C18 (250 x 4.6mm), 5 µm	Gradient	A: 2% (v/v) CH_3COOH in H_2O, B: 0.5% H_2O in H_2O/CH_3CN (50:50 (v/v))	1 mL/min	80 min	Room	52
RP	UG120 C18 (250 x 10 mm)	Isocratic	$CH_3CN/MeOH/H_2O$ (5:30:65 (v/v/v))	3.0 mL/min	-	-	54
RP	UG120 C18 (250 x 10 mm)	Isocratic	CH_3CN/H_2O (18:82 (v/v))	3.0 mL/min	-	-	54
RP	NH2P-50, (250 x 4.6-mm), 9 µm	Isocratic	CH_3CN/H_2O (75:25 (v/v))	1 mL/min	-	40 ºC	54
RP	ODS-80TM (30 x 21.5 mm)	Isocratic	$H_2O/CH_3CN/HCOOH$ (95:5:1 (v/v/v)) and/or $H_2O/CH_3CN/HCOOH$ (85:15:1 (v/v/v))	-	-	-	55, 57
RP	C18 (250 x 4.6 mm), 5µm	Gradient	A: 0.2% (v/v) H_3P0_4 in H_2O, B: CH_3CN	1 mL/min	75 min	40 ºC	58
RP	Phenomenex Luna C18 (150 x 2 mm), 3 µm	Gradient	A: H_2O, B: CH_3CN. Both acidified 0.1% HCOOH	0.2 mL/min	75 min	-	60

After separation of the compounds from each other, the chemical screening is done through **detection techniques** coupled to HPLC. The signal generated in the detector is transformed and represented against time, giving place to the chromatogram. If the separation step is well developed, each analytical detection signal corresponds to a target compound, and it is represented by a peak at a specific retention time, being the intensity of the peak proportional to the quantity of the analyte in the sample. Thus, characteristic like high sensitivity, stability and reproducibility, specificity, detectability, linearity, and repeatability are specific properties that a good detector might have. Nevertheless, there is no single HPLC-hyphenated detector which is capable to identify all the compounds. Consequently, the use of two or more detectors could solve this gap.

From the perspective of **guava leaves**, a number of different tools including ultraviolet-visible detection (UV-Vis) or photodiode array detection (DAD), mass spectrometry (MS), and nuclear magnetic resonance (NMR), coupled or not to LC, have been used for detecting guava components. In many instances, the structural elucidation was done with NMR, and HPLC coupled to spectrophotometric or MS detection was only applied to obtain complementary information such as mass, molecular formula and spectral information of **guava leaves** compounds (Table 7). In addition, **quantification** was traditionally done measuring different structural groups by spectrophotometric detection. However, these assays (e.g. total phenolic content (TPC), flavonoid content (TFC), among others) give an over-estimation of the content because of their lack of specificity[69]. Therefore, in **guava leaves**, HPLC-DAD has also been employed for this purpose in many instances[26,48,49,51,52]. Chang et al.[60] reported the most comprehensive identification and quantification of several phenolic compounds by HPLC-DAD-MS. Nonetheless, 7 of 27 compounds were not assigned. Due to the lack of information about the whole characterization of **guava leaves** extract, the following detectors (coupled or not to HPLC) have been employed in the current thesis.

3.4.2. Detection techniques

3.4.2.1. HPLC coupled to UV-Vis detector

UV-Vis detection has demonstrated to be an efficient complementary tool throughout tentative characterization, and is routinely used in LC-based analyses[89]. Despite of being the detector with best characteristics (sensitivity, linearity, versatility, and reliability)[90], one of its main limitation is that UV-Vis detection only enabled the identification of the same sub-class of compounds. Poor chromophores, such glycosides and acyl substituents could not be distinguished[89].

[90] Wolfender, J.-L. HPLC in natural product analysis: the detection issue. *Planta Med.* **2009**, *75*, 719–734.

Instrumentally, three kinds of detectors are available. The most sensitive one, and also the most limited, is the fixed-wavelength detector, followed by the multiple-wavelength detector, which can emit more than one specific wavelength. Finally, DAD provides a complete UV-Vis spectrum, which can be registered during the analysis when it is hyphenated with HPLC[90].

The property of absorbing UV or Vis light, from 200 to 560 nm, is because the phenolic compounds have one or more double bonds and unshared electrons[90]. More specifically, phenolic acids present a single maximum in the range of 200-290 nm due to the benzoic acid carbon framework [61], whereas flavonoids have two absorption bands. Band II (240-290 nm) is due to the conjugation and substitution of A ring, and band I is affected by the substitution of C ring and conjugation between B and C rings. The maximum of band I is at 310-370 nm for flavonols (Figure 17a) and at 460-560 nm for anthocyanins. The lack of conjugation among B-C rings (flavanols and flavanones) gives a spectrum like Figure 17b[91].

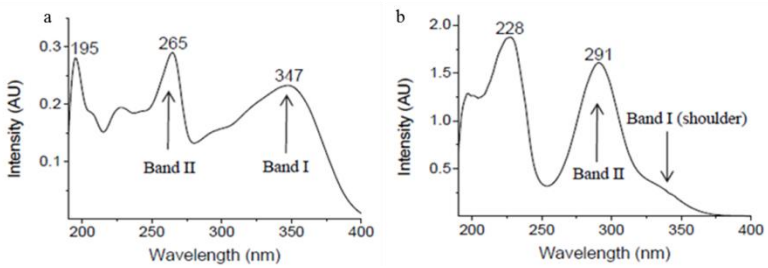

Figure 17. Examples of UV spectra: a) flavonols and b) flavanols

3.4.2.2. HPLC coupled to fluorescence detector (FLD)

Fluorescence mechanism (Figure 18) is based on the molecular absorption of a photon (λ_{exc}) which triggers the emission of another photon with longer wavelength (λ_{em})[90].

[91] Vihakas, M. Flavonoids and other phenolic compounds: characterization and interactions with Lepidopteran and Sawfly larvae, 2014.

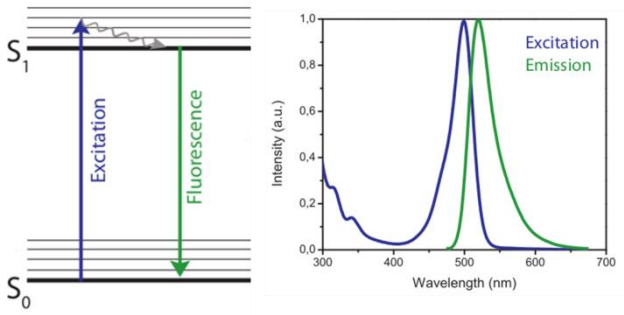

Figure 18. FLD mechanism

Fluorimetric detection has demonstrated to be suitable in molecules which exhibit native fluorescence like flavonols and their higher oligomers. Compared to UV-Vis detection, FLD affords two main benefits. For instance, greater sensitivity is achieved, resulting in detection limits about an order of magnitude lower, and an enhanced selectivity because of the difference in wavelengths (absorption-emission)[89,90]. It is important to keep in mind that solvents used as mobile phases must not quench with phenol fluorescence.

3.4.2.3. Mass spectrometry (MS)-based detection

The hyphenation of MS with HPLC has become such a relevant technique in comprehensive analyses of phenolic compounds. In addition, it has supposed a breakthrough in qualitative and quantitative analyses since it provides great sensitivity and selectivity. Besides, it affords reliable information about the compounds like molecular formula (depending on the mass analyzer) and weight, and fragments[90]. The principle behind MS is the analysis of gas-phase ions (anions and cations) according to their mass-to-charge ratios (*m/z*). Each MS system is composed by three main parts: the ionization source, analyzer and detector.

The coupling of MS to HPLC makes essential the use of an interface which allows the conversion of the sample molecules into gas-phase ions. This step is compound dependent, so sensitivity would be affected if the selection of the interface, the ion mode polarity, and the eluent are not well chosen. The most employed ionization sources work at atmospheric pressure, and are able to be fixed at positive or

negative ionization mode depending on the target compound. Within them, electrospray ionization (ESI) is a soft ionization technique suitable for phenolic compounds. The nebulization of the column effluent is produced applying a high-voltage (3-5 kV) to the sample needle, causing charged droplet formation. After that, droplet size decreases as the solvent is evaporated aided by a drying gas. Finally, ions emerge, because the electrostatic repulsion breaks down the droplets ('Coulombic explosion')[90,92]. (Figure 19)

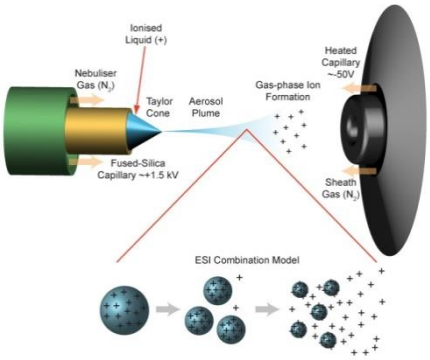

Figure 19. ESI source[93]

Once the ions have been formed, they go to the mass analyzer. Different kind of analyzer, in terms of resolution, mass range, and the way of performing tandem mass-spectrometry experiments (MS/MS), can be found. However, this subsection concentrates on three different mass analyzers, single quadrupole (Q-MS), triple quadrupole (QqQ-MS), and Q-time of flight (QTOF-MS), which have been applied for the identification and quantification of phenolic compounds in the current thesis.

a. HPLC coupled to single quadrupole (Q-MS)

Despite of affording the lowest resolution within the MS available (max. 3000), the use of Q analyzer is frequent due to its economical prize[90]. In fact, it can be considered a mass filter since it does not allow the fragmentation of ions by its self[92].

[92] Steinmann, D.; Ganzera, M. Recent advances on HPLC/MS in medicinal plant analysis. *J. Pharm. Biomed. Anal.* **2011**, *55*, 744–757.
[93] Awad, H.; Khamis, M. M.; El-Aneed, A. Mass spectrometry, review of the basics: ionization. *Appl. Spectrosc. Rev.* **2015**, *50*, 158–175..

The mass spectrum is produced by a certain range of *m/z* values (limited *m/z* mass of 4000 Da), which pass through four parallel aligned metal rods (9.5 mm diameter), with pre- and post-filters, aided by an oscillating electric field which operated at 880 kHz. Ions out of the ratio selected, collide with the rods (Figure 20)[91,92].

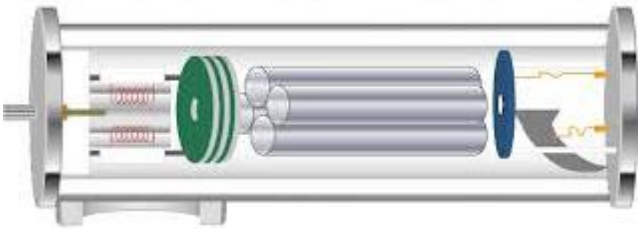

Figure 20. Q analyzer

b. HPLC coupled to triple quadrupole (QqQ-MS)

Another low resolution analyzer is the QqQ. However, it affords the major sensitivity and its linear dynamic range is larger compared with the other analyzers. Consequently, it is principally applied for the quantification of compounds in complex matrices and for precise determinations[90,91]. These applications are possible thanks to the data acquiring mode, known as selected reaction monitoring (SRM) or multiple reactions monitoring (MRM), which allow working as tandem mass spectrometry (MS/MS)[91,92].

Figure 21 shows the schematic data acquiring mode of a QqQ analyzer. The MRM mode consisted on the selection of one or more precursor ions which produce more than one specific product ion. For that, three quadrupoles are arranged in parallel. The first one act as mass filter, like single Q-MS, only desired precursor ions pass through to the collision cell (q), where the fragmentation takes place helped by a collision gas and specific energy. Finally, the product ions that reach the third Q are analyzed according to their *m/z* value[91,92].

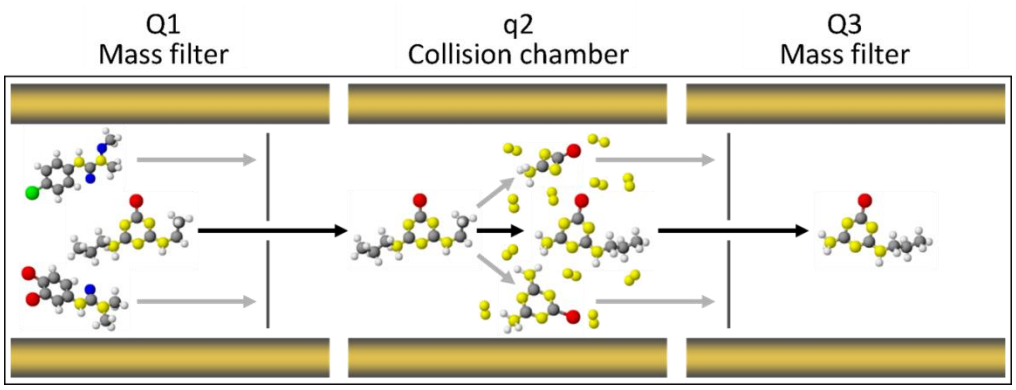

Figure 21. QqQ analyzer

c. HPLC coupled to Q-time of flight (QTOF-MS)

Currently, the popularity of TOF analyzer, and even coupled to a single Q, is increasing due to its high resolution power (Figure 22). This feature lets provide the accurate masses of compound ions and their fragments, verifying in this way the elemental composition of each compound[91,92]. Besides, this tool has been successfully applied for detection and quantification of phenolic compounds[90].

This hybrid technique relies on the fact that the velocity of the ions in the TOF analyzer differs due to their molecular mass. In fact, the velocity of an ion is inversely proportional to its m/z value. The first part of the QTOF-MS, resembles the Qq of the previous analyzer, and TOF operates as follows. After the collision cell, ions with different masses are accelerated into the flight tube and reach the detector at slightly different times. These means that ions with higher m/z value will cross the analyzer later than smaller ions[91,92].

The orthogonal position of the ion source confers to this analyzer the possibility of analyzing a wide range of m/z (50-20000 Da) with high resolution. Crucial parameters are position of the ion source (orthogonal or linear), length of the flight tube and the reflectron. The increase flight tube provides accuracies of 5 ppm for external calibration, and 3 ppm for internal calibration[92].

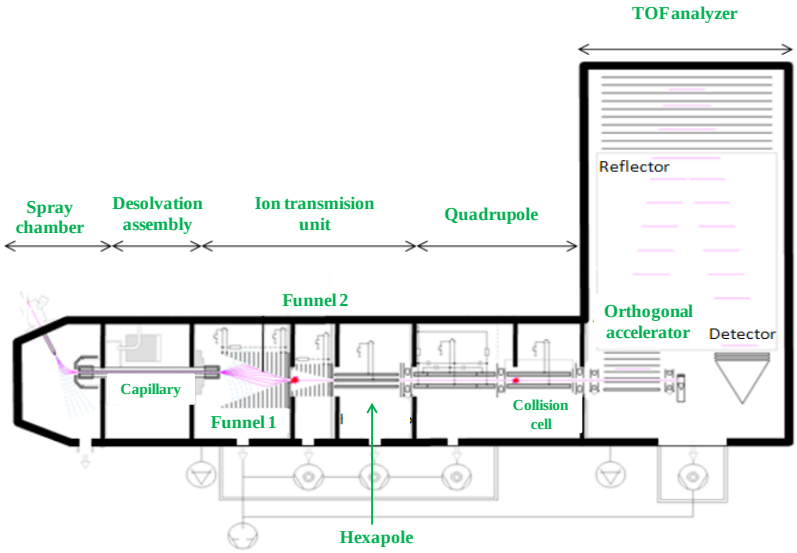

Figure 22. QTOF analyzer

3.4.2.4. Nuclear magnetic resonance (NMR)

NMR is, no doubt, the technique that provides more structural information about a molecule and it is a helpful complement to HPLC[90]. NMR relies on the ability of nuclear spin to absorb energy (radiofrequencies) when are exposed to oscillating magnetic field[94]. The spectrums of the most representative nuclei (^{1}H and ^{13}C) that can be resolved are ^{1}H and ^{13}C NMR. Two-dimensional homonuclear (2D ^{1}H–^{1}H) experiments are achieved by correlation spectroscopy (COSY) and totally correlation spectroscopy (TOCSY), while two-dimensional heteronuclear spectrums are provided by chemical shift correlation NMR (C–H HECTOR), single-quantum correlation, and multiple bond correlation experiments (HSQC and HMBC, respectively), nuclear over-hauser spectroscopy, in which cross relaxation from an initial state z-magnetization (NOESY), rotating-frame over-hauser spectroscopy, where equilibrium magnetization is rotated onto the x axis (ROESY) experiments[75].

In practice, it is a simple sample handling and stable technique. However, its equipment is very expensive and its sensitivity is very low[75], although this property

[94] Hümmer, W.; Schreier, P. Analysis of proanthocyanidins. *Mol. Nutr. Food Res.* **2008**, *52*, 1381–1398.

could be enabled by the magnet (100 MHz - 1 GHz) or the flow probe use[90]. In addition, one advantage of using off-line NMR is that, apart from phenolic compounds, other metabolites like lipids, amino and organic acids, and sugars can be detected. These metabolites make up the fingerprint of the plant samples, useful for doing comparisons and classifications scopes, and also to link properly the pharmacological effect[75,90].

EXPERIMENTAL PART.
Results and Discussion

Section I. Analytical approaches for the determination of phenolic compounds in guava leaves

CHAPTER 1

Determination of polar compounds in guava leaves infusions and ultrasound aqueous extract by HPLC-ESI-MS

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Research Article

Determination of Polar Compounds in Guava Leaves Infusions and Ultrasound Aqueous Extract by HPLC-ESI-MS

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Abstract

Literature lacks of publications about polar compounds content in infusion or guava leaves tea. Because of that, a comparison between different times of infusion and a conventional ultrasound aqueous extract was carried out. Several polar compounds have been identified by HPLC-ESI-MS and their antioxidant activity was evaluated by FRAP and ABTS assays. Four different classes of phenolic compounds (gallic and ellagic acid derivatives, flavonols, flavanones, and flavan-3-ols) and some benzophenones were determined. The quantification results reported that the order, in terms of concentration of the classes of polar compounds in all samples, was flavonols > flavan-3-ols > gallic and ellagic acid derivatives > benzophenones > flavanones. As expected, the aqueous extract obtained by sonication showed the highest content in the compounds studied. Significative differences were noticed about the different times of infusion and five minutes was the optimal time to obtain the highest content in polar compounds using this culinary method. All the identified compounds, except HHDP isomers and naringenin, were positively correlated with antioxidant activity.

1. Introduction

The studies on antioxidant activity of plants have increased dramatically in recent years, because they are identified as natural antioxidant resources by traditional Chinese medicine [1]. Medicinal plants have usually been applied to control the blood glucose or reduce the diabetic complications; they have the potential to increase the life span and quality of life in these patients [2]. The increasing prevalence of type 2 diabetes mellitus and the negative clinical outcomes observed with the commercially available antidiabetic drugs have led to the investigation of new therapeutic approaches focused on controlling postprandial glucose levels. The use of carbohydrate digestive enzyme inhibitors from natural resources could be a possible strategy to block dietary carbohydrate absorption with less adverse effects than synthetic drugs. In fact, some authors [3] reported *in vitro* and *in vivo* studies in relation to pancreatic alpha-amylase inhibitors of plant origin and presented bioactive compounds of phenolic nature that exhibit anti-amylase activity.

Guava leaves (*Psidium guajava* L.), are considered native to Mexico but today they are extended throughout South America, Europe, Africa, and Asia. Different studies considerate these leaves as a promising source of phenolic compounds for diabetes treatment [4]. Several authors noticed that oral administration of capsules containing aqueous leaf extract from *Psidium guajava* L. showed hypoglycemic effect [5].

Recently, Eidenberger and coworkers [6] investigated the effect of extracts from *Psidium guajava* L. leaves, particularly, the effects of main flavonol-glycoside components on dipeptidyl-peptidase IV (DP-IV), a key enzyme of blood glucose homoeostasis, and, finally, indicated that guava extract has a potential to exert the effect observed *in vitro* also in humans after oral administration.

In vivo experiments carried out by Cheng et al. [7] reported that quercetin in the aqueous extract of guava leaves promotes glucose uptake in liver cells and as a consequence contributes to the alleviation of hypoglycemia in diabetes.

Usually, guava leaf tea was consumed after infusion; however different infusion times were advice from production company. Because of that, in the present work, the antioxidant activities of infusions obtained at different infusion times and conventional ultrasound aqueous extracts of guava leaves were evaluated and compared in terms of their composition in polar compounds.

2. Material and methods

2.1 Chemicals

Double-deionised water with conductivity lower than 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Methanol LC-MS "optima" grade and acetonitrile were obtained from Fisher Scientific (Leicestershire, UK). Acetic acid and the standards gallic acid, catechin, ellagic acid, naringenin, quercetin, and rutin were all from Sigma-Aldrich (Steinheim, Germany). The reagents used to measure the antioxidant capacity, TPTZ (2,4,6-tripyridyl-S-triazine), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate)), potassium persulfate, and ferric sulfate, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate, ferric chloride, and hydrochloric acid were obtained from Panreac (Barcelona, Spain).

2.2 Plant material and sample preparation

Fresh guava leaves were harvested in Motril, Spain (36°44′43″N 3°31′14″O). They were middle age intense green leaves and they were collected in February 2014. The environmental conditions had mean max/min temperature of 18/10 ºC, precipitation of 0 mm, and saturated light duration that ranged from 9.55 to 10.50 h day $^{-1}$.

The samples were air-dried and ground before the analyses. Two different extraction methodologies, such as ultrasound extraction and infusion, were carried out.

Conventional ultrasound extraction. 0.5 g of dry guava leaves were extracted with 15 mL of water (x3) using a sonicator Branson B3510 for 10 min at room temperature. Then, samples were centrifuged for 15 min at 6000 rpm using a centrifuge to remove solids. The supernatants were pooled, evaporated, and dissolved in 2 mL of 50% methanol. This solution was filtered through a 0.20 µm syringe filter and kept at -20ºC in amber bottles to avoid degradation until analysis.

Infusion extraction. For the infusion, 1 g of dried guava leaves and 50 mL of boiling water were used. The extract was prepared according to the method previously described by Chen et al. [8], where they prepared 5 min infusion and compared with infusion for 3 and 7 min. After the extraction by infusion for 3, 5 and 7 min, the solution was raised to 50 mL with water, filtered through a 0.20 µm syringe filter and kept at -20ºC in amber bottles to avoid degradation until analysis.

All extractions were made in triplicate.

2.3 Trolox equivalent antioxidant capacity (ABTS) assay

The ABTS assay, which measures the reduction of the radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by antioxidants, was performed by using a method previously described by Laporta and coworkers [9]. Concisely, ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate in the dark at room temperature for 12–24 h before use. The absorbance of ABTS radical cation was adjusted to 0.70 (±0.02) at 734 nm. A calibration curve was prepared with different concentrations of Trolox (0–20 µM).

2.4 Ferric-reducing antioxidant power (FRAP)

The reducing power was evaluated according to the method validated by Benzie and Strain [10]. Briefly, 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM aqueous $FeCl_3$, were prepared and mixed (10:1:1), to obtain the FRAP reagent. The FRAP reagent was warmed to 37 ºC, before reading its absorbance. Then, the samples were added. The change in absorbance (593 nm)

between the samples and the blank was related to the absorbance of an aqueous solution of known Fe (II) concentration, prepared for calibration.

2.5 HPLC-ESI-MS analysis

Phenolic and other polar compounds in the extracts obtained from guava leaves were identified using a method introduced by Chang et al. [11], with slight modifications. Briefly, HPLC analyses were performed using a HP 1100 Series instrument (Hewlett Packard, Wilmington, DE, USA) equipped with a binary pump delivery system, a degasser (model G1322A), an autosampler (Automatic Liquid Sampler, ALS, model G1312A), a HP diode-array UV-VIS detector (DAD, model G1315A) and a quadrupole HP-Mass Spectrometer Detector (MSD, model G1946A); integration and data elaboration were performed using Chemstation software (Hewlett Packard). A Phenomenex Luna C18 analytical column (150 mm × 2.0 mm, particle size 3 μm) (Phenomenex Inc., Torrance, CA, USA) was used for polar compounds separation. All analyses were carried out at room temperature using the gradient proposed by Chang et al. [11]. MS analysis were carried out using an electrospray ionization (ESI) interface in negative ionization mode at the following conditions: drying gas flow (N2), 9.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 350°C; capillary voltage, 3500 V; fragmentor voltage and scan range, 100 V and *m/z* 50-1000, respectively.

Phenolic standards of interest such as gallic acid, catechin, ellagic acid, naringenin, and rutin were used for quantification of phenolic compounds in guava leaf extracts. The identified compounds were quantified on the basis of their peak area and compared with calibration curves obtained with the corresponding standards and then expressed as μg/g of extract.

2.6 Statistical analysis

The results reported in this study are the averages of three repetitions (n=3). Fisher's least significance difference (LSD) test and Pearson's linear correlations, both at $P < 0.05$, were evaluated using Statistica 6.0 (2001, StatSoft, Tulsa, OK).

3. Results and discussion

3.1 Identification of polar compounds

Phenolic and other polar compounds were identified by their elution order, UV/vis spectra, and MS characteristics, compared with reported literature values, and by coinjection with available standards (Table 1).

Table 1: Identification of polar compounds in guava leaves by HPLC-DAD-ESI-MS.

No.	Compound	λ_{max} (nm)	$[M-H]^-$	Fragments
1	HHDP glucose isomer	290	481	301
2	HHDP glucose isomer	290	481	301
3	HHDP glucose isomer	290	481	301
4	Gallic acid	272 ,225	169	125
5	Prodelphinidin B2 isomer	280, 360	609	423, 441, 305
6	Pedunculagin/casuariin isomer	253, 377	783	481, 301
7	Prodelphinidin dimer isomer	280, 340	593	407, 425
8	Gallocatechin	236, 270sh	305	179, 221
9	Prodelphinidin dimer isomer r	280, 340	593	407, 425
10	Geraniin isomer	270	951	783, 481, 301
11	Pedunculagin/casuariin isomer	253, 377	783	481, 301
12	Geraniin isomer	270	951	783, 481
13	Procyanidin B isomer	278, 234	577	289
14	Procyanidin B isomer	278, 235	577	289
15	Tellimagrandin I isomer	279, 340	785	615, 301
16	Catechin	236, 281	289	245, 205, 179
17	Casuarinin/casuarictin isomer	238, 275sh	935	783
18	Tellimagrandin I isomer	277, 338	785	615, 301
19	Gallocatechin	236, 270sh	305	179, 221
20	Myricetin-arabinoside/xylopyranoside isomer	264, 231sh, 356	449	316, 317
21	Procyanidin B isomer 2	268, 350	577	425, 289
22	Myricetin hexoside isomer	261, 231sh, 358	479	316, 317
23	Myricetin hexoside isomer	264, 235sh, 356	479	316, 317
24	Myricetin-arabinoside/xylopyranoside isomer	264, 231sh, 356	449	316, 317
25	Quercetin-galloylhexoside isomer	268, 350	615	463, 301
26	Quercetin-galloylhexoside isomer	280, 345	615	463, 301
27	Myricetin-arabinoside/xylopyranoside isomer	256, 234sh, 356	449	316, 317
28	Morin	232sh, 257, 374	301	151
29	Myricetin -arabinoside/xylopyranoside isomer	257, 231sh, 356	449	316, 317

No.	Compound	λ_{max} (nm)	$[M-H]^-$	Fragments
30	Ellagic acid	254, 370	301	257, 185
31	Hyperin	259, 355, 235sh	463	301
32	Quercetin glucoronide	265, 355, 233sh	477	301, 433
33	Isoquercitrin	258, 355, 235sh	463	301
34	Reynoutrin	258, 356, 231sh	433	301
35	Guajaverin	257, 356, 231sh	433	301
36	Guavinoside A	218, 288	543	-
37	Avicularin	257, 355, 231sh	433	301
38	Quercitrin	264, 353	447	300
39	Guavinoside C	211, 265, 355	585	-
40	Guavinoside B	218, 283	571	-
41	Guavinoside A isomer	218, 288	543	-
42	Prodelphinidin B2 isomer 2	282, 340	609	423, 441, 305
43	Guavinoside B isomer	218, 283	571	-
44	Guavinoside B isomer	218, 283	571	-
45	Guavinoside B isomer	218, 283	571	-
46	Guavin B	208, 221, 283	693	-
47	Quercetin	232sh, 257, 374	301	151
48	Naringenin	280	271	-

About phenolic compounds, four different classes identified as gallic and ellagic acid derivatives, flavonols, flavanones and flavan-3-ols were determined.

Thirteen gallic and ellagic acid derivatives were determined. Three compounds (**1**, **2**, and **3**) with molecular ion at *m/z* 481 and fragment at *m/z* 301 were identified as hexahydroxydiphenic acid (HHDP) glucose and its presence in guava was previously reported by Okuda and co-workers [12]. Gallic acid (compound **4**) was identified according to its MS data (*m/z* 169 and *m/z* 125) and by coelution with a chemical standard. Two compounds (**6** and **11**) with $[M-H]^-$ at *m/z* 783 and two fragments at *m/z* 481 and 301 corresponding to loss of ellagic acid were detected. This fragmentation pattern was assigned to peduncalagin/casuariin compounds; these compounds were described in guava leaves by Okuda et al. [12].

Two compounds (**10** and **12**) with molecular ion at *m/z* 951 and fragments at *m/z* 783 and 481 were also determined. These compounds were identified as geraniin isomers [13].

Two compounds (**15** and **18**) showing significant $[M–H]^-$ signals at *m/z* 785 with fragment ions at *m/z* 615 and at *m/z* 301 were found. This fragmentation pattern corresponded to a digalloyl-HHDP-glucose structure, probably tellimagrandin I isomer. This compound was previously detected in guava leaves by Okuda et al. [12]. Compound **17** at *m/z* 935 reported a fragment ion at *m/z* 783; this fragmentation pattern was assigned to casuarinin/casuarictin and this compound was described in guava tea by Yamanaka and coworkers [13]. Compound **30** was identified as ellagic acid due its coelution with commercial standard. Finally, guavin B (compound **46**) that reported a molecular ion at *m/z* 693 was identified according to Okuda et al. [14].

Moreover, ten flavan-3-ol derivatives were determined. Compounds **5** and **42** showed a molecular ion at *m/z* 609 and three fragments at *m/z* 441, 423 and 305 (gallocatechin unit); these compounds were identified as prodelphinidin B2 and its isomer and their presence in guava leaves was noticed by Qa'Dan et al. [15].

Compounds **7** and **9** showed a molecular ion at *m/z* 593 and two fragments at *m/z* 425 and 407. According to Qa'Dan et al. [15] these compounds were identified as prodelphinidin dimer (4α-8).

Two compounds (**8** and **19**) with $[M-H]^-$ at *m/z* 305 and fragment ions at *m/z* 221 and 179 were identified as gallocatechin isomers and their presence in guava leaves was reported by Qa'Dan et al. [15]. Three procyanidin dimers ($[M-H]^-$ at *m/z* 577) were also described (compounds **13**, **14**, and **21**).

Catechin compound (**16**) was identified by mass spectra data elaboration and co-elution with a commercial standard.

The flavonols were the most representative phenolic compounds; in fact, eighteen flavonol-derivatives were identified. Four compounds (compounds **20**, **24**, **27**, and **29**) reported a molecular ion at *m/z* 449 and two major fragments at *m/z* 316 and 317. According to Chang et al. [11], these compounds were identified as myricetin-arabinoside/ xylopyranoside isomers.

Two flavonol compounds (compounds **22** and **23**) with molecular ion at *m/z* 479 and fragment at *m/z* 317 and 316 were identified as myricetin-hexoside isomers [11]. Two compounds (**25** and **26**) corresponding to $[M–H]^-$ signals at *m/z* 615 were also detected. Based on their molecular weight and the presence of two fragments at *m/z* 463 and 301, they were assigned to quercetin-galloylhexoside isomers and their presence in guava leaves was reported by Park et al. [16].

Two compounds at *m/z* 301 were detected (**28** and **47**); moreover, they showed the same fragment ion at *m/z* 151. Quercetin standard solution was injected and because of that, compound **28** was assigned to morin and compound **47** was assigned to quercetin; their presence in guava leaves was reported by several authors [11,17].

Compounds detected at *m/z* 463 (**31** and **33**) with fragment ion at *m/z* 301 corresponded to hyperin and isoquercitrin, respectively. They have previously been found in leaves of guava by Eidenberger et al. [6].

Quercetin glucuronide (32) with molecular ion at *m/z* 477 and fragment ions at *m/z* 433 and 301 was identified according to Chang et al. [11]

Three compounds (**34**, **35** and **37**) reported the same molecular ion (*m/z* 433) and a fragment ion at *m/z* 301 (corresponding to quercetin aglycone); according to their retention times they were identified as reynoutrin, guajaverin and avicularin as reported by Chang et al. [11].

Quercitrin (**38**) was identified at *m/z* 447 and fragment ion *m/z* 300, according to Park et al. [16].

A flavonone compound, namely naringenin (*m/z* 271) was detected and identified by analyzing the mass spectra and by coelution with a chemical standard [18]. Compound **39** with *m/z* 585 was identified as guavinoside C [19].

Six benzophenone compounds were also determined in guava leaves sample. The $[M-H]^-$ ion at *m/z* 543 revealed the presence of two compounds, namely,

guavinoside A isomers; their presence in guava leaves was noticed by Matsuzaki and coworkers [19].

Finally, four compounds with *m/z* 571 (**40**, **43**, **44**, and **45**) were identified as guavinoside B isomers according to Matsuzaki et al. [19].

3.2 Quantification of polar compounds

Comparison between different times of infusion and a conventional ultrasound aqueous extract was carried out due to several publications about phenolic and other polar compounds content in infusion or guava leaves tea.

Quantification of polar compounds was performed by preparing five calibration curves with the standards available: gallic acid, catechin, ellagic acid, naringenin, and rutin. For those with no commercial standard available, quantification was carried out comparing with compounds bearing similar structures.

It is important to underline that the quantification results reported that the order, in terms of concentration of the families of polar compounds in all samples, decreased in the following order: flavonols > flavan-3-ols > gallic and ellagic derivatives > benzophenones > flavanones.

In general, the results given in Table 2 show that the concentration of each compound is greater in the ultrasound aqueous extract (AE), except the compounds identified as HHDP glucose that was higher in the infusion of 3 min (I3) and in the 5 min (I5) samples and naringenin, which presented the largest concentration in I3. Similar results were obtained by Nantitanon and co-workers [20] using ethanol as extraction solvent. In fact, they extracted the guava leaves by maceration and ultrasounds, and the highest recovery of phenolic compounds was obtained by sonication.

The high extraction of HHDP and naringenin in some infusions than ultrasound extraction could be justified by the temperature that has been reached during the two extraction methodologies. As reported by Zhang and co-workers [21]

the solubility of naringenin gradually increases as the temperature increases; based in these results it is expected to obtain lower extraction of this compounds during ultrasound extraction instead of that infusion. This hypothesis can be confirmed with the results obtained by Wen et al. [22] that noticed that naringenin is insoluble in water at room temperature.

However, naringenin content in infusion samples reported a decreasing trend when increased the time of infusion; these results should be attributed to a degradation of this compound when the thermal treatment was prolonged.

To our knowledge, there is no literature about the water solubility and the effect of temperature on HHDP compound. Nevertheless, taking into account the results reported in Table 2, a similar trend that the one reported for naringenin compound could be supposed for HHDP.

Table 2: Quantification (mean ± SD, n=3) of the compounds identified in guava leaves infusions and ultrasound aqueous extract. The different letter in the same line means that they are significantly different ($P \leq 0.05$).

No.	Compounds	Quantification (µg analyte/g leaves)			
		AE	I3	I5	I7
1	HHDP glucose isomer	1146 ± 34^{c}	2256 ± 37^{a}	2253 ± 27^{a}	2021 ± 67^{b}
2	HHDP glucose isomer	228 ± 40^{b}	441 ± 72^{a}	$368 \pm 59^{a,b}$	$397 \pm 93^{a,b}$
3	HHDP glucose isomer	1424 ± 48^{c}	1756 ± 39^{a}	1762 ± 12^{a}	1546 ± 27^{b}
4	Gallic acid	719 ± 33^{a}	240 ± 5^{b}	260 ± 5^{b}	254 ± 9^{b}
5	Prodelphinidin B2 isomer	665 ± 55^{a}	212 ± 11^{b}	220 ± 12^{b}	126 ± 8^{c}
6	Pedunculagin/casuariin isomer	2405 ± 38^{a}	573 ± 14^{c}	688 ± 5^{b}	533 ± 6^{c}
7	Prodelphinidin dimer isomer	1768 ± 90^{a}	444 ± 26^{b}	467 ± 20^{b}	335 ± 11^{b}
8	Gallocatechin	5887 ± 273^{a}	2419 ± 19^{b}	$2301 \pm 43^{b,c}$	1960 ± 77^{c}
9	Prodelphinidin dimer isomer	5452 ± 308^{a}	1526 ± 4^{b}	1688 ± 40^{b}	1309 ± 34^{b}
10	Geraniin isomer	1396 ± 47^{a}	304 ± 1^{b}	318 ± 12^{b}	201 ± 8^{c}
11	Pedunculagin/casuariin isomer	2894 ± 46^{a}	687 ± 9^{c}	814 ± 19^{b}	624 ± 6^{c}
12	Geraniin isomer	2333 ± 160^{a}	433 ± 26^{b}	457 ± 8^{b}	304 ± 3^{b}
13	Procyanidin B isomer	17659 ± 785^{a}	6703 ± 124^{b}	7106 ± 51^{b}	6105 ± 34^{b}
14	Procyanidin B isomer	1751 ± 150^{a}	398 ± 10^{b}	413 ± 10^{b}	321 ± 9^{b}
15	Tellimagrandin I isomer	728 ± 26^{a}	141 ± 2^{c}	184 ± 5^{b}	129 ± 2^{c}
16	Catechin	12875 ± 705^{a}	6127 ± 80^{b}	5960 ± 19^{b}	5192 ± 2^{b}
17	Casuarinin/casuarictin isomer	8725 ± 216^{a}	482 ± 1^{c}	859 ± 21^{b}	$590 \pm 17^{b,c}$
18	Tellimagrandin I isomer	1492 ± 46^{a}	$186 \pm 6^{b,c}$	243 ± 9^{b}	164 ± 3^{c}
19	Gallocatechin	5866 ± 362^{a}	$2205 \pm 134^{b,c}$	2446 ± 21^{b}	1896 ± 13^{c}
20	Myricetin-arabinoside/xylopyranoside isomer	608 ± 18^{a}	<LOQ	<LOQ	<LOQ

No.	Compounds	Quantification (µg analyte/g leaves)			
		AE	I3	I5	I7
21	Procyanidin B isomer 2	1206 ± 56[a]	207 ± 14[b,c]	224 ± 7[b]	136 ± 6[c]
22	Myricetin hexoside isomer	1301 ± 44[a]	<LOQ	<LOQ	<LOQ
23	Myricetin hexoside isomer	331 ± 2[a]	245 ± 2[c]	299 ± 3[b]	232 ± 4[d]
24	Myricetin-arabinoside/xylopyranoside isomer	639 ± 29[a]	<LOQ	<LOQ	<LOQ
25	Quercetin-galloylhexoside isomer	566 ± 20[a]	148.3 ± 0.1[b]	171 ± 2[b]	149 ± 1[b]
26	Quercetin-galloylhexoside isomer	452 ± 15[a]	97.25 ± 0.02[b]	102.3 ± 0.4[b]	92,25 ± 0.07[b]
27	Myricetin-arabinoside/xylopyranoside isomer	592 ± 24[a]	<LOQ	<LOQ	<LOQ
28	Morin	8377 ± 464[a]	3235 ± 53[b,c]	3676 ± 12[b]	3003 ± 18[c]
29	Myricetin-arabinoside/ xylopyranoside isomer	986 ± 28[a]	<LOQ	<LOQ	<LOQ
30	Ellagic acid	4338 ± 234[a]	1082 ± 16[b]	1367 ± 14[b]	1040 ± 9[b]
31	Hyperin	7798 ± 280[a]	2492 ± 6[c]	2891 ± 7[b]	2031 ± 18[d]
32	Quercetin glucoronide	2293 ± 91[a]	1249 ± 19[c]	1612 ± 40[b]	1132 ± 7[c]
33	Isoquercitrin	4408 ± 182[a]	1111 ± 19[b,c]	1306 ± 5[b]	996 ± 3[c]
34	Reynoutrin	5849 ± 173[a]	1386 ± 21[b,c]	1611 ± 20[b]	1229 ± 9[c]
35	Guajaverin	12843 ± 421[a]	3169 ± 40[b,c]	3595 ± 4[b]	2713 ± 36[c]
36	Guavinoside A	1920 ± 40[a]	453 ± 3[c]	522 ± 1[b]	413 ± 2[c]
37	Avicularin	13666 ± 421[a]	3825 ± 48[b]	4183 ± 34[b]	3232 ± 25[c]
38	Quercitrin	6822 ± 274[a]	1825 ± 39[b]	2078 ± 24[b]	1705 ± 12[b]
39	Guavinoside C	2298 ± 40[a]	<LOQ	<LOQ	<LOQ
40	Guavinoside B	1456 ± 34[a]	370 ± 3[c]	423 ± 4[b]	328.6 ± 0.1[c]
41	Guavinoside A isomer	558 ± 4[a]	177 ± 1[b]	160 ± 4[c]	140.0 ± 0.3[d]

No.	Compounds	Quantification (µg analyte/g leaves)			
		AE	I3	I5	I7
42	Prodelphinidin B2 isomer 2	<LOQ	<LOQ	<LOQ	<LOQ
43	Guavinoside B isomer	<LOQ	<LOQ	<LOQ	<LOQ
44	Guavinoside B isomer	<LOQ	nd	nd	nd
45	Guavinoside B isomer	<LOQ	nd	nd	nd
46	Guavin B	460 ± 6^{a}	34 ± 1^{b}	32.2 ± 0.2^{b}	20 ± 1^{c}
47	Quercetin	408 ± 18^{a}	nd	nd	nd
48	Naringenin	538 ± 14^{c}	941 ± 3^{a}	815 ± 24^{b}	558 ± 9^{c}

n.d. = not detected, AE= aqueous extract obtained by ultrasound, I3, I5 and I7: infusion obtained at 3, 5 and 7 minutes of infusion time, respectively

Flavan-3-ols, gallic and ellagic acid derivatives, benzophenones and flavonols in the ultrasound aqueous extract were from 3 to 5 times more concentrated than leaves infusions. Compared to the ultrasound aqueous extract (AE) and infusion of 7 min (I7) samples, naringenin was 1.5 and 1.7 times higher in the infusion of 3 min (I3) and in the 5 min (I5) samples, respectively.

Flavonols represent about 50 percent of total polar compounds in each sample. Avicularin and guajaverin were the major flavonol components and their concentrations varied from 13.7 to 3.2 mg/g and from 12.8 to 2.7 mg/g, respectively. Similar trend was showed by Chang et al. [11]. Morin was also found in high concentration with a range that varies from 3.0 and 8.4 mg/g in I7 and AE sample, respectively. Other flavonol compounds presented in all samples in higher quantities and in the same order of magnitude were hyperin, quercitrin, reynoutrin, and isoquercitrin. Myricetin-arabinoside was detected in all samples, but it was quantified only in AE sample; instead quercetin was only detected and quantified in the AE sample. These data could promote the use of guava leaves extract for nutraceutical scopes because, as reported by Wang et al. [23], myricetin and quercetin have high inhibitory activities against some enzymes that are involved in diabetes. Guavinoside C was quantified in ultrasound aqueous extract; it was identified in infusion samples, but its content was lower than LOQ.

The second class of polar compounds was represented by flavan-3-ols, which correspond to 26-30% of total polar compounds. Procyanidin was the first polar compound and its amounts ranged from 6.1 to 17.7 mg/g. Catechin was the second flavan-3-ol ranging between 5.1 and 12.9 mg/g.

Two (epi)-gallocatechin isomers and prodelphinidin dimer were the third flavan-3-ols and their amounts were about 5.4-5.9 mg/g.

Gallic and ellagic acid derivatives account for 20% of the total concentration of polar compounds in each sample. In this case, ultrasound aqueous extract and infusions reported different extraction power. Effectively, ultrasound aqueous extract

showed casuarinin/casuarictin as first ellagic acid derivative (8.7 mg/g); contrary, infusion samples reported HHDP glucose compounds in highest amounts (2.0-2.3 mg/g). Benzophenones were 2-4 % of total polar compounds. Guavinoside A was the first benzophenone and it was represented by two isomers. Finally, four guavinoside B isomers were also detected in the extract, but only one was quantified; their content in infusion samples was less than LOQ or, in some cases, they were not detected.

At last, a flavanone, namely, naringenin was presented in all samples; I3 sample showed the higher content, contrary aqueous extract and I7 samples reported the lowest quantities.

3.3 Comparison between phenolic content and antioxidant activity

As shown in Figure 1, the amount of total polar compounds is significantly higher in the ultrasound aqueous extract than in the infusions. Comparing the results obtained for the infusions, the quantity of these compounds is quite higher for I5 than for the others, I3 and I7. In fact, I3 sample reported a lower content probably due to an incomplete extraction of polar compounds; instead, I7 sample showed lower amounts, probably due to a degradation of these compounds during maceration.

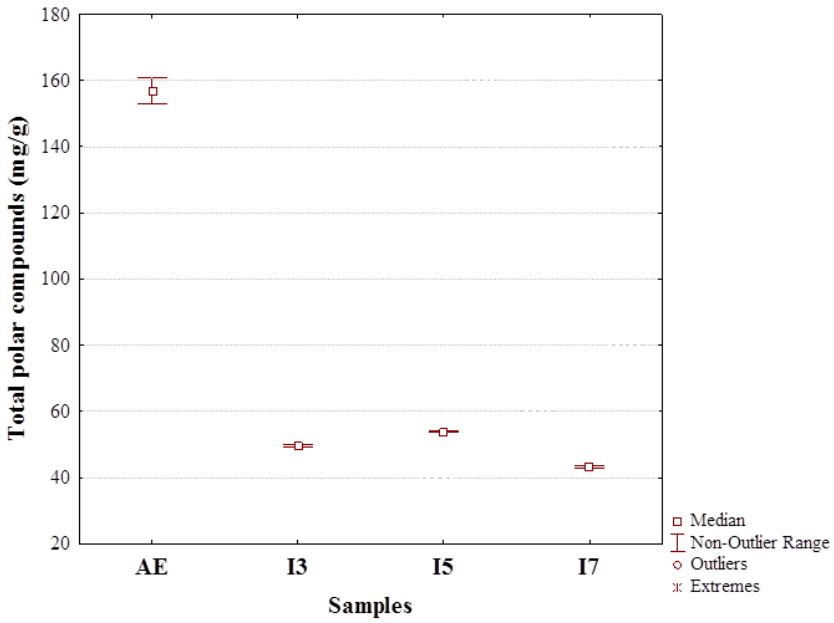

Figure 1: Total content (mg/g) of total polar compounds by HPLC in analysed samples.

To evaluate the antioxidant activity of the extract and to corroborate the correlation between phenolic content and antioxidant activity, two different assays were developed: TEAC evaluated by $ABTS^{\bullet+}$ test and FRAP.

The choice of these two methods was assessed based on their different mechanisms: the radical scavenging capacity demonstrated by ABTS and ferric reducing capacities evaluable by FRAP method. Moreover, the results obtained by Thaipong et al. [24] demonstrated that ABTS and FRAP reported higher correlation with total phenolic content in guava fruit compared to other antioxidant activity assays.

Total polar compounds by HPLC are in concordance with the values obtained for the FRAP and ABTS assays (Table 3). Besides, the reducing power and radical scavenging capacity displayed a significative difference between the samples obtained by infusion and the ultrasound aqueous extract. Positive correlations with $R = 0.9883$ and $P < 0.001$ and $R = 0.9973$ and $P < 0.001$ were noticed between total polar compounds content and FRAP and ABTS, respectively.

Table 3: Comparison between total polar compound (mg/g) determined by HPLC and antioxidant activity evaluated by FRAP (μM of $FeSO_4$ equivalents/mg) and ABTS (μM of Trolox equivalents/mg).

SAMPLE	TPC by HPLC	FRAP	ABTS
AE	157 ± 6 [a]	3027 ± 586 [a]	1128 ± 69 [a]
I3	49.6 ± 0.5 [c]	314 ± 15 [b]	156 ± 1 [b]
I5	53.8 ± 0.2 [b]	285 ± 7 [b]	218 ± 19 [b]
I7	43.1 ± 0.4 [d]	286 ± 7 [b]	178 ± 1 [b]

Means in the same column with different letter are significantly different ($P < 0.05$).

FRAP and ABTS did not report significative differences ($P < 0.05$) among infusion samples; however, ultrasound aqueous extract values were higher than infusions values.

Moreover, a simple linear regression analysis was carried out to compare the correlation between all compounds identified and the antioxidant activity (Table 4).

Table 4. Correlation between the antioxidant activity and polar compounds

	Compounds	FRAP		ABTS	
		R value	*P* value	*R* value	*P* value
1	HHDP glucose isomer	-0.9644	***	-0.9721	***
2	HHDP glucose isomer	-0.8199	*	-0.8321	*
3	HHDP glucose isomer	-0.7507	*	-0.7700	*
4	Gallic acid	0.9920	***	0.9993	***
5	Prodelphinidin B2 isomer	0.9817	***	0.9845	***
6	Pedunculagin/casuariin isomer	0.9849	***	0.9980	***
7	Prodelphinidin dimer isomer	0.9895	***	0.9962	***
8	Gallocatechin	0.9887	***	0.9922	***
9	Prodelphinidin dimer isomer	0.9909	***	0.9977	***
10	Geraniin isomer	0.9747	***	0.9911	***
11	Pedunculagin/casuariin isomer	0.9850	***	0.9979	***
12	Geraniin isomer	0.9922	***	0.9911	***
13	Procyanidin B isomer	0.9907	***	0.9978	***
14	Procyanidin B isomer	0.9949	***	0.9973	***
15	Tellimagrandin I isomer	0.9870	***	0.9985	***
16	Catechin	0.9900	***	0.9916	***
17	Casuarinin/casuarictin isomer	0.9874	***	0.9990	***
18	Tellimagrandin I isomer	0.9881	***	0.9989	***
19	Gallocatechin	0.9881	***	0.9940	***
20	Myricetin-arabinoside/xylopyranoside isomer	0.9889	***	0.9978	***
21	Procyanidin B isomer 2	0.9891	***	0.9967	***
22	Myricetin hexoside isomer	0.9893	***	0.9979	***
23	Myricetin hexoside isomer	0.7694	*	0.8073	*
24	Myricetin-arabinoside/xylopyranoside isomer	0.9907	***	0.9981	***
25	Quercetin-galloylhexoside isomer	0.9893	***	0.9994	***
26	Quercetin-galloylhexoside isomer	0.9900	***	0.9986	***
27	Myricetin-arabinoside/xylopyranoside isomer	0.9901	***	0.9980	***
28	Morin	0.9889	***	0.9967	***
29	Myricetin-arabinoside/xylopyranoside isomer	0.9887	***	0.9977	***
30	Ellagic acid	0.9684	***	0.9915	***
31	Hyperin	0.9830	***	0.9938	***
32	Quercetin glucoronide	0.9622	***	0.9794	***
33	Isoquercitrin	0.9887	***	0.9983	***
34	Reynoutrin	0.9874	***	0.9982	***
35	Guajaverin	0.9878	***	0.9978	***
36	Guavinoside A	0.9866	***	0.9982	***

	Compounds	FRAP		ABTS	
		R value	*P* value	*R* value	*P* value
37	Avicularin	0.9874	***	0.9970	***
38	Quercitrin	0.9897	***	0.9989	***
39	Guavinoside C	0.9873	***	0.9974	***
40	Guavinoside B	0.9864	***	0.9979	***
41	Guavinoside A isomer	0.9842	***	0.9928	***
46	Guavin B	0.9865	***	0.9968	***
47	Quercetin	0.9906	***	0.9981	***
48	Naringenin	-0.5619	NC	-0.5855	NC
	TPC by HPLC	0.9883	***	0.9973	***

*** $P < 0.001$; * $P < 0.05$; NC: not correlated

Most of the polar compounds were highly correlated with FRAP assay ($R = 0.98$; $P < 0.001$) except compounds 10, 30, and 32 that reported an R value ranging between 0.96 and 0.97 ($P < 0.001$). Compound 23 showed a lower correlation ($R = 0.76$; $P < 0.05$). HHDP glucose isomers resulted inversely correlated with FRAP assay. Moreover, naringenin did not show any correlation.

ABTS assay confirmed data reported by FRAP assay; in fact, the two antioxidant assays showed a good correlation between them, that reported an R value of 0.9916 and $P < 0.0001$. These results agreed with the data reported by Thaipong et al. [24].

4. Conclusions

Several polar compounds have been identified and quantified in guava leaves extracts (ultrasound aqueous extract and infusions). According to the amount of polar compounds and also to the FRAP and ABTS assays, the water ultrasound assisted extraction provided better results than the infusion. Significative positive correlations $R > 0.98$ $P < 0.001$ were detected between total polar content and antioxidant activity assays. Moreover, positive correlation was also detected for single compounds, except for HHDP and naringenin.

The results suggested that aqueous ultrasound extract can represent a valuable strategy to obtain nutraceuticals using a green technology. About infusions, the 5-minute infusion is advisable for guava leaves culinary uses because reported higher polar compounds content.

Conflicts of interest

The authors declare no competing financial interest.

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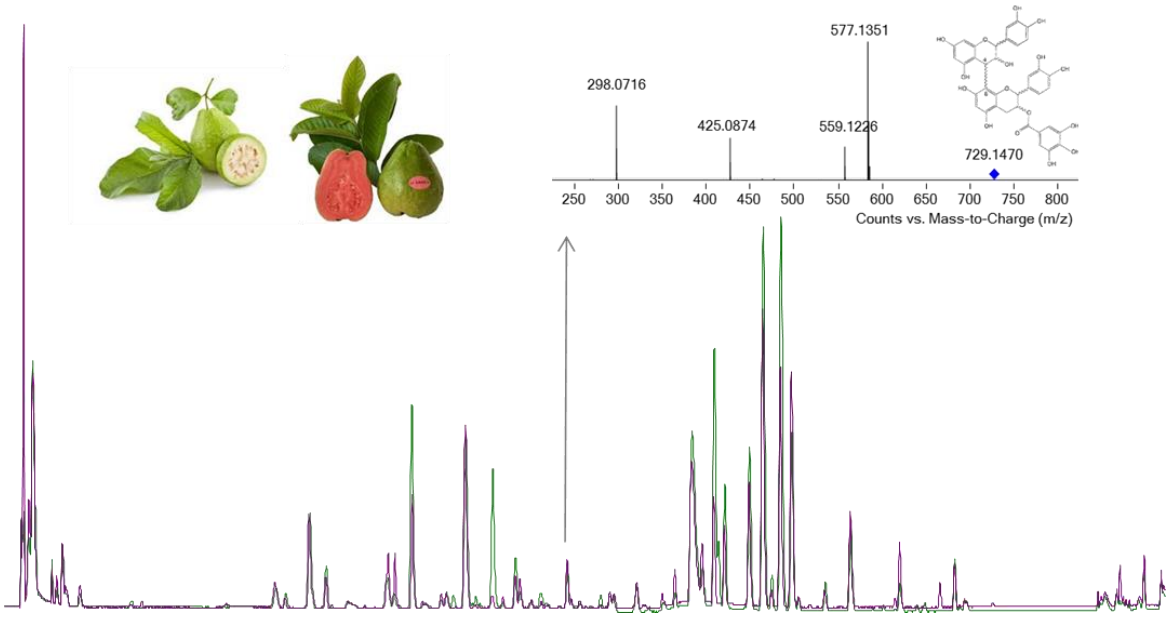
CHAPTER 2



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Determination of guava (*Psidium guajava* L.) leaf phenolic compounds using HPLC-DAD-QTOF-MS

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Abstract

Markets of different countries have proposed guava tea infusions as drink that can modulate the glycemic index in blood. This property has been attributed to the phenolic compounds contained in guava leaves. However, phenolic profile of guava leaves is still not well-known. Based on this information, different ethanol/water mixtures were used to extract the phenolic compounds in guava leaves. Phenolic identification was carried out by HPLC-ESI-QTOF-MS in guava leaves from *pomifera* and *pyrifera* varieties; moreover, the antioxidant activities of the ethanolic extracts was determined by TEAC and FRAP methods. To sum up, seventy-two phenolic compounds were identified. To our knowledge, twelve of them were determined for the first time in guava leaves. The highest amount of phenolic compounds was found in EtOH/H_2O 80:20 (v/v) mixture. Furthermore, *pyrifera* var. showed higher concentration of phenolic compounds than *pomifera* var. (113.34 vs. 86.12 mg/g leaf d.w.) and also greater antioxidant capacity.

Keywords: Guava (*Psidium guajava* L.) leaves, flavan-3-ols, flavonols, gallic and ellagic derivatives, phenolic compounds, HPLC-DAD-QTOF-MS

1. Introduction

Diabetic complications are now a global health problem without effective therapeutic approach. Hyperglycemia and oxidative stress are important components for the development of diabetic complications due to an excessive production of free radicals (Singh, Kaur, Kishore, & Gupta, 2013). It is known that plants are a rich source of secondary metabolites that have been implicated in several therapeutic methologies like flavonoids, alkaloids, terpenoids and tannins. Thus, for diabetic complications, an antioxidant treatment coupled with other approaches could be effective in ameliorating these complications (Scott & King, 2004).

Psidium guajava L. is a small tree native to Central America from Southern Mexico to Northern South America. It is popularly known as guava and belongs to the myrtle family (Myrtaceae). Today, guava tree has been distributed through many countries as a result of its capacity to grow in tropical and subtropical conditions (Morton, 1987).

Extracts from the leaves of this plant have traditionally been used in folk medicine around the world. They are mainly known for its antispasmodic and antimicrobial properties in the treatment of diarrhoea and dysentery, although they also exhibit antioxidant, hepatoprotection, anti-allergy, antimicrobial, antigenotoxic, antiplasmodial, cytotoxic, antispasmodic, cardioactive, anticough, anti-inflamatory and antinociceptive properties. Besides, they have extensively been used as a hypoglycaemic agent for diabetes, due to their high concentration of total phenolic compounds (Gutiérrez, Mitchell, & Solis, 2008).

Several authors (Wang, Jiao, Liu & Hong, 2007; Haida, Baron, Haida, de Faci, & Haas, 2011) reported that the leaves of white (*P. guajava* L. var. *pyrifera*) and red guava (*P. guajava* L. var. *pomifera*) presented higher amounts of phenolic compounds with antioxidant activity comparing with other vegetable species.

In the last years, traditional and advanced techniques have been applied to extract phenolic compounds from natural product matrices (Stalikas, 2007). Nantitanon, Yotsawimonwat, and Okonogi (2010) found that ultrasonication was the best method, followed by Soxhlet extraction and maceration. The same authors affirmed that ultrasound assisted extraction is also simpler, faster and cheaper than conventional methods. Thus, in this study, pure ethanol and different ethanol: water mixtures were used to extract commercial guava leaves. The extracts were analyzed and evaluated by HPLC-DAD-ESI-QTOF. Moreover, the phenolic content and the antioxidant capacity of *P. guajava* L. var. *pyrifera* leaves extracts were compared with those of *P. guajava* L. var. *pomifera*.

2. Experimental

2.1 Chemicals

Double-deionised water with conductivity lower than 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Methanol LC-MS "optima" grade and acetonitrile were obtained from Fisher Scientific (Leicestershire, UK). Acetic acid, TPTZ (2,4,6-tripyridyl-S-triazine), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate)], potassium persulfate, ferric sulfate and the standards namely gallic acid, catechin, ellagic acid, naringenin, quercetin and rutin were all from Sigma-Aldrich (Steinheim, Germany). Ethanol, sodium acetate, ferric chloride, and hydrochloric acid were obtained from Panreac (Barcelona, Spain).

2.2 Plant material

Commercial *P. guajava* L. leaves were used for the optimization of solvent extraction. Then, *P. guajava* L. var. *pyrifera* and *pomifera* harvested in Motril (Spain) (36°44′43″N 3°31′14″O) were collected. They were middle age intense green leaves and they were collected in February 2015. The environmental conditions had mean

max/min temperature of 23/8 ºC, precipitation of 0-0.8 mm, and saturated light duration that ranged from 9.45 to 10.40 h day $^{-1}$.

2.3 Guava leaves extraction

The phenolic compounds extraction was performed using an ultrasound bath and a mixture of ethanol: water 70/30 (v/v) as extractant solvent. Briefly, 0.5 g of air-dried and crushed guava leaves were extracted with 15 mL of solvent (x3) using a sonicator Branson B3510 for 10 min at room temperature. Then, samples were centrifuged for 15 min at 6000 rpm using a centrifuge to remove solids. The supernatants were pooled, evaporated and dissolved in 2 mL of methanol/water 1/1 (v/v). This solution was filtered through a 0.20-µm RC syringe filter and kept at -20ºC in amber vials until analysis to avoid degradation. The analysis were run in triplicate (n=3) and results expressed as mg of phenolic content/g leaf dry weight (d.w.).

2.4 Preparation of standards

Phenolic standards of interest such as gallic acid, catechin, ellagic acid, naringenin, and rutin were used for quantification of phenolic compounds in guava leaf extracts. The standard stock solutions were prepared at 250 mg/L in methanol, except for ellagic acid, that was solved in water. Then, each solution was diluted from 50 mg/L to 0.01 mg/L.

2.5 HPLC-DAD-ESI-QTOF-MS analysis

Chromatographic analyses were performed using an HPLC Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, an online degasser, an autosampler and a thermostatically controlled column compartment, and a UV-Vis Diode Array Detector (DAD). The column was maintained at 25ºC. Phenolic compounds from *P. guajava* L. leaves were separated at room temperature using a method previously reported by López-Cobo, Gómez-Caravaca, Švarc-Gajić, Segura-Carretero, and Fernández-Gutiérrez (2014) with slight modifications. Briefly, a Poroshell 120 EC-C18 (4.6 mm x 100 mm, particle size 2.7

μm) (Agilent Technologies) was used to separate the compounds. The gradient elution was carried out using water containing 1% acetic acid as solvent system A and acetonitrile as solvent system B, and applied as follows: 0 min, 0.8% B; 2.5 min, 0.8% B; 5.5 min, 6.8% B; 11 min, 14.4% B; 17 min, 24% B; 22 min, 40% B; 26 min, 100% B, 30 min, 100% B; 32 min, 0.8% B; 34 min, 0.8% B. The sample volume injected was 5 μL and the flow rate used was 0.8 mL/min.

MS analysis were carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass Q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in negative ionization mode at the following conditions: drying gas flow (N_2), 12.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 370°C; capillary voltage, 3500 V; fragmentor voltage and scan range were 3500 V and *m/z* 50-1500, respectively. Automatic MS/MS experiments were carried out using the followings collision energy values: *m/z* 100, 30 eV; *m/z* 500, 35 eV; *m/z* 1000, 40 eV; and *m/z* 1500, 45 eV.

Integration and data elaboration were performed using MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

2.6 Antioxidant assays

Two different antioxidant assays were carried out to evaluate the antioxidant capacity of guava leaves extracts. The reducing power (FRAP) was evaluated according to the method validated by Benzie and Strain (1996) using a Fe (II) solution as a standard.

The TEAC assay, was performed by using a method previously described by Laporta, Perezfons, Mallavia, Caturla, and Micol (2007), using the radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and Trolox as standard.

2.7 Statistical analysis

The results reported in this study are the averages of three repetitions (n=3). Fisher's least significance difference (LSD) test and Pearson's linear correlations, both at $p < 0.05$, were evaluated using Statistica 6.0 (2001, StatSoft, Tulsa, OK).

3. Results and Discussion

3.1 Identification of phenolic compounds

As first step, to identify the phenolic compounds, a conventional extraction described by Seo and coworkers (2014) using an ethanol:water mixture (70:30 (v/v)) was carried out in commercial guava leaves. After that, the extracts were analyzed by HPLC-DAD-QTOF-MS and seventy two phenolic compounds could be identified. An overview of all the compounds tentatively identified in the extract is given in Table 1. Peak identification was performed on the basis of their retention times, their UV–Vis and mass spectra together with the information previously reported in the literature. Table 1 summarizes the information about identification: retention times, experimental and calculated *m/z*, maximum absorbance, fragments, molecular formula, score and error (ppm). Forty eight of these compounds (number **1-8**; **10**;**12-15**; **17**; **18**; **23**; **27**; **31**; **32**; **36**; **39**; **41**; **42**; **44-48**; **50-57**; **59-63**; **50-57**; **59-63**; **65-68**; and **70-72**) had previously been identified by Díaz-de-Cerio, Verardo, Gómez-Caravaca, Fernández-Gutiérrez, and Segura-Carretero (2015) by HPLC-ESI-Q-MS. These compounds were also verified by HPLC-ESI-QTOF-MS as is shown in Table 1.

Table 1. Identification of phenolic compounds in *Psidium Guajava* L. leaves by HPLC-DAD-ESI-QTOF-MS

No.	Compound	tr (min)	*m/z* exp	*m/z* calculated	Molecular Formula	λ (nm)	Fragments	Score	error (ppm)
1	HHDP glucose Isomer 1	1.861	481.0640	481.3406	C20H18O14	290	421.0406, 300.9991, 275.0202	96.51	-2.55
2	HHDP glucose Isomer 2	2.061	481.0638	481.3406	C20H18O14	290	421.0406, 300.9991, 275.0202	99.09	-0.19
3	HHDP glucose Isomer 3	2.393	481.0639	481.3406	C20H18O14	290	421.0406, 300.9991, 275.0202	97.21	-2.24
4	Prodelphinidin B2 Isomer	3.506	609.1276	609.5111	C30H26O14	272, 225	441.0838, 423.0701, 305.0687, 125.0226	97.84	-1.7
5	Gallic acid	3.749	169.0142	169.1116	C7H6O5	280, 360	125.0243	99.27	0.37
6	Pedunculagin/ Casuariin Isomer	6.733	783.0699	783.5332	C34H24O22	253	481.0606, 391.0307, 300.9999, 275.0191	98.57	-1.29
7	Prodelphinidin Dimer Isomer	6.955	593.1311	593.5117	C30H26O13	280, 340	425.0875, 289.0715,	96.51	-2.35
8	Gallocatechin	7.497	305.0698	305.2595	C15H14O7	270	125.0241, 179.0347, 219.0661, 261.0774	95.55	-3.32
9	Vescalagin/castalagin Isomer	7.52	933.0649	933.6216	C41H26O26	260, 280	466.0299, 300.9968	99.19	-0.79
10	Prodelphinidin Dimer Isomer	7.863	593.1316	593.5117	C30H26O13	280, 340	305.0667, 423.0719, 441.0841	96.51	-2.35
11	Uralenneoside	9.203	285.0624	285.2268	C12H14O8	270	153.0193, 109.0279	97.8	-2.69
12	Geraniin Isomer	9.214	951.0749	951.6369	C41H28O27	270	907.0825, 783.0785, 481.0606, 300.9999	99.56	-0.2
13	Pedunculagin/ Casuariin Isomer	9.241	783.0699	783.5332	C34H24O22	253	481.0606, 391.0307, 300.9999, 275.0191	98.39	-1.36
14	Geraniin Isomer	9.391	951.0752	951.6369	C41H28O27	270	907.0825, 783.0785, 481.0606, 300.9999	99.56	-0.2
15	Procyanidin B Isomer	9.784	577.1367	577.5123	C30H26O12	278	425.0881, 407.0777, 289.0718, 125.0243	95.68	-2.55
16	Galloyl(epi)catechin-(epi)gallocatechin	10.051	745.1420	745.6160	C37H30O17	280, 340	593.1302, 575.1214, 423.0694, 305.0688	96.9	-0.62
17	Procyanidin B Isomer	10.066	577.1367	577.5123	C30H26O13	278	425.0881, 407.0777, 289.0718, 125.0243	99.41	-0.61
18	Tellimagrandin I Isomer	10.432	785.0851	785.5491	C34H26O22	279, 340	615.0674, 392.0396, 300.9985, 169.0144	99.13	-0.96
19	Pterocarinin A	10.703	1067.1220	1067.7521	C46H36O30	280	533.0585, 377.0313, 301.0330, 249.0377	99.82	-0.11
20	Pterocarinin A Isomer	10.913	1067.1220	1067.7521	C46H36O30	280	533.0585, 377.0313, 301.0330, 249.0377	98.39	-1.26
21	Stenophyllanin A	10.941	1207.1495	1207.8903	C56H40O31	278	917.0763, 603.0735	98.64	-1.08
22	Procyanidin trimer Isomer	10.958	865.1998	865.7645	C45H38O18	278	739.1593, 577.1337, 449.0888, 289.0745	97.53	-1.59
23	Catechin	10.974	289.0727	289.2601	C15H14O6	281	245.0821, 203.0718, 179.0349, 125.0242	96.76	-3.18

No.	Compound	tr (min)	*m/z* exp	*m/z* calculated	Molecular Formula	λ (nm)	Fragments	Score	error (ppm)
24	Procyanidin tretamer	11.041	1153.2612	1153.0246	C60H50O24	280	576.1291	99.60	-0.50
25	Procyanidin trimer Isomer	11.129	865.1998	865.7645	C45H38O18	278	739.1593, 577.1337, 449.0888, 289.0745	97.53	-1.59
26	Guavin A	11.201	1223.1423	1223.8897	C56H40O32	277	611.0724	99.05	0.85
27	Casuarinin/ Casuarictin Isomer	11.578	935.0810	935.6375	C41H28O26	275	783.0637, 633.0735, 300.9979, 275.0189	97.67	-1.43
28	Galloyl(epi)catechin-(epi)gallocatechin	11.773	745.1420	745.6160	C37H30O17	280, 340	593.1302, 575.1214, 423.0694, 305.0688	96.90	-0.62
29	Procyanidin pentamer	11.845	1441.3234	1441.2688	C75H62O30	280	720.1604	95.66	1.97
30	Galloyl-(epi)catechin trimer Isomer	11.855	1017.2097	1017.8687	C52H42O22	280	508.104	99.72	-0.01
31	Gallocatechin	12.087	305.0702	305.2595	C15H14O7	270	125.0241, 179.0347, 219.0661, 261.0774	95.55	-3.32
32	Tellimagrandin I Isomer	12.198	785.0855	785.5491	C34H26O22	277, 338	615.0674, 392.0396, 300.9985, 169.0144	98.44	-1.38
33	Vescalagin	12.425	933.0649	933.6216	C41H26O26	260, 280	466.0295, 457.0781, 300.9968	96.33	-0.80
34	Stenophyllanin A Isomer	12.635	1207.1472	1207.8903	C56H40O31	280	917.0763, 603.0735	98.37	0.89
35	Galloyl-(epi)catechin trimer Isomer	12.985	1017.2097	1017.8687	C52H42O22	280	508.104	98.17	-1.35
36	Myricetin hexoside Isomer	13.067	479.0836	479.3678	C21H20O13	261, 358	317.0294, 316.0226, 271.0255	98.36	-0.92
37	Stachyuranin A	13.073	1225.1587	1225.9055	C56H42O32	276	612.0779	95.54	1.35
38	Procyanidin gallate Isomer	13.25	729.1476	729.6166	C37H30O16	280	577.1356, 559.1226, 425.0874, 407.0798, 298.0716	96.89	-1.91
39	Myricetin hexoside Isomer	13.46	479.0835	479.3678	C21H20O13	261, 358	317.0294, 316.0226, 271.0255	97.89	-0.08
40	Vescalagin/castalagin Isomer	13.538	933.0645	933.6216	C41H26O26	260	466.0299, 300.9968	88.32	-1.57
41	Myricetin -arabinoside/ xylopyranoside Isomer	13.759	449.0728	449.3418	C20H18O12	264, 356	317.0291, 316.0241, 271.0249	98.39	-1.65
42	Myricetin -arabinoside/ xylopyranoside Isomer	13.992	449.0726	449.3418	C20H18O12	264, 357	317.0291, 316.0241, 271.0249	98.02	-1.65
43	Procyanidin gallate Isomer	14.263	729.1476	729.6166	C30H26O12	280	577.1356, 559.1226, 425.0874, 407.0798, 298.0716	98.17	-1.73
44	Myricetin -arabinoside/ xylopyranoside Isomer	14.761	449.0726	449.3418	C20H18O12	264, 356	317.0291, 316.0241, 271.0249	98.66	-1.65

No.	Compound	tr (min)	*m/z* exp	*m/z* calculated	Molecular Formula	λ (nm)	Fragments	Score	error (ppm)
45	Myricetin hexoside Isomer	14.806	479.0839	479.3678	C21H20O13	261, 358	317.0294, 316.0226, 271.0255	97.08	-1.92
46	Myricetin hexoside Isomer	14.999	479.0841	479.3678	C21H20O13	264, 356	317.0288, 316.0241, 271.0253	97.08	-1.92
47	Myricetin -arabinoside/ xylopyranoside Isomer	15.376	449.0743	449.3418	C20H18O12	264, 356	317.0291, 316.0241, 271.0249	98.39	-1.65
48	Quercetin -galloylhexoside Isomer	15.42	615.1008	615.4726	C28H24O16	268, 350	463.0886, 300.0283	99.16	-0.98
49	Ellagic acid deoxyhexoside	15.653	447.0578	447.3259	C20H16O12	265, 350	300.9974,	91.25	-3.19
50	Quercetin -galloylhexoside Isomer	15.83	615.0999	615.4726	C28H24O16	280, 345	463.0886, 300.0283	99.16	-0.98
51	Myricetin -arabinoside/ xylopyranoside Isomer	15.968	449.0736	449.3418	C20H18O12	256, 356	317.0291, 316.0241, 271.0249	98.39	-1.65
52	Morin	16.024	301.0362	301.2278	C15H10O7	257, 374	178.9978, 151.0032	97.46	-2.5
53	Myricetin -arabinoside/ xylopyranoside Isomer	16.24	449.0735	449.3418	C20H18O12	257, 356	317.0291, 316.0241, 271.0249	98.39	-1.65
54	Ellagic acid	16.262	300.9996	301.1847	C14H6O8	254, 360	283.9921, 257.0088, 229.0169, 185.0233	98.88	-1.71
55	Hyperin	16.522	463.0895	463.3684	C21H20O12	259, 355	301.0350, 300.0279, 178.9980, 151.0032	96.41	-2.65
56	Quercetin glucoronide	16.616	477.0659	477.3519	C21H18O13	265, 355	301.0359, 151.0026	98.10	-1.83
57	Isoquercitrin	16.76	463.0893	463.3684	C21H20O12	258, 355	301.0353, 300.0281, 178.9983, 151.0090	97.04	-2.33
58	Procyanidin gallate Isomer	16.777	729.1476	729.6166	C37H30O16	280	577.1356, 559.1226, 425.0874, 407.0798, 298.0716	96.89	-1.91
59	Reynoutrin	17.303	433.0792	433.3424	C20H18O11	258, 356	301.0356	95.94	-2.90
60	Guajaverin	17.607	433.0795	433.3424	C20H18O11	257, 356	301.0352	97.99	-1.91
61	Guavinoside A	17.801	543.1159	543.4610	C26H24O13	218, 288	313.0568, 229.0503, 169.0148	98.10	-1.77
62	Avicularin	18.000	433.0803	433.3424	C20H18O11	257, 355	301.0359	96.70	-2.20
63	Quercitrin	18.244	447.0947	447.3690	C21H20O11	264, 353	301.0348, 271.0247, 178.9988, 151.0028	95.23	-3.02
64	Myrciaphenone B	18.991	481.0999	481.3836	C21H22O13	280, 340	313.0570, 169.0141	97.20	-2.23
65	Guavinoside C	19.556	585.0898	585.4466	C27H22O15	265, 355	433.0757, 301.0351, 283.0449, 169.0142	97.19	-1.92
66	Guavinoside B	20.658	571.1470	571.5062	C28H28O13	218, 283	313.057, 257.0829, 169.0142	97.26	-2.05

No.	Compound	tr (min)	*m/z* exp	*m/z* calculated	Molecular Formula	λ (nm)	Fragments	Score	error (ppm)
67	Guavinoside A Isomer	20.702	543.1159	543.4530	C26H24O13	218, 288	313.0568, 229.0503, 169.0148	98.10	-1.77
68	Guavinoside B Isomer	21.549	571.1470	571.5062	C28H28O13	218, 283	313.057, 257.0829, 169.0142	97.26	-2.05
69	2,6-dihydroxy-3-methyl-4-O-(6″-O-galloyl-β-D-glucopyranosyl)benzophenone	21.881	557.1318	557.4796	C27H26O13	280	313.0575, 243.0670, 169.0146	96.93	-2.12
70	Guavin B	22.103	693.1110	693.5414	C33H26O17	283	391.1468	97.82	-1.67
71	Quercetin	22.147	301.0358	301.2278	C15H10O7	257, 374	178.9985, 151.0036	98.90	-1.34
72	Naringenin	26.726	271.0622	271.2448	C15H12O5	280	118.6395, 150.5022	96.09	-3.67

However, HPLC-ESI-QTOF-MS platform allowed the identification of several more phenolic compounds in the same analysis. Six of the identified compounds were noticed by several authors in guava leaves before. Compounds number **9** and **40**, with molecular ion at *m/z* 933.0649 and two fragments at *m/z* 466.0299 and 300.9968, corresponding to the $[M-2H]^{2-}$ and to a loss of ellagic acid, respectively, were identified as vescalagin/castalagin isomers. Compound **33**, also with molecular ion at *m/z* 933.0649 and based on the fragment at *m/z* 457.0781, due to $[M-2H-H_2O]^{2-}$, was also described as vescalagin. These compounds had previously been described in fresh guava leaves using HPLC-DAD and ^1H NMR by Yamanaka et al. (2008) and via HPLC-MicroTOF-Q by Moilanen, Sinkkonen, and Salminen (2013).

Two compounds (**21** and **34**) were identified as stenophyllanin A at *m/z* 1207.1472 MS/MS spectra yielded ions at *m/z* 917.0763 (M–H-289, loss of catechin), 603.0735 ($[M-2H]^{2-}$). These compounds have previously been reported in guava leaves by Yamanaka et al. (2008).

Guavin A (compound **26**) that reported a molecular ion at *m/z* 1223.1423 was identified according to Okuda (1987) by NMR.

As far as we are concerned, twelve compounds were identified in *P. guajava* L. leaves for first time using this methodology. Compound **11**, with a molecular ion at *m/z* 285.0624, and fragments at *m/z* 153.0193, due to the loss of hydroxybenzoic acid unit, and *m/z* 109.0279, because of the loss of a CO_2 group from the carboxylic acid moiety, was assigned as uralenneoside according to the fragmentation pattern reported by Yu, Chen, and Liang (2000).

Two isomers were detected at *m/z* 745.1420 (compounds **16** and **28**) According to the MS/MS fragmentation pattern proposed by Jaiswal, Jayasinghe, and Kuhnert (2012), the isomers were identified as galloyl(epi)catechin-(epi)gallocatechin.

Isomers **19** and **20** (*m/z* 1067.1220) provided four fragments at *m/z* 533.0585 ($[M-2H]^{2-}$), 377.0313, 301.0330, and 249.0377, which matched with the MS/MS

fragments identified by Regueiro et al. (2014) for pterocarinin A. This compound has been identified in Myrtaceae family by several authors (Nonaka, Ishimaru, Azuma, Ishimatsu, & Nishioka, 1989; Tanaka, Orii, Nonaka, & Nishioka, 1993); however, it has not been described in guava leaves previously.

Procyanidin trimer isomers (**22** and **25**) with $[M-H]^-$ at *m/z* 865.1998 provided a fragmentation pattern in concordance with Regueiro et al. (2014). Procyanidin tetramer (**24**) and procyanidin pentamer (**29**) showed significant $[M–2H]^{2-}$ signals at *m/z* 576.1291 and 720.1604, respectively (Regueiro et al., 2014).

Two isomers (**30** and **35**) were identified as galloyl-(epi)catechin trimer isomers based on their molecular formula, on their molecular ion at *m/z* 1017.2097, and on their $[M–2H]^{2-}$ fragment at *m/z* 508.1040 (De Freitas, Glories, Bourgeois, & Vitry, 1998).

Compound **37** provided a molecular ion at *m/z* 1225.1587, an $[M–2H]^{2-}$ at *m/z* 612.0779 and a molecular formula ($C_{56}H_{42}O_{32}$). Thus, it was identified as stachyuranin A.

Three isomers (compounds **38**, **43** and **58**), at *m/z* 729.1476, corresponded to procyanidin gallate isomers. The isomers produced fragments at *m/z* 577.1356 resulted from the loss of a galloyl residue, at *m/z* 559.1226 by the loss of a gallic acid and at 425.0874 and 407.0798 from a retro-Diels-Alder fragmentation (Jaiswal et al., 2012). Moreover, they provided a fragment at *m/z* 289.0716 corresponded to an (epi)-catechin unit.

Compound **49** was identified as ellagic acid deoxyhexoside. It showed $[M-H]^-$ ion at *m/z* 447.0578 and yielded a fragment at *m/z* 300.9974 from a ellagic acid unit. It was described in *Psidium* fruits by several authors (Gordon, Jungfer, da Silva, Maia, & Marx, 2011; Ribeiro et al., 2014).

Compound **64** corresponding to $[M-H]^-$ signal at *m/z* 481.0999 was detected. Based on its molecular formula and its fragments at *m/z* 313.0570, due to a loss of a

dihydroxybenzoic acid unit, and 169.0141, due to a gallic acid unit, was assigned as Myrciaphenone B. This compound was previously reported in *Myrcia multiflora* (Myrtaceae) by Yoshikawa and coworkers (1998).

Compound **69** was identified as 2,6-dihydroxy-3-methyl-4-O-(6″-O-galloyl-β-D-glucopyranosyl)-benzophenone according to its molecular ion at *m/z* 557.1318, and to its MS/MS spectrum, that showed fragments at *m/z* 313.0575 (loss of $C_{14}H_{12}O_4$), 243.0670 (loss of $C_{13}H_{15}O_{10}$), and 169.0146 (loss of $C_7H_5O_5$). It was described in *P. guajava* L. fruit (Shu, Chou, & Wang, 2010).

3.2 Method parameters

Table 2 summarises the parameters of the method for each standard: calibration curve (y = a + bx), R^2, linear range and limits of detection (LOD) and quantification (LOQ). Standard calibration curves were done in the range of concentration from the quantification limit (LOQ) to 50 mg/L and five calibration points for each standard were run in triplicate (n=3). The values of the regression coefficients varied from 0.9964 to 0.9996. The limits of detection (LOD) and quantification (LOQ) were calculated as the concentration corresponding to 3 and 10 times, respectively, the standard deviation of the background noise. The LOD values ranged from 0.023 mg/L for naringenin to 0.062 mg/L for gallic acid. LOQs ranged from 0.08 mg/L for naringenin to 0.21 mg/L for gallic acid.

Quantification of phenolic compounds was done with the calibration curves showed in Table 2. For the compounds with no standard available, quantification was done using compounds with similar structure.

Table 2. Analytical parameters of the method

Standard	Equation	R^2	Linear range (mg/L)	LOD (mg/L)	LOQ (mg/L)
Gallic acid	y = 2.20E5 X + 3.85E5	0.9855	LOQ-50	0.062	0.21
Catechin	y = 2.82E5X + 4.71E5	0.9986	LOQ-50	0.049	0.16
Rutin	y = 4.24E5 X - 5.33E5	0.9996	LOQ-50	0.032	0.11
Ellagic acid	y = 4.30E5X - 6.95E5	0.9975	LOQ-50	0.032	0.11
Naringenin	y = 6.09E5 X + 2.46E6	0.9664	LOQ-50	0.023	0.08

3.3 Effect of solvent mixture on phenolic extraction

Extraction is the most important step to obtain extracts enriched in phenolic compounds. Ethanol and water are the most widely applied extraction solvents in food systems because of the hygiene, low cost and abundance in addition to being compatible with health (Moure et al., 2001). In this study, pure ethanol and different hydroethanolic mixtures, such as ethanol/water in the ratios of 90:10, 80:20, 70:30, 60:40, and 50:50 (v/v), were evaluated with the same extraction procedure described in "Guava leaves extraction" section. Total phenolic compounds quantified by HPLC-MS using the different mixtures are shown in Fig. 1. The phenolic content of the pure ethanol extract (201 ± 5 mg/g leaf d.w.) was lower than the content of the extracts obtained with the different mixtures, due to a less solubility of polar compounds in pure organic solvents. The best recoveries of phenolic compounds, without significative differences ($P < 0.05$), were provided by EtOH/H_2O 80:20 (v/v) (304 ± 7 mg/g leaf d.w.), 70:30 (v/v) (301 ± 1 mg/g leaf d.w.) and 50:50 (v/v) (298 ± 4 mg/g leaf d.w.) mixtures. EtOH/H_2O 60:40 (v/v) (284 ± 9 mg/g leaf d.w.) and 90:10 (v/v) (255 ± 5 mg/g leaf d.w.) mixtures provided significative lower contents of phenolic compounds than the other mixtures ($P < 0.05$). As can be seen, water content enhances the extraction due a better solubility of the target compounds in the solvent systems (Seo et al., 2014). TPC value increased from 100 to 80% ethanol, where the highest content was reached, a fact also observed for palm kernel cake (Kua et al., 2015). Then, the extraction of phenolic compounds remained nearly constant. Zhu, Lian, Guo, Peng, and Zhou (2011) compared the total phenolic content, using Folin-Ciocalteu

assay, of 30, 50, 70 and 100% ethanol, and aqueous extracts of defatted wheat germ, and found that the 70 and the 50% ethanol extract exhibited relatively higher antioxidant capacity than the others. In contrast to these results, Taha, Mohamed, Mohamed, Mohamed, and Kamil (2011) examined different ethanol concentrations (80, 70, 60, and 50%) and their results indicated that 60% was the most effective solvent to extract total phenolic compounds from sunflower meal. In general, ethanol/water mixtures, particularly from 40 to 80% ethanol, had greater efficiency in the extraction of phenolic compounds compared to water or pure ethanol or methanol (Fatiha et al., 2012).

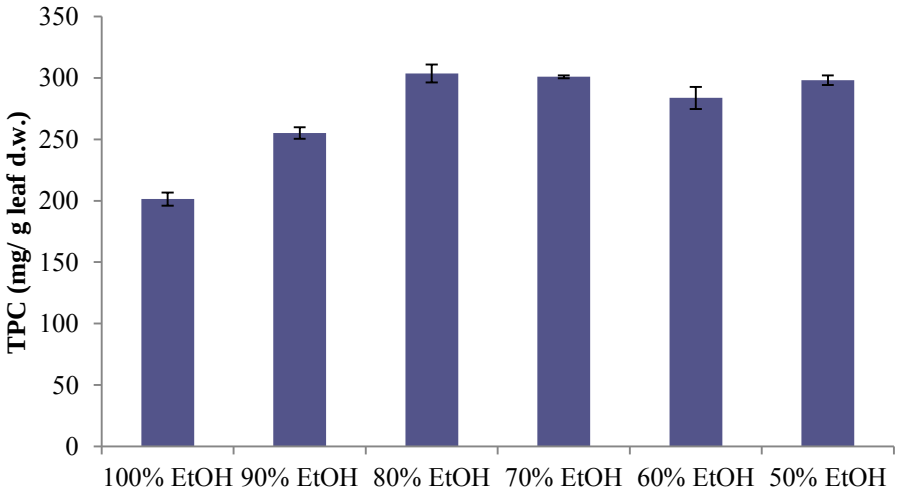

Figure 1. Total phenolic content (mean ± SD, n=3) by HPLC-DAD-QTOF-MS (mg/g leaf d.w.) present in each extract

Fig. 2 indicates the variability of the different families of phenolic compounds quantified with all solvent systems tested. These results showed that the concentration of the families of phenolic compounds decreased in the following order: flavonols > flavan-3-ols > gallic and ellagic derivatives > flavanones. The same order was noticed by Chang et al. (2013) for a methanolic extract of guava budding leaves, due to these compounds emerge at the early stage of budding. Seo et al. (2014) also noticed that the content of flavonoids was higher in 70% hydroethanolic extract than in 50 and 90%.

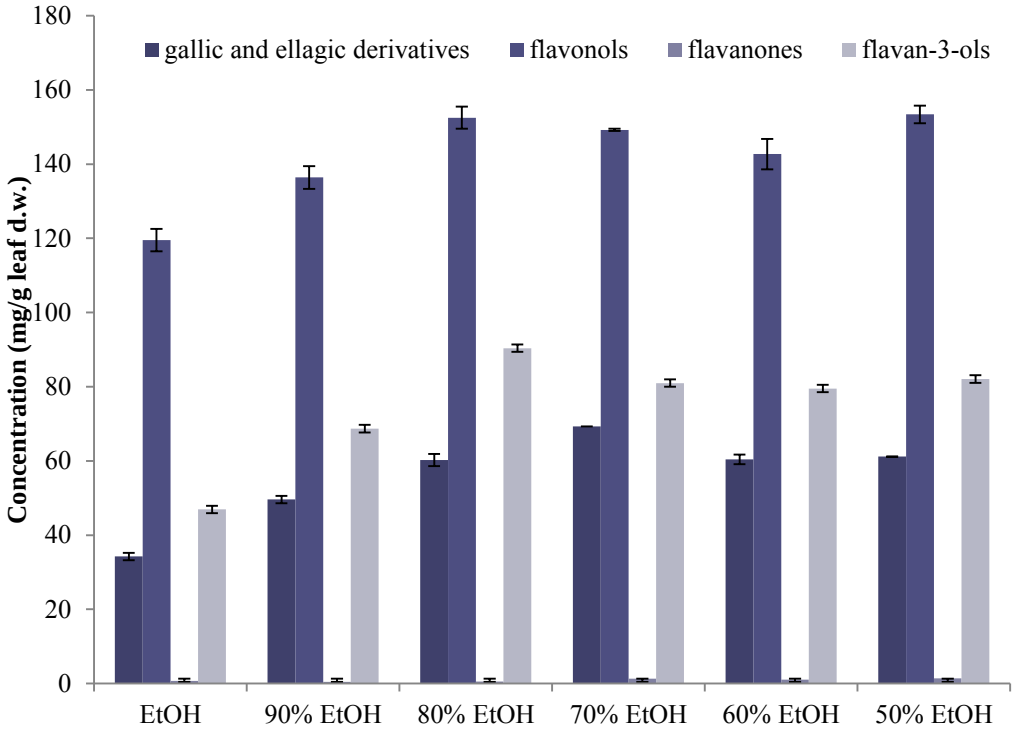

Figure 2. Variability of the different families of phenolic compounds (mg/g leaf d.w.) quantified (mean ± SD, n=3) in each solvent system

This trend could also be applied to the amount of each family of phenolic compounds that increased when the content of water increased between 0 and 20%, due to a higher solubility of the compounds in hydroalcoholic mixtures rather than in pure ethanol, and remained with slight variations when the presence of water is higher. These results are in concordance with Fatiha et al. (2012) who found that the highest recovery of phenolic compounds can be achieved at 75% ethanol compared with pure ethanol or with an aqueous mixture at 50% ethanol. This is because both the polar and less polar compounds are co-extracted together (Wu, Hsieh, Wang, & Chen, 2009).

Moreover, flavonols, the main class of phenolic compounds, varied between 53.5 and 76% of total polar compounds in each extraction mixture. The second class of polar compounds was represented by flavan-3-ols, which correspond to 23-45% of total polar compounds. Gallic and ellagic acid derivatives ranged from 17 to 35% of

the total concentration of polar compounds, and flavanones, accounted for a percentage lower than 1%.

Among the better extraction mixtures, EtOH/H_2O (80:20 (v/v)) was chosen as the best solvent mixture to extract the target compounds. Despite the fact that there were no significant differences in the extraction of total phenolic compounds among 80:20 (v/v), 70:30 (v/v) and 50:50 (v/v), EtOH/H_2O (80:20 (v/v)) extraction mixture showed better recoveries for flavonols and flavan-3-ols.

Several works demonstrate beneficial effects of compounds that are also present in guava leaves in the control of diabetes mellitus. Flavonol-glycosides, such as quercetin-glycosides, exert a dose-dependent inhibition of dipeptidyl peptidase *in vitro* (Eidenberger, Selg, & Krennhuber, 2013). Catechins protect type 2 diabetic erythrocytes from t-BHP-induced oxidative stress, providing some protection against the development of long-term diabetic complications (Rizvi, Zaid, Anis, & Mishra, 2005). Myrciaphenone B, exhibited inhibitory activity on aldose reductase and α-glucosidase (Yoshikawa et al., 1998). Geraniin, also possess *in vitro* hypoglycaemic activity and has the ability to prevent the formation of advanced glycation end-products (AGE), at more significant levels than the positive controls acarbose, quercetin and green tea (Palanisamy, Ling, Manaharan, & Appleton, 2011). Wu et al. (2009) compared the potential of guava leaves, with Polyphenol 60 and with an antiglycation agent, as inhibitor of glycation of proteins. They found that guava extract presented higher activity than the supplements due to a greater concentration of phenolic compounds such as quercetin, catechin and gallic acid, and suggested their daily consumption for prevention of diabetes complication. Moreover, in Japan, Guava Leaf Tea containing the aqueous leaf extract from guava has been approved as one of the Foods for Specified Health Uses and is now commercially available (Deguchi & Miyazaki, 2010).

3.4 Comparison between Psidium guajava L. leaves phenolic content and antioxidant capacity

The phenolic content (Table 3) and antioxidant capacity (Table 4) were determined in two guava leaves varieties; moreover, the guava leaves phenolic content was compared with guava fruit phenolic content (Table 5).

Table 3. Quantification (mg/g leaf d.w.) by HPLC of the phenolic compounds identified in guava leaves var. *pomifera* and *pyrifera.* Means in the same line with different letter are significantly different ($P < 0.05$)

Compound	var. *pomifera*	var. *pyrifera*
HHDP glucose Isomer 1	1.27 ± 0.01^{a}	0.77 ± 0.03^{b}
HHDP glucose Isomer 2	1.42 ± 0.04^{a}	0.94 ± 0.01^{b}
HHDP glucose Isomer 3	1.20 ± 0.01^{a}	0.73 ± 0.05^{b}
Prodelphinidin B2 Isomer	0.347 ± 0.002^{b}	0.417 ± 0.005^{a}
Gallic acid	0.223 ± 0.003^{a}	0.060 ± 0.008^{b}
Pedunculagin/ Casuariin Isomer	0.999 ± 0.003^{a}	0.807 ± 0.006^{b}
Prodelphinidin Dimer Isomer	0.502 ± 0.001^{a}	0.59 ± 0.01^{a}
Gallocatechin	3.89 ± 0.02^{a}	3.62 ± 0.01^{b}
Vescalagin/castalagin	0.529 ± 0.020^{a}	0.191 ± 0.001^{b}
Prodelphinidin Dimer Isomer	1.450 ± 0.004^{b}	1.60 ± 0.01^{a}
Uralenneoside	0.368 ± 0.005^{b}	0.97 ± 0.01^{a}
Geraniin Isomer	1.04 ± 0.02^{a}	0.459 ± 0.006^{b}
Pedunculagin/ Casuariin Isomer	1.424 ± 0.006^{a}	1.16 ± 0.03^{b}
Geraniin Isomer	1.15 ± 0.01^{a}	0.567 ± 0.005^{b}
Procyanidin B Isomer	5.111 ± 0.003^{b}	7.67 ± 0.03^{a}
Galloyl(epi)catechin-(epi)gallocatechin	<LOQ	<LOQ
Procyanidin B Isomer	0.575 ± 0.002^{b}	0.63 ± 0.01^{a}
Tellimagrandin I Isomer	0.641 ± 0.006^{a}	0.444 ± 0.001^{b}
Pterocarinin A	<LOQ	0.65 ± 0.01^{a}
Pterocarinin A Isomer	<LOQ	0.37 ± 0.02^{a}
Stenophyllanin A	1.20 ± 0.04^{b}	1.67 ± 0.14^{a}
Procyanidin trimer Isomer	0.40 ± 0.01^{b}	0.978 ± 0.005^{a}
Catechin	7.22 ± 0.04^{a}	7.14 ± 0.01^{a}
Procyanidin tetramer	<LOQ	<LOQ

Compound	var. *pomifera*	var. *pyrifera*
Procyanidin trimer Isomer	<LOQ	0.233 ± 0.003[a]
Guavin A	0.43 ± 0.03[b]	0.63 ± 0.02[a]
Casuarinin/ Casuarictin Isomer	0.368 ± 0.001[b]	4.15 ± 0.06[a]
Galloyl(epi)catechin-(epi)gallocatechin	0.494 ± 0.004[a]	0.225 ± 0.005[b]
Procyanidin pentamer	<LOQ	<LOQ
Galloyl(epi)catechin trimer Isomer	<LOQ	0.007 ± 0.001[a]
Gallocatechin	0.93 ± 0.01[b]	1.944 ± 0.004[a]
Tellimagrandin I Isomer	1.01 ± 0.01[a]	0.68 ± 0.01[b]
Vescalagin/castalagin	0.191 ± 0.004[a]	0.192 ± 0.003[a]
Stenophyllanin A Isomer	0.32 ± 0.01[b]	0.77 ± 0.03[a]
Galloyl(epi)catechin trimer Isomer	<LOQ	<LOQ
Stachyuranin A	0.25 ± 0.01[b]	0.31 ± 0.01[a]
Myricetin hexoside Isomer	0.127 ± 0.001[b]	0.182 ± 0.007[a]
Myricetin hexoside Isomer	0.130 ± 0.001[b]	0.184 ± 0.004[a]
Procyanidin gallate	2.35 ± 0.05[a]	1.81 ± 0.03[b]
Vescalagin/castalagin	0.194 ± 0.001[a]	0.199 ± 0.003[a]
Myricetin-arabinoside/ xylopyranoside Isomer	0.152 ± 0.004[b]	0.22 ± 0.01[a]
Myricetin-arabinoside/ xylopyranoside Isomer	0.231 ± 0.005[b]	0.50 ± 0.02[a]
Procyanidin gallate	<LOQ	0.060 ± 0.003
Myricetin-arabinoside/ xylopyranoside Isomer	0.198 ± 0.002[b]	0.512 ± 0.005[a]
Myricetin hexoside Isomer	0.75 ± 0.01[b]	0.82 ± 0.01[a]
Myricetin hexoside Isomer	0.251 ± 0.003[a]	0.216 ± 0.001[b]
Myricetin- arabinoside/ xylopyranoside Isomer	0.500 ± 0.003[a]	0.412 ± 0.001[b]
Quercetin- galloylhexoside Isomer	0.335 ± 0.001[b]	0.391 ± 0.004[a]
Ellagic acid deoxyhexoside	0.97 ± 0.02[a]	0.697 ± 0.005[b]
Quercetin- galloylhexoside Isomer	0.258 ± 0.002[a]	0.309 ± 0.008[a]
Myricetin-arabinoside/ xylopyranoside Isomer	0.780 ± 0.017[a]	0.617 ± 0.008[b]
Morin	3.8 ± 0.1[b]	5.50 ± 0.06[a]
Myricetin-arabinoside/ xylopyranoside Isomer	0.711 ± 0.009[b]	0.76 ± 0.01[a]
Ellagic acid	2.15 ± 0.06[a]	2.08 ± 0.03[b]
Hyperin	2.86 ± 0.01[b]	7.19 ± 0.06[a]
Quercetin glucoronide	<LOQ	1.86 ± 0.02[a]
Isoquercitrin	2.11 ± 0.02[b]	3.27 ± 0.03[a]
Procyanidin gallate	0.22 ± 0.01[a]	0.111 ± 0.002[b]
Reynoutrin	3.13 ± 0.05[b]	4.73 ± 0.06[a]
Guajaverin	8.0 ± 0.1[b]	11.92 ± 0.05[a]

Compound	var. *pomifera*	var. *pyrifera*
Guavinoside A	0.54 ± 0.01[b]	1.08 ± 0.01[a]
Avicularin	6.2 ± 0.1[b]	12.40 ± 0.03[a]
Quercitrin	6.5 ± 0.1[a]	5.15 ± 0.05[b]
Myrciaphenone B	0.616 ± 0.001[b]	0.98 ± 0.01[a]
Guavinoside C	2.46 ± 0.07[a]	2.63 ± 0.02[a]
Guavinoside B	1.45 ± 0.03[a]	0.875 ± 0.003[b]
Guavinoside A Isomer	0.147 ± 0.001[b]	0.398 ± 0.004[a]
Guavinoside B Isomer	0.652 ± 0.008[a]	0.154 ± 0.001[a]
2,6-dihydroxy-3-methyl-4-O-(6″-O-galloyl-β-D-glucopyranosyl)-benzophenone	0.98 ± 0.02[b]	1.35 ± 0.02[a]
Guavin B	0.357 ± 0.008[a]	0.362 ± 0.004[a]
Quercetin	0.320 ± 0.009[b]	0.460 ± 0.003[a]
Naringenin	0.65 ± 0.02[b]	1.51 ± 0.02[a]

Guava leaves var. *pyrifera* and *pomifera* were extracted with a hydroethanolic mixture (80:20 (v/v)), and then analyzed by HPLC-DAD-QTOF-MS. The comparison of the concentration of the different phenolic families present in both leaves is shown in Fig. 3 and for each compound in Table 3. The quantification results reported that significant differences ($P < 0.05$) for each family were found when both varieties were compared, being higher in *pyrifera* than in *pomifera* variety. The comparison in terms of single phenolic compounds and also total phenolic content showed that *pyrifera* variety concentrations were higher than *pomifera* variety concentrations (Table 4). Total phenolic content in the two varieties ranged between 86.1 and 113.3 mg/g leaf d.w. for *P. guajava* L. var. *pomifera* and *pyrifera*, respectively. These data are in the same order of magnitude of those obtained in a previous work (Diaz-de-Cerio et al., 2015).

Table 4. Comparison (mean ± SD, n=3) between TPC by HPLC, FRAP and TEAC of *Psidium guajava* L. leaves var. *pyrifera* and *pomifera*. Means in the same column with different letter are significantly different ($P < 0.05$)

Sample	TPC[a]	FRAP [b]	TEAC[c]
var. *pyrifera*	113.3 ± 0.5[a]	5.8 ± 0.1[a]	3.27 ± 0.09[a]
var. *pomifera*	86.1 ± 0.9[b]	5.55 ± 0.02[a]	2.66 ± 0.06[b]

[a] Expressed in mg of polar compound/g leaf d.w.; [b] Expressed in mM of $FeSO_4$ equivalents /mg leaf d.w; [c] Expressed in mM of Trolox equivalents /mg leaf d.w

For both varieties, the most abundant phenolic family was flavonols that represented 49.5% for *pomifera* var. and 55.3% for *pyrifera*, followed by flavan-3-ols, which exhibited a percentage of 27.3 and 23.8%, respectively. The sum of gallic and ellagic acid derivates supposed 19.6 and 22.4%, and flavanones 0.8 and 1.3% for *pomifera* and *pyrifera* varieties, respectively.

Higher amounts of gallic acid (0.060 and 0.223 mg/g d.w.), gallocatechin (5 and 5.6 mg/g d.w.) and catechin (7.2 and 7.1 mg/g d.w.) were noticed compared to the data obtained by Jang et al. (2014), who reported 0.09, 2.88 and 0.72 mg/g d.w., respectively. This discordance could be due to the different origin of samples and/or the method of extraction.

Haida et al. (2011) also compared the phenolic content of *P. guajava* L. var. *pomife*ra and *pyrifera* leaves. Contrary to the present results, where concentrations obtained for *pomifera* and *pyrifera* were 86.1 and 113.3 mg/g d.w., respectively, they noticed that total phenolic content of *P. guajava* L. var. *pomifera* was higher than TPC of *pyrifera* variety, between 161-175 and 159-164 mg/g extract, respectively; however they analyzed total phenolic content by Folin-Ciocalteu assay.

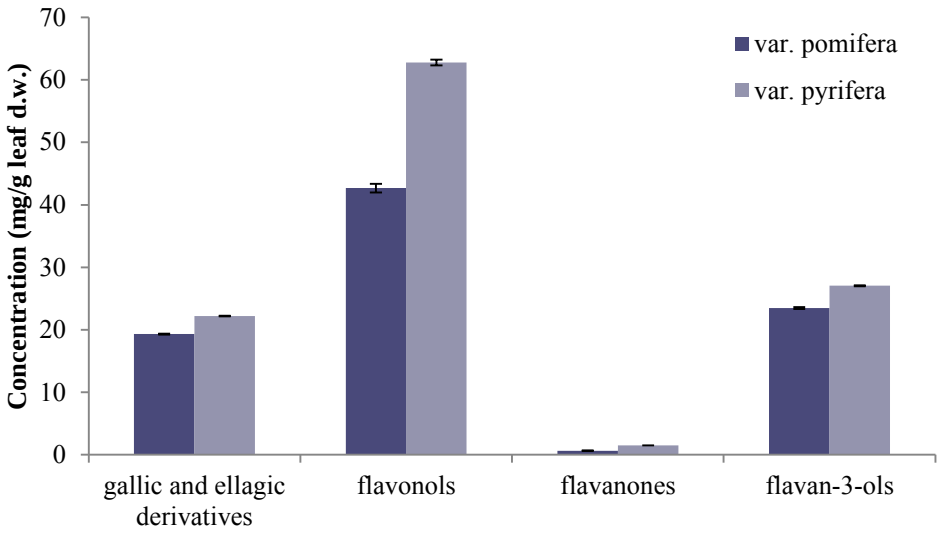

Figure 3. Comparison between the quantification (mean ± SD, n=3) of the different families of compounds (mg/g leaf d.w.) identified in *Psidium guajava* L. leaves var. *pyrifera* and *pomifera*.

Chen and Yen (2007) also studied leaves from different guava cultivars (*cv*. Hong Ba, Shi Ji Ba, Shui Jing Ba and Tu Ba) and no concordances were noticed with the data showed in this work. This could be justified by the different determination methods used to quantify the phenolic compounds; in fact, they analyzed different varieties of guava leaves and fruit by Folin-Ciocalteu assay and HPLC-UV. Because of that, not all the phenolic compounds could be identified. As matter of fact, they identified only gallic and ferulic acid because there were no other available commercial standards. As reported in the literature (Table 5), the concentration of gallic acid in guava leaves varied from 0.8 to 1.6 mg/g leaf and total phenolic contents ranged between 267 and 313 mg/g leaf, expressed as (+)-catechin equivalents and from 414 to 483 mg/g leaf expressed as gallic acid equivalents. In contrast, in the present work, the concentration of gallic acid recovered was lower than those shown in Table 5.

Table 5. Comparison between guava leaves and fruits

Origin (type)	Quantification method	Identified compounds	Content	Reference
FRUIT				
Mexico (white guava by-products)	Folin–Ciocalteu	TPC	7.5 ± 0.3 GAE mg/g d.w.	(Amaya-Cruz et al., 2015)
	$AlCl_3$	Flavonoids	2.3 ± 0.1 CE mg/g d.w.	
	HPLC-DAD-MS	Gallic acid	< LOD	
		Ellagic acid	0.6 ± 0.1 ng/g d.w.	
		Catechin	0.3 ± 0.0 ng/g d.w.	
		Quercetin	0.1 ± 0.0 ng/g d.w.	
Taiwan	Folin–Ciocalteu	TPC	69.6 ± 2.8 CE mg/g d.w.	(Chen & Yen, 2007)
	Folin–Ciocalteu	TPC	115 ± 4.2 GAE mg/g d.w.	
China (peel and pulp)	HPLC-DAD	Gallic acid	1.24 - 1.58 mg/100g	(Li et al., 2013)
Brazil (pulp)	Folin–Ciocalteu	TPC	17.2 ± 1.1 mg/g d.w	(Ribeiro da Silva et al., 2014)
Thailand (white cv. Pansithong)	Folin–Ciocalteu	TPC	145.52 ± 0.08 GAE mg/g d.w.	(Thuaytong & Anprung, 2011)
	$AlCl_3$	Flavonoids	19.06 ± 0.18 CE mg/g d.w.	
Thailand (red cv. Samsi)	Folin–Ciocalteu	TPC	163.36 ± 0.05 GAE mg/g f.w.	
	$AlCl_3$	Flavonoids	35.85 ± 0.13 CE mg/g f.w.	
USA (white cv. Allahabad Safeda)	Folin–Ciocalteu	TPC	344.97 ± 33.6 GAE mg/100 g f.w.	(Thaipong et al., 2006)
USA (red cv. Fan Retief', 'Ruby Supreme' and an advanced selection)	Folin–Ciocalteu	TPC	170 -300 GAE mg/100 g f.w.	

Origin (type)	Quantification method	Identified compounds	Content	Reference
Mauritius (white)	Folin–Ciocalteu	TPC	2473 ± 45 GAE µg /g f.w.	(Luximon-Ramma et al., 2003)
	$AlCl_3$	Flavonoids	209 ± 10 Q µg /g f.w.	
Mauritius (pink)	Folin–Ciocalteu	TPC	1264 ± 60 GAE µg /g f.w.	
	$AlCl_3$	Flavonoids	110 ± 21 Q µg/g f.w.	
LEAVES				
Spain (young leaf)	HPLC-MS	TPC	157 ± 6 mg/g d.w.	(Díaz-de-Cerio et al., 2015)
Mexico (young leaf)	HPLC-DAD	Quercetin	0.620 mg/g d.w.	(Vargas-Alvarez et al., 2006)
Thailand (young leaf)	HPLC-DAD	Gallic acid	3.67 ± 0.12 mg/g e	(Nantitanon et al., 2010)
		Quercetin	26.12 ± 0.98 mg/g e	
		Ellagic acid	13.82 ± 0.34 mg/g e	
Taiwan (Shi Ji Ba, Tu Ba)	HPLC-DAD	Gallic acid	11-22 mg/g e	(Wu et al., 2009)
		Quercetin	9-11 mg/g e	
		Catechin	4-5 mg/g e	
Korea	HPLC-DAD	Gallic acid	0.09 ± 0.00 mg/g d.w.	(Jang et al., 2014)
		Gallocatechin	2.88 ± 0.02 mg/g d.w.	
		Catechin	0.72 ± 0.04 mg/g d.w.	
Paraná (*Pomifera* var.)	Folin–Ciocalteu	TPC	161-175 GAE mg/g e	(Haida et al., 2011)
Paraná (*Pyrifera* var.)			159-164 GAE mg/g e	
Taiwan (Hong Ba, Shi Ji Ba, Shui Jing Ba, Tu Ba)	Folin–Ciocalteu	TPC	267-313 CE mg/g l	(Chen & Yen, 2007)
			414-483 GAE mg/g l	

GAE-gallic acid equivalent; CE-catechin equivalent; Q-quercetin; LOD- detection limit; f.w., fresh weight; d.w., dry weight; e, extract; l, leaf

The differences noticed among the different works in the literature could be explained by the fact that flavonoids vary substantially among genotypes, seasons, age and level of damaged leaves, and location (Salazar et al., 2006; Vargas-Alvarez, Soto-Hernández, González-Hernández, Engleman, & Martínez-Garza, 2006). Moreover, the guava tree (*P. guajava* L.) shows different phenological stages throughout its vegetative period in response to environmental conditions (Eidenberger et al., 2013). In addition, Folin-Ciocalteu method represent a none selective spectrophotometric estimation of total phenolic compounds, because other substances (such as sugars and proteins) can react with Folin-Ciocalteu reagent and can overestimate the phenolic content.

Briefly, comparing the results obtained in this work for guava leaves with data reported by several authors for guava fruits (Luximon-Ramma, Bahorun, & Crozier, 2003; Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006; Thuaytong & Anprung, 2011), the present data confirms that guava leaves have higher content of phenolic compounds than guava fruits.

Moreover, Chen and Yen (2007) found that all the leaves had higher amounts of phenolic compounds than the fruit. Values exhibited for the dried fruit were 69.9 mg/g d.w., expressed as (+)-catechin equivalents and 115 mg/g d.w. expressed as gallic acid equivalents.

The same phenolic classes detected in guava leaves, were also detected in guava fruits and guava juice by-products and they demonstrate anti-inflammatory and antiproliferative activity, and reduced hepatic steatosis (Amaya-Cruz et al., 2015; Chen & Yen, 2007; Li et al., 2013). In fact, Li et al. (2013) only detected and quantified gallic acid in pulp and peel of guava fruits; its content varied from 1.2 to 1.6 mg/100g in fresh peel and pulp, respectively. The obtained data in this work for guava leaves showed higher content of gallic acid (0.060 and 0.223 mg/g dry weight) compared to the peel and the pulp. Moreover, the present data are in the same order of magnitude of those obtained for cv. Shui Jing Ba (0.79 mg/g leaf) by Chen and Yen

(2007) in guava leaves and higher if they are compared with guava juice, where the concentration was lower than the detection limit (Amaya-Cruz et al., 2015). In addition, Amaya-Cruz et al. (2015) identified and quantified several phenolic compounds in guava juice by-products, and some of them such as ellagic acid, quercetin and catechin were also found in leaves. However, the content of these phenolic compounds in guava juice by-products was lower than the amounts of the same compounds shown in the studied guava leaves. It is important to highlight that several phenolic compounds identified in guava fruits and fruit by-products (Amaya-Cruz et al., 2015; Flores et al., 2015) are not described in guava leaves.

About guava fruits, several authors reported that red guava (*P. guajava* L.) cv. Samsi has higher antioxidant activity, higher total phenolics and also higher total flavonoid contents than white guava cv. Pansithong (Thuaytong & Anprung, 2011). In contrast, other authors (Thaipong et al., 2006) analyzed one white guava (cv. Allahabad Safeda) and three pink guava fruits (cv. Fan Retief, Ruby Supreme and an "advanced selection") clones of guava fruits and reported that white pulp guava had higher antioxidant capacity and total phenolic content than pink pulp guavas; Luximon-Ramma et al. (2003) found the same trend in their analysis. Data from these works are summarized in Table 5. As it can be seen, a wide range is displayed for TPC. In this case, TPC varies from 1.3 to 163 GAE mg/g f.w., and from 7.5 to 115 GAE mg/g d.w. in the fruit. Instead, guava leaf phenolic content showed less variation (from 157 mg/g in d.w. and 159 GAE mg/g extract to 483 GAE mg/g leaf). This trend could be justified not only by genotypes, seasonal changes, and location, but also by the determination method that the authors used for phenolic determination. In fact, Folin-Ciocalteu reagent can react with sugars that are naturally present in fruits and the phenolic content could be overestimated.

The health benefits of phenolic composition of guava fruits and leaves have been studied by several authors. Singh et al. (2013) reported that flavonoids are one of major chemical constituents of plant species used in the management of diabetic complications. Eidenberger and coworkers (2013) evaluated the inhibition of

dipeptidyl peptidase activity by flavonol glycosides of guava (*P. guajava* L.) and found that these compounds have a potential to exert the effect observed *in vitro*. Moreover, species like *Murraya koenigii* L. and *Mentha piperitae* L. shown antidiabetic properties (Narendhirakannan, Subramanian, & Kandaswamy, 2006). The results of phytochemical analysis of the leaves of these species (Samarth & Samarth, 2009; Uraku & Nwankwo, 2015), revealed a lower content of flavonoids than guava leaves, thus, *P. guajava* L. leaves (var. *pyrifera*) could serve as an effective extract for ameliorating certain disease complications.

Furthermore, the antioxidant capacity was tested. To our knowledge, this is the first time that these varieties are compared by FRAP and TEAC. Table 4 shows the data obtained by TEAC, FRAP and TPC (total phenolic compounds expressed as the sum of total compounds determined by HPLC-MS) for the two varieties of guava leaves. *P. guajava* L. leaves var. *pyrifera* provided higher amount of total phenolic compounds, and consequently, a higher antioxidant capacity than *pomifera* variety. Correlation analysis was carried out between TPC, TEAC and FRAP assay, and the results showed the highest correlation between TPC and TEAC ($R > 0.97$ $P < 0.03$). Moreover, positive correlation was also detected for TEAC and each family of phenolic compounds ($R > 0.97$ $P < 0.03$).

Flores et al. (2015) studied seven *P. guajava* cultivars that varied in color from white to pink. They found that pink-pulp guavas had higher antioxidant capacity than white-pulp ones, and even though the major compounds were common to all cultivars, important differences existed in the accumulation of a significant number of compounds. Briefly, the antioxidant capacity and the phytochemical composition of *P. guajava* vary significantly according to the cultivar and pulp color. TEAC data obtained in this work for guava fruits reported lower TEAC values compared to guava leaves.

4. Conclusions

Several compounds were verified and twelve new compounds were identified in *P. Guajava* L. leaves by HPLC-DAD-ESI-QTOF-MS. Moreover, the hydroethanolic mixture (80:20, v/v) was selected as the best solvent to extract phenolic compounds, especially for flavonols and flavan-3-ols. Significant differences ($P < 0.05$) between varieties *pyrifera* and *pomifera* were found in the quantification of total phenolic compounds and their families by HPLC-MS. FRAP and TEAC assays were carried out for the first time to compare the varieties of guava leaves. High correlation was noticed among TPC, FRAP and TEAC. Due to its composition, *P. guajava* L. var. *pyrifera* extract is a good source of antioxidants that could be employed in diabetic therapeutic approaches. Further studies will be carried out to obtain flavonols and flavan-3-ols enriched extracts by green technologies.

Conflicts of interest

The authors declare no competing financial interest.

Aknowledgment

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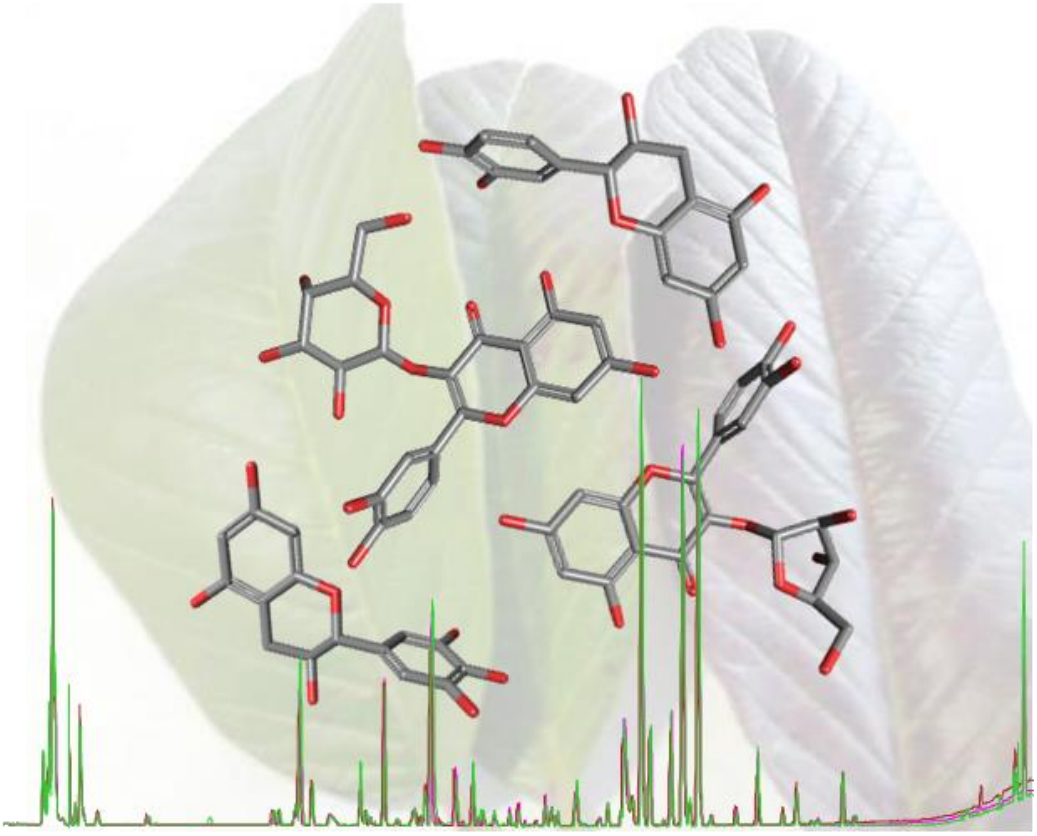
CHAPTER 3



Exploratory characterization of phenolic compounds with demonstrated anti-diabetic activity in guava leaves at different oxidation states

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Article

Exploratory Characterization of Phenolic Compounds with Demonstrated Anti-Diabetic Activity in Guava Leaves at Different Oxidation States

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Abstract

Psidium guajava L. is widely used like food and in folk medicine all around the world. Many studies have demonstrated that guava leaves have anti-hyperglycemic and anti-hyperlipidemic activities, among others, and that these activities belong mainly to phenolic compounds, although it is known that phenolic composition in guava tree varies throughout seasonal changes. Andalusia is one of the regions in Europe where guava is grown, thus, the aim of this work was to study the phenolic compounds present in Andalusian guava leaves at different oxidation states (low, medium, and high). The phenolic compounds in guava leaves were determined by HPLC-DAD-ESI-QTOF-MS. The results obtained by chromatographic analysis reported that guava leaves with low degree of oxidation had a higher content of flavonols, gallic, and ellagic derivatives compared to the other two guava leaf samples. Contrary, high oxidation state guava leaves reported the highest content of cyanidin-glucoside that was 2.6 and 15 times higher than guava leaves with medium and low oxidation state, respectively. The QTOF platform permitted the determination of several phenolic compounds with anti-diabetic properties and provided new information about guava leaf phenolic composition that could be useful for nutraceutical production.

Keywords: *Psidium guajava* L.; HPLC-DAD-ESI-QTOF-MS; phenolic compounds; gallic and ellagic derivatives; flavonols; cyanidin-glucoside

1. Introduction

Psidium guajava (*P. guajava*) L., from the *Myrtaceae* family, is common throughout tropical and subtropical areas [1] and Andalusia is one of the regions in Europe where guava is grown. Moreover, it is widely used like food and in folk medicine all around the world. Many studies have demonstrated that guava leaves have anti-hyperglycemic and anti-hyperlipidemic activities [2–4], among others, and these biological activities have mainly been related to the phenolic compounds [5].

Nowadays, alternative therapeutic strategies based on the use of phenolic compounds in food products as "functional foods" and "nutraceuticals" are being developed. In fact, the capacity of plant-derived foods to reduce the risk of chronic diseases has been demonstrated [6].

It is known that *P. guajava* L. shows different phenological stages throughout its vegetative period in response to environmental conditions [7], because of that, it has been seen that the accumulation of specific compounds such as anthocyanins changes [8]. Furthermore, the response of the different classes of phenolic compounds, especially flavonoids, also vary substantially [9]. This fact plays an important role in finding the best conditions of the leaf in order to obtain the best recovery of the target compounds for the development of a promising alternative source for ameliorating diabetes complications [2–4].

In this sense, spectrophotometric analyses are still helpful for a preliminary identification and quantification [10]; however, LC-MS has opened up new approaches for the structural characterization of target compounds. Moreover, LC-TOF-MS can provide tentative identification of unknown peaks, due to accurate-mass measurement [11]. So, both UV-VIS diode array and mass spectrometry coupled to HPLC have been proved as most appropriate analytical techniques for phenolic

compounds in many matrices [10,11]. Concerning guava leaves, most of the literature shows that quantification of the different classes of phenolic compounds is generally done via spectrophotometric analysis [12–15], although different analytical techniques, such as LC-DAD and LC-DAD-MS, are used to characterize the bioactive compounds present in guava leaves [3,4,16,17].

Despite these facts and to our knowledge, there is no literature taking into account the change that climatic conditions cause in phenolic composition and this information would be useful to choose the best raw material for nutraceutical scopes. Thus, the aim of this work was to study the phenolic compounds present in Andalusian guava leaves at different oxidation states (low, medium, and high) by HPLC-DAD-ESI-QTOF-MS.

2. Results and discussion

2.1. Characterization of phenolic compounds

The HPLC-DAD-ESI-QTOF-MS analyses in negative mode permitted the identification and quantification of seventy-three phenolic compounds in guava leaves [18]. The individual compounds were quantified on the basis of their peak area and compared with calibration curves obtained with the corresponding standards and then expressed as μg/g of leaf dry weight (d.w.). Moreover, quantification of compounds for which no commercial standards were available, was achieved comparing with standard compounds bearing similar structures (Table 1).

Furthermore, the analysis in positive mode allowed the identification of an anthocyanin compound. The compound, with *m/z* 449.1090 presented its maximum of absorption at 280, 350, and 520 nm on the UV spectrum. The MS/MS analysis produced a fragment ion at 287.0557 *m/z* corresponding to the loss of hexose unit (Figure 1). Due to the UV and MS spectrum and by co-elution with a commercial standard, this component has been identified as cyanidin-3-O-glucoside. To our

knowledge, this compound has been identified for the first time in guava leaves. Limits of detection (LOD) and quantification (LOQ) calculated for the cyanidin-3-O-β-galactopyranoside standard were 0.007 and 0.024 mg/L.

In terms of concentration of the individual compounds (Table 1), in negative mode, leaves with lower oxidation state exhibited the highest amounts for almost all compounds quantified, followed by moderate oxidized leaves and, finally, highly oxidized leaves. Concentrations of several compounds tentatively identified [18], such as procyanidin tetramer and pentamer, galloyl-(epi)catechin trimer isomers 1 and 2, and quercetin glucuronide were found to be lower than the quantification limit for all samples. In contrast, and as it was expected due to the red coloration of the leaves at high oxidative state, in positive mode, opposite results were found (Table 1); the concentration of cyanidin-3-O-glucoside increased as the oxidation state of the leaves increased. At low oxidative state, the concentration of cyanidin-3-O-glucoside was 29.5 ± 0.2 μg/g leaf d.w. and it raised until 441.28 ± 0.04 μg/g leaf d.w. for the highest oxidation state.

Table 1. Quantification (mean ± standard deviation (SD), $n = 3$) by HPLC-DAD-ESI-QTOF-MS in negative and positive mode of individual compound tentatively identified in *P. guajava* leaves for the different oxidative states.

No.	Compound	High	Medium	Low
	Negative mode	Concentration (μg compound/g leaf d.w.)		
1	HHDP glucose Isomer	526 ± 2 ^{c}	651 ± 19 ^{b}	936 ± 10 ^{a}
2	HHDP glucose Isomer	505 ± 3 ^{c}	645 ± 3 ^{b}	823 ± 16 ^{a}
3	HHDP glucose Isomer	510 ± 11 ^{c}	645 ± 20 ^{b}	934 ± 2 ^{a}
4	Prodelphinidin B Isomer	447.1 ± 0.1 ^{c}	515.7 ± 0.4 ^{b}	715 ± 13 ^{a}
5	Gallic acid	153.52 ± 0.09 ^{c}	164 ± 3 ^{b}	175.9 ± 0.7 ^{a}
6	Pedunculagin/Casuariin Isomer	158.8 ± 0.6 ^{b}	163.84 ± 0.06 ^{b}	175 ± 3 ^{a}
7	Pedunculagin/Casuariin Isomer	464.0 ± 0.8 ^{c}	475.5 ± 0.5 ^{b}	557 ± 2 ^{a}
8	Prodelphinidin Dimer Isomer	497 ± 1 ^{b}	529 ± 6 ^{b}	603 ± 30 ^{a}
9	Gallocatechin	4913 ± 47 ^{a}	4435 ± 7 ^{b}	4098 ± 84 ^{c}
10	Vescalagin/castalagin Isomer	157.59 ± 0.01 ^{a}	136.6 ± 0.3 ^{c}	143 ± 2 ^{b}
11	Prodelphinidin Dimer Isomer	1365 ± 7 ^{c}	1560 ± 14 ^{b}	1739 ± 25 ^{a}

No.	Compound	High	Medium	Low
12	Uralenneoside	2464 ± 4 [a]	1911 ± 24 [b]	1872 ± 81 [b]
13	Geraniin Isomer	241 ± 1 [b]	264.9 ± 0.5 [b]	343 ± 25 [a]
14	Pedunculagin/Casuariin Isomer	466 ± 3 [c]	575 ± 16 [b]	683 ± 20 [a]
15	Geraniin Isomer	260 ± 3 [b]	290 ± 7 [a,b]	356 ± 48 [a]
16	Procyanidin B Isomer	4262 ± 12 [c]	4742 ± 15 [b]	5514 ± 69 [a]
17	Galloyl(epi)catechin-(epi)gallocatechin	<LOQ	12.60 ± 0.07 [b]	38 ± 3 [a]
18	Procyanidin B Isomer	650 ± 3 [c]	708 ± 11 [b]	757 ± 23 [a]
19	Tellimagrandin I Isomer	347 ± 4 [c]	367.2 ± 0.7 [b]	397 ± 2 [a]
20	Pterocarinin A Isomer	569 ± 31 [b]	617 ± 9 [b]	679 ± 7 [a]
21	Pterocarinin A Isomer	316 ± 2 [c]	360 ± 4 [b]	376 ± 4 [a]
22	Stenophyllanin A	853 ± 13 [c]	1036 ± 50 [b]	1318 ± 24 [a]
23	Procyanidin trimer Isomer	781 ± 1 [a]	706 ± 1 [c]	738 ± 4 [b]
24	Catechin	8486 ± 10 [b]	8957 ± 11 [a]	6845 ± 24 [c]
25	Procyanidin tetramer	<LOQ	<LOQ	<LOQ
26	Procyanidin trimer Isomer	89 ± 2 [c]	108 ± 3 [b]	128 ± 1 [a]
27	Guavin A	263 ± 9 [c]	357 ± 8 [b]	518 ± 15 [a]
28	Casuarinin/Casuarictin Isomer	1297 ± 5 [c]	1568 ± 10 [b]	2089 ± 11 [a]
29	Galloyl(epi)catechin-(epi)gallocatechin	61 ± 5 [c]	135 ± 1 [b]	211 ± 12 [a]
30	Procyanidin pentamer	<LOQ	<LOQ	<LOQ
31	Galloyl-(epi)catechin trimer Isomer	<LOQ	<LOQ	<LOQ
32	Gallocatechin	2074 ± 2 [b]	1526 ± 2 [c]	2613 ± 55 [a]
33	Tellimagrandin I Isomer	463 ± 2 [c]	516 ± 6 [b]	737 ± 24 [a]
34	Vescalagin	160 ± 6 [b]	159 ± 3 [b]	187 ± 1 [a]
35	Stenophyllanin A Isomer	355.36 ± 0.07 [c]	425 ± 13 [b]	548 ± 20 [a]
36	Galloyl-(epi)catechin trimer Isomer	<LOQ	<LOQ	<LOQ
37	Myricetin hexoside Isomer	432.10 ± 0.05 [c]	555 ± 2 [b]	572 ± 7 [a]
38	Stachyuranin A	207.40 ± 0.04 [a]	207 ± 6 [a]	216 ± 1 [a]
39	Procyanidin gallate Isomer	533.2 ± 0.07 [c]	799 ± 3 [b]	1036 ± 32 [a]
40	Myricetin hexoside Isomer	213 ± 2 [c]	288 ± 2 [b]	307.8 ± 0.8 [a]
41	Vescalagin/castalagin Isomer	152 ± 3 [b]	155 ± 2 [b]	191 ± 3 [a]
42	Myricetin arabinoside/xylopyranoside Isomer	241 ± 5 [c]	286 ± 2 [b]	306 ± 5 [a]
43	Myricetin arabinoside/xylopyranoside Isomer	608 ± 1 [c]	839 ± 8 [b]	946 ± 11 [a]
44	Procyanidin gallate Isomer	11 ± 1 [a]	3.7 ± 0.2 [b]	<LOQ
45	Myricetin arabinoside/xylopyranoside Isomer	688 ± 16 [c]	816.0 ± 0.5 [b]	874 ± 9 [a]
46	Myricetin hexoside Isomer	1186 ± 13 [a]	1010 ± 3 [b]	1012 ± 65 [b]
47	Myricetin hexoside Isomer	200 ± 3 [b]	208 ± 5 [b]	224 ± 6 [a]
48	Myricetin arabinoside/xylopyranoside Isomer	276.0 ± 0.9 [a,b]	266 ± 3 [b]	282 ± 8 [a]
49	Quercetin galloylhexoside Isomer	375.0 ± 0.6 [b]	380 ± 5 [b]	438 ± 18 [a]
50	Ellagic acid deoxyhexoside	700 ± 1 [a]	702 ± 12 [a]	733 ± 32 [a]
51	Quercetin galloylhexoside Isomer	180 ± 2 [b]	194 ± 7 [a]	205 ± 1 [a]
52	Myricetin arabinoside/xylopyranoside	544.3 ± 0.4 [b]	525 ± 2 [b]	588 ± 18 [a]

No.	Compound	High	Medium	Low
	Isomer			
53	Morin	2619 ± 4 [c]	3206 ± 11 [b]	4474 ± 98 [a]
54	Myricetin arabinoside/xylopyranoside Isomer	611 ± 4 [a]	581 ± 6 [b]	559 ± 3 [c]
55	Ellagic acid	1229 ± 26 [c]	1345 ± 34 [b]	1759.6 ± 0.9 [a]
56	Hyperin	11305 ± 27 [c]	11906 ± 57 [b]	12528 ± 83 [a]
57	Quercetin glucuronide	<LOQ	<LOQ	<LOQ
58	Isoquercitrin	2254 ± 10 [b]	2471 ± 16 [b]	3410 ± 38 [a]
59	Procyanidin gallate Isomer	<LOQ	7.3 ± 0.3 [b]	73.3 ± 0.2 [a]
60	Reynoutrin	2641 ± 11 [b]	2762 ± 2 [b]	3210 ± 104 [a]
61	Guajaverin	8864 ± 8 [b]	9668 ± 64 [b]	11813 ± 64 [a]
62	Guavinoside A	783 ± 5 [a,b]	770 ± 4 [b]	793 ± 4 [a]
63	Avicularin	10353 ± 18 [a,b]	10173 ± 54 [b]	11441 ± 63 [a]
64	Quercitrin	213 ± 2 [b]	208 ± 2 [b]	223 ± 1 [a]
65	Myrciaphenone B	546 ± 6 [c]	621 ± 1 [b]	715 ± 20 [a]
66	Guavinoside C	2069 ± 1 [b]	1966 ± 21 [c]	2209 ± 21 [a]
67	Guavinoside B	872 ± 17 [c]	1035 ± 23 [b]	1273 ± 30 [a]
68	Guavinoside A Isomer	137 ± 1 [a]	135.1 ± 0.6 [a]	137 ± 3 [a]
69	Guavinoside B Isomer	120 ± 2 [b]	119,6 ± 0.2 [b]	129 ± 1 [a]
70	2,6-dihydroxy-3-methyl-4-*O*-(6″-*O*-galloyl-β-D-glucopyranosyl)-benzophenone	1179 ± 12 [b]	1242 ± 33 [b]	1365 ± 20 [a]
71	Guavin B	220.51 ± 0.03 [b]	230.1 ± 0.7 [a,b]	241 ± 7 [a]
72	Quercetin	258 ± 4 [a]	253 ± 3 [a]	255 ± 5 [a]
73	Naringenin	487 ± 3 [c]	638 ± 24 [b]	705 ± 6 [a]
	Positive mode	Concentration (µg compound/g leaf *d.w.*)		
74	Cyanidin-3-*O*-glucoside	441.28 ± 0.04 [a]	169.3 ± 0.5 [b]	29.5 ± 0.2 [c]

LOQ: limits of quantification; Means in the same line with different letter ([a] ; [b] ;[c]) are significantly different ($p < 0.05$).

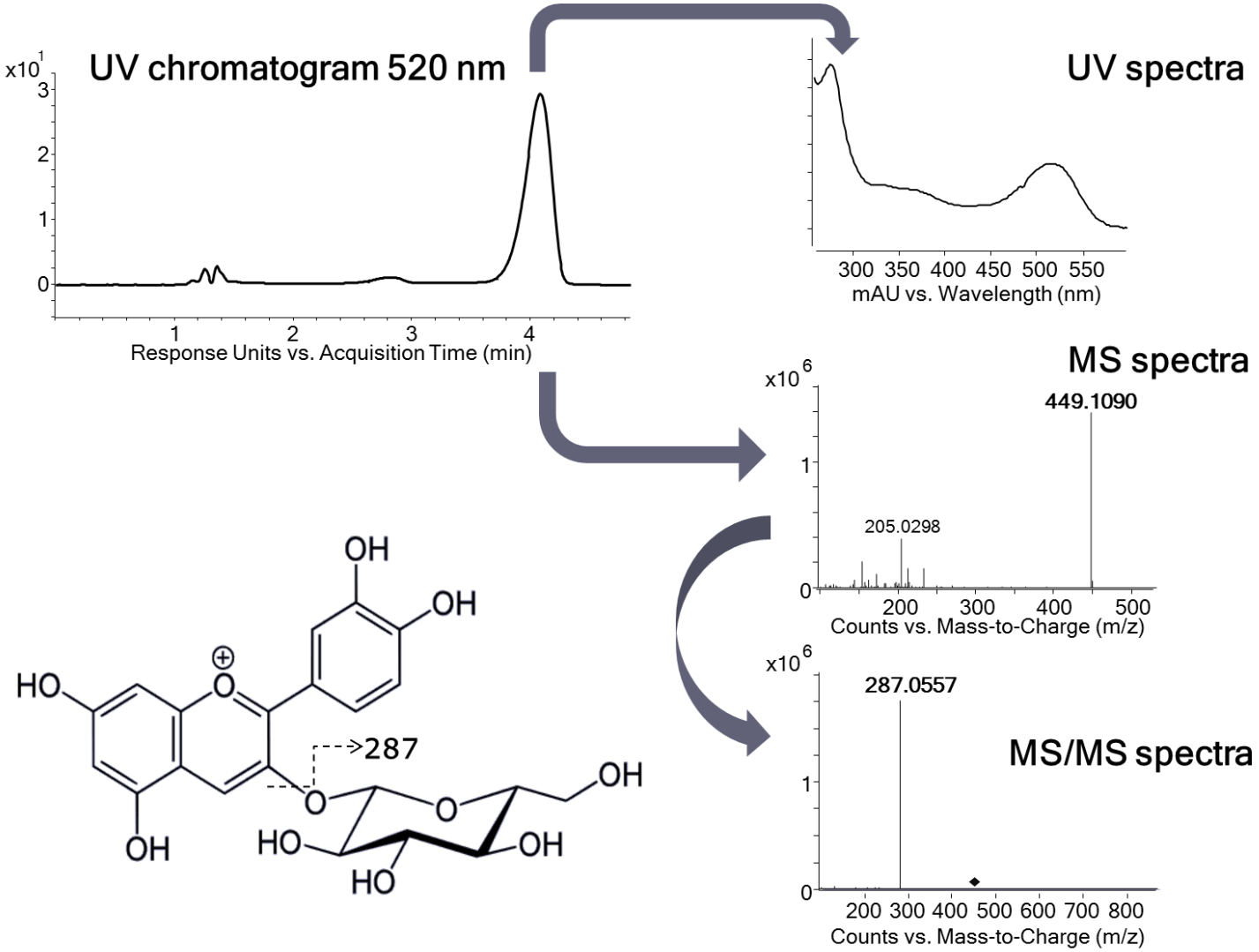

Figure 1. Fragmentation pattern of cyanidin-3-*O*-glucoside. MS/MS spectra has been obtained by auto MS/MS fragmentation.

Several works reported the quantification of some compounds such as ellagic acid, quercetin, gallic acid, catechin, and gallocatechin by HPLC-DAD in guava leaves [3,9,12,19]. Comparing the results, compounds in dried leaves are found in lower quantities than in the extract [3,12], although values in the same order of magnitude are noticed for quercetin [9] and greater amounts of catechin and gallocatechin are found comparing values with those obtained for dried leaves from Korea [19]. Furthermore, Zhu and coworkers [20] also isolated the major compounds in Chinese guava leaves: hyperoside, isoquercitrin, reynoutrin, guajaverin, avicularin, and also 2,4,6-trihydroxy-3,5-dimethylbenzophenone 4-*O*-(6″-*O*-galloyl)-β-D-glucopyranoside. Concentrations of reynoutrin, guajaverin, and avicularin isomers followed the same order as in the present leaves (reynoutrin < guajaverin < avicularin) and the opposite order was observed for the other isomers (isoquercitrin < hyperin). The compound 2,4,6-trihydroxy-3,5-dimethylbenzophenone 4-*O*-(6″-*O*-galloyl)-β-D-glucopyranoside was not detected in the present samples, in contrast, 2,6-dihydroxy-3-methyl-4-*O*-(6″-*O*-galloyl-β-D-glucopyranosyl)-benzophenone could be found. Differences noticed between these works could be because phenolic composition vary substantially among genotypes, seasons changes, ages, and damaged leaves, and location sites [7]. Regarding the different families present in leaves, extracts reported significant differences ($p < 0.05$), the lowest content of the different classes of phenolic compounds were found in leaves at the highest oxidation state, whereas the highest content was found in leaves at the lowest oxidation state (Figure 2). The major class of phenolic compounds in guava leaves samples was flavonols that ranged between 48.1 and 50.6 mg/g leaf d.w. The second class of polar compounds was represented by flavan-3-ols (24.2–24.7 mg/g leaf d.w.), followed by gallic and ellagic acid derivatives (14.8–15.8 mg/g leaf d.w.) and finally, by flavanones, that varied from 0.49 to 0.63 mg/g leaf d.w. Indeed, the contribution of reynoutrin, guajaverin, and avicularin isomers to total phenolic content (TPC) was predominant in this work, corresponding, on average, to 25% and 26% of the TPC, followed by hyperin and isoquercitrin, which supposed about 15%. Furthermore, myricetin derivatives contributed on 5% to TPC, morin and quercetin account between 3% and 5% of TPC from the different samples.

Moreover, catechin was ranged between 7% and 10%, gallocatechin isomers varied between 7% and 8% of the total amount, and procyanidin B isomers represented a 6%.

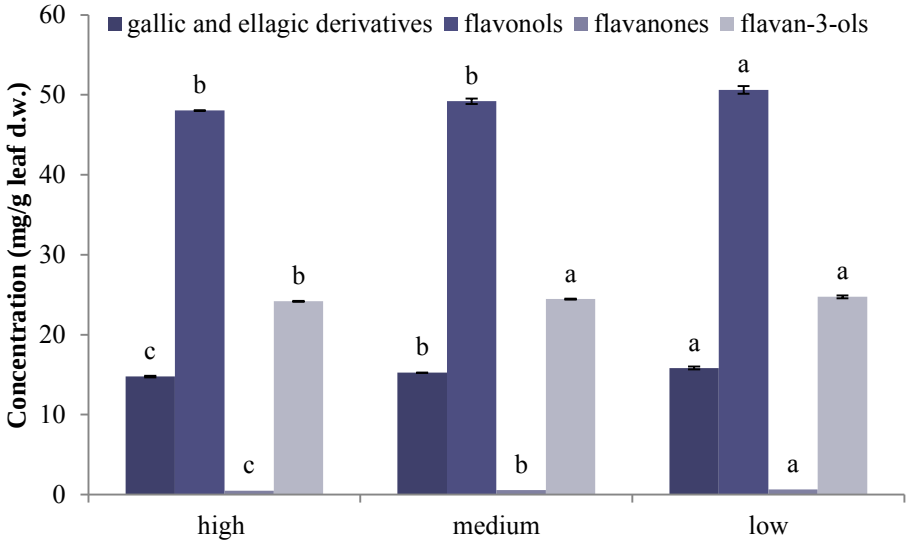

Figure 2. Quantification of different families of phenolic compounds present in guava leaves at different oxidative states. The different letter (a, b, c) in the same phenolic class means a significant difference ($p \leq 0.05$).

The variance in concentration of the individual compounds (Table 1), and the differences between the families (Figure 2) is probably resulting from the different synthesis of secondary metabolites as response to the oxidative state of the leaves [9] and it could be explained by the formation of the compounds in the flavonoid pathway [21,22]. Most of the compounds present in guava leaves derivate from dihydroquercetin, precursor of both anthocyanins and flavonols. In fact, it has been noticed that leaves with low oxidation state presented greater amounts of flavonols, such as quercetin and myricetin derivatives, whereas the contents of the flavan-3-ols catechin and gallocatechin, and the content of cyanidin-3-*O*-glucoside were lower. In contrast, the highest oxidative state showed lower amounts of quercetin and myricetin derivatives than low oxidation state, and higher concentration of catechin, gallocatechin, and cyanidin-3-*O*-glucoside than low oxidized leaves (Table 1). Chang *et al.* [16] also found for guava budding leaf tea that the presence of quercetin and its

glycosides was larger than catechins and myricetin derivatives. Moreover, the increasing concentration of cyanidin-3-*O*-glucoside explained the dramatic red coloration of the leaves at higher oxidative states [21–23].

P. guajava leaves have traditionally been used in many countries to manage, control, and treat the diabetes [24], and its potential against diabetes mellitus type 2 has also been demonstrated in several works by the different parts of the plant, such as fruit, peel, pulp, seeds, and stem bark [25–28]. Singh *et al.* [29] reported that flavonoids are one of the major chemical constituents of plant species used in the management of diabetic complications. In fact, the anti-diabetic activity has mainly been attributed to a synergistic effect of the phenolic compounds present in the leaves [30]. This effect has been observed in half ripen guava fruit for *in vitro* and *in vivo* assays [25]. Authors reported that the effect is due to the total phenolic and flavonoid content in the fruit that was 40.13 ± 2.12 and 18.43 ± 1.22 mg/g of dry weight sample, respectively. Moreover, the recovery of total phenolic compounds in the peel was 58.7 ± 4.0 g gallic acid equivalent (GAE)/kg dry matter [31]. Ribeiro da Silva and coworkers [32] reported that the phenolic contents in the pulp was 1723.06 ± 111.58 mg GAE/100 g dry basis; in the seeds varied between 14.54 and 91.05 mg total phenols (TP)/100g of defatted ground seeds in several solvents [33]; and the TPC of the stem bark, determined by Folin–Ciocalteu, was 1.15 ± 0.12 g GAE/100g dry weight [34]. These values are lower than the ones summarized in the present work, so it could be supposed that guava leaves could be better anti-diabetic agents than the other parts of the plant.

Comparing the phenolic content of guava leaves to other plant leaves, different evidence has been found. The ethanol extract from *Telfairia occidentalis* leaves has also exhibited anti-hyperglycemic activity and has demonstrated strong inhibition of α-glucosidase and mild inhibition of α-amylase [35]. Authors related its activity to the phenolic and flavonoid content; however, its content was less than three order of magnitude than that reported in the present work. Additionally, other plants such as *Teucrium polium*, cinnamon and garlic, have generally presented lower phenolic

contents than guava leaves [36–38] are also widely used in folk medicine for the treatment of diabetes [25]. In fact, different extracts from *Teucrium polium* (leaves, flowers, and stems) reported a TPC between 14.6 to 157.8 mg of GAE/g of extract [36], different parts of *Cinnamomum cassia* (barks, buds, and leaves) exhibited values for TPC from 6.3 to 9.5 g/100 g d.w. [37], and 3.4–10.8 mg GAE/g d.w. was the range of TPC found for different cultivars of garlic [38]. Additionally, several individual compounds isolated from different sources that have also been found in guava leaves have demonstrated anti-diabetic properties (Table 2). The principal activities related to these compounds are the inhibition of carbohydrate-hydrolysing enzymes due to the presence of myrciaphenone B [39], casuarictin and tellimagrandin I [40], flavonol glycosides (hyperin, isoquercitrin, reynoutrin, guajaverin, avicularin) [30,41], geraniin and catechin [42], quercetin [30], and cyanidin-3-*O*-β-glucoside [43]. Insulinomimetic activity has been attached to casuarinin, casuariin [40], procyanidin oligomers [44], and pedunculagin [45]. Moreover, geraniin, vescalagin, gallic acid, naringenin, morin, quercetin, catechin, epicatechin, and procyanidin B2 [42] exhibited anti-glycation activity, due to the inhibition of the formation of Amadori products and advanced glycation end-products (AGEs). At last, the improvement of postprandial hyperglycemia has been related to catechin and gallocatechin, among others [46].

Table 2. Guava leaves' bioactive compounds related with anti-diabetic properties.

Compound	Assay	Activity	Ref.
Myrciaphenone B	*in vivo*	Inhibition of aldose reductase α-glucosidase	[39]
Casuarictin, tellimagrandin I	*in vitro*	Inhibition of α-glucosidase	[40]
Cyanidin-3-*O*-β-glucoside	*in vitro/ in silico*	Inhibition of α-amylase	[43]
Flavonol glycosides	*in vitro*	Inhibition of dipeptidyl-peptidase IV, and α-glucosidase and α-amylase	[30,41]
Geraniin	*in vitro*	Hypoglycemic activity; inhibition of carbohydrate-hydrolysing enzymes (α-glucosidase and α-amylase); effective in preventing advanced glycation end-products (AGEs) formation	[42]
Vescalagin	*in vivo*	Retard AGEs formation	[42]
Gallic acid	*in vitro*	Inhibitory effect on the formation of α-dicarbonyl compounds and protein glycation: inhibitory effects on the production of Amadori products and AGEs	[3,42]

Compound	Assay	Activity	Ref.
Naringenin	*in vitro*	Anti-glycation activity	[42]
Morin	*in vitro*	Protective activity against glycation	[42]
Quercetin	*in vitro*	Inhibitory effect on protein glycation, on the formation of α-dicarbonyl compounds, and on the production of Amadori products and AGEs	[3,30,42]
Catechin	*in vitro/ in vivo/ clinical trial*	Inhibitory effect on the formation of α-dicarbonyl compounds and protein glycation: inhibitory effects on the production of Amadori products and AGEs; improvement of postprandial hyperglycaemia	[42,46]
Procyanidin B2	*in vitro/ in vivo*	Inhibitory effects on the formation of AGEs	[42]
Casuarinin, casuariin	*in vitro/*	Inhibition of insulin-like glucose uptake	[40]
Procyanidin oligomers	*in vitro/ in vivo*	Insulinomimetic properties	[44]
Pedunculagin	*in vivo*	Improvement sensitivity of insulin	[45]
Gallocatechin	*clinical trial*	Improvement of postprandial hyperglycaemia	[46]

2.2. Antioxidant capacity and total phenolic content

The evaluation of the antioxidant capacity is usually done comparing different methods in order to take into account the large number of factors that can influence the antioxidant action [47]. The choice of the two methods used in this work were assessed based on their different mechanisms: Trolox Equivalent Antioxidant Capacity (TEAC) assay estimates the ability to scavenge 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) radicals and ferric reducing capacity is evaluated by the Ferric Reducing Antioxidant Power (FRAP) method. As is shown in Table 3, significant differences have been found among the different oxidation states ($p < 0.05$), the lowest oxidation states reported the highest values for TEAC and FRAP (3.1 ± 0.1 mM eq Trolox/mg leaf *d.w.* and 5.4 ± 0.1 mM $FeSO_4$/mg leaf *d.w.*, respectively) and decrease as the oxidation state increases. Comparing the results with those reported by Tachakittirungrod and coworkers [48] for guava leaves, similar values were accomplished for TEAC and higher values for FRAP. This can be due to the fact that phenolic compounds in Spanish leaves are more involved in the mechanism of reduction of oxidized intermediates in the chain reaction.

Moreover, high correlation was found between the antioxidant assays (r = 0.9978 and $p < 0.001$), in concordance with the data obtained for guava leaves [49], fruits [50], and in 30 plant extracts of industrial interest [51]. However, TEAC value seems to be higher than FRAP value for guava leaves [14,12,48].

Table 3. Comparison (mean ± SD, n = 3) among total phenolic content (TPC) by HPLC-DAD-ESI-QTOF-MS, Trolox Equivalent Antioxidant Capacity (TEAC) and Ferric Reducing Antioxidant Power (FRAP) of *Psidium guajava* L. leaves at different oxidative states.

Oxidation State	TPC (mg/g leaf *d.w.*)	TEAC (mM eq Trolox/mg leaf *d.w.*)	FRAP (mM $FeSO_4$/mg leaf *d.w.*)
High	87.91 ± 0.05 [c]	2.2 ± 0.2 [c]	3.69 ± 0.03 [c]
Medium	92.0 ± 0.4 [b]	2.44 ± 0.05 [b]	4.20 ± 0.06 [b]
Low	103 ± 2 [a]	3.1 ± 0.1 [a]	5.4 ± 0.1 [a]

Means in the same column with different letter ([a, b, c])are significantly different ($p < 0.05$).

Quantification of total phenolic compounds by HPLC-DAD-ESI-QTOF-MS revealed that the three extracts showed significant differences ($p < 0.05$) as is displayed in Table 3. The lowest oxidation state provided the highest content of total phenolic compounds (103 ± 2 mg/g leaf d.w.), followed by the medium and the highest oxidation state (92.0 ± 0.4 and 87.91 ± 0.04 mg/g leaf d.w., respectively). The values obtained were lower than those reported by several authors that employed Folin–Ciocalteu method to quantify total phenolic compounds [12,14]. Even though the variance noticed is not great, it could be because the determination by HPLC presented only 50–60 percentage of total phenolic content [12]. Additionally, high correlation among TPC by HPLC, FRAP, and TEAC assays were found in the present work. In fact, positive correlation with r = 0.9921 ($p < 0.001$) was noticed between TPC by HPLC and FRAP and r = 0.9867 ($p < 0.001$) for TPC by HPLC and TEAC. Good correlations were also found between TPC by Folin–Ciocalteu and antioxidant activity was also noticed for guava leaves in literature [14,12,48].

3. Materials and methods

3.1. Plant material and sample preparation

Fresh guava leaves were harvested in Motril, Spain (36°44′43″N 3°31′14″W). The leaves were collected in February 2014 at different oxidation states (low, medium, and high) based on the difference of leaf color according to Hao [8]. The samples were air-dried at room temperature, ground, and extracted with ethanol:water 80/20 (v/v) by ultrasonics as was previously reported by Díaz-de-Cerio *et al.* [18].

3.2. Antioxidant capacity analysis

Trolox Equivalent Antioxidant Capacity (TEAC) and Ferric Reducing Antioxidant Power (FRAP) analysis were used to measure the antioxidant capacity.

For TEAC, ABTS radical cation was generated by reacting ABTS stock solution with 2.45 mM potassium persulfate in the dark at room temperature for 12–24 h before use. A calibration curve was prepared with different concentrations of Trolox (0–20 μM). The absorbance of ABTS radical cation was adjusted to 0.70 (± 0.02) at 734 nm, and its change was measured [52].

To evaluate the reducing power, FRAP reagent (containing 2,4,6-tripyridyl-S-triazine (TPTZ), $FeCl_3$ and acetate buffer) was prepared. An aqueous solution of Fe (II) was used for calibration (12.5–200 μM). The reduction was measured at 593 nm [53].

Results are expressed as mM eq Trolox/mg leaf d.w. and mM $FeSO_4$/mg leaf d.w., respectively.

3.3. HPLC-DAD-ESI-QTOF-MS analysis

Chromatographic analyses were performed using an HPLC Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, an online degasser, an autosampler, a thermostatically-controlled column compartment, and a UV-VIS diode array detector (DAD). The column was maintained at 25 °C.

Phenolic compounds from *P. guajava* L. leaves were separated using a method previously reported by Gómez-Caravaca *et al.* [54], in positive mode, slightly modified. Briefly, a fused-core Poroshell 120, SB-C18 (3.0 mm × 100 mm, 2.7 μm) from Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA) was used. The mobile phases were water plus 1% acetic acid (A) and acetonitrile (B). A multi-step linear gradient was applied as follows; 0 min, 5% B; 2 min, 7% B; 4 min, 9% B; 6 min, 12% B; 8 min, 15% B; 9 min, 16% B; 10 min, 17% B; 11 min, 17.5% B; 12 min, 18% B; 13 min, 100% B; 17 min, 100% B; 18 min, 5% B. The initial conditions were maintained for 5 min. The sample volume injected was 3 μL and the flow rate used was 0.8 mL/min.

MS analyses were carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass Q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface, at the following conditions: drying gas flow (N_2), 12.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 370 °C; capillary voltage, 3500 V; fragmentor voltage and scan range were 3500 V and *m/z* 50-1700. In positive mode, auto MS/MS experiments were carried out using the followings collision energy values: *m/z* 100, 40 eV; *m/z* 500, 45 eV; *m/z* 1000, 50 eV; and *m/z* 1500, 55 eV.

Additionally, phenolic compounds were also analyzed in negative mode using the chromatographic and the detection method described by Díaz-de-Cerio *et al.* [18].

Standard calibration curves for cyanidin-3-*O*-β-galactopyranoside, gallic acid, catechin, ellagic acid, naringenin, and rutin were prepared in the range of concentrations from the limit of quantification (LOQ) to 50 mg/L and five calibration points for each standard were run in triplicate ($n = 3$).

Integration and data elaboration were performed using MassHunter Workstation software (Agilent Technologies).

3.4. Statistical analysis

The results reported in this study are the averages of three repetitions (n = 3). Fisher's least significance difference (LSD) test and Pearson's linear correlations, both at $p < 0.05$, were evaluated using Statistica 6.0 (2001, StatSoft, Tulsa, OK).

4. Conclusions

HPLC coupled to QTOF-MS detector, which provides a molecular formula and the MS/MS data, permitted the analysis of the major phenolic compounds of guava leaves. The method performed in negative mode has proven to be successful in determining 73 compounds in the different guava leaves. Moreover, in positive mode, the analysis with QTOF analyzer and the co-elution with a standard solution allowed the identification of the cyanidin-glucoside. To our knowledge the cyanidin-glucoside, was identified for the first time in guava leaves. Quantification data, in negative mode, reported that leaves with low oxidation state presented the highest concentration of these compounds and decreased when the oxidation state raise. On the contrary, the state of oxidation affected significantly the cyanidin content. In fact, highest amount was detected in the leaves with high oxidation state.

Guava leaves seem to be a good source of phenolic compounds with described anti-diabetic properties since several compounds present in the leaves have been related for ameliorating the effects of diabetes mellitus disease, although this content varies due to the oxidative state of the leaf, so further studies should be carried out in order to evaluate the influence of the phenolic composition on the bioactivity of the extract.

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Conflicts of Interest

The authors declare no conflict of interest.

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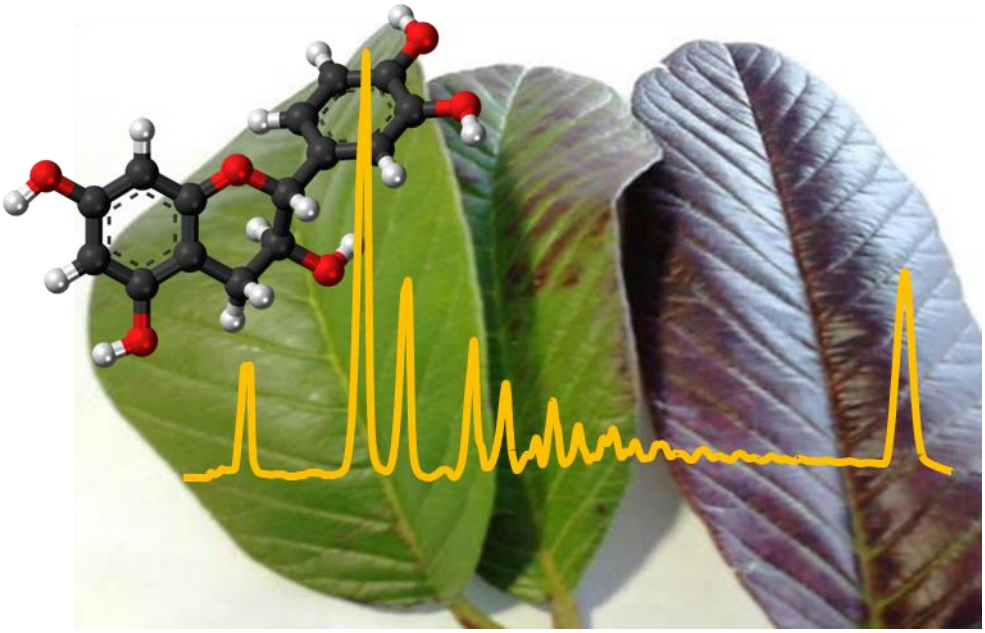
CHAPTER 4



Psidium guajava L. leaves as source of proanthocyanidins: a study of the degree of polymerization in different guava leaves by NP-HPLC-FLD-ESI-MS

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Psidium guajava L. leaves as source of proanthocyanidins: Optimization of the extraction method by RSM and study of the degree of polymerization by NP-HPLC-FLD-ESI-MS

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Abstract

Due to the importance of the proanthocyanidins (PAs) bioactivity and its relationship with the PAs degree of polymerization (DP), an experimental design was carried out to establish the best extraction conditions in order to evaluate the proanthocyanidins content and their degree of polymerization in *Psidium guajava* L. leaves at different oxidation state. Optimal conditions achieved by response surface methodology (RSM) were 50% acetone/water (v/v), 48 ºC, 30 min, and 0% acetic acid (v/v). The highest DP has been found in the low oxidized state (DP 13 plus the polymers). Medium and high oxidized state leaves reported a DP 11 plus the polymers. The total amounts of proanthocyanidins (sum of PAs by HPLC-FLD-ESI-MS) decreased when oxidation state of leaves increased (15.8 ± 0.4, 12.6 ± 0.4, and 10.5 ± 0.3 mg /g leaf dry weight (d.w.) in low, medium and high oxidized state leaves, respectively). Guava leaves present an interesting source of low DP-PAs.

Keywords: Guava (*Psidium guajava* L.) leaves, proanthocyanidins, oligomers ultrasound assisted extraction, response surface methodology, NP-HPLC-FLD-ESI-MS.

1. Introduction

Psidium guajava L., a tree native from Mexico, has been widely used in folk medicine. Its leaves have gain attention since they exhibited higher amounts of phenolic compounds than the other parts of tree [1]. A recent study of the leaves [2], showed the presence of some proanthocyanidins (PAs) oligomers in *pyrifera* and *pomifera* varieties, although some of them were unable to be quantified; this could be due to the unselective extraction method and/or to the chromatographic and detection conditions used. Furthermore, it has been demonstrated that the phenolic composition in guava leaves, especially of flavonols, flavan-3-ols and gallic and ellagic acid derivatives, changes as a result of its phenological stages [3,4]. In addition, these alterations cause an oxidative change in leaves resulting in a color change [5].

PAs are compounds formed by chemical bond of monomeric flavan-3-ol units, leading to different classes commonly formed by (epi)-catechin units (procyanidins) and less common by (epi)-afzelechin (propelargonidins) or (epi)-gallocatechin (prodelphinidins) units (Fig. 1) [6].

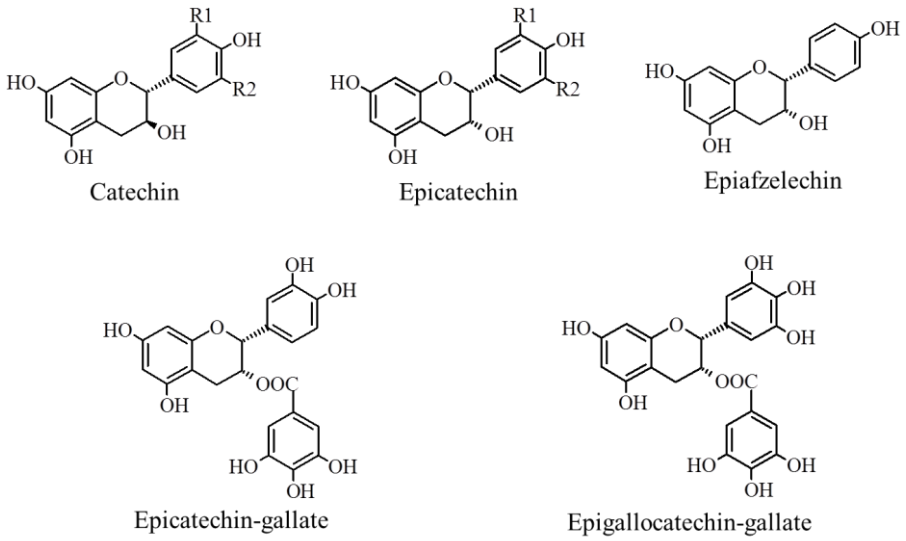

Figure 1. Principal flavan-3-ols units forming procyanidins ((epi)-catechin oligomers), propelargonidins ((epi)-afzelechin + (epi)-catechin monomers); prodelphinidins ((epi)-gallocatechin and/or (epi)-gallocatechin-gallate + (epi)-catechin monomers)

PAs have an important number of pharmacological effects [6,7], being the anti-diabetic and anti-obesity activity some of the most interesting actions [8,9]. Actually, analysis of PAs to extract maximum amount of these substances and isolation in pure form from natural sources, as from plant extracts are still being done due to the necessity for further uses in food and nutraceutical [10–12]. In spite of this fact, the technique most used for the extraction is called one-variable-at-a-time and there is a lack of literature concerning about multivariate optimization strategies such as response surface methodology (RSM) for PAs, a tool that allows the evaluation of the variables of the process as well as the interaction effects between them via statistical analysis of a mathematical model [13], although it has been applied for the extraction of phenolic compounds in several matrices, such as *Coriandrum sativum* seeds [14] and blackberry leaves [15].

Taking into account these premises, RSM was employed to optimize the extraction conditions of PAs via sonication in *P. guajava* L. leaves. Moreover, best extraction conditions were tested on guava leaves, var. *pyrifera*, at different oxidations states (low, medium and high) to find out the source with highest amounts of the target compounds for further uses as nutraceuticals.

2. Materials and methods

2.1 Chemicals and plant material

HPLC-grade solvents: acetonitrile, water, methanol, and acetone were purchased from Merck (Darmstadt, Germany). Double-deionised water was from Millipore (Bedford, MA, USA). Catechin and procyanidin B2 and other reagents were from Sigma-Aldrich (St. Louis, MO).

Commercial *Psidium guajava* L. leaves were used for the optimization of proanthocyanidins extraction. Afterwards, fresh *P. guajava* L. var. *pyrifera* leaves were harvested in Motril (Spain) (36°44′43″N 3°31′14″W). The leaves have been collected in December 2014 at different oxidation states (low, medium, and high)

based on the leave color [5]. Then, the leaves were air-dried at room temperature, powdered, and stored at -20°C until extraction.

2.2 Experimental design

Experimental conditions of extraction to recover PAs from *P. guajava* L. leaves were selected by Box-Behnken design (BBD), which is considered to be more efficient than other designs and allows avoiding extreme situations [13]. The complete design consisted on 27 experimental runs, arranged in 3 orthogonal blocks, with three levels (-1, 0, 1) for each factor, in order to normalize parameters, and three center points, and was applied to evaluate the effects of acetone/water ratio (% (v/v)) (X_1), temperature of the bath (ºC) (X_2), time (min) (X_3) and acetic acid percentage (% (v/v)) (X_4) on ultrasound assisted extraction (UAE). The coded and natural values of the factors are shown in Table 1.

Table 1. BBD with natural and coded values for PAs extraction conditions and experimental values for SPAs quantified by NP-HPLC-FLD.

Run	Block	Independent factors				Response
		X_1	X_2	X_3	X_4	SPAs (mg/ g leaf d.w.)
1	1	40 (-1)	20 (-1)	17.5 (0)	0.5 (0)	14.4
2	1	100 (1)	20 (-1)	17.5 (0)	0.5 (0)	6.0
3	1	40 (-1)	50 (1)	17.5 (0)	0.5 (0)	21.7
4	1	100 (1)	50 (1)	17.5 (0)	0.5 (0)	5.8
5	1	70 (0)	35 (0)	5 (-1)	0 (-1)	18.6
6	1	70 (0)	35 (0)	30 (1)	0 (-1)	24.0
7	1	70 (0)	35 (0)	5 (-1)	1 (1)	15.9
8	1	70 (0)	35 (0)	30 (1)	1 (1)	16.5
9	1	70 (0)	35 (0)	17.5 (0)	0.5 (0)	16.7
10	2	40 (-1)	35 (0)	17.5 (0)	0 (-1)	14.6
11	2	100.0	35 (0)	17.5 (0)	0 (-1)	5.8
12	2	40 (-1)	35 (0)	17.5 (0)	1 (1)	17.0
13	2	100 (1)	35 (0)	17.5 (0)	1 (1)	6.4
14	2	70 (0)	20 (-1)	5 (-1)	0.5 (0)	15.2
15	2	70 (0)	50 (1)	5 (-1)	0.5 (0)	21.8
16	2	70 (0)	20 (-1)	30 (1)	0.5 (0)	15.4

Run	Block	Independent factors				Response
		X_1	X_2	X_3	X_4	SPAs (mg/ g leaf d.w.)
17	2	70 (0)	50 (1)	30 (1)	0.5 (0)	16.2
18	2	70 (0)	35 (0)	17.5 (0)	0.5 (0)	16.5
19	3	40 (-1)	35 (0)	5 (-1)	0.5 (0)	13.3
20	3	100 (1)	35 (0)	5 (-1)	0.5 (0)	4.4
21	3	40 (-1)	35 (0)	30 (1)	0.5 (0)	15.1
22	3	100 (1)	35 (0)	30 (1)	0.5 (0)	6.5
23	3	70 (0)	20 (-1)	17.5 (0)	0 (-1)	15.4
24	3	70 (0)	50 (1)	17.5 (0)	0 (-1)	20.8
25	3	70 (0)	20 (-1)	17.5 (0)	1 (1)	15.5
26	3	70 (0)	50 (1)	17.5 (0)	1 (1)	13.4
27	3	70 (0)	35 (0)	17.5 (0)	0.5 (0)	16.2

$X_{1\text{-}4}$: acetone/water ratio (% v/v), temperature of the bath (ºC), time (min), and acetic acid percentage (% v/v).

The response variables were fitted to a second-order polynomial model equation (Eq. (1)) estimated by the RSM:

$$\Upsilon = \beta_0 + \sum_{i=0}^{4} \beta_i \chi_i + \sum_{i=0}^{4} \beta_{ii} \chi_{ii}^2 + \sum_{i=0}^{4} \sum_{j=0}^{4} \beta_{ii} \chi_i \chi_j$$

where Y represents the response variable, sum of proanthocyanidins (SPAs via NP-HPLC-FLD), X_i and X_j are the independent factors affecting the response, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the model (intercept, linear, quadratic and interaction term).

The model building, experimental results and designs were processed using Statistica 7.0 (2002, StatSoft, Tulsa, OK). An analysis of variance (ANOVA) with 95% confidence level was carried out for the response variable. The suitability of the regression model was evaluated by the regression coefficient (R^2), the predicted variation (Q^2), the *p*-value of the regression model and the *p*-value of the lack of fit (LOF). The significance of the model was evaluated with *p*-value obtained for the intercept, linear, quadratic and interaction terms of the model. Optimal conditions were chosen considering the response surfaces (3D plots).

2.3 Ultrasound assisted extraction

PAs were extracted by UAE from guava leaves according to a previous work carried out in our laboratory (data not shown) a solid to solvent ratio of 1:80 (w/v) was established for the experiments, and the extractions were accomplished in an Elmasonic S 120 (H) bath, with a frequency and power fixed at 37 kHz and 200 W, respectively. The other variables that affect the extraction process: acetone/water ratio (40 - 100 % (v/v)) (X_1), temperature of the bath (20 - 50 ºC) (X_2), time (5 - 30 min) (X_3) and acetic acid percentage (0 - 1 % (v/v)) (X_4) were fixed taking into account the experimental design (Table 1). Moreover, optimal conditions reached by RSM (50 % acetone/water (v/v), 48 ºC, 30 min, and 0 % acetic acid (v/v)) were employed to compare *P. guajava* L. leaves var. *pyrifera* at three oxidative states (low, medium and high).

2.4 Determination of proanthocyanidins via NP-HPLC-FLD-ESI-MS analysis

The material used in this work for the separation, detection and quantification was the same as Verardo et al. [16]. The injection volume was 10 µL and the gradient elution was the same as reported by Robbins and coworkers [17]. Calibration curves of (+)-catechin and procyanidin B2 were arranged from LOQ to 500 and from LOQ to 125 mg/L, respectively, at six concentration levels for each compound. The maximum concentration of calibration curves were established taken into account the linearity.

In order to consider the matrix effect, the calibration curves were made in the matrix; briefly, the standard solutions were added in a guava leaf extract and the calibration curves were calculated. LOD and LOQ of catechin and procyanidin B2 were 0.051 and 0.169 mg/L, and 0.036 and 0.121 mg/L, respectively.

The repeatability of the method was assessed for an extract obtained from low oxidized guava leaf. The extract was injected 3 on the same day (intraday precision) and for three consecutive days (interday precision, n=9). The percent relative standard

deviations (% RSD) of the peak areas and retention times were determined for each peak detected.

The intraday repeatability (expressed as % RSDs) of the retention times was from 0.11 to 0.61 %, whereas the interday repeatability was from 0.28 to 0.86 %.

The intraday repeatability (expressed as % RSDs) of the total peak area was from 0.58 to 1.24%, whereas the interday repeatability was from 1.12 to 3.04 %.

The intraday and interday precision (expressed as RSD %) of each peak are showed in Table 2.

Correction factors suggested by Prior and Gu [18] were used to quantify the oligomer proanthocyanidins. Results for quantification of SPAs are expressed as mg/g leaf dry weight (d.w.).

Table2. Intraday and interday precision (RSD %) evaluated on retention time (RT) and peak area of low oxidized guava leaf extract

Peak	Intraday RT	Interday RT	Intraday area	Interday area
1a	0.21	0.39	0.85	1.64
1b	0.18	0.28	0.76	1.12
2a	0.23	0.37	0.91	1.49
2b	0.11	0.30	1.06	2.03
2c	0.19	0.42	1.20	1.96
3a	0.24	0.49	0.77	1.24
3b	0.31	0.57	0.94	1.47
3c	0.42	0.69	0.83	1.89
4a	0.29	0.44	1.03	2.22
4b	0.36	0.52	1.10	2.46
4c	0.54	0.81	0.90	1.85
5a	0.47	0.77	0.85	1.73
5b	0.22	0.48	0.68	1.64
6a	0.51	0.64	0.95	2.14
6b	0.39	0.59	0.81	2.09
7a	0.28	0.46	0.76	1.57
7b	0.19	0.37	1.04	2.60
8a	0.61	0.86	1.10	2.81
8b	0.44	0.77	0.84	1.84
9a	0.37	0.53	0.72	2.42
9b	0.60	0.81	1.09	2.73

Peak	Intraday RT	Interday RT	Intraday area	Interday area
10a	0.29	0.45	1.15	3.04
10b	0.48	0.74	1.24	2.43
11	0.36	0.61	0.96	1.99
12	0.27	0.52	0.58	1.68
13	0.35	0.73	0.67	2.34
n	0.20	0.46	0.81	2.17

2.5 Statistical analysis

The results related to the second part of the study on different oxidation states of guava leaves are the averages of three repetitions (n=3). Fisher's least significance difference (LSD) test was evaluated using Statistica 7.0 (2002, StatSoft, Tulsa, OK).

3. Results and Discussion

Extraction is the most important step to recover the highest amount of the target compounds. To our knowledge, the extraction of monomeric and oligomer PAs is generally done with anhydrous organic solvents, which only can remove low molecular mass compounds, with water, where high molecular mass compounds are partially insoluble, or with aqueous organic solvents [6], particularly, aqueous acetone. This last solvent mixture usually provides a better recovery of PAs [19,20] in proportions from 50:50 to 80:20 of acetone/water (v/v) [21]. Besides, ratios lower than 40% of acetone were not tested since it was reported to yield less amounts of procyanidins [22]. Thus, acetone/water ratio was selected as the most important factor to optimize in UAE of PAs, and varied from 40 to 100% of acetone.

Likewise, temperature and extraction time also affected the yield of chemical extraction. Wissam et al. [11] evaluated these single factors in pomegranate peel and found that temperature for phenolic compounds and PAs should range from 20 to 50 ºC because higher values cause degradation of the compounds. The authors also investigated the extraction time at 50 ºC and concluded that times longer than 30 minutes caused a decrease on the yield. Therefore, to carry out the study of the

combined effect of these variables, the following ranges were selected; temperature was set between 20 and 50 ºC and the extraction time was established from 5 to 30 minutes.

Furthermore, acidified acetone has been employed for the extraction of PAs [6], so the effect of acetic acid percentage (0 - 1 % (v/v)) was tested in the present work.

3.1 Fitting the model

A RSM approach based on a BBD was purposed in order to optimize the extraction of PAs and to determine the combined effect of acetone/water ratio (X_1), temperature of the bath (X_2), time (X_3), and acetic acid percentage (X_4). The experimental values obtained for the quantification of the sum of PAs (SPAs) via NP-HPLC-FLD for each run are presented in Table 1.

The observed values ranged from 4.4 to 24.0 mg/g leaf d.w. which corresponded to the following conditions of extraction: 100% (v/v) of acetone, 35 ºC, 5 min, and 0.5% (v/v) of acetic acid, and 70% (v/v) of acetone, 35 ºC, 30 min, 0% (v/v) of acetic acid, respectively. In fact, every run at 100% (v/v) of acetone reported the lowest recovery of PAs, in concordance with Counet and Collin [23] who found that pure acetone yielded a much lower content.

The experimental data (Table 1) were analyzed via RSM using an approach called least squares method (LSM), a regression model that provides the lowest residual value [24], and fitted to a second-order polynomial equation (Eq. (1)) using for this the regression coefficients that are listed in Table 3. On the basis of Fisher test, with a level of significance (α) of 0.05, most of the single factors, interactions between them and their cross-products reported a significant effect ($p < 0.05$) on the response variable, being both the linear and quadratic term of acetone/water ratio (X_1) the most influent, succeeded by the linear term of temperature of the bath (X_2) and the following cross-products: β_{12}, β_{23}, β_{24}, β_{34}. At higher α ($0.05 < \alpha < 0.06$) one term, β_{14},

appeared to be significant ($p < 0.05$), and β_4, β_{13} and β_{33} had no effect at the significances chosen.

After discarding the non-significant terms at $p < 0.05$, the predicted model was recalculated only with significant terms and was tested by ANOVA and the data are summarized in Table 4 (data for β_4, β_{13} and β_{33} are included from the previous model). The validity of the model is generally accepted if it presented a non-significant LOF and a significant regression [24]. As can be seen, the present model revealed a high correlation between the factors and the response variable ($R^2 = 0.997$; $Q^2 = 0.961$) and a good fit to the regression model ($p < 0.05$). Moreover, insignificant effect of LOF ($p > 0.05$) confirmed the adequacy of the predicted model.

Table 3. Estimated regression coefficients of the fitted second-order polynomial equation.

Regression coefficients	Response			
	Coefficients	Standard error	t- value	*p*-value
β_0^*	13.36131	0.11500	116.19003	<0.00001
Linear				
β_1^*	-5.58750	0.13148	-42.49748	<0.00001
β_2^*	2.14167	0.18594	11.51815	0.00003
β_3^*	-0.75000	0.18594	-4.03359	0.00685
β_4	0.13333	0.12583	1.05963	0.40037
Cross product				
β_{12}^*	-1.87500	0.18594	-10.08398	0.00006
β_{13}	0.07500	0.12583	0.59604	0.61162
β_{14}^{**}	-0.45000	0.18594	-2.42016	0.05185
β_{23}^*	-1.45000	0.18594	-7.79828	0.00023
β_{24}^*	-1.87500	0.18594	-10.08398	0.00006
β_{34}^*	-1.20000	0.18594	-6.45375	0.00066
Quadratic				
β_{11}^*	3.00265	0.09575	31.36090	<0.00001
β_{22}^*	-0.34886	0.08864	-3.93562	0.00766
β_{33}	-0.12292	0.07777	-1.58061	0.25476
β_{44}^*	-0.29174	0.07862	-3.71075	0.00996

*, ** Significant at 0.05 and 0.06 level

Table 4. Analysis of variance of the fitted model.

	Sum of Squares	*Degree of freedom*	Mean Square	F- value	*p*- value
Model (R^2 = 0.997; Q^2 = 0.961)					
Regression	737.6957	18	40.9831	149.6280	<0.0001
Residuals	2.1910	8	0.2739		
Lack of fit	2.0643	6	0.3441	5.4320	0.1636
Pure error	0.1267	2	0.0633		
Total	739.8867	26			

3.2 Optimization of PAs extraction conditions

Taking into account the validity of the model predicted optimization of the conditions for proanthocyanidins extraction was performed. The software used to analyze the statistic did not provide the critical values for the model predicted. Thus, to obtain the optimal conditions to maximize the PAs value, a compromise between the minimum possible values of each independent factor has to be established. In this regard, and to understand better the influence of independent factors on SPAs, three dimensional response surfaces were plotted fixing at middle point the other factors (Fig. 2). Fig. 2a represents the combined effect between acetone/water ratio (X_1) and temperature of the bath (X_2) showing that SPAs decreases when the amount of acetone increases because of the negative coefficient (Table 3), while the positive term for X_2 results in an increase of SPAs, as it was reported in previous works [11,23]. In addition, the positive influence of the quadratic term of X_1 and the negative interaction between X_1 and X_2 produces a maximum range at temperatures higher than 40 ºC and a content of acetone lower than 60% (v/v). According to the visual analysis for acetone/water ratio (X_1) - time (X_3) and acetone/water ratio (X_1) - acetic acid percentage (X_4), similar conclusions could be made due to the same shape of the plots (Fig. 2b and c). Both situations provided a saddle point resulting from the low effect of the terms X_3 and X_4 compared to the values obtained for X_1 and since some of them were not significant (Table 3), that is why high recovery of PAs could be achieved in these cases between 40 and 80% (v/v) of acetone through the full range of these

factors, although is preferable larger times and low amounts of acid to recover the maximum PAs.

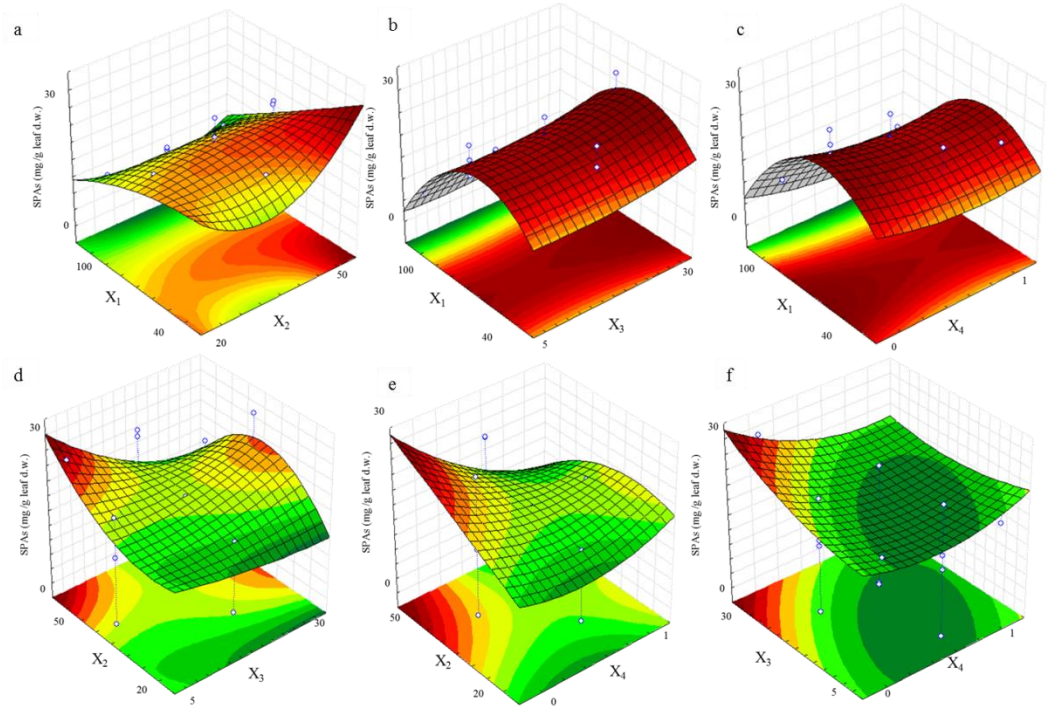

Figure 2. Response surface plots showing combined effects of process variables: acetone/water ratio (% (v/v)) - temperature of the bath (ºC) (a); acetone/water ratio (% (v/v)) - time (min) (b); acetone/water ratio (% (v/v)) - acetic acid percentage (% v/v) (c); temperature of the bath (ºC) - time (min) (d); temperature of the bath (ºC) - acetic acid percentage (% v/v) (e); time (min) - acetic acid percentage (% v/v) (f).

Regarding the combined effect of temperature of the bath (X_2) with time (X_3) and acetic acid percentage (X_4) (Fig. 2d and e, respectively), it could be seen that higher amounts of PAs could be obtained, on one hand, at the highest values of X_2 as a result of the positive influence of the regression coefficient (Table 3), and on the other hand, at lowest values of X_3 and X_4, since the interaction effects are negative. However, a second maximum seems to be possible for the effect between X_2 and X_3 as previously observed in the extraction of flavonoids from coriander seeds [14]. Despite it should be further studied, earlier research reported that times longer than 30 min caused PAs degradation [11]. About the X_2 - X_3 interaction, the negative effect could be due to the degradation of the compounds upon heat treatment and acidic pH [25]. Finally, Fig. 2f clearly shows a minimum as a result of the term β_{34}. As a consequence,

large values of SPAs are obtained at the highest level of X_3 and the lowest for X_4, concluding that the content of acetic acid should be kept at the minimum, because despite the acid increased the polarity, its sensitiveness towards the inter-flavanoid bond could cause structural modifications [6].

These observations made possible to fit the best conditions as follows: 50% (v/v) of acetone, 48 ºC, 30 min and 0% (v/v) of acetic acid, which provided a predicted value of 25 ± 5 mg/g leaf d.w. for SPAs. In addition, verification of the predicted model was done employing these conditions; observed value (20.9 ± 0.2 mg/g leaf d.w.) did not reported significant differences ($p < 0.05$) compared to the predicted value.

Several authors have reported similar conditions for PAs extraction, for example, the best acetone content achieved was in agreement with Dailey and Vuong [26], and in contrast, they found that temperature and time were no significant in the PAs extraction. However, 40 ºC and 35 min were proposed as best conditions at 200 W, although temperatures between 40 - 50 ºC reported higher content of PAs, and extraction time between 30 - 40 min showed non-significant differences.

3.4 PAs composition of guava leaves at different oxidative states

Extraction at the optimized conditions was performed on *P. guajava* leaves var. *pyrifera* at three oxidative states (low, medium and high) to compare their PAs content. *Pyrifera* var. was selected for the extraction since it exhibited greater amounts of PAs than *pomifera* var. [2]. The results obtained by chromatographic analysis reported that guava leaves have a high DP of flavan-3-ols (Table 5 and Fig. 3). As it could be seen in Fig. 3, guava leaves have two different classes of PAs, no-galloylated (nG) and galloylated (G), that could be differentiated into mono-galloylated (mG) and di- galloylated (dG) in some cases; the identification has been carried out by MS spectrometry detector (Table 5) and confirmed the presence of these types of procyanidins and prodelphinidins as was previously reported [2,27], although the

present method allowed the quantification of every oligomer and even of the polymers present in the leaves; in fact flavan-3-ols ranged between monomers to undecamer plus the polymers for all the samples, and until tridecamer for leaves at the lowest oxidation state.

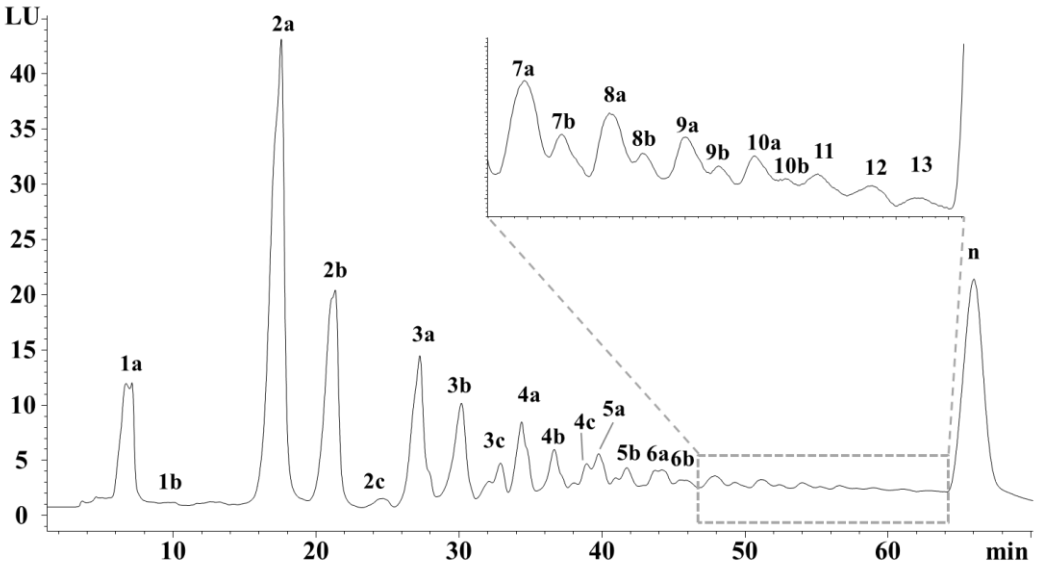

Figure 3. HPLC-FLD chromatogram of the PAs in guava leaves. Each number corresponds to the DP and n to the polymers. Letters a, b and c correspond to the compounds described in Table 4.

Among the different oxidative states, significant differences ($p < 0.05$) were noticed (Table 5). The highest value was found in the leaves with low degree of oxidation (15.8 ± 0.4 mg/g leaf d.w.), followed by medium and high oxidation states (12.6 ± 0.4 and 10.5 ± 0.3 mg/g leaf d.w., respectively). Differences between the oxidative states could be due to the fact that procyanidins cleaved into anthocyanins [25], which would explain the dramatic red coloration of the leaves as the oxidative state raise, decreasing in this way PAs concentration [28,29]. Moreover, it was noticed that higher values of anthocyanins were found in red colored guava leaves [27].

Table 5. Quantification of SPAs, degree of polymerization (DP), and percentages of no-galloylated (nG%) and galloylated (G%), which could be divided into mono-(mG%) and di-galloylated (dG%), flavan-3-ols present in guava leaves at different oxidative state (low, medium, and high) at optimized conditions.

Peak	DP	Mass ions	LOW	MEDIUM	HIGH
			PAs (mg/g leaf d.w.)		
	Monomers		1.59 ± 0.07[a]	1.68 ± 0.08[a]	1.56 ± 0.02[a]
1a	nG%	289	99	99	99
1b	mG%	305	1	1	1
	Dimers		6.6 ± 0.1[a]	5.18 ± 0.07[b]	4.06 ± 0.07[c]
2a	nG%	577	66	68	69
2b	mG%	593, 729	32	30	28
2c	dG%	609, 745	2	2	3
	Trimers		2.9 ± 0.1[a]	2.44 ± 0.07[b]	2.32 ± 0.08[b]
3a	nG%	865	53	53	52
3b	mG%	881	34	35	35
3c	dG%	897, 1017	13	12	13
	Tetramers		1.18 ± 0.03[a]	0.91 ± 0.05[b]	0.90 ± 0.04[b]
4a	nG%	1153	52	48	49
4b	mG%	1305	31	33	35
4c	dG%	1457	17	19	16
	Pentamers		0.512 ± 0.004[a]	0.40 ± 0.04[b]	0.35 ± 0.04[b]
5a	nG%	1441	54	58	50
5b	G%	797, 873	46	42	50
	Hexamers		0.31 ± 0.02[a]	0.24 ± 0.01[b]	0.246 ± 0.008[b]
6a	nG%		72	64	63
6b	G%		28	36	37
	Heptamers		0.26 ± 0.01[a]	0.21 ± 0.02[b]	0.14 ± 0.01[c]
7a	nG%		68	65	63
7b	G%		32	35	37
	Octamers		0.150 ± 0.002[a]	0.100 ± 0.005[b]	0.071 ± 0.003[c]
8a	nG%		68	66	61
8b	G%		32	34	39
	Nonamers		0.074 ± 0.003[a]	0.050 ± 0.001[b]	0.031 ± 0.001[c]
9a	nG%		68	67	59
9b	G%		32	33	41
	Decamers		0.083 ± 0.006[a]	0.048 ± 0.002[b]	0.036 ± 0.004[b]
10a	nG%		65	61	55
10b	G%		35	39	45
11	Undecamers		0.038 ± 0.001[a]	0.0229 ± 0.0001[b]	0.016 ± 0.003[b]
12	Dodecamers		0.034 ± 0.002	n.d.	n.d.
13	Tridecamers		0.0268 ± 0.0007	n.d.	n.d.
n	Polymers		1.94 ± 0.04[a]	1.29 ± 0.01[b]	0.77 ± 0.06[c]

Peak	DP	Mass ions	LOW	MEDIUM	HIGH
			PAs (mg/g leaf d.w.)		
	SPAs (mg/g leaf d.w.)		15.8 ± 0.4[a]	12.6 ± 0.4[b]	10.5 ± 0.3[c]

[a, b,c] The different letter in the same raw means that they are significantly different ($p < 0.05$). Peak numbers are the same reported in Figure 2.

Concerning the data showed in this work, *P. guajava* fruit showed low content of PAs; between 3.5 and 6.3 mg gallic acid equivalents/kg depending on the stage of ripening and the time of the treatment with hot water [30]. To our knowledge, there is not published work on the quantification of PAs in other guava leaves. Moreover, the PAs content found in guava leaves was also higher than those reported for different barley samples which varied from 0.29 to 0.65 mg/g of flour [16], for whole grain rice with red-colored bran that exhibited an amount of 1.3 mg/g grain [31], and for apple skin (7.3 mg/g d.w.) [32]. The same order of magnitude was obtained in the leaves at different oxidative states compared to grape seed after different extrusion conditions which ranged between 20.0 ± 0.2 and 26.4 ± 1.8 mg/g d.w., or to the value achieved for a cocoa extract (38.7 mg/g dried fraction) [9] or compared to tannin content in sorghum (10.0 - 68.0 mg/g d.w.) [33].

According to the results listed in Table 5, similar trend was found in the three leaves, most nG-oligomers varied from 61 to 72% of the total content, while the percentage of G-flavan-3-ol derivatives ranged between 28 and 39%. Only for DP 2 - 4 mG- and dG-flavan-3-ol derivatives were found, contributing these last in greater amounts (12-19 %) in DP 3 and 4 respectively. Moreover, as well as for DP 3 and 4, similar percentages of nG and G were found for DP 9 and 10 at the highest oxidative state, in contrast to tea leaves and roots, where the galloylated catechins and PAs were dominant [34] or to grape seeds, where the galloylated compounds were found in lower percentages (1.8 - 5.3%) [35].

Furthermore, the amount of PAs decreases from DP 2 to DP 9. In this sense, the content of oligomers (DP 2-10) in Hawthorn dried leaf and flower was also studied

and a linear decrease was noticed as the DP increase, which varied from less than 3.5 to less than 1 mg/g plant material, except for DP 8 [36]. According to our data (Table 5), three oxidative states revealed greater amounts of DP 2, similar values of DP 3 and lower for the others oligomers compared to Hawthorn extract. In addition, higher values were obtained for monomers, dimers and trimers when guava leaves are compared with the skins of 'Shiraz', 'Cabernet Sauvignon' and 'Marselan' grape varieties [37], although they presented higher amounts of polymer than guava leaves.

However, the importance of PAs depends on their bioavailability that is determined by the degree of polymerization: monomers have the highest absorption, followed by dimers, trimers and tetramers, and oligomers with higher DP are less or even not absorbable, so the bioactivity of these might not be significant. Moreover, nG-monomers are more bioavailable than G-monomers. Consequently, there is an interest in low PAs sources [38–40].

4. Conclusions

BBD was applied for the optimization of PAs extraction in *P. guajava* leaves and showed the adequacy of the model due to the significance of the factors (acetone/water ratio (% (v/v)), temperature of the ultrasound bath, time of extraction, and acetic acid percentage) tested in the response variable (SPAs via NP-HPLC-FLD). The optimized conditions were used to extract PAs from guava leaves at different oxidative states (low, medium, and high) and significant differences ($p < 0.05$) were found among them. NP-HPLC-FLD platform allowed the identification and quantification of the monomers, several oligomers (DP 2-13) and polymers. Interesting differences were noticed among the different oxidation state of guava leaves; the total amounts of proanthocyanidins decreased when oxidation state of leaves increased. In the end, guava leaves represent a potential source of PAs with low degree of polymerization (comparable with cocoa and apple) that could be used for nutraceutical formulation.

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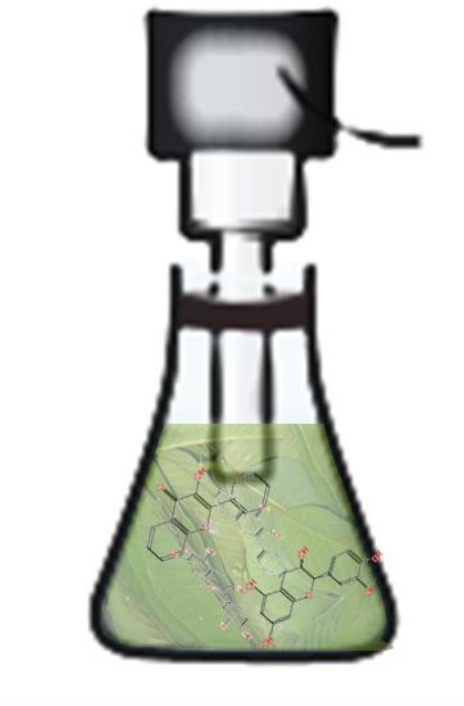
CHAPTER 5



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Design of Sonotrode Ultrasound-Assisted Extraction of Phenolic Compounds from *Psidium guajava* L. Leaves

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Abstract

Psidium guajava L. has gained a special attention as health plant due to the presence of phenolic compounds. Box-Behnken design (BBD) has been applied for the extraction of target compounds from guava leaves via sonotrode ultrasound-assisted extraction (UAE). Different extraction times (5, 30 and 55 min), ratios of ethanol/water (50, 75, and 100% (v/v)) and ultrasound (US) power (80, 240 and 400 W) were tested to find their effect on the sum of phenolic compound (SPC), flavonols and flavan-3-ols via HPLC-ESI-QqQ-MS, and antioxidant activity (DPPH and TEAC assays). The best process conditions were as follows: 40 min, 60% ethanol/water (v/v), and 200 W. Established method has been used to extract phenolic compounds in two guava leaves varieties (*pyrifera* and *pomifera*). P*yrifera* var. showed greater values of the SPC via HPLC-ESI-QqQ-MS (49.7 mg/g leaf dry weight (d.w.)), flavonols (12.51 mg/g d.w.), flavan-3-ols (7.20 mg/g d.w.), individual phenolic compounds, and antioxidant activity (8970 ± 5 and 465 ± 6 μmol Trolox/g leaf d.w, respectively) than *pomifera* var. Conventional extraction showed lower amounts of phenolic compounds (7.81 ± 0.03 and 4.64 ± 0.01 mg/g leaf d.w. for flavonols and flavan-3ols, respectively) in comparison to the ultrasound-assisted ones.

Keywords: Guava (*Psidium guajava* L.) leaves, Ultrasound assisted extraction, Response surface methodology, Phenolic compounds, HPLC-ESI-QqQ-MS, Antioxidant activity.

1. Introduction

Psidium guajava L., one of the most gregarious fruit trees from the *Myrtaceae* family, is common throughout tropical and subtropical areas. Different parts of the tree, e.g. the fruit, flowers, shoots, bark, and leaves have been widely used for the treatment of several diseases (Morton 1987).

In the last years, the leaves of this plant have gained a special attention which has caused an increase in pharmacological studies displaying many effects on health such as antispasmodic and antimicrobial properties, antioxidant, anti-cough, anti-diabetic, anti-inflamatory and anti-nociceptive activities, among others (Gutiérrez et al. 2008; Barbalho et al. 2012).

Several authors reported that these effects are due to the presence of phenolic compounds in the plant. For instance, Liu et al. (2015) evaluated the effects of aqueous guava leaf extract (GvEx) on insulin resistance in high glucose-induced insulin-resistant mouse FL83B cells, and suggested that single and/or synergistic effects of the multiple bioactive components present in GvEx promote the anti-hyperglycemic activity, and Mailoa et al. (2013) reported that tannin extract of guava leaves have antimicrobial activities against *E. coli*, *S. aureus*, *P. aureginosa*, *A. niger* and *C. albicans*.

Since last years, recovery of valuable ingredients from by-products and side streams has inspired the scientific community to initiate several projects across scientific disciplines. The challenge for standardization of natural products leads the investigation for developing the scale-up of laboratory or pilot-plant processes due to the need to maintain the functional properties of the bioactive compounds for nutraceutical applications (Galanakis and Schieber 2014). The emergence of nutraceuticals from agricultural by-products is possible due to the existence of methodologies, which allow not only the recovery, but also their reutilization inside food (Galanakis 2013).

In order to obtain the target compounds is important to select the proper extraction technology also optimizing the related process parameters. The traditional extraction techniques at a laboratory scale, commonly used for the chemical standardization of plants, generally require long extraction time, large amounts of samples and organic solvents that may have potentially negative effects on the environment and human health, and the employment of high temperature (Deng et al. 2014). Hence, non-conventional methods, known as emerging extraction technologies with various potential advantages have been proposed to overcome the disadvantages of conventional techniques, as well as achievement of higher efficiency and lower energy consumption (Galanakis 2012; Galanakis 2013; Deng et al. 2014). Among these, ultrasound-assisted extraction (UAE) could suit more properly in the field of food science due to their direct incorporation with the extraction process (Galanakis 2013), although it could be also found as pre-treatment technique (Barba et al. 2015), and seems to be the best choice as it is the most economical technique, can be performed at atmospheric pressure and room temperature on an ultrasound (US) bath or even with an US probe (or sonotrode) (Heng et al. 2013).

Moreover, US waves after interaction with subjected plant material alter its physical and chemical properties. Their cavitational effect facilitates the release of extractable compounds and enhances the mass transport by disrupting the plant cell walls. UAE is a clean method that allows obtaining high reproducibility, reducing the consumption of solvent, simplifying manipulation and work-up and giving higher purity of the final product (Chemat et al. 2011).

In a previous report, the suitability of this technique to recover phenolic compounds from different sources, such as fruits and leaves, has been reported. It was found that the variation of the treatment conditions and the solvent ratio increased the yield of polyphenols (Roselló-Soto et al. 2015). The effect of US treatment on phenolic compounds has also been studied in fruit juices, concluding that the different conditions of the process must be optimized for each food matrix (Zinoviadou et al. 2015). Additionally, Jacotet-Navarro et al. (2015) compared three different US

apparatus (US bath, US reactor and US probe) and microwave for different antioxidant compounds extraction from rosemary. On the basis of extraction yield, energy consumption, and CO_2 emissions, US probe was chosen as the most efficient process. The scale up of ultrasonic extractor has already successfully applied in the food industry (Tao and Sun 2015).

The method most used in guava leaves as far to know the effect of a variable in a process is called one-variable-at-a-time technique. However, several factors influence the UAE; so, it is better to employ a technique which includes interactive effects among the variables. This could be allowed by response surface methodology (RSM) that is useful for developing, improving, and optimizing process outcomes in which a response of interest is influenced by several variables (Baş and Boyacı 2007; Wong et al. 2015).

Based on these premises, the purposes of this work were to find out the best process conditions to extract phenolic compounds from guava leaves via sonotrode UAE. RSM was applied in order to optimize the extraction conditions for all the phenolic compounds previously described in guava leaves and particularly to flavonols and flavan-3-ols that are described as potential nutraceutical compounds versus Diabetes Mellitus T2 disease. The best conditions were tested to compare two varieties of *Psidium guajava* leaves.

2. Material and methods

2.1 Chemicals and plant material

LC-MS grade solvents were purchased from Merck (Darmstad, Germany). Double-deionised water was from Millipore (Bedford, MA, USA). Standards and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Middle age intense green leaves *Psidium guajava* L. var. *pyrifera* and *pomifera* were harvested in March 2015 in Motril (Spain) (36°44′43″N 3°31′14″O).

The samples were air dried at 25ºC until constant weight was achieved, ground and sieved (0.6 – 0.8 mm particle size) before the analyses.

2.2 Sonotrode ultrasound assisted extraction

2.2.1 Selection of process conditions

The first effort of this work was to find mild conditions for the extraction step. This goal was encouraged due to the eco-friendly advantages of UAE, such as high reproducibility in shorter times, reduced solvent consumption and temperature and lower energy input. Besides, it is necessary to take into account the variables that affect the behavior of the system studied, which can be divided into US factors (ultrasound frequency, duration, acoustic power/intensity, and treatment mode) and non-US factors (solvent type, solvent/sample ratio, particle size, temperature) (Tao and Sun 2015).

Several studies demonstrated that a higher solid-to-solvent ratio, ranged between 1:60 and 1:80 (w/v), seemed to be favorable in extraction of phenolic compounds (Wong et al. 2013; Tay et al. 2014). Moreover, the ratio 1:80 (w/v) was selected since solid to solvent ratio higher than 1:80 (w/v) were investigated and no significant differences were found (data not shown). Ethanol has been selected as most preferable solvent to extract phenols because it is rather cheap and possesses “GRAS” status (Generally-Recognized-As-Safe according to American Food and Drug Administration) (Galanakis 2012). The ethanol/water (50, 75, and 100% (v/v)) ratio chosen, was the same as the one previously used for the optimization of this single parameter (Díaz-de-Cerio et al. 2016). As resumed by Tao and Sun (2015), the maximum extraction yield employing UAE could be reached within 1 hour; so, in the present work, different extraction times (5, 30 and 55 min) at different US powers (80, 240 and 400W) were tested. Despite of the classification of US technique as non-thermal method, long time treatments allow the temperature increase. For this reason, the temperature is strictly depending from the extraction time used and is preferable to use low temperatures. Because of that, in this work temperature was kept constant at

20 ºC to avoid solvent evaporation, which may vary the solid/solvent ratio, and because low temperature is beneficial to keep the bioactivity and value of the compounds during extraction (Tao and Sun 2015). In addition, pulsed UAE treatment mode was evaluated since it has been proved to save 50 % of electrical energy (Pan et al. 2012)

2.2.2 Extraction procedure

Powdered air-dried guava leaves were ultrasonically extracted. Solid to solvent ratio was 1:80 (w/v). The extraction was achieved with an US sonotrode (Startec S.r.l., Milano, Italy). The frequency was fixed at 24 kHz and the number of cycles was fixed at 0.5 cycles/s. Temperature data were recorded every 15 s during the experiment using a digital multimeter mod. SCC-TC02 (National Instruments, Assago (MI), Italy) coupled with thermocouples and a personal computer to ensure the constant temperature at 20 ± 3 ºC employing a temperature controller. In the first step of the experiment, carried out on var. *pyrifera*, extraction time (5, 30 and 55 min), ethanol/water ratio (50, 75, and 100 % (v/v)), and ultrasound power (80, 240 and 400W) were changed in order to select the best process conditions. Other parameters were not taken into account because they have been previously established. After extraction, each run was concentrated under vacuum, re-dissolved in 2 mL of 50% methanol/water (v/v), filtered through a 0.20-µm RC syringe filter, and kept at −20 °C until HPLC analysis.

2.3 Experimental design

Box-Behnken design (BBD) was used as the most appropriate to select the best sonotrode UAE process conditions, since it is a simple design that allows avoiding extreme situations (Ferreira et al. 2007). The complete design consisted on 15 experimental runs, three levels (-1, 0, 1) for each factor, in order to normalize parameters, and three center points, and was applied to evaluate the effects of extraction time (min) (X_1), ethanol/water ratio (% (v/v)) (X_2), and US power (W) (X_3) on sonotrode UAE. The coded and natural values of the factors are shown in Table 1.

The response variables were fitted to a second-order polynomial model equation (Eq. (1)) obtained by the RSM:

$$\Upsilon = \beta_0 + \sum_{i=1}^{3} \beta_i \chi_i + \sum_{i=1}^{3} \beta_{ii} \chi_{ii}^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ii} \chi_i \chi_j$$

where Y represents the response variables sum of phenolic compound (SPC), sum of flavonols, and sum of flavan-3-ols via HPLC-ESI-QqQ-MS, DPPH, and TEAC. X_i and X_j are the independent factors affecting the response, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the model (intercept, linear, quadratic and interaction term).

The model building, experimental results and designs were processed using STATISTICA 7.0 (2002, StatSoft, Tulsa, OK). An analysis of variance (ANOVA) with 95 % confidence level was carried out for each response variable. The suitability of the regression model was decided by the regression coefficient (R^2), the *p*-value of the model, and the lack of fit. The significance of the model was evaluated with t-value obtained for the intercept, linear, quadratic, and interaction terms of the model. Optimal conditions were chosen considering the response surfaces (3D plots) for all variables.

2.4 Sonotrode UAE versus Conventional agitation extraction

The selected best conditions of sonotrode UAE were employed to extract phenolic compounds from leaves of both guava varieties (*pyrifera* and *pomifera*) in comparison with a conventional extraction method. The last was carried out as described by Wang et al. (2015). Briefly, *pyrifera* leaves were subjected to mechanical agitation (250 rpm) during 30 minutes and 75% ethanol/water (v/v) at room temperature.

Table 1. BBD with natural and coded values for sonotrode UAE conditions and experimentally values for antioxidant assays (DPPH and TEAC), SPC quantify by HPLC-ESI-QqQ-MS and different classes of phenolic compounds present in guava leaves.

Exp. No.	Independent factors			Independent Responses					
	X_1	X_2	X_3	DPPH (µmol Trolox/g leaf d.w.)	TEAC (µmol Trolox/g leaf d.w.)	SPC (mg/g leaf d.w.)	Flavonols (mg/g leaf d.w.)	Flavan-3-ols (mg/g leaf d.w.)	GED (mg/g leaf d.w.)
1	5 (-1)	50 (-1)	240 (0)	9142.90	473.04	49.89	11.97	6.74	31.14
2	55 (1)	50 (-1)	240 (0)	7850.35	469.43	49.54	12.19	6.86	30.45
3	5 (-1)	100 (1)	240 (0)	3886.00	235.87	23.09	7.18	1.36	14.54
4	55 (1)	100 (1)	240 (0)	5774.79	310.54	26.46	8.19	1.87	16.38
5	5 (-1)	75 (0)	80 (-1)	8125.74	407.59	40.78	10.26	5.29	25.21
6	55 (1)	75 (0)	80 (-1)	8616.30	448.84	43.73	11.31	5.79	26.60
7	5 (-1)	75 (0)	400 (1)	8631.18	439.91	45.70	11.01	5.70	28.95
8	55 (1)	75 (0)	400 (1)	8593.38	424.33	50.39	12.43	6.64	31.28
9	30 (0)	50 (-1)	80 (-1)	8896.04	444.99	48.33	12.23	6.40	29.66
10	30 (0)	100 (1)	80 (-1)	4191.54	235.43	25.51	8.01	1.69	15.80
11	30 (0)	50 (-1)	400 (1)	8842.17	441.33	41.57	11.08	5.26	25.19
12	30 (0)	100 (1)	400 (1)	6351.97	333.55	27.28	8.71	2.42	16.14
13	30 (0)	75 (0)	240 (0)	8758.65	431.86	48.53	11.99	6.65	29.85
14	30 (0)	75 (0)	240 (0)	9009.52	447.03	50.73	12.82	6.63	31.25
15	30 (0)	75 (0)	240 (0)	9084.42	429.74	46.79	12.07	7.15	27.53

X_1: Time, X_2: EtOH/water ratio, X_3: US power SPC: Sum of total phenolic compounds, GED: gallic and ellagic acid derivatives

2.5 HPLC-ESI-QqQ-MS analysis

Chromatographic analysis was performed on HPLC Agilent 1290 Infinity series (Agilent Technologies, Santa Clara, CA, USA) equipped with an online vacuum degasser, an autosampler, a binary pump, a thermostated column compartment. The chromatographic method used was previously reported by Díaz-de-Cerio et al. (2016). Briefly, separation was accomplished on a Poroshell 120 SB-C18 (3.0 mm x 100 mm, 2.7 μm, (Agilent Technologies)) at 25 °C. The mobile phase consisted of water containing 1% acetic acid (A) and acetonitrile (B), and applied as follows: 0 min, 0.8% B; 2.5 min, 0.8% B; 5.5 min, 6.8% B; 11 min, 14.4% B; 17 min, 24% B; 22 min, 40% B; 26 min, 100% B, 30 min, 100% B; 32 min, 0.8% B; 34 min, 0.8% B. The sample volume injected was 10 μL and the flow rate was kept at 0.8 mL/min.

Quantification analysis was performed on a 6420 Triple Quadrupole (QqQ) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an electrospray ionization (ESI) source in negative mode. The following conditions were fixed: flow rate 0.5 mL/min: gas temperature 350 °C, nebulizer pressure 50 psi, sheath gas flow 9 L/min. All compounds were monitored in the multiple reaction monitoring mode (MRM). The compound specific parameters of MRM (fragmentor voltage and collision energy) were automatically established with MassHunter Optimizer for each target compound.

2.5.1 Standard solutions

Standards of interest such as gallic acid, catechin, ellagic acid, quercetin, and rutin were used for quantification of phenolic compounds in guava leaf extracts. The standard stock solutions were prepared from the quantification limit (LOQ) to 500 or 1000 mg/L in methanol, except for ellagic acid, that was dissolved in water.

Linear range and limits of detection (LOD) and LOQ were determined as corresponding to 3 and 10 times, respectively, the standard deviation of the background noise; values were ranged from 0.002 to 0.03 mg/L for LOD and from

0.005 to 0.099 mg/L for LOQ. Results for the SPC, flavonols, and flavan-3ols via HPLC-ESI-QqQ-MS are expressed as mg/g leaf dry weight (d.w.) and quantification of individual compounds as mg/kg leaf d.w.

2.6 Antioxidant assays

In order to evaluate the antioxidant capacity of the 15 experiments of the BBD and both samples (*pyrifera* and *pomifera* at the optimized conditions), two different antioxidant assays were carried out. The TEAC assay, was performed by using a method previously described by Re et al. (1999) where the radical monocation $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate (2.45 mM) in the dark at room temperature for 12–24 h. The absorbance was adjusted to 0.70 ± 0.02 at 734 nm. Afterwards, 1 mL of $ABTS^{\bullet+}$ is reduced in the presence of hydrogen-donating antioxidants or with a standard (10 µL). Trolox was used as standard (calibration range: 1-15 µM).

The DPPH radical scavenging activity was assayed with a method proposed by several authors (Brand-Williams et al. 1995; Parejo et al. 2000). 100 µL of the sample were added to 2.9 mL of DPPH. The reduction of DPPH was measured by the decrease of the absorbance of its radical at 517 nm. Trolox was used as standard (calibration range: µM).

Results for both assays are expressed as µmol Trolox/g leaf d.w.

2.7 Statistical analysis

The results related to the second part of the study on both varieties of guava leaves are the averages of three repetitions (n=3). Fisher's least significance difference (LSD) test and Pearson's linear correlations, both at $p < 0.05$, were evaluated using STATISTICA 7.0 (2002, StatSoft, Tulsa, OK).

3. Results and discussion

3.1 Fitting the models

Box-Behnken experimental design elaborated for the optimization of sonotrode UAE conditions, considering experimental values obtained for the investigated responses, is exhibited in Table 1.

Experimental values for the sum of phenolic compounds quantified by HPLC-ESI-QqQ-MS (SPC) ranged from 23.09 to 50.73 mg/g leaf dry weight (d.w.). Moreover, the phenolic compounds were grouped in three different classes namely flavonols, which varied from 7.18 to 12.82 mg/g leaf d.w.; flavan-3-ols, ranged from 1.36 and 6.86 mg/g leaf d.w.; and gallic and ellagic acid derivatives (GED) that were between 14.54 and 31.25 mg/g leaf d.w. For antioxidant capacity, results varied from 3886.00 to 9142.90 and from 235.27 to 473.04 μmol Trolox/g leaf d.w., by using DPPH and TEAC method, respectively. Prior to analyze the model the correlation between the effects was carried out. Since good linear correlation was observed (R values between 0.8257-0.9763 and $p < 0.001$), it is expected a similar pattern of the independent variables on the responses.

RSM employed the data from Table 1 to find the combined effect of extraction time, ethanol/water ratio, and ultrasound power on the response variables during the sonotrode UAE. The regression coefficients of the model that describe DPPH, TEAC, SPC, flavonols, flavan-3-ols and GED responses and the results of the analysis of variance (ANOVA) are summarized in Table 2.

Table 2. Regression coefficients and analysis of variance (ANOVA) of the model.

Regression coefficients	Responses					
	DPPH	TEAC	SPC	Flavonols	Flavan-3-ols	GED
β_0	1501.2524*	228.3866*	-9.0909	1.8829*	-6.5008*	-4.4534*
Linear						
β_1	-40.6955	-1.1252**	0.0098	0.0449**	0.0256	-0.0608
β_2	301.0026*	9.6070*	1.9026*	0.3245*	0.4198*	1.1565*
β_3	-6.8759*	-0.1553	0.0097	0.0003	0.0026	0.0068
Cross product						
β_{12}	1.2725*	0.0313**	0.0015	0.0003	0.0002	0.001
β_{13}	-0.033	-0.0035**	0.0001	0.0002	0.0004	0.0001
β_{23}	0.1384*	0.0063*	0.0005	0.0001	0.0001**	0.0003
Quadratic						
β_{11}	-0.6929*	0.0019	-0.0016	-0.0009	-0.0006**	-0.0001
β_{22}	-2.9669*	-0.1043*	5.2294*	-0.0029*	-0.0036*	-0.0102*
β_{33}	-0.001	-0.0002	0.0001	0.0001	0.0002**	-0.0001
Adequacy of the model						
R^2	0.9865	0.9682	0.9556	0.8939	0.9576	0.9000
p (model)	< 0.0001	0.0002	0.0058	0.001	0.0001	0.0003
p (Lack of fit)	0.2627	0.1771	0.1741	0.3193	0.2099	0.4889

*Significant at $\alpha \leq 0.05$ ** Significant at $\alpha \leq 0.1$; 1: Time, 2: EtOH/water ratio, 3: US power SPC: Sum of total phenolic compounds, GED: gallic and ellagic acid derivatives

On the basis of Fisher test, ethanol/water ratio (X_2) presented both linear and quadratic effect ($p < 0.05$) on all responses. According to *p*-values, for SPC, flavonols, flavan-3-ols, GED, and TEAC all other effects were not significant. Furthermore, for DPPH, linear effect of US power (X_3), quadratic of time (X_{11}), and cross product between time (X_1) – ethanol/water ratio (X_2) and ethanol/water ratio (X_2) - US power (X_3) were also found. Significant variables are different for DPPH response; this could be due to a less sensitivity of the DPPH method with hydrophilic antioxidants, considering that the interaction of a potential antioxidant with DPPH depends on its structural conformation. These results confirm that this assay system may not give the true picture of total antioxidant capacity of a matrix (Kaur and Kapoor 2001).

Considering that few significant terms were found, according to Zeković et al. (2014) and Ramić et al. (2015), it was decided to increase the level of significance (α) to 0.1. The increase in the significance level achieved a greater effect of the factors, as can be seen in Table 2. Briefly, according to *p* values, for DPPH no variations were found in the model. Moreover, for TEAC, the linear term of time (X_1) and the interaction between all variables (X_{12}, X_{13}, and X_{23}) exhibited significant effect. Comparing the data, the response of SPC was influenced by the high concentration of GED, which represented a 60%. For those, as for SPC, the extraction was only affected by both terms of ethanol/water ratio (X_2), while for flavonols and flavan-3-ols extraction was influenced by more terms ($\alpha \leq 0.1$). On the one hand, positive effect of time (X_1) was found for flavonols, and on the other hand, interaction between ethanol/water ratio (X_2) - US power (X_3) and the quadratic terms of time (X_{11}) and US power (X_{33}) were also significant for flavan-3-ols.

Analysis of variance for each variable, with a level of significance (α) of 0.1 and 95% confidence intervals and only with significant effects, was carried out to find the adequacy of the responses to a second-order regression model. ANOVA revealed that the models presented high correlation between independent factors and response variables with coefficients of determination (R^2) between 0.9000 and 0.9862, except for the model predicted for flavonols, which exhibited a good correlation but lower

than the others. Moreover, models were statistically acceptable since *p* value was lower than 0.05 for all cases. The validity of the model was also confirmed by the *p* value of the lack of fit as non-significant lacks of fit were found ($p > 0.05$).

Three-dimensional response surface plots for the variables are presented in Fig. 1 related to DPPH and TEAC and Fig. 2 for flavonols and flavan-3-ols. SPC and GED were not represented because there is only one factor (ethanol/water ratio) that showed significant differences. However, it could be seen that ethanol/water ratio influence was dominant comparing to other factors for all the variables.

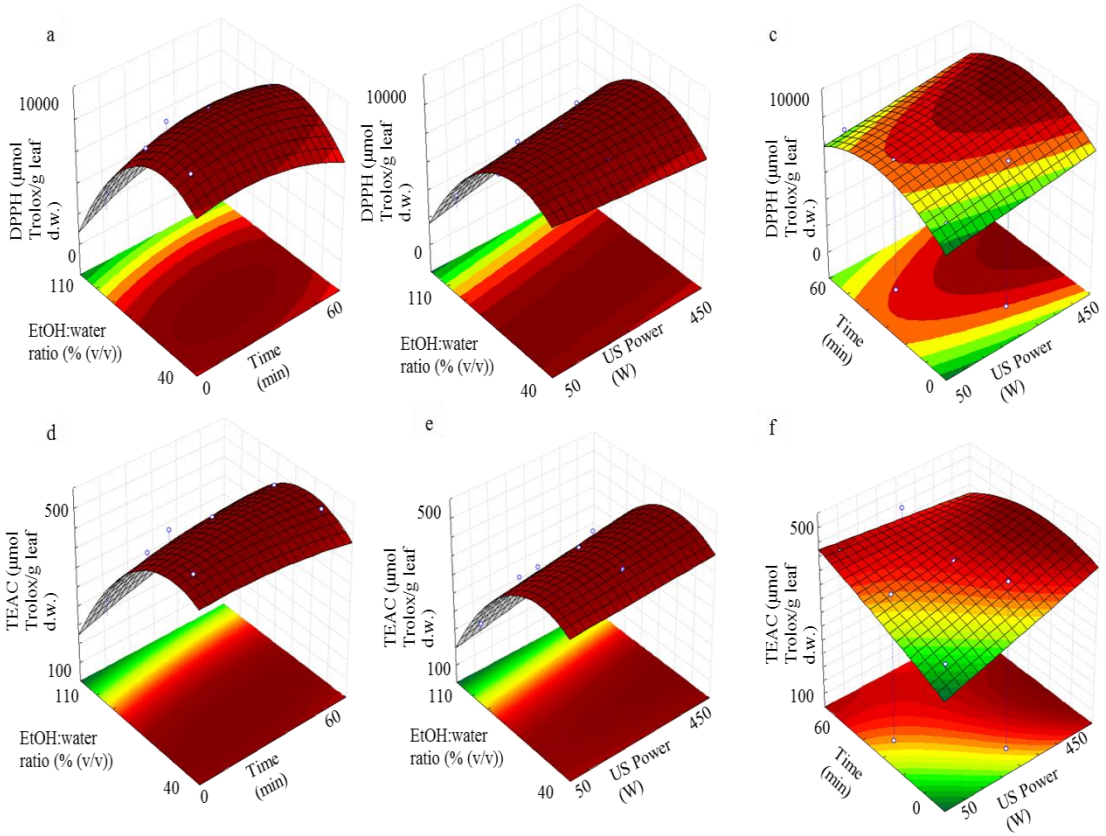

Fig. 1 Response surface plots showing combined effects of process variables: (a and d) ethanol/water ratio (% (v/v)) and time; (b, and e) ethanol/water ratio (% (v/v)) and ultrasonic power; and (c and f) ultrasonic power and time on DPPH (a-c) and TEAC (d-e).

When the US power (X_3) was hold, the negative effect of the quadratic term (X_{22}) had less meaning than the positive effect of the linear one (X_2), also lower than the effect of (X_1) for TEAC and flavonols, and than (X_{11}) for flavan-3-ols (Figs. 1a,

1d, 2a and 2d). Moreover, the positive effect of the interaction between these variables (X_{12}) is well represented in Figs. 1 a and d. The same trend was notice when the time (X_1) was fixed (Figures 1b, 1e, 2b and 2e), although in this case, the positive effect of ethanol/water ratio (X_2) is noticeable for DPPH (Fig. 1b), the low value of the quadratic terms (X_{22} and X_{33}) in the response of the flavan-3-ol content (Fig. 2e) and the positive interaction among the factors (X_{23}) for both variables (Figs. 1b and 2e) and for TEAC (Fig. 1e). Furthermore, for all the variables, it is observable that the relation between (X_1) and (X_3) supposed no much variation when (X_2) was set (Figs. 1 and 2c, f). However, the linear effect of (X_1) for flavonols (Fig. 2c) and TEAC is evident, with a slight negative effect of the interaction between the factors (X_{13}) in this case (Fig. 1f), and the small influence of the quadratic term (X_{11}) for DPPH (Figure 1c) and (X_{11}, X_{33}) for flavan-3-ols (Figure 2f). Furthermore, in most cases, it is notable that higher values of considered parameters are obtained nearby the medium level of the factors, as is expected for a BBD (Hibbert 2012).

3.2 Optimization of sonotrode UAE parameters

RSM provided the optimal conditions for the different variables, and as it was expected, they differ due to the differences values obtained for the regression coefficients of the desirability function (Table 3). Because of that, a compromise has to be found to choose the optimized conditions in order to obtain the maximum area for all responses, and it is possible studying the response surface plots (Figs. 1 and 2).

Since the time and US power effects have less significance than the ethanol content in each response, it is desirable to carry out the process on lower possible conditions in order to reduce the extraction time and the use of the sonotrode, reducing operational costs and avoiding the raise of temperature of it, respectively. Taking into account these premises, the optimized conditions that allow obtaining the highest values for the response variables are 40 min, 60% ethanol/water (v/v) and 200 W, values that are close to the optimal conditions predicted. In fact, as is shown in the 3D

plots (Figs. 1 and 2), DPPH, TEAC, flavonols and flavan-3-ols are still at the maximum range.

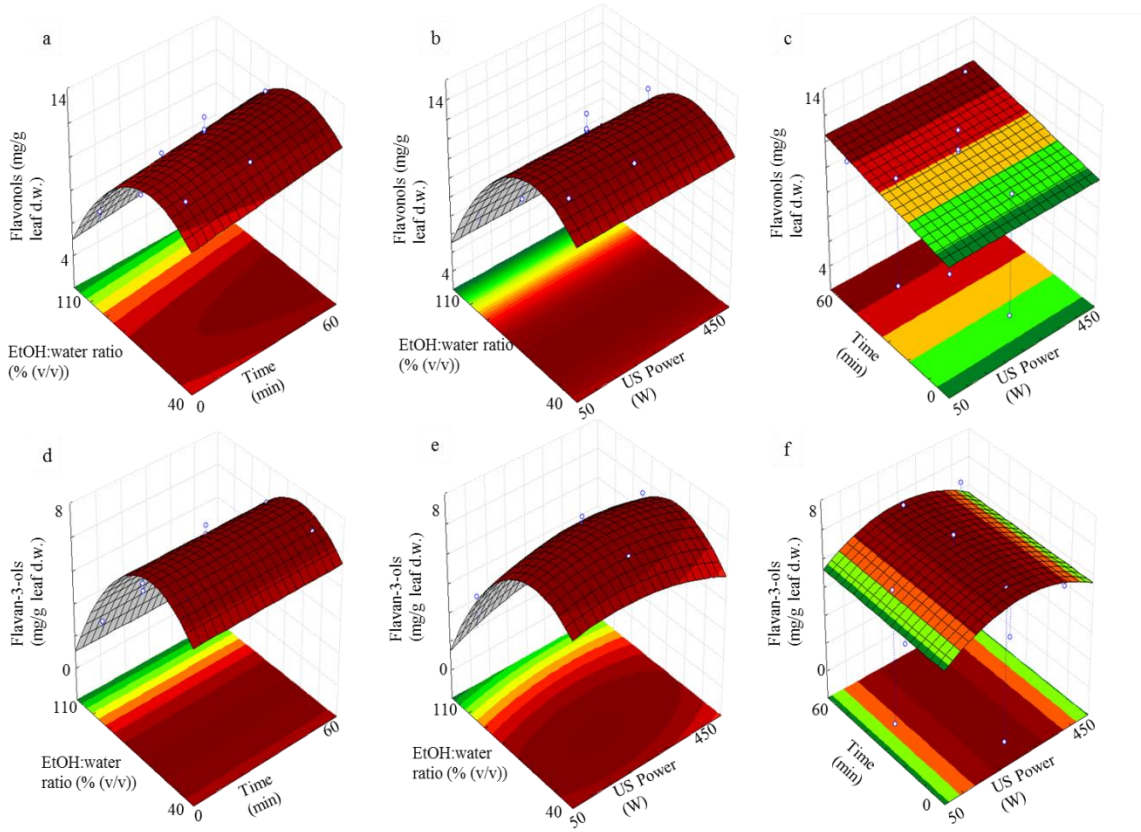

Fig. 2 Response surface plots showing combined effects of process variables: (a and d) ethanol/water ratio (% (v/v)) and time; (b, and e) ethanol/water ratio (% (v/v)) and ultrasonic power; and (c and f) ultrasonic power and time on flavonols (a-c) and flavan-3-ols (d-e).

Verification of the predicted model was done employing the optimized conditions. Only significant differences ($p < 0.05$) were found when predicted and observed values were compared for TEAC and flavonol content in *pomifera* var. which reported lower content for most of the variables (Table 3). Moreover, conventional extraction was compared with the optimized conditions. In concordance with Pingret et al. (2012), extraction by agitation reported more than 30% less amount of sum of phenolic compounds and for individual components than those for the UAE. The authors attributed this improvement of the extraction to ultrasonic cavitation. Similar percentages were found with Khan et al. (2010), which reported that UAE increased in a 35-40% in TPC versus solvent extraction.

Table 3. Optimal conditions for sonotrode UAE. Predicted and experimental values obtained for both varieties of guava leaves extracted by sonotrode UAE and also experimental values for extraction by agitation with *Pyrifera* var.

Optimal conditions	DPPH (µmol Trolox/g leaf d.w.)	TEAC (µmol Trolox/g leaf d.w.)	SPC (mg/g leaf d.w.)	Flavonols (mg/g leaf d.w.)	Flavan-3-ols (mg/g leaf d.w.)
RSM optimal conditions					
Time (min)	22	45	41	38	37
EtOH/water ratio (% (v/v))	54	58	62	62	63
US Power (W)	80	180	230	235	228
Optimized conditions					
Predicted values	9128 ± 385^{a}	463 ± 18^{a}	50.8 ± 5^{a}	13 ± 1^{a}	7 ± 2^{a}
Observed *Pomifera*	8730 ± 9^{a}	434 ± 6^{b}	46.2 ± 0.1^{a}	9.40 ± 0.02^{b}	6.35 ± 0.03^{a}
Observed *Pyrifera*	8970 ± 5^{a}	465 ± 6^{a}	49.7 ± 0.3^{a}	12.51 ± 0.01^{a}	7.20 ± 0.01^{a}
Conventional extraction *Pyrifera*	6638 ± 8^{b}	338 ± 7^{b}	26.2 ± 0.9^{b}	7.81 ± 0.03^{c}	4.64 ± 0.01^{b}

*Mean ± CI (95 % confidence interval); ** Mean ± SD (standard deviation, n=3)
The different letter in the same column means that they are significantly different ($p \leq 0.05$).

Finally, MRM methodology was used to quantify each compound (the transitions used for quantification are showed in Table 4), and the area of the peak was compared with calibration curves obtained with the corresponding standard. For the compounds with no standard available, quantification was done using compounds with similar structure.

Quantification results reported higher concentration of sum of phenolic compound in *pyrifera* variety than in *pomifera* var. ($p < 0.05$), 49.7 and 46.2 mg/g leaf d.w., respectively. Wong Paz et al. (2014) employed ultrasound-assisted extraction to extract polyphenols from *E. camaldulensis* leaves, and reported values of TPC ranged from 3.9 to 15.71 mg/g of dry plant material using the Folin-Ciocalteu method. In another study TPC of commercialized *Lawsonia inermis* L. dried leaves and processed powder were compared and showed high polyphenolic content estimated to 20.22 ± 0.23 and 31.35 ± 2.05 mg/g of dray starting material, respectively (Dhaouadi et al. 2015). Also, *Hibiscus sabdariffa* leaves were found to contain high levels of TPC by Zhen et al. (2016), with values from 18.98 ± 2.7 to 29.9 ± 0.5 mg GAE/g of plant material. Because of that, higher phenolic content was noticed in guava leaves compared to other mentioned leaves.

The amounts of individual phenolic compounds were found to be higher in the most of the cases for *pyrifera* var. (Table 4). Major constituent of ellagic acid derivatives represented a 43% of the SPC in *pyrifera* var. while *pomifera* showed a 35 %. For both varieties, two benzophenones and major quercetin, catechin derivatives and gallic acid derivatives were present in similar percentage: 24%, 13%, 6% and 5% respectively.

Table 4. Optimized HPLC-ESI-QqQ-MS parameters in MRM for the quantification (mean ± SD, n=3) of the compounds identified in guava leaves.

Compound	Precursor Ion $[M-H]^-$	Product Ion $[M-H]^-$	Fragmentor (V)	Collision Energy (eV)	Var. *Pomifera* (µg/g leaf d.w.)	Var. *Pyrifera* (µg/g leaf d.w.)
HHDP glucose Isomer 1	481	301	108	12	2.490 ±0.006^b	4.45 ± 0.06^a
HHDP glucose Isomer 2	481	301	108	12	6.8 ± 0.1^b	11.63 ± 0.05^a
HHDP glucose Isomer 3	481	301	108	12	14.42 ± 0.02^b	19.0 ± 0.4^a
Prodelphinidin B Isomer 1	609	441	141	12	90 ± 2^a	58 ± 2^b
Gallic acid	169	125	108	12	37.5 ± 0.8^b	84.4 ± 0.6^a
Prodelphinidin B Isomer 2	609	441	141	12	38.0 ± 0.7^a	38 ± 2^a
Pedunculagin/ Casuariin Isomer	783	301	169	28	1448 ± 44^b	2340 ± 195^a
Gallocatechin Isomer 1	305	125	141	12	468 ± 4^b	530 ± 17^a
Prodelphinidin Dimer Isomer	593	305	141	12	353 ± 2^a	316 ± 1^b
Gallocatechin Isomer 2	305	125	141	12	1781 ± 6^a	1814 ± 21^a
Vescalagin/castalagin Isomer 1	933	301	169	28	16 ± 1^b	143 ± 14^a
Prodelphinidin Dimer Isomer 2	593	305	141	12	22 ± 2^a	36 ± 4^a
Uralenneoside Isomer 1	285	153	98	12	16.4 ± 0.2^a	7.3 ± 0.1^b
Uralenneoside Isomer 2	285	153	98	12	17.69 ± 0.02^a	8.2 ± 0.1^b
Geraniin Isomer 1	951	907	169	28	796 ± 13^b	2532 ± 22^a
Pedunculagin/ Casuariin Isomer 2	783	301	169	28	3175 ± 13^b	5205 ± 40^a
Geraniin Isomer 2	951	907	169	28	610 ± 6^b	1783 ± 89^a
Procyanidin B Isomer 1	577	289	141	12	265 ± 3^a	208 ± 1^a
Galloyl(epi)catechin-(epi)gallocatechin	745	593	141	12	12 ± 5^a	24.5 ± 0.4^a
Procyanidin B Isomer 2	577	289	141	12	2367 ± 44^a	2496 ± 59^b
Tellimagrandin I Isomer 1	785	301	169	28	738 ± 1^b	1460 ± 29^a
Pterocarinin A Isomer 1	1067	377	169	28	107 ± 4^a	<LOQ
Pterocarinin A Isomer 2	1067	377	169	28	14.0 ± 0.5^a	<LOQ
Stenophyllanin A	1207	917	169	28	564 ± 5^a	486 ± 3^b

Compound	Precursor Ion $[M-H]^-$	Product Ion $[M-H]^-$	Fragmentor (V)	Collision Energy (eV)	Var. *Pomifera* (µg/g leaf d.w.)	Var. *Pyrifera* (µg/g leaf d.w.)
Procyanidin trimer Isomer	865	577	141	12	64 ± 1^a	29 ± 6^b
Catechin	289	245	141	12	2240 ± 69^a	2131 ± 47^a
Casuarinin/ Casuarictin Isomer 1	935	633	169	28	1152 ± 7^a	230 ± 5^b
Casuarinin/ Casuarictin Isomer 2	935	633	169	28	3259 ± 89^a	408 ± 3^b
Tellimagrandin I Isomer 2	785	301	169	28	2639 ± 29^b	5082 ± 32^a
Vescalagin	933	301	169	28	<LOQ	6.7 ± 0.7^a
Epi-catechin	289	245	141	12	2.82 ± 0.03^a	0.61 ± 0.02^b
Procyanidin gallate Isomer	729	577	141	12	291 ± 4^a	361 ± 24^a
Myricetin hexoside Isomer 1	479	316	131	16	60.4 ± 0.8^a	25 ± 1^b
Vescalagin/castalagin Isomer 2	933	301	169	28	<LOQ	21.3 ± 0.8^a
Myricetin pentoside Isomer 1	449	316	131	16	142 ± 2^a	64 ± 2^b
Myricetin pentoside Isomer 2	449	316	131	16	389 ± 1^a	171.7 ± 0.7^b
Procyanidin gallate Isomer	577	289	141	12	180.9 ± 0.5^a	116 ± 8^a
Myricetin pentoside Isomer 3	449	316	131	16	220 ± 3^a	80.6 ± 0.7^b
Myricetin hexoside Isomer 2	479	316	131	16	235 ± 2^b	278 ± 5^a
Myricetin hexoside Isomer 3	479	317	131	16	81.6 ± 0.5^a	94 ± 5^a
Myricetin pentoside Isomer 4	449	316	131	16	329.5 ± 0.8^a	348 ± 17^a
Quercetin galloylhexoside Isomer 1	615	463	131	16	27.5 ± 0.8^a	28 ± 2^a
Ellagic acid deoxyhexoside	447	301	169	28	411 ± 2^b	636 ± 5^a
Quercetin galloylhexoside Isomer 2	615	463	131	16	14.7 ± 0.6^a	13.4 ± 0.2^a
Myricetin pentoside Isomer 5	449	316	131	16	204 ± 7^a	204 ± 15^a
Morin	301	151	131	16	210 ± 4^a	120 ± 31^a
Myricetin pentoside Isomer 6	449	316	131	16	176 ± 7^a	151 ± 7^a
Ellagic acid	301	284	169	28	2255 ± 40^a	2229 ± 172^a
Hyperin	463	300	131	16	725 ± 3^a	503 ± 6^b

Compound	Precursor Ion $[M-H]^-$	Product Ion $[M-H]^-$	Fragmentor (V)	Collision Energy (eV)	Var. *Pomifera* (µg/g leaf d.w.)	Var. *Pyrifera* (µg/g leaf d.w.)
Quercetin glucoronide	477	301	131	16	1256 ± 10^a	<LOQ
Isoquercitrin	463	300	131	16	603 ± 5^a	557 ± 8^b
Procyanidin gallate Isomer	729	425	141	12	4.1 ± 0.6^a	7.3 ± 0.3^a
Reynoutrin	433	301	131	16	964 ± 6^a	805.1 ± 0.3^b
Guajaverin	433	301	131	16	443 ± 8^a	401 ± 4^b
Guavinoside A Isomer 1	543	169	213	60	97.8 ± 0.8^a	61 ± 3^b
Avicularin	433	301	131	16	1792 ± 36^a	1814 ± 15a
Quercitrin	447	301	131	16	985 ± 11^b	1112 ± 35^a
Myrciaphenone B	481	313	169	28	4050 ± 76^a	3970 ± 36^a
Guavinoside C	585	169	213	60	87.9 ± 0.2^b	92 ± 1^a
Guavinoside B Isomer 1	571	169	213	60	116 ± 1^b	203 ± 3^a
Guavinoside A Isomer 2	543	229	213	60	24.3 ± 0.2^a	7.1 ± 0.6^b
Guavinoside B Isomer 2	571	169	213	60	1.72 ± 0.01^b	120 ± 3^a
2,6-dihydroxy-3-methyl-4-O-(6″-O-galloyl-β-D-glucopyranosyl)-benzophenone	557	243	169	28	7454 ± 123^a	7514 ± 91^b
Quercetin	301	151	131	16	35.8 ± 0.4^a	33.4 ± 0.3^b
Guavinoside B Isomer 3	571	169	213	60	7.4 ± 0.2^b	14.3 ± 0.3^a

The different letter in the same line means that they are significantly different ($p \leq 0.05$).

Engström et al. (2015) quantified different polyphenol subclasses in twelve different leaves extracts by UPLC–QqQ–MS/MS. Comparing the sum of the different subclasses, guava leaves content is higher than *Rosa pimpinellifolia*, *Sorbus hybrid*, *Betula pubescens*, *Sorbus aucuparia*, and *Vincetoxicum hirundinaria* leaves. Briefly, almost all the leaves studied presented higher concentration of total ellagitannin than guava leaves. In contrast, few of them had more content of total gallic derivatives, only two extract showed total quercetin quantity higher and one if total myricetin is compared.

Finally, guava leaves exhibited similar DPPH radical scavenging capacity as *E. camaldulensis* leaves (Wong Paz et al. 2014). Aybastier et al. (2013) found for blackberry leaves, extracted by optimized ultrasound-assisted extraction, an experimental value for the antioxidant capacity of the extract, determined with TEAC, of 87.96 ± 0.35 mg Trolox/g dried plant, while for guava leaves is between 115 and 123 mg Trolox/g leaf d.w.

4. Conclusions

Box-Behnken experimental design was used for the optimization of immersion ultrasound assisted extraction of phenolic compounds from guava leaves. Results showed in a good way the influence of the factors chosen in the different responses tested, being the ethanol/water ratio the most important for each model, followed by US power for DPPH, time for TEAC and flavonols. Moreover, the flavan-3ols response is affected by the quadratic terms of each factor and TEAC is highly influenced by the interaction among the factors. Studying response surface plots, highest values for the response variables were found at 40 min, 60% ethanol/water (v/v), and 200 W. Suitability of the predicted model was proved comparing predicted and observed values for optimized conditions. Quantification results reported higher concentration of sum of phenolic compound in *pyrifera* variety than in *pomifera* var. ($p < 0.05$), 49.7 and 46.2 mg/g leaf d.w., respectively, and also, higher amount of flavonols and flavan-3-ols were found which supposed a 25 and 15 percentage from

the SPC, respectively. *Pyrifera* variety had also the highest amount of ellagic acid derivatives. Finally, ultrasound assisted extraction is a more effective technique than conventional agitation extraction for the recovery of phenolic compounds from guava leaves.

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Compliance with Ethical Standards

Funding

Not applicable

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

This article does not contain any studies with human or animal subjects.

Informed Consent

Informed consent was not applicable.

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Section II. Guava leaves bioactivity

CHAPTER 6

Health effects of *Psidium guajava* L. leaves: An overview of the last decade

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Review

Health Effects of *Psidium guajava* L. Leaves: An Overview of the Last Decade

Elixabet Díaz-de-Cerio [1,2], Vito Verardo [3], Ana María Gómez-Caravaca [1,*], Alberto Fernández-Gutiérrez [1,2] and Antonio Segura-Carretero [1,2]

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Abstract

Today, there is increasing interest in discovering new bioactive compounds derived from ethnomedicine. Preparations of guava (*Psidium guajava* L.) leaves have traditionally been used to manage several diseases. The pharmacological research *in vitro* as well as *in vivo* has been widely used to demonstrate the potential of the extracts from the leaves for the co-treatment of different ailments with high prevalence worldwide, upholding the traditional medicine in cases such as diabetes mellitus, cardiovascular diseases, cancer, and parasitic infections. Moreover, the biological activity has been attributed to the bioactive composition of the leaves, to some specific phytochemical subclasses, or even to individual compounds. Phenolic compounds in guava leaves have been credited with regulating blood-glucose levels. Thus, the aim of the present review was to compile results from *in vitro* and *in vivo* studies carried out with guava leaves over the last decade, relating the effects to their clinical applications in order to focus further research for finding individual bioactive compounds. Some food applications (guava tea and supplementary feed for aquaculture) and some clinical, *in vitro,* and *in vivo* outcomes are also included.

Keywords: *Psidium guajava* L. (guava) leaves; traditional medicine; *in vitro*; *in vivo*; phenolic compounds; pharmacology

1. Introduction

Ethnomedicine, which refers to the study of traditional medical practice, is an integral part of the culture and the interpretation of health by indigenous populations in many parts of the world [1]. For example, Indian Ayurveda and traditional Chinese medicine are among the most enduring folk medicines still practiced. These systems try to promote health and improve the quality of life, with therapies based on the use of indigenous drugs of natural origin [2]. Given that plants have been widely used as herbal medicines, several approaches are now being carried out to discover new bioactive compounds [3].

Psidium guajava L., popularly known as guava, is a small tree belonging to the myrtle family (*Myrtaceae*) [4]. Native to tropical areas from southern Mexico to northern South America, guava trees have been grown by many other countries having tropical and subtropical climates, thus allowing production around the world [5]. Traditionally, preparations of the leaves have been used in folk medicine in several countries, mainly as anti-diarrheal remedy [6]. Moreover, other several uses have been described elsewhere on all continents, with the exception of Europe [6–8]. Figure 1 summarizes the main traditional uses of guava leaves in the main producer countries. Depending upon the illness, the application of the remedy is either oral or topical. The consuption of decoction, infusion, and boiled preparations is the most common way to overcome several disorders, such as rheumatism, diarrhea, diabetes mellitus, and cough, in India, China, Pakistan, and Bangladesh [6–9], while in Southeast Asia the decoction is used as gargle for mouth ulcers [6,8,9] and as anti-bactericidal in Nigeria [8,9]. For skin and wound applications, poultice is externally used in Mexico, Brazil, Philippines, and Nigeria [6–9],. Also, chewing stick is used for oral care in Nigeria [9].

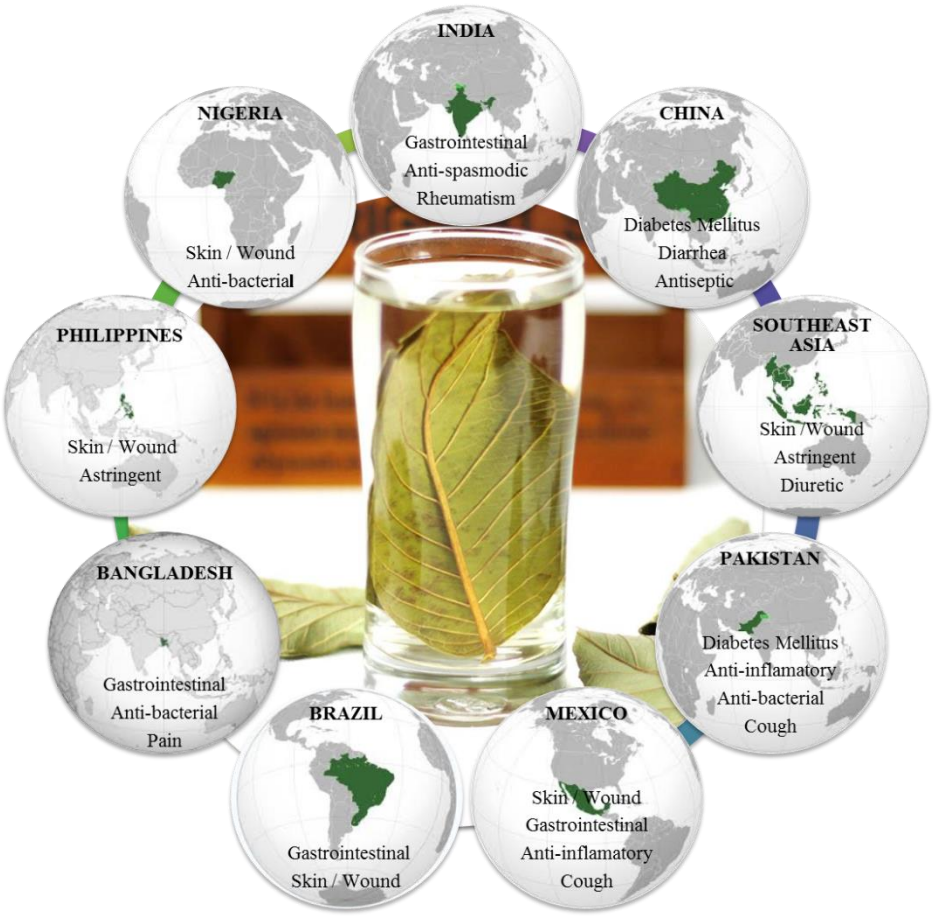

Figure 1. Main traditional uses of guava leaves in the principal producer countries.

Currently, there is increasing interest in studying of plants regarding their chemical components of bioactive compounds, their effects on several diseases, and their use for human health as functional foods and/or nutraceuticals [10]. In recent years, guava leaves tea and some complementary guava products are available in several shops in Japan as well as on the internet [11], because guava leaf phenolic compounds have been claimed to be food for specified health use (FOSHU), since they have beneficial health effects related to the modulation of blood-sugar level [12]. Thus, the aim of this review was to summarize the biological activities, *in vitro* and *in vivo*, studied in the last decade on *P. guajava* L. leaves, relating them to the international classification of diseases provided by the World Health Organization. In addition, the beneficial effects of some applications of guava leaves are also been

examined. For this purpose, a comprehensive review of the literature from 2004 to 2016 was done, although more recent studies have also been included. Reviewed journals, website, books, and several databases as "Scopus", "Google Scholar", "PubMed", and "ScienceDirect", were used to compile them. To ensure that relevant works are included, terms such as "*Psidium guajava*", "guava", "leaves", "*in vitro*", "*in vivo*", "clinical", "trial", "food application", and those related with the diseases like "bacteria", "cancer", "blood", "glycaemia", and "oral", among others were matched in the search. Besides, only complete available works published in English, Spanish, and even in Portuguese have been included.

2. Pharmacological properties

2.1. *In vitro* studies

2.1.1. Infectious and parasitic diseases

Aqueous and organic extracts of guava leaves have been demonstrated to have antibacterial activity due to an inhibitory effect against antibiotics-resistant clinical isolates of *Staphylococcus aureus* strains [13,14]. Despite using the same diffusion method, differences are noticed in their inhibition zones as is sown in Table 1, probably due to extraction method or to the dose assayed. A methanol extract exerted antibacterial effects, preventing the growth of different strains from several bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* spp., and *Shigella* spp. [15]. Furthermore, different extracts of the leaves such as aqueous, acetone-water, methanolic, spray-dried extracts, and the essential oil, showed potential inhibitory activity against gram-positive and gram-negative bacteria and fungi [16–20]. In these works is noticeable that gram-positive bacteria exhibited greater inhibition zones and minimum inhibitory concentrations (MICs) than gram-negative. Concerning the anti-fungal activity, less inhibition than bacteria is reported [16,17], except for *Candida krusei* and *Candida glabrata* which was higher [18], and for Aspergillus spp. for which no activity was found [16] (Table 1). Moreover, Bezerra et al. [21] evaluated the effect of guava leaves on different bacterial strains, concluding

that the synergistic action between the leaves and various antibiotics boosted its anti-bacterial activity. This effect was also observed by Betoni et al. [22] with target drugs for the protein synthesis, cell-wall synthesis, and folic acid. However, the latter did not found synergic effect with gentamicin, perhaps because the time of maceration was lower than the time used by Bezerra et at. [21], and also the solvent is different (Table 1).

Metwally et al. [23] associated the antimicrobial activity against some bacteria and fungi with five flavonoids isolated from the leaves. This effect was also related to the concentration of tannins in the leaves [24] and to the content of gallic acid and catechin [19]. Additionally, the activity against bacterial and fungal pathogens was traced to betulinic acid and lupeol [25]. In fact, these works are focused on the activity of these compounds, rather than on the effect of the whole extract of the leaves.

In addition, leaf acetone extract of *P. guajava* has also exhibited moderate acaricidal and insecticidal activities causing the dead of *Hippobosca maculata* adult fly [26].

Furthermore, Adeyemi et al. [27] suggested that an ethanol extract from the leaves function as a trypanocide agent, since its inhibition of *Trypanosoma brucei brucei* growth proved similar to that of the reference drugs. Kaushik et al. [28] proposed the leaves as an anti-malaria agent, due to its inhibitory activity and the resistance indices. Furthermore, the effect of guava leaf essential oil against toxoplasmosis caused by the growth of *Toxoplasma gondii* were reported [29]. Additionally, guava leaves were proposed for the treatment of diarrhea caused by enteric pathogens, since it showed significant inhibitory activity against *Vibrio cholerae* and *V. parahemolyticus*, *Aeromonas hydrophila, Escherichia coli, Shigella* spp. and *Salmonella* spp. [30–32]. It is supposed that the same plant origin and similar extraction procedure make that these works show comparable inhibition zones for the bacteria tested [30,31], in contrast to the leaves of India and Bangladesh, where MIC values did not show any concordance [31,32] (Table 1). In addition, a reduction was

described for *S. flexneri* and *V. cholera* invasion and for their adherence to the human laryngeal epithelial cells, and for the production of *E. coli* heat labile toxin and cholera toxin, as well as their binding to ganglioside monosialic acid enzyme [33]. Moreover, other studies also demonstrated the antimicrobial effect of some bacteria that cause gastrointestinal disorders by different methods [34,35]. In contrast to previous results [20,31], no inhibition of the hydrodistillation and n-hexane extract was found against E. coli *Salmonella* spp., respectively [31] (Table 1).

Furthermore, guava leaf tea helped control of the growth of influenza viruses, including oseltamivir-resistant strains, via the prevention of viral entry into host cells, probably due to the presence of flavonols [36].

Table 1 *In vitro* assays against infectious and parasitic diseases

Origin	Extraction method	Major constituent	Microorganisms	Assay	Main results	Ref
Saudi Arabia	Decoction (30 min)	-	*Staphylococcus aureus* strains	Agar well diffusion assay	At 200 µL: iz ≤ 30 mm	[13]
India	Soxhlet with MeOH (12 h), maceration in H_2O (4 h)	-	*S. aureus* strains	Agar well diffusion assay, time-kill of bacterial cell, SDS-PAGE analysis, and cellular toxicity to human erythrocytes assays	At 20 mg/L: iz ≤ 20 mm, MIC: 25 µg/mL (MeOH) and 7.5 mg/mL (H_2O). MBC: 1.25 and 12.5 mg/mL, respectively, 10 h to kill bacteria, ↑ degradation of protein, no hemolysis	[14]
Nigeria	Maceration in MeOH (48 h)	-	*S. aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus* spp., and *Shigella* spp	Agar well diffusion assay	At 20 mg/mL: iz ≤ 18 mm; 81.8% prevention growth	[15]
India	Maceration with agitation in MeOH, Ac, and DMF (12 h)	-	G-p and G-n bacteria and fungi (91 clinically important strains)	Disc diffusion assay	At 25 mg/mL: against g-p 70% MeOH > 80% Ac > 50% DE, ↓ 76.36% g-n bacteria. Fungi 56% Ac > 38% ME > 31% DMF. No activity against *Citrobacter* spp., *Alcaligenes fecalis*, and *Aspergillus* spp.	[16]
India	Soxhlet with MeOH (4 h)	Phytochemical screening: mainly flavonoid-glycosides and tannins	Bacteria (*Bacillus subtilis, S. aureus* and *E. coli*), and fungi (*Candida albicans* and *Aspergillus niger*)	Paper disc diffusion assay	At 50 µg/mL: iz ≤ 12.6 mm and 10 mm for bacterial and fungi strains, respectively. *E. coli*: MIC 0.78 µg/mL, MBC 50 µg/mL, and MFC 12.5 µg/mL	[17]
Brazil	Maceration with stirring in EtOH:H_2O 70% (v/v) (50°C, 1 h)	TPC: 25.93 (% m/m, dry base), TFC: 23.48 (mg/g, dry base)	Fungi (*C. albicans, Candida krusei*, and *Candida glabrata*), G-p (*S. aureus*) and G-n (*E. coli* and *P. aeruginosa*)	Microdilution assay	MIC = 80-100 µg/mL (*C. krusei, C. glabrata* and *S. aureus*)and MBC, MFC ≤ 250-1000 µg/mL (the others)	[18]

Origin	Extraction method	Major constituent	Microorganisms	Assay	Main results	Ref
Brazil	Turbo-extraction with water or Ac:H_2O 70% (v/v) (20 min)	Gallic acid: 0.065 µg/g, Catechin: 1.04 µg/g	G-p strains (*S. aureus, Staphylococcus epidermidis,* and *Enterococcus faecalis*) G-n (*E. coli, Salmonella enteritidis, Shigella flexneri*, and *Klebsiella pneumoniae*)	Agar-diffusion and microdilution assays	At 5 mg/mL:iz ≤ 20 mm, MIC = 39 µg/mL (*S. epidermis*), MIC < 600 µg/ml (the others)	[19]
India	Soxhlet with n-hexane	Methyl 2, 6, 10 – trimethyltridecanoate (28.86%) and Methyl octadecanoate (22.18%)	G-p: *S. aureus, Streptoccocus faecalis, Bacillus subtillis, Lactobacillus* spp., *Enterococcus aerogenes, Acinetobacter* spp. G-n: *E. coli, Proteus vulgari, Enterobacter aerogenes , Salmonella typhimurium, P. aeruginosa*, and *K. pneumoniae*	Agar well diffusion assay	At 80 µL: iz ≤ 27 mm, MIC = 3-10 µL	[20]
Brazil	Maceration in EtOH:H_2O 70% (v/v) (72 h)	-	*E. coli, P. aeruginosa,* and *S. aureus*	Microdilution assay	Only *S. aureus* (MIC = 256 mg/mL). Synergic effect with ciprofloxacin and gentamicin at 1024 mg/mL	[21]
Brazil	Maceration in MeOH:H_2O 70% (v/v) (48h)	-	*S. aureus* strains	Disc diffusion assay	MIC 90% = 0.52 mg/mL, at 131.75 mg/mL synergic effect with tetracycline, chloramphenicol, erythromycin, vancomycin, oxacillin, cephalothin, ampicillin, cefoxitin, cotrimoxazole	[22]
Egypt	Maceration in EtOH:H_2O 50% (v/v)	Quercetin, avicularin, guajaverin, isoquercitrin, hyperin	*S.aureus, E.coli, P. aeruginosa,*and *C.albicans*	Agar well diffusion assay	*S. aureus*: ↑ iz quercetin (28 mm), MIC (µg/mL) guajaverin (0.09-0.19) < avicularin (0.09-0.38) < quercetin (1.25) for all the microorganism tested	[23]

Origin	Extraction method	Major constituent	Microorganisms	Assay	Main results	Ref
Indonesia	Maceration in EtOH:H_2O 30% (v/v) (3 days)	Tannins (2.35 mg/g)	*E. coli, P. aureginosa, S. aureus, A. niger* and *C. albicans*	Paper disc diffusion method	iz ≤ 15 mm	[24]
India	Soxhlet with toluene (72 h)	Betulinic acid and lupeol	Fungi: *Calletotricheme camellie, Fussarium equisitae, Alterneria alternate, Curvularia eragrostidies,* and *Colletrichum Gleosproides*. Bacteria: *E, Coli, B. Subtillis, S. aureus,* and *Enterobactor*	Slide germination method	Bacteria: MIC < 100-200 µg/mL, fungi: MIC < 2.5-10 µg/mL	[25]
India	Soxhlet with Ac (8 h)	-	*H. bispinosa* Neumann (Acarina: *Ixodidae*) and *H. maculata* Leach (Diptera: *Hippoboscidae*)	Antiparasitic activity method of FAO (2004)	At 3 mg/mL: mortality 100% *H. maculate*, 78% *H.bispinosa* , parasite dead *H. maculata* (LC_{50} = 0.646 mg/mL)	[26]
Nigeria	Maceration with agitation in EtOH:H_2O 20% and 80% (v/v) (24 h)	-	*Trypanosoma brucei brucei* and HEK293	Alamar Blue assays	At 238.10 µg/mL: IC_{50} (*T. b. brucei*) = 6.3 µg/mL and 48.9 µg/mL for 80% and 20% extracts, respectively, IC_{50} (HEK293) 30.1 and 24.16%, respectively	[27]
India	Soxhlet with ethyl acetate and MeOH(8 h)	-	*Plasmodium falciparum* strains	SYBR green assay	IC_{50} 9-18 µg/mL, resistance indices = 0.6 and 1.4 in MeOH and ethyl acetate, respectively	[28]
Malaysia	Hydrodistillation (3 h)	-	*Toxoplasma gondii*	MTT assay with Vero cells	At 200 µg/mL: No cytotoxic effect (EC_{50} = 37.54 µg/mL), anti-parasitic activity (EC_{50} of 3.94 µg/mL)	[29]

Origin	Extraction method	Major constituent	Microorganisms	Assay	Main results	Ref
India	Soxhlet with EtOH, and maceration in H_2O (6 days)	-	*E. coli*, *Shigella* spp., *Salmonella* spp., *Aeromonas* spp., *S. aureus*, and *Candida* spp.	Agar well diffusion assay	H_2O: iz ≤ 30 mm (max *C. albicans*). EtOH: iz ≤ 31 mm (max *Aeromonas hydrophila*)	[30]
India	Soxhlet with EtOH:H_2O 70% (v/v), MeOH, ethyl acetate, and H_2O	Phytochemical analysis: tannins, saponins, flavonoids, terpenoids, sugars	*E. coli*, *Salmonella* spp., and *Vibrio cholerae*	Agar well diffusion assay	At 1000 µg/mL: iz ≤ 30 mm. MeOH: MIC (100%) > 250 µg/mL. EtOH:H_2O: MICs (38-65%) > 500 µg/mL and > 750 µg/mL. Ethyl acetate and H_2O: MICs > 750 µg/ml.	[31]
Bangladesh	Maceration in H_2O and MeOH:H_2O 75% (v/v) (48 h)	-	*V. cholerae*	Agar well diffusion assay	MICs = 1,250 µg/mL (H_2O), 850 µg/mL for (MeOH:H_2O). Antibacterial resistance to trimethoprim/sulfomethoxazole, furazolidone, tetracycline, and erythromycin	[32]
India	Decoction	Major component: quercetin (2 mg/g)	*E. coli* (heat labile (HLT) and cholera toxin (CT)), *V. cholerae*, *Shigella flexneri*	Microtitre plate based assay. Assays for bacterial colonization (adherence and invasion) and enterotoxins	At 2.7 mg/mL: (EC_{50} = 0.98 (*S. flexneri*) and 2.88% (*V. cholerae*). ↓ adherence and invasion to epithelial cells (EC_{50} = 0.37-1.25% and 0.04-0.25%, respectively). The effect on adherence is not due to quercetin and the invasion is lower than with the extract. ↓ production of HLT and CT (EC_{50} = 1.03 and 2.69%) and binding to glioside monosialic acid enzyme (EC_{50} = 0.06 and 2.51%)	[33]
Brazil	Soxhlet with hexane, ethyl acetate, MeOH, H_2O (24 h)	-	*S. aureus*, *Salmonella* spp.,and *E. coli*	Disc diffusion method	At 1938 µg/disc: iz = 7.00-11.25 mm (shoxlet), and 11-18 mm (H_2O). No inhibition to *E. coli* (H_2O)and *Salmonella* spp. (hexane and ethyl acetate)	[34]
Nigeria	Soxhlet with EtOH:H_2O 60% (v/v) (5 h), and H_2O (3 h)	-	*E. coli* and *S. aureus*	Agar well diffusion assay	At 10 mg/mL: H_2O: iz = 9-16 mm and 8-11 mm, MICs = 5 and 2.5 mg/mL (*E. coli* and *S. aureus*, respectively). EtOH:H_2O: iz 12-21 and 11-14 mm, MICs = 1.25 and 0.625 mg/mL, respectively.	[35]
Japan	Infusion (8 min)	Tannin content: 1.11 mg/mL	H1N1 virus strains	19-h influenza growth inhibition assay	At 0.4 mg/mL: inhibition growth (IC_{50} = 0.05-0.42%)	[36]

Acetone (Ac); N, N-dimethylformamide (DMF); dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); effective concentration (EC_{50}); inhibition zone (iz); inhibitory concentration (IC_{50}); lethal concentration (LC_{50}); minimum bactericidal concentration (MBC); minimum fungicidal concentration (MFC), minimum inhibitory concentration (MIC); total flavonoid content (TFC); total phenolic content (TPC); Tetrazolium (MTT); ↑ increases the effect; ↓ decreases the effect

2.1.2. Neoplasms

All the results published regarding anti-cancer properties has been summarized in Table 2.

Kawakami et al. [37] evaluated the anti-proliferative activity of guava leaf extract in human-colon adenocarcinoma cell line (COLO320DMA). These authors found that the extract depressed the proliferation rate due to the presence of quercetin and quercetin glycosides. Moreover, different extracts were tested on three cancer-cell lines (cervical cancer (HeLa), breast cancer (MDA-MB-231), and osteosarcoma (MG-63)). The extracts showed no anti-proliferative activity towards HeLa cells, although they displayed activity against MDA-MB-231 and MG-63, the ether extract being the most effective, followed by methanol and water extracts. However, ether and methanol extracts presented a cytotoxic effect on non-malignant cell Madine Darby canine kidney (MDCK) [38]. In contrast, an ethanol extract from the stem and leaves reported significant anti-tumor activity on HeLa and colorectal carcinoma (RKO-AS45-1), whereas its effect was less significant for a lung fibroblast cell line (Wi-26VA4) [39]. This difference could be due to the origin of the leaves, compounds in the steam, or even to the extraction method selected. In this context, an organic guava leaf extract provided molecular evidence of cytotoxic or anti-tumor activity in human breast carcinoma benign cells (MCF-7) and also in murine fibrosarcoma (L929sA) [40]. A fact worthy to comment is that the difference noticed in the cytotoxic effect on MDA-MB-231 cell line might be because the extraction differs [38,40]. Furthermore, the aqueous extract of budding guava leaves displayed an anti-tumor effect against human prostate epithelial (PZ-HPV-7) and carcinoma (DU-145) cells in view of the cell-killing-rate coefficients, as well as anti-angiogenesis and anti-migration activities, respectively [41,42].

Regarding the bioactivity of terpenes from guava, an enriched mixture of guajadial, psidial A, and psiguadial A and B proved anti-proliferative effect for nine human cancer lines: leukemia (K-562), breast (MCF-7), resistant ovarian cancer

(NCI/ADR-RES), lung (NCI-H460), melanoma (UACC-62), prostate (PC-3), colon (HT-29), ovarian (OVCAR-3), and kidney (786-0) [43]. The apoptotic effect of β-caryophyllene oxide (CPO) on MCF-7 and PC-3 cell lines was also demonstrated because of its ability to interfere with multiple signaling cascades involved in tumor genesis [44]. Moreover, the essential oil from guava leaves exerted an anti-proliferative effect on human-mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines [45], while a hexane fraction of the leaves showed a cytotoxic effect against leukemia (Kasumi-1) cancer-cell line at higher half maximal inhibitory concentration (IC_{50}), probably due to a less concentration of the bioactive compounds of the leaves[46]. Finally, cytotoxic and apoptotic effect in PC-3 cells and apoptotic effect in LNCaP cells was reported. The lack of cytotoxic effect in LNCaP might be because the cell growth is androgen-dependent, while in PC-3 is androgen-independent. [47]. Comparing these data with those reported by Park et al. [44], high concentration is needed for causing cell death, and a weak effect is found on early apoptotic cell. The main difference between these works is the composition of the extract, so it could be conclude that an antagonist effect is produced amongst the isolated compounds by Ryu et al. [47].

Table 2 *In vitro* studies against neoplams

Origin	Extraction method	Major constituent	Cell line	Assay	Main results	Ref
Japan	Maceration in EtOH:H_2O 50% (v/v)	TPC: 71 g/100g	Human colon adenocarcinoma (COLO320DMA)	Cyclooxygenase and cell proliferation assays	At 1 mg/mL: ↓ human cyclooxygenase activity (IC_{50} 55 and 560 µg/mL PGHS-1 and 2, respectively), ↓ IC_{50} 5.1 µg/mL (PGSH) and 4.5 µg/mL (cyclooxygenase). At 100 µg/mL: Quercetin ↓ IC_{50}= 5.3 (PGSH-1) and 250 µg/mL (PGSH-2), ↓ DNA synthesis rate	[37]
Malaysia	Soxhlet with ether, MeOH, and H_2O	-	Cervical cancer (HeLa), breast cancer (MDA-MB-231) and osteosarcoma (MG-63). Control: non-malignant Madin-Darby canine kidney (MDCK)	Methylene blue assay	At 10 mg/mL: HeLa: No anti-proliferative activity. MDA-MB-231: IC_{50} ether extract (4.2 µg/mL) > MeOH (18.6 µg/mL) > H_2O (55.7 µg/mL). MG-63: same order (IC_{50} of 5.42, 23.25, and 61.88 µg/mL, respectively). MDCK: cytotoxic effect of ether and MeOH extract (IC_{50} = 5.03 and 11.55 µg/mL, respectively)	[38]
Brazil	Maceration in EtOH	TPC: 766.08 mg/g, TFC: 118.90 mg/g	HeLa, colorectal carcinoma (RKO-AS45-1), and lung fibroblast (Wi-26VA4)	MTT assay	At 1 mg/mL: IC_{50} = 15.6 µg/mL (HeLa), 21.2 (RKO) µg/mL, and 68.9 µg/mL (Wi-26VA4)	[39]
Palestine	Maceration in DCM:MeOH 50% (v/v) (24 h)	-	Murine fibrosarcoma (L929sA), and human breast cancer (MDA-MB-231 and MCF-7)	MTT assay	IC_{50} = 55 µg/mL (L929sA), 820 µg/mL (MCF7 cells), no cytotoxic effect on MDA-MB-231 cells.	[40]

Origin	Extraction method	Major constituent	Cell line	Assay	Main results	Ref
Taiwan	Decoction (30 min)	-	Human prostate carcinoma (DU-145)	MTT, ELISA, gelatinolytic zymography, wound scratch, and chicken chorioallantoic membrane assays	At 0.25 mg/mL: cell suppression (IC_{50} 0.57 mg/mL). ↓ expressions of VEGF (76.9%), IL-6 (98.8%) and IL-8 (98%), and MMP-2 (100%) and MMP-9 (100%). Suppressed the cell migration (30.9%) and the angiogenesis	[41]
Taiwan	Decoction (1 h)	TPC: 470.0 mg/g Individual compounds: gallic acid (348), catechin (102), epicatechin (60), rutin (100), quercetin (102), and rutin (100) in mg/g	Human prostate epithelial (PZ-HPV-7) and DU-145	MTT assay	At 1 mg/mL: 100% suppression DU-145 cells. PZ-HPV-7 cells followed an auto-decaying process. Cell-killing rate coefficient (kapp) =.03x10^3 phenolic compounds cells/mg h.	[42]
Brazil	Soxhlet with DCM. Maceration with EtOH	Guajadial, psidial A, and psiguajadial A and B	Leukemia (K-562), MCF-7, ovarian cancer (NCI/ADR-RES), lung (NCI-H460), melanoma (UACC-62), prostate (PC-3), colon (HT-29), ovarian (OVCAR-3), and kidney (786-0)	protocol established by NCI (ELISA test)	At 250 µg/mL: Anti-proliferative activity DE > EtOH, inhibition growths: 26 (OVCAR-3)-65 (UACC-62) µg/mL due to the major compounds	[43]
Japan	Maceration with sonication in MeOH:H_2O 80% (v/v) (3 h) and isolation	CPO	PC-3 and MCF-7	MTT, annexin V antibody, TUNEL, and western blot assays	At 50 µg/mL: ↓ cell proliferation, ↑ early and late apoptotic effect, down-regulation of PI3K/AKT/mTOR/ S6K1 pathway , up-regulation of MAPKs, JNK, ERKs, and p38 MAPK	[44]

Origin	Extraction method	Major constituent	Cell line	Assay	Main results	Ref
Thailand	Hydrodistillation		Human mouth epidermal carcinoma (KB) and murine leukemia (P388)	MTT assay	At 0.15 mg/mL: KB: 75% cytotoxic effect, IC_{50} = 0.04 mg/mL At 0.08 mg/mL: P388: 80% cytotoxic effect, IC_{50} = 0.05 mg/mL	[45]
Jamaica	Maceration in hexane (4 days)	-	Leukemia (Kasumi-1)	MTT assay	IC_{50} = 200 µg/mL	[46]
Japan	Maceration with sonication in MeOH:H_2O 80% (v/v) (3 h). Fractionation with hexane	60 compounds (in hexane fraction): β-eudesmol (11.98%), α-copaene (7.97%), phytol (7.95%), α-patchoulene (3.76%), and CPO (3.63%)	Human prostate cancer (PC-3 and LNCaP)	MTT, annexin V antibody, TUNEL, and western blot assays	At 150 µg/mL: PC-3: ↑ apoptotic effect of the hexane fraction (15%), ↓ effect on early apoptotic cells, ↑effect for late apoptosis, via the suppression of PI3K/AKT/mTOR/S6K1 and MAPK signalling cascades in both cell lines	[47]

β-Caryophyllene oxide (CPO); c-jun NH2-terminal kinases (JNK); dichloromethane (DCM); inhibitory concentration (IC_{50}); mammalian target of rapamycin (mTOR); mitogen-activated protein kinases (MAPKs); phosphatidylinositol 3-kinase (PI3K); prostaglandin endoperoxide H synthase (PGHS); protein kinase B (AKT); ribosomal protein S6 kinase beta-1 (S6K1); signal-related kinases (ERKs); tetrazolium (MTT); total flavonoid content (TFC); total phenolic content (TPC); ↑ increases the effect; ↓ decreases the effect

2.1.3. Diseases of the blood and immune system

A fermented guava leaf extract was tested in mouse macrophage (RAW 264.7) cells. The results confirmed its potential to decrease the expression of lipopolysaccharide-inducible nitric oxide synthase and cyclooxygenase-2 proteins level, two pro-inflammatory mediators, through the down-regulation of nuclear factor-κB transcriptional activity (NF-κB) [48]. This biological activity was also reported in other works [40,49,50]. Briefly, Jang et al. [49] evaluating the prostaglandin E_2 production found that the inhibitory effect was highly correlated to the total phenolic content. Kaileh et al. [40] suggested that the suppression of the nuclear factor-κB could be at the transcriptional level because of the lack of binding between nuclear factor-κB and DNA in murine fibrosarcoma (L929sA) and two breast-cancer cell lines (MDA-MB231 and MCF7). At the same time, Jang et al. [50] found that the lipopolysaccharide-induced production of nitric oxide and prostaglandin E_2 was due to the ability of guava leaf extract to suppress phosphorylation in protein expression. Moreover, Sen et al. [51] verified the inhibition of nuclear factor-κB activation in *Labeo rohita* head-kidney macrophages by the flavonoid fraction of guava leaf extract and Jang et al. [52] improved the inhibition of lipopolysaccharide-induced prostaglandin E_2 and nitric oxide production by optimizing of the extraction conditions. Furthermore, methanol and ethanol leaf extracts also showed the inhibition of hypotonicity-induced lysis of erythrocyte membrane [53]. Meanwhile, Laily et al. [54] suggested the use of guava leaves as immune-stimulant agent because they modulated the lymphocyte proliferation response.

The results for this activity, confirm the potential of guava leaves as an anti-inflammatory treatment and as immune-system stimulatory agent. As is shown in Table 3, a general trend is reported in every work, although the differences noticed in the data, probably are due to the different extraction method and to the doses assayed, or even the harvesting time of the leaves. However, the mechanism should be further studied since two different pathways are suggested for the down-regulation of NF-kB.

Table 3 *In vitro* assays against diseases of the blood and immune system

Origin	Extraction method	Major constituent	Cells	Assay	Main results	Ref
Korea	Maceration in MeOH:H_2O 70% (v/v) (5 days)	-	LPS-stimulated RAW 264.7 (Mouse macrophage)	Griess, MTT, ELISA kit, western blot, transient transfection, and luciferase assays	At 125 µg/mL: no cytotoxic effect, ↑ 44 -62% inhibition rates. ↓ LPS-induced NO and PEG_2 ↓ iNOS and COX-2(↓ I-κBα degradation, ↓ activation NF-κB)	[48]
Palestine	Maceration in DCM:MeOH 50% (v/v) (24 h)	-	L929sA fibroblast	Transfection and luciferase assays	At 62.5 µg/mL: ↓ expression of IL-6 and NF-κB luciferase reporter gene construct via the NF-κB transactivation level, since no ↓ inhibition of NF-κB/DNA binding	[40]
Korea	Extraction in MeOH:H_2O 70% (v/v) (6 h)	TPC: 426.84 mg (GAE)/g	LPS-stimulated RAW 264.7	MTT, Griess, and ELISA test assays	At 30 µg/mL: no cytotoxic effect. ↓ LPS-induced NO (52.58%) and the production of PGE_2 (43.45)	[49]
Korea	Extraction in EtOH:H_2O 55% (v/v) (4.9 h, 47°C)	Gallic acid (0.2) and catechin (4.4) in mg/g	LPS-stimulated RAW 264.7	MTT, Griess, ELISA test, RT-PCR, and total western blot assays	At 50 µg/mL: no cytotoxic effect. ↓ LPS-induced NO (> 65%) by ↓ iNOS, ↓ PGE_2 (to basal level) via ↓ COX-2 mRNA. ↓ IL-6. ↓ iNOS and COX-2 due to the down-regulation of ERK1/2 pathway, because no effect was found to other proteins at the dose tested	[50]
India	Maceration in MeOH:H_2O 90% (v/v) (x3)	-	LPS-stimulated in Labeo rohita head-kidney macrophages.	MTT, Greiss, ELISA, RT-PCR, and western blot assays	At 200 µg/mL, ↓ LPS-induced NO (75%) by ↓iNOS-mRNA, ↓PGE2 (45%) via ↓ production COX-2-mRNA, TNF-α, IL-1β, IL-10, and mRNA expression. Suppressed phosphorylation of MAPK (↓ I-κBα degradation ↓ activation NF-κB)	[51]
Korea	Soxhlet with EtOH:H_2O 55% (v/v) (4.9 h, 47°C)	Gallic acid (0.09) and catechin (0.72) in mg/g	LPS-stimulated RAW 264.7	MTT, Greiss and ELISA test assays	At 30 µg/mL: no cytotoxic effect. ↓ LPS-induced NO (47.5%) and PGE_2 (45.8)	[52]
India	Maceration with agitation in MeOH and EtOH(24 h)	-	Human blood	HRBC membrane stabilization method	At 200 µg/mL: ↑ 13.8- 14.4% prevention of lysis of the membrane	[53]
Indonesia	Maceration with agitation in EtOH:H_2O 96% (v/v) (6h)	TPC: 101.93 mg GAE/g	Human lymphocyte	MTT assay	0.5 µg/mL: stimulation index 1.54%	[54]

Cyclooxygenase-2 (COX-2); dichloromethane (DCM); gallic acid equivalent (GAE); human red blood cell (HRBC); inducible nitric oxide synthase (iNOS); inhibitor of kappa B (I-κBα); interleukin-1β (I-1β); lipopolysaccharide (LPS); mitogen-activated protein kinases (MAPKs); nitric oxide (NO); prostaglandin E_2 (PEG_2); reverse transcription-polymerase chain reaction RT-PCR; tetrazolium (MTT); total phenolic content (TPC); transcriptional nuclear factor-κB (NF-κB); Tumour necrosis factor alpha (TNF-α); ↑ increases the effect; ↓ decreases the effect

2.1.4. Endocrine and metabolic diseases

Several works have focused on elucidating the anti-diabetic compounds present in guava leaves (Table 4). Although the origin of the leaves remains different, the presence of these compounds has demonstrated the hyperglycemic effect of the leaves via different assays. However, the main mode of action seems to be due to an inhibition of the enzymes related to this activity.

The anti-glycative potential of the guava leaves was evaluated, with the conclusion that the extract inhibited, *in vitro*, the formation of advanced glycation end-products formation [55]. Moreover, the aqueous guava leaf extract, in an albumin/glucose model system, also exerted the same effect and indeed inhibited Amadori products. Gallic acid, catechin and quercetin exhibited over 80% inhibitory effects whereas ferulic acid showed no activity [56]. In another study, seven pure flavonoid compounds (quercetin, kaempferol, guaijaverin, avicularin, myricetin, hyperin, and apigenin) showed strong inhibitory activities against sucrase, maltase, and α-amylase, and an clear synergistic effect against α-glucosidase [57]. Moreover, Deguchi and Miyazaki [58] suggested that the component that inhibited the *in vitro* activities of α-glucosidase enzymes in guava extract was a polymerized polyphenol. In addition, polysaccharides from guava leaves also exhibited α-glucosidase inhibition [59].

Eidenberger et al. [60] demonstrated the dose-dependent inhibition of guava leaf ethanol extracts on dipeptidyl-peptidase-IV due to the individual flavonol-glycosides: peltatoside, hyperoside, methylquercetin hexoside, isoquercitrin, quercetin/morin pentoside, guaijaverin, and quercetin/morin pentoside. Additionally, the individual flavonol-glycosides found in the guava extract reported no significant differences compared with the uptake of the whole guava extract into epithelial cells (CaCo-2) [60]. In the same cell line, the inhibition of fructose uptake was also tested by Lee et al. [61], who confirmed that catechin and quercetin contributed to the inhibition of glucose transporters. Also, the enhancement of aqueous guava leaf extract

was investigated with regard to glucose uptake in rat clone 9 hepatocytes. Moreover, quercetin was proposed as the active compound responsible for promoting glucose uptake in liver cells and contributing to the alleviation of hypoglycemia in diabetes [62]. Furthermore, Basha and Kumari [63] also estimated the glucose uptake of different extracts. The methanol extract of guava leaves was found to be the most efficient in lowering glucose levels. Basha et al. [64] demonstrated the ability of guavanoic-acid-mediated gold nanoparticles to inhibit the protein tyrosine phosphatase 1B activity.

Table 4 Compounds in guava leaves with anti-diabetic properties in *in vitro* assays.

Origin	Compound	Assay	Main results	Ref
India	Ethyl acetate fraction	*In vitro* glycation of BSA-fluorescence mesurement	*In vitro* AGEs formation with IC_{50} of 38.95 ± 3.08 µg/mL	[55]
Taiwan	Gallic acid, catechin and quercetin	*In vitro* glycation of BSA-fluorescence mesurement; Fructosamine assay and Girard-T assay	At 100 µg/mL: 80% inhibitory effects on the formation of α-dicarbonyl compounds at a concentration of 50 µg/ml, inhibitory effects on AGEs formation in BSA glycation systems	[56]
China	Quercetin, kaempferol, myricetin	Rat intestinal sucrase and maltase inhibitory activities; Porcine pancreatic α-amylase inhibitory activity	At 1.5 mg/mL: inhibitory activities with IC_{50} values of 3.5 mM, 5.2 mM and 3.0 mM against sucrase, with IC_{50} values of 4.8 mM, 5.6 mM and 4.1 mM against maltase and with IC_{50} values of 4.8 mM, 5.3 mM and 4.3 mM against α-amylase, respectively. Synergistic effect against α-glucosidase	[57]
China	Water-soluble polysaccharides, including GP90 and P90	α-glucosidase inhibition assay	α-glucosidase inhibition activity with an EC_{50} of 2.27 µg/mL and 0.18 mg/mL	[59]
-	Peltatoside, hyperoside, isoquercitrin, guaijaverin and flavonol-glycosides	Spectrophotometric assay; absorption assay into CaCo-2 cells	Concentration of the compounds (0.01 to 0.06 µmol/mL). Individual flavonol-glycosides inhibited DP-IV dose-dependently. The ethanolic guava leaves extract (380 µg/mL) showed a dose-dependent inhibition of DP-IV, with an IC_{50} of 380 µg/mL test assay solution; the highest uptake was from Guaijaverin	[60]
Korea	Quercetin and catechin	Fructose transport in CaCo-2 cell systems	At 1 mg/mL: inhibition of fructose uptake (55%). At 30 µg/mL: quercetin contributed to both, GLUT2 and 5 transporters, and catechin to GLUT5-mediated fructose uptake inhibition	[61]
India	Guavanoic acid	Spectrophotometric assay	At 27 µg/mL: remarkable PTP1B inhibitory activity (90%) and in vitro stability in various physiological medium including saline, histidine, cysteine, BSA, HSA and buffers (pH 5, 7 and 9). IC_{50} = 1.14 µg/mL	[64]
India	N-hexane, methanol, ethanol and aqueous leaf extracts	Inhibitory glucose diffusion	At 50 g/L: the methanol extract was the most potent with the lowest mean glucose concentration of 201 ± 1.69 mg/dl at the end of 27 hours (↓93% uptake)	[63]

Origin	Compound	Assay	Main results	Ref
Japan	70% ethanol extract	Oil Red O Assay; Real-Time RT	At 100 µg/mL: inhibition of 3T3-L1 differentiation via down-regulation of adipogenic transcription factors and markers (mRNA levels of PPAR-γ, C/EBP-α, and aP2), and suppression of mitotic clonal expansion (at day 4 and 8)	[65]
Taiwan	Aqueous extract	Glucose uptake test; bicinchonic acid method; Western-blot analysis	At 400 µg/mL: ↑IR (25.1%), p-IR (46.2%), p-IRS (51.2%), PI3K (32.2%), Akt (46.1%), p-Akt (36.3%), GLUT-2 (46.8%), and total glycogen synthase (45.5%)	[66]
Taiwan	Vescalagin	Glucose-uptake test	At 100 µg/mL: Enhancement of glucose uptake in TNF-α-induced insulin-resistant	[67]

Advanced glycation end products (AGEs); bovine serum albumin (BSA), dipeptidyl peptidase (DP); effective concentration (EC_{50}); glucose transporter 2 and 5 (GLUT-2; GLUT-5); human serum albumin (HSA); inhibitory concentration (IC_{50}); insulin receptor (IR); insulin receptor substrate (p-IRS (Tyr)); p85 regulatory subunit of phospho-inositide 3 kinase (PI3K (p85)); phosphorylation of the insulin receptor (p-IR (Tyr)); protein kinase B (p-Akt (Ser)); tumor necrosis factor (TNF); ↑ increases the effect; ↓ decreases the effect

Indeed, a guava leaf ethanol extract was tested in pre-adipocyte cell line (3T3-L1), which showed its ability to inhibit adipocyte differentiation via down-regulation of adipogenic transcription factors and markers, and hence may prevent obesity *in vivo* [65]. To evaluate the potential of the leaves on glucose uptake and glycogen synthesis, an aqueous extract was used in insulin-resistant mouse (FL83B) cells. The results confirmed the improved expression and phosphorylation of insulin signaling-related proteins, promoting glycogen synthesis and glycolysis pathways. In fact, this work provides new insights into the mechanisms through which the guava extract improves insulin resistance in the hepatocytes [66]. In the same cell line, vescalagin was postulated as the active component that may alleviate the insulin resistance in mouse hepatocytes [67].

In this sense, the latest study made in L6 myoblasts and myotubes cells confirmed that the glucose uptake recruitment followed a wortmannin-dependent pathway. In addition, guava leaves also inhibited aldose reductase activity, up-regulated gene- and protein-level expression of several insulin receptors and also improved cellular-level glucose uptake [68].

2.1.5. Diseases of the circulatory system

Cardiovascular disorders have been related to the endothelial cell damage that causes atherosclerosis. In this sense, extracts from budding guava leaves demonstrated a protective, *in vitro*, effect in bovine aortal endothelial cells, delaying low-density lipoprotein oxidation and preventing oxidized low-density lipoprotein cytotoxicity [69]. A similar effect was also noted in human umbilical-vein endothelial cell due to the ability of saving cell-viability reduction, suppressing reactive oxygen species production and nitric oxide release, as well as inhibiting the expression of NF-κB [70]. Moreover, budding guava leaves also showed their ability as an anticoagulant in plasma, since they reduced thrombin clotting time and inhibited the activity of antithrombin III. Thus, they could help to reduce the development of cardiovascular complications [71].

In addition, flavonoids and phenolic acids in the leaves could contribute to the prevention and amelioration of gout and hypertension, since, in rat-tissues homogenates, they inhibit the activity of two enzymes related to the development of both diseases (xanthine oxidase and angiotensin 1-converting enzymes) [72].

2.1.6. Diseases of the digestive system

Guaijaverin, isolated from guava leaves, displayed high inhibitory activity against *Streptococcus mutans*. In fact, guaijaverin exhibited its ability as an anti-plaque agent, becoming an alternative for oral care [73]. Furthermore, guava leaves showed greater bactericidal effect on early (*Streptococcus sanguinis*) and late (*S. mutans*) colonizers compared to *Mangifera indica* L. and *Mentha piperita* L. leaves; whereas, when they are compared with the plant extract mixture, the effect is slightly lower. By contrast, guava leaves showed similar and higher anti-adherence effect than the plant mixture [74]. In another study, the whole extract was tested on the cell-surface hydrophobicity of selected early settlers and primary colonizers of dental plaque, showing its ability to alter and disturb the surface characteristics of the agents, making them less adherent [75–77], and also delayed in the generation of dental biofilm by targeting growth, adherence, and co-aggregation [78]. This property could be due to the presence of flavonoids and tannins detected in *P. guajava* [79]. Shekar et al. [80] also confirmed the use of the leaves as anti-plaque agents against *Streptococcus mutans*, *S. sanguinis*, and *S. salivarius*. Kwamin et al. [81] discovered the effectiveness of guava leaf extract in the leukotoxin neutralization of *Aggregatibacter actinomycetemcomitans*, leading it to be considered as a possible agent for the treatment of aggressive forms of periodontitis. In addition, extracts rich in guava flavonoids have demonstrated their potential for preventing dental caries due to the growth inhibition of the oral flora [82]. Moreover, its soothing of toothache has been verified based on the analgesic, anti-inflammatory, and anti-microbial activity properties [83] and it has been reviewed positively as an adjutant for treating periodontal disease [84].

Concerning the liver disorders, the cytotoxic and hepato-protective effects of guava leaves were reported. Studies carried out in clone 9 cells treated with different extracts of the leaves showed that only ethanol and acetone extracts tend to have cytotoxicity effect at high concentrations. Moreover, the ethanol extract showed hepato-protective activity, although the hot-water extract reported greater effect and lower cytotoxicity [85].

Table 5 compiles the methodology followed and the results reported in the present works. It is important to keep in mind that the origin, the selection of the extraction method or solvent, and the concentration of the extract tested, generally provide different data. For example, comparing data for inhibition zones, best results are noticed at long maceration time in acetone, which seems to be better extracting solvent than ethanol [77,78,80,82]. Hydrophobicity, depends on the origin of the leaves, the extraction method, and the concentration of the extract tested, and also it depends on the lipophilic (index > 70%) or hydrophilic nature of the strain [73,75,79]. Finally, minimum inhibitory concentration relies on all factors.

2.1.7. Diseases of the skin and subcutaneous tissue

Qa'dan et al. [86] described the antimicrobial effect of a leaf extract against the main developer of acne lesions, *Propionibacterium acnes*, and other organisms isolated from acne lesions. The antimicrobial activity was also displayed against pathogenic bacteria associated with wound, skin, and soft-tissue infections [87]. Furthermore, antifungal properties have also been studied by Padrón-Márquez et al. [88]. The acetone and methanol extracts displayed relevant activity against dermatophytic fungi, and thus could be considered as new agents against skin disease. Furthermore, phenols from the leaves were tested on human-skin fibroblast cells and showed antifungal properties [89].

In addition, the tyrosinase inhibitory activities of 4 different parts (branch, fruit, leaf, and seed) of guava, extracted with acetone, ethanol, methanol, and water were tested by You et al. [90] who reported that the ethanol extract from the leaves

reached the highest activity. Therefore, the leaves might be appropriate for both boosting the whitening of skin and inhibiting browning. Also, in a human keratinocyte cell line, an ethyl acetate extract showed a positive effect on atopic dermatitis via the inhibition of cytokine-induced Th2 chemokine expression [91].

Lee et al. [92] carried out the first electrophysiological study based on ultraviolet (UV)-induced melanogenesis with guava leaves. The authors suggested the use of guava leaves for both direct and indirect prevention of skin melanogenesis caused by UV radiation. In fact, they demonstrate that methanolic guava leaves extract inhibits tyrosinase, that is the key enzyme in melanin synthesis, and ORAI1 channel that has shown to be associated with UV-induced melanogenesis.

2.1.8. Other activities related to several diseases

An aqueous guava extract showed its ability to decrease the radiolabeling of blood constituent due to an antioxidant action and/or because it alters the membrane structures involved in ion transport into cells [93]. Guava leaves also have been demonstrated to possess anti-allergic effects in rat mast (RBL-2H3) cell line by the inhibition of degranulation and cytokine production, as well as blocking high-affinity immunoglobulin E-receptor signaling [94].

Table 5 *In vitro* assays against diseases related to the digestive system

Origin	Extraction method	Microorganism(s)/cells	Assay	Main results	Ref
India	Soxhlet with MeOH (4.5 h)	*S. mutans* strains	Agar well diffusion assay, effect on acid production, on sucrose-dependent adherence to smooth glass surfaces, and on sucrose-induced cellular aggregation, and MATH assays	MIC > 5 mg/mL (MeOH). MIC = 2-4 mg/mL (guaijaverin) At sub-MIC (0.125-2 mg/mL): ↑ pH (5 to 6-7), hydrophobicity indexes (3.2-72%), ↓ sucrose-dependent adherence (34-84%) and aggregation	[73]
Malaysia	Decoction	*S. sanguinis* and *S. mutans*	NAM model system	At 60.95 mg/mL: MIC = 7.62 (*S. sanguinis*) and 3.81(*S. mutans*.) mg/mL. MBC values = 15.24 and 30.48 mg/mL, respectively. at 0.5 mg/mL: ↓ adherence 57 and 60% (single-species) and 88-89% (dual-species)	[74]
Malaysia	Sonication with H_2O (10 min)	*S. sanguinis, S. mitis*, and *Actinomyces* spp.	MATH assay	At 1 mg/mL: ↓ 54.1%, 49.9% and 40.6%, respectively, cell-surface hydrophobicity. At 20 mg/mL: was 64.7, 60.5, and 55.5%, respectively	[75]
Malaysia	Decoction	*S. sanguinis, S. mitis*, and *Actinomyces* spp.	Bacterial growth and generation time rates determinations	At 4 mg/mL: Time growth = 1.22 (*S. sanguinis, Actinomyces* spp) and 2.06 h (*S. mitis*) ↓ growth 42.6, 51.2 and 55%	[76]
India	Maceration with stirring in EtOH (2 days)	*S. mutans, S. sanguinis*, and *S. salivarius*	Agar well diffusion assay	At 10 mg/mL: inhibition zones of 21.17, 18.58, and 23.00 mm, respectively	[77]
India	Maceration (2 days) and soxhlet (6 h) with EtOH, H_2O, and EtOH:H_2O 50% (v/v)	*S. mutans* and *S. mitis*	Agar well diffusion assay, sucrose-dependent adherence and cellular co-aggregation activities, and biofilm formation sterile acrylic tooth determinations	At 15 mg/mL : inhibition zone for H_2O (11.8 mm) to EtOH:H_2O (25 mm), both by shoxlet. MIC = 1 mg/mL. EtOH:H_2O extract: at > 0.05 mg/mL: ↓ adherence and co-aggregation, at MIC, ↓ the viable count of dental biofilm (3.50 $\log_{10}$ CFU/mL)	[78]

Origin	Extraction method	Microorganism(s)/cells	Assay	Main results	Ref
India	Soxhlet with EtOH:H_2O 50% (v/v) (6 h)	*S. mutans* and *S. mitis*	MATH assay	At > 1 mg/mL ↓ hydrophobicity (index < 40%)	[79]
India	Maceration with stirring (2 days) and soxhlet with EtOH	S. mutans, S. sanguinis, and S. salivarius	Agar well diffusion assay	At 10 mg/mL: ↑ inhibition zones for maceration extracts (19-23 mm)	[80]
Ghana	Maceration with agitation in EtOH:H_2O 70% (v/v) (24 h)	*Aggregatibacter actinomycetemco mitans* strains	Agar well diffusion assay, release of the cytosol enzyme lactate dehydrogenase, fluorescence assisted cell sorter, and ELISA assays	No growth inhibitory effect, although neutralized the cell death and pro-inflammatory response, and restored the morphological alterations induced by the leukotoxin . These effects were due to the direct binding of guava compounds and the leukotoxin	[81]
India	Maceration in Ac, EtOH, chloroform, MeOH and H_2O (15 days at 22°C)	*Neisseria catarrhalis, S. mutans, S. salivarius, Streptococcus viridans, Bacillus megaterium,* and *P. aeruginosa*	Agar well diffusion assay	↑ inhibition zones in Ac (15-29 mm), except for *N. catarrhalis* (20 mm in MeOH)	[82]
India	Maceration in MeOH (72 h). Fractionation with ethyl acetate	*S. aureus* and *S. mutans*	HRBC membrane stabilization method, disc and agar well diffusion assays	MeOH and ethyl acetate fraction ↑ protection (84-99%) to the inflammatory response. Inhibition zones (25-100 µg/mL) = 10.5 to 22 mm by both methods. MICs = 0.48 (ethyl acetate)and 0.62 (MeOH)mg/mL	[83]
Taiwan	Maceration EtOH, Ac, H_2O (room temperature and 60°C) (24 h)	Clone 9 rat liver cells	WST-1 and ALT assays	At > 500 µg/mL cytotoxic effect of EtOH and Ac and 600 µg/mL for H_2O. At < 200 µg/mL normal values were observed for H_2O and Ac, and EtOH (< 500 µg/mL). At < 100 µg/mL,h Hepato-protective effect in EtOH and H_2O	[85]

Alanin aminotransferase (ALT); colony forming unit (CFU); human red blood cell (HRBC); microbial adhesion to hydrocarbon test (MATH); minimum bactericidal concentration (MBC); minimum fungicidal concentration (MFC); minimum inhibitory concentration (MIC); nordini's Artificial Mouth (NAM); Tetrazolium (WST-1); ↑ increases the effect; ↓ decreases the effect

2.2. In vivo studies

2.2.1. Infectious and parasitic diseases

After checking the effect of guava leaf extract, *in vitro*, against *Aeromonas hydrophila*, *in vivo* experiments were carried out in tilapia (*Oreochromis niloticus*), indicating the potential use of *P. guajava* as environmentally friendly antibiotic [95]. The leaves also had anti-viral and anti-bacterial activity towards shrimp (*Penaeus monodon*) pathogens such as yellow-head virus, white spot syndrome virus, and *Vibrio harvey*. Also, guava leaf extract improved the activities of prophenoloxidase and nitric oxide synthase in serum, and of superoxide dismutase, acid phosphatase, alkaline phosphatase, and lysozyme in serum and hepatopancreas [96].

Furthermore, guava leaves have been suggested for managing sleeping sickness, since they exhibited trypanocidal effect in albino rats [97]; the extract ameliorate the tissue-lipid peroxidation associated to trypanosomosis, as well as raising the level of the glutathione concentration [98]. The leaves also showed anti-malarial effect in BALB/c mice infected with *Plasmodium berghei* via parasitemia suppression [99]. Moreover, guava leaves are also recommended for treatening infectious diarrhea since they prevented intestinal colonization of *Citrobacter rodentium* in Swiss albino mice [100].In chicks, guava leaf extract enabled the control of diarrhea produced by *E. coli* and reduced the severity of its symptomatology [101]. In mice, the improvement of cholera symptoms caused by *V. cholerae*, a human pathogen, was also confirmed by Shittu et al. [102].

In addition, anti-helminthic properties towards gastro-intestinal nematodes have been found, as a result of the presence of condensed tannins in the guava plant, which raised the levels of hemoglobin, packed cell volume, total protein, globulin, glucose, and calcium, and lowered the levels of blood urea [103].

All the results published regarding *in vivo* anti-bacterial properties have been summarized in Table 6.

Table 6 *In vivo* anti-bacterial effect

Origin	Extraction method	Subject	Treatment	Main results	Ref
Thailand	Maceration in H_2O, EtOH, and ether (24 h)	*Oreochromis niloticus*	*Aeromonas hydrophila*	LD_{50} = 3.44×106 CFU/mL. ↓ mortality of the subjects	[95]
China	-	*Penaeus monodon*	Yellow-head virus, white spot syndrome virus, and *Vibrio harveyi*	Survival rate = 80-95% (↑ Weight (2 to6 g)). In serum (↑ feed): ↓ PO (7.50 U/mL) and SOD (178.33 U/mL), ↑ NOS (64.80 U/mL). In hepato-pancreas : ↑ SOD (57.32 U/mg), ACP (23.28 U/mg), AKP (19.35 U/mg), and LSZ (3459.946 U/mg)	[96]
Nigeria	Maceration with agitation in EtOH:H_2O 80% (v/v) (24 h)	Albino rats	*T. b. brucei*	At 300 mg/kg: ↓ parasitemia; ↑ survival in 24 days	[97]
Nigeria	Maceration with agitation in EtOH:H_2O 80% (v/v) (24 h)	Albino rats	*T. b. brucei*	Administration 1-7 days. ↑ GSH: liver (5.4 to 8.1), kidney (3.3 to 6.0), and serum (0.8 to 2.4), restored in kidney and serum. In the brain, no effect was found. ↓ MDA: serum (13.9 to 5.9), brain (42.8 to 18.1), kidney (27.3 to 17.6), and liver (38.2 to 19.2)	[98]
India	Decoction of the leaves (10 min)	BALB/c mice	*Plasmodium berghei*	At 350 and 1,000 mg/kg ↓ parasitemia (73.7% and 85.8%); ↑ survival 15 and 18 days	[99]
India	Extraction in EtOH:H_2O 50% (v/v)	Swiss mice	*Citrobacter rodentium*	At 300 mg/kg: ↓ infection (day 4) of the treatment, and no infection at day 19 (control group at day 24)	[100]
Nigeria	Hidrodistillation and fractionation with ethyl acetate	ISA brown male chicks	*E. coli*	At 100 mg/kg: In 10 days ↓ signs of villous collapse (stunting, matting and fusion of villi), number of wet droppings (12-6); ↑ activity, weight gaining, and feed intake (from 27 to 45 g) in contrast to the infected ones (from 30 to 18 g); ↓ bacterial shedding load (from 60 to 45 CFU/mL)	[101]
Nigeria	Decoction of the leaves	Adult mice	*V. cholerae*	At 250mg/kg: Histopathological observations: mild degenerative, secretory, and inflammatory changes with goblet cells and with most of the exudate (neutrophils and lymphocytes)	[102]

Origin	Extraction method	Subject	Treatment	Main results	Ref
India	-	Adult male goat	*Haemonchus contortus*	90 days feeding: ↑ Hb (7.2 to 8.6 g/dL), PCV (20.2 to 29.3%), total protein (4.8 to 6.3 g/dL), GLO (2.3 to 3.8 g/dL) (↑ control (2.8)), glucose (43.9 to 52.6 g/dL), and calcium (8.7 to 9.6 mg/dL); ↓ blood urea (47.9 to 29.8 mg/dL) (↓ control (41)). Phosphorus balance, serum albumin levels and serum enzyme activity did not show variation.	[103]

Acid phosphatase (ACP); alkaline phosphatase (AKP); colony forming unit (CFU); globulin (GLO); glutathione (GSH); hemoglobin (Hb); lysozyme (LSZ); malondialdehyde (MDA); median lethal dose (LD_{50}); nitric oxide synthase (NOS); packed cell volume (PCV); prophenoloxidase (PO); superoxide dismutase (SOD); ↑ increases the effect; ↓ decreases the effect

2.2.2. Neoplasms

Only one study is available on the anti-tumor effect that could be related to the phenolic composition of guava leaves. An ethanol extract of the leaves was administrated to B6 mice after inoculation of melanoma cells. The results suggested that the extract had a vaccine effect, but not a therapeutic effect, against tumors through by depressing T regulatory cells [104].

Moreover, the meroterpene-enriched fraction of guava leaves, containing guajadial, psidial A, and psiguadial A and B, was evaluated *in vivo* in a solid Ehrlich murine breast-adenocarcinoma model. The results suggested that these compounds may act as phytoestrogens, presenting tissue-specific antagonistic and agonistic activity on estrogen receptors [43]. These data partially confirmed the results *in vitro* obtained by Ryu et al. [47].

2.2.3. Diseases of the blood and immune system

Among blood diseases, anemia indicates a failure in the immune system. In this sense, guava extract presented an anti-anemic effect in trypanosomosis-infected Wistar rats, improving the values of hemoglobin, packed cell volume, red-blood cell counts, mean corpuscular volume, and mean concentration hemoglobin count while decreasing white-blood cell and neutrophil levels [105]. Moreover, the same trend in the hematological analyses was also recorded in mice. After the administration of guava leaf extract, no alterations on the erythron were detected [106]. Nevertheless, results differ because subjects under study are different, also the duration of the treatment, the extraction method, and the dose assayed (Table 7).

The anti-inflammatory response of the leaves was dose-dependent in induced hyperalgesia in Sprague-Dawley rats, decreasing in paw-withdrawal latency, and significantly improving the survival rate of mice with lethal endotoxemia [50]. Moreover, the anti-inflammatory activity of aqueous and acetone-water extracts of the leaves was also confirmed in Swiss mice by reducing the amount of leukocyte

migration. The acetone-water extract also exhibited peripheral analgesic activity, probably by blocking the effect or the release of endogenous substances that excite pain-nerve endings [19]. The analgesic effect in albino rats was also reported. The ethanol extract reduced the writhing response [107], and a jumping response was found after the administration of a distilled extract (combination of methanol and aqueous extracts) [108]. In this case, the writhing response for both Swiss mice and Wistar rats seems to be comparable, although the dose assayed is completely different (Table 7).

2.2.4. Endocrine and metabolic diseases

Guava leaves have shown their potential against one of the diseases with the highest incidence level worldwide, diabetes mellitus, and also towards biochemical changes caused by the disease. In spite of being leaves from different countries, treatments in different subjects or even different data, the same trend is followed in these works (Table 8).

The effect of aqueous guava leaf extract was investigated in rabbits, fed a high-cholesterol diet. Treatment with guava leaves reduced the plasma-cholesterol level, caused a remarkable spike in high-density lipoprotein, a dip in low-density lipoprotein levels, and significantly reduced the associated hyperglycemia. In addition, the extract showed hypolipidemic and hypoglycemic potentials in hypercholesterolemic rabbits [109]. Furthermore, guava leaves reduced oxidative stress induced by hypercholesterolemia in rats [110].

In addition, the anti-diabetic effect was also evaluated in $Lepr^{db}/Lepr^{db}$ mice and significant blood-glucose-lowering effects were observed. In addition, histological analysis revealed a significant reduction in the number of lipid droplets, which, furthermore, at least in part, could be mediated via the inhibition of protein tyrosine phosphatase 1B [111].

In streptozotocin-induced diabetic rats, the administration of oral doses of aqueous and ethanol extracts from guava leaves could alter the Ca:Mg ratio [112]; however, in low-dose streptozotocin and nicotinamide-induced Sprague-Dawley diabetic rats, long-term administration of guava leaf extracts raised the plasma-insulin level, the glucose utilization, and the activity of hepatic enzymes [113]. Moreover, the leaves also lowered blood glucose levels and decreased protein glycation [55].

In agreement with the above, a lower blood-glucose level was also reported in alloxan-induced diabetic rats. Additionally, no side effects were observed in certain liver enzymes (alkaline phosphatase and aspartate aminotransferase) whereas alanine aminotransferase activity declined [114]. In alloxan-induced diabetic rats, a decrease was also found in blood glucose, total cholesterol, triglycerides, low-density lipoprotein cholesterol, very low-density lipoprotein cholesterol, and a significant increase in high-density lipoprotein cholesterol after 21 days of treatment with guava leaf ethanolic extract [115].

Among the works that evaluated only biochemical parameters, guava leaf extract promoted changes due to an alteration on the activity of alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and acid phosphatase in the kidney, liver, and serum [106,116]. In addition, Adeyemi and Akanji [117] evaluated the effect of daily administration of guava leaves, demonstrating the alteration of the serum homeostasis and the pathological variations in rat tissues.

Table 7 *In vivo* studies against diseases of the blood and immune system

Origin	Extraction method	Subject	Treatment	Main results	Ref
Nigeria	Maceration with agitation in EtOH:H_2O 80% (v/v) (24 h)	Wistar rats	*T. b. brucei* /no infected	Treatment (1-7 days) at 150 mg/kg: ↑ Hb (6.5 to 10.7 g/dL), PCV (28.6 to 34.4%), RBCC (4.1 to 5.0 x10^{12} /L), MCV (53.6 to 64.3 fL), and MCHC (21.4 to 31.4 g/dL); ↓ WBC (23.2 to 19.4 x10^{9} /dL) and neutrophil levels (28.9 to 27.3 x10^{3} /mL). Compared to no infected subjects: similar values that obtained in treated-infected animals but with opposite conclusions	[105]
Nigeria	Extraction in chloroform (24 h)	Mice	No infected	Treatment (28 days) at 45.9 mg/mL: no differences in Hb (12 to 11 g/dL), PCV (37 to 35%), RBCC (6.1 to 5.1 x10^{6} /L), and MCHC (33 to 32 g/dL), and neutrophil levels (13 to 12%); ↑ lymphocyte levels (85 to 92%) and MCV (61 to 69 fL)	[106]
Korea	Extraction in EtOH:H_2O 55% (v/v) (4.9 h, 47°C)	Sprague-Dawley rats and mice	Freund's complete adjuvant-induced hyperalgesia/LPS-induced endotoxic shock	At 400 mg/kg: PWL restored; ↑ 67% survival rate (72 h) by ↓ TNF-α (500 to 325 pg/mL) and IL-6 (80 to 58 ng/mL)	[50]
Brazil	Turbo-extraction in water and acetone: H_2O 70% (v/v) (20 min)	Swiss mice	Carrageenan-induced peritonitis, acetic acid-induced abdominal writhing and hot plate test	At 50mg/kg: number of leukocyte migration into the peritoneal cavity H_2O < H_2O -acetone extract. No central analgesic activity. Peripheral analgesic activity: ↓ number writhing response (from 50 to 15 count)	[19]
India	Maceration in EtOH (7 days)	Wistar rats	Acetic acid-induced writhing	At 2 mg/kg ↓ 66% number writhing response (from 67 to 54 count). Comparable to diclofenac sodium (75%)	[107]
India	Distillation with MeOH and H_2O	Wistar rats	Acetic acid-induced writhing and hot plate test	At 10 and 30 mg/kg ↓ responses time (at 9.4 and 10.6 s) compared to the analgesic drug Pentazocine (14 s)	[108]

Hemoglobin (Hb); interleukin-6 (IL-6); lipopolysaccharide (LPS); mean concentration hemoglobin count (MCHC); mean corpuscular volume (MCV); packed cell volume (PCV); paw withdrawal latency (PWL); red-blood cell counts (RBCC); tumor necrosis factor alpha (TNF-α); white-blood cell (WBC); ↑ increases the effect; ↓ decreases the effect

Table 8. Endocrine and metabolic *in vivo* assays with guava leaves.

Origin	Subject	Treatment	Main results	Ref
Nigeria	Rabbits	High-cholesterol diet	At 250 mg/kg: ↓TC (15%); ↑HDL (69%); ↓LDL (74%); ↓hyperglycemia 43%	[109]
Brazil	Wistar rats	High-cholesterol diet	At 369.89 mg phenolic compound in the extract/g: ↓TC (29-35%), TG (59-73%);↑HDL (46%); ↓VLDL+LDL; ↓enzyme activity (SOD (6.2 to 5.7 U/mg protein), GP (4.6 to 2.3 µmol/g protein))	[110]
Korea	*Lepr*^{db}/*Lepr*^{db} juvenile and adult mice	Diabetes spontaneous mutation	At 10 mg/kg: 87% inhibition PTP1B;↓ glucose levels 31 and 42% respectively	[111]
Iran	Wistar rat	Streptozotocin-induced diabetes	At 1mg/L: ↓Ca/Mg ratio (18 to 12), glucose level, TG (100 to 65 mg/dL), TC (68 to 48 mg/dL), ↑ HDL (18 to 40 mg/dL), ↓ LDL, and VLDL to normal levels; ↓alteration in vascular reactivity (110 to 50 mmHg)	[112]
Taiwan	Sprague-Dawley rats	Low-dose streptozotocin and nicotinamide-induced diabetes	At 400 mg/kg: ↓blood glucose level (230 to 140 mg/dL); ↑plasma insulin level and glucose utilization (normal levels); ↑enzyme activity (hepatic hexokinase (8 to 11 U/mg protein), phosphofructokinase (18 to 25 U/mg protein) and glucose-6-phosphate dehydrogenase (11 to 25 U/mg protein))	[113]
India	Sprague-Dawley rats	Streptozotocin-induced diabetes	At 100 mg/kg: ↓blood glucose level (4 to 1 mg/mL) and lipid peroxidation (2 to 1 mmol/100 g tissue); ↑enzyme activity (CAT (6 to 10 x10^3 U/mg protein), SOD (6 to 10 U/mg protein), GPx (0.4 to 0.6 U/mg protein), GRd (0.1 to 0.3 U/mg protein)	[55]
Nigeria	Albino rats	Alloxan-induced diabetes	At 200 mg/kg: ↑average weight (99 to 209g); ↓blood glucose level (15 to 8 mmol/L); ↓alanine aminotransferase activity (32 to 24 U/L)	[114]
India	Albino rats	Alloxan-induced diabetes	At 500 mg/kg: ↓blood glucose level, TC (231 to 163 mg/dL), TG (133 to 69 mg/dL), LDL (186 to 126 mg/dL), VLDL (26 to 13 mg/dL);↑HDL (18 to 23 mg/dL)	[115]

Origin	Subject	Treatment	Main results	Ref
Nigeria	Wister rats	-	At 150 mg/kg: ↑ALP (300, 175and 650 IU), AST (500, 400, 450 IU), ALT (1200, 1200, 1800 IU), ACP (750, 650, 900 IU) activity in the kidney, liver, and serum, respectively	[116]
Nigeria	Mice	-	At 49.3 mg/mL: ↑AST (93 to 126 iμ/L), ALT (30 to 35 iμ/L), ALP (57 to 66 iμ/L), conjugate bilirubin (0.2 to 0.3 mg/dL) and creatinine (0.9 to 1.2 mg/dL)	[106]
Nigeria	Albino rats	-	At 150 mg/kg: ↑serum urea (2.9 to 6 mmol/L) and creatinine (2.7 to 4 mmol/L); ↓concentration of serum Na^+ (122 to 99 mmol/L)	[117]

Acid phosphatase (ACP); alanine aminotransferase (ALT); alkaline phosphatase (ALP); aspartate aminotransferase (AST); catalase (CAT); glutathione peroxidase (GPx); glutathione reductase (GRd); high-density lipoprotein (HDL) cholesterol; low-density lipoprotein (LDL) cholesterol; protein tyrosine phosphatase 1B (PTP1B); superoxide dismutase enzyme (SOD); total cholesterol (TC); triglycerides (TG); very low-density lipoprotein (VLDL) cholesterol; ↑ increases the effect; ↓ decreases the effect

2.2.5. Diseases of the circulatory system

Ademiluyi et al. [118] assessed the lipid peroxidation in rats after checking the antihypertensive effect, *in vitro*, of red and white guava leaves. The work concluded that the activity may be related to rosmarinic acid, eugenol, carvacrol, catechin, and caffeic acid since they were the major constituents of their extracts. Also, this activity was supported by the biphasic and contractile effect on rat vascular smooth muscles [119,120].

In addition, atherosclerosis development was reduced in apoE-knockout mice by guava leaf extracts. In fact, the effect was connected to the presence of ethyl gallate and quercetin [121,122]. In streptozotocin-induced diabetic rats, vascular reactivity to vasoconstrictor agents was reduced, as was vessel atherosclerosis [112]. Furthermore, Soman et al. [123] found that an ethyl acetate fraction of guava leaves reduced cardiac hypertrophy in streptozotocin-induced diabetic rats due to an anti-glycative effect.

2.2.6. Diseases of the digestive system

In the digestive system, formed by the gastrointestinal tract plus the group organs necessary for the digestion, guava leaves have demonstrated activity towards different parts.

On the one hand, the leaves have shown the ability to protect the stomach against ulceration by inhibiting gastric lesions, reducing gastric secretory volume, and acid secretion, and raising the gastric pH [124–126]. This anti-ulcer activity, resulting from the protection of the mucosa, was related to the flavonoids in the leaves [127]. Despite of the subject employed for the assay, similar data are reported in these works (Table 9). The anti-diarrheal activity of guava leaf aqueous extract was evaluated on experimentally induced diarrhea in rodents. The extract performed in the same way as the control drugs, offering protection, inhibiting intestinal transit, and delaying gastric emptying [128]. Another study attributed this activity to a dual action between the antimicrobial effect and the reduction in gastrointestinal motility ability of the extract

[129]. In rabbits the anti-spasmodic effects were connected to a calcium channel blocking activity, which explains the inhibitory effect on gut motility. The anti-diarrheal protection was also tested in mice [130]. As is shown in Table 9, the anti-diarrheal activity is dose-dependent, although the protection varied depending on the subject.

On the other hand, guava leaves exhibited hepato-protective effect due to the reduction of serum parameters of hepatic enzymes markers and histopathological alterations in the acute liver damage induced in rats [131–135]. Here, a dose-dependent effect is also found. However, decoction of the leaves seems to be the best option for the extraction of the compounds that exhibited this activity (Table 9).

Table 9 *In vivo* assays for digestive system related diseases

Origin	Extraction method	Subject	Treatment	Main results	Ref
India	Extraction with MeOH	Wistar rats	ASP, PL, and EtOH-induced ulcers	At 200 mg/kg: PL-induced ulcers: ↓ 64% ulcer formation (ui =2.1), ↓ GV (5 to 2 mL), acid secretion (88 to 64 mEq/L/100 g), ↑ pH (2 to 5).Comparable to omeprazole. ASP (↓ 70.5%, ui = 2.5) and EtOH (↓ 70.4%, ui = 8.7)-induced systems	[124]
Nigeria	Maceration in H_2O (24 h)	Albino rats	EtOH-induced ulcers	At 1000 mg/kg: ↓ MNL (9.4 to 2) ui (4.7 to 1)	[125]
Nigeria	Maceration with agitation in MeOH (24 h)	Wistar rats	EtOH-induced ulcers	At 1000 mg/kg: ↓ ui (17.7 to 6.3), ↑ protection (64.4%)	[126]
India	Maceration in EtOH: H_2O 90% (v/v) (72 h).	Wistar rats	PL and EtOH-induced ulcers	At 200 mg/kg: PL-induced: ↓ ulcer formation (77 to 84%), ui (5 to 1.3), GV (1.4 to 0.5 mL/100g), and acid secretion (28 to 23 mEq/L); ↑ pH (2.0 to 3.4). EtOH-induced: ↓ (63 to 79%, ui = 1.6 to 5.6), and gastric lesions (5.6-1.9)	[127]
South Africa	Maceration in H_2O (48h)	Wistar rats and BALB/c mice	Castor oil-induced diarrhea and castor oil-induced enteropooling	At 400 mg/kg: ↑ 83.3% rat protection, ↓ 75% fluid accumulation in rats; ↓ 87.73% transit in rats and 77.2% in mice; ↓ 64.35% of contractions in mice	[128]
Nigeria	Soxhlet with EtOH: H_2O 70% (v/v)	Wistar rats	Castor oil-induced diarrhea	At 80 mg/kg: ↓ 53.03% transit in rats and ↓ 67.70% intestinal contractions	[129]
Pakistan	Maceration with EtOH	BALB/c mice, rabbit jejunum	Castor oil-induced diarrhea, K^+-induced motility	At 1 g/kg: ↑ 81.1% mice protection; Spasmolytic effect (0.3-1 mg/mL) ↓ spontaneous contractions EC_{50} = 0.66 mg/mL in rabbits	[130]
India	Decoction (1h)	Wistar rats	CCl_4, PCM, and TAA-induced liver injury	At 500 mg/kg: CCl_4: ↓ ALT (384 to 17 U/L), AST (642 to 152 U/L), ALP (750 to 489 U/L), and bilirubin (1.6 to 0.3 mg/dL), ↓ control levels; PCM: ↓ ALT (384 to 87 U/L), AST (642 to 179 U/L), ALP (750 to 338 U/L), and bilirubin (1.6 to 0.6 mg/dL); TAA: ↓ ALT (337 to 32 U/L), AST (438 to 237 U/L), and ALP (770 to 479 U/L)	[131]

Origin	Extraction method	Subject	Treatment	Main results	Ref
India	Soxhlet with EtOH	Wistar rats	PCM-induced liver injury	At 400 mg/kg: ↓ SGOT (475 to 370), SGPT (158 to 128), ALP (814 to 729), and bilirubin (0.7 to 0.6); ↑ total protein (5.15 to 5.6), albumin (2.6 to 3.1), and GLO (2.1 to 2.4). Histopathological observations: less diffuse granular degeneration and mild periportal lymphocytic infiltration	[132]
India	Decoction (1h)	Wistar rats	Acetaminophen-induced liver injury	At 500 mg/kg: ↓ AST (121 to 77 IU/L), ALT (80 to 57 IU/L), ALP (115 to 67 IU/L), and total bilirubin (4 to 2 mg/dL). Restored: total protein (5 to 7 g/dL), LPO (7 to 2 nmol/mg protein), GPx (13 to 19 µmol/mg protein), GSH (15 to 23 µmol/mg protein), CAT (14 to 24 µmol/mg protein), and SOD (48 to 63 µmol/mg protein). Histopathological observations: normal lobular structure	[133]
Egypt	Maceration with agitation in EtOH:H_2O 70% (v/v) (24 h)	Albino rats	CCl_4-induced liver injury	At 500 mg/kg: ↓ ALT (94 to 55 U/mL), AST (199 to 82 U/mL), GGT (71 to 23 U/mL), lysosomal enzymes (50%), and LPO (7 to 3 nmol/mg protein); ↑ SOD (15 to 39 U/mg protein), CAT (5 to 15 µg/mg protein), GSH (6 to 8 µg/mg protein), GST (13 to 25 mM/min/mg protein), total protein (48 to 58 g/L), albumin (29 to 38 g/L), GLO (19 to 21 g/L)	[134]
Egypt	Decoction (1h)	Wistar rats	PCM-induced liver injury	↓ AST (342 to 156 U/L), ALT (359 to 80 U/L), ALP (288 to 263 U/L), LDH (207 to 143 U/L), GGT (11 to 7 U/L), and total bilirubin (0.3 to 0.2 mg/dL). Restored SOD (13 to 24 U/g) and CAT (5 to 17 U/g)	[135]

Alkaline phosphatase (ALP); Alanine aminotransferase (ALT); aspirin (ASP); aspartate aminotransferase(AST); catalase (CAT); carbon tetrachloride (CCl4); ethanol (EtOH); gamma glutamyl transferase (GGT); gastric volume (GV); globulin (GLO); glutathione (GSH); glutathione peroxidase (GPx); glutathione S-transferase (GST); lactate dehydrogenase (LDH); lipid peroxidation (LPO); mean number lesions (MNL); paracetamol (PCM); pyloric ligation (PL), Serum glutamic oxaloacetic transaminase (SGOT); Serum glutamic pyruvic transaminase (SGPT), superoxide dismutase (SOD); thioacetamide (TAA); ulcer index (ui); ↑ increases the effect; ↓ decreases the effect

2.2.7. Diseases of the skin and subcutaneous tissue

Guava leaves have been suggested as a therapeutic agent to control pruritus in atopic dermatitis. The improvement of the skin lesions was due to a reduction in serum immunoglobulin E level and in the eczematous symptoms [136]. Moreover, the epithelium was repaired with connective tissue and absence or moderate presence of inflammatory cells by the leaves. As a result, the leaves exhibited wound healing properties [137]. Furthermore, guava leaf extract was tested on rat skin, and exhibited inhibitory activity towards an active cutaneous anaphylaxis reaction [138].

2.2.8. Other activities related to several diseases

Triterpenoids from guava leaves were suggested as a potential therapeutic approach for treating diabetic peripheral neuropathy, as they enhanced physical functions and offered neuronal protection towards the suppression of the expression of pro-inflammatory cytokines [139]. Also, the leaves can act as radio modulators for cancer patients because by preventing DNA damage and apoptosis. [140], and also as protective agents by restoring the normal values of sperm viability, sperm count, sperm motility, and sperm-head abnormality caused by caffeine-induced spermatotoxicity [141].

Moreover, the consumption of guava leaf tea was evaluated, *in vivo*, in the inhibition of cytochrome P450 (CYP) 3A-mediated drug metabolism by the interaction between guava tea and several drugs [11,142]. Matsuda et al. [11] investigated the consequence of the ingestion of guava tea for two weeks in rats, and the effect with an anxiolytic drug. The short-term consumption of the tea had little effect on the assays performed. This weak influence was due to the absence of interaction between the tea and midazolam in the metabolism studied. Also, two *in vivo* studies were made in rats, to evaluate the interaction of guava leaf tea with an anti-coagulant drug (warfarin) [142]. Kaneko et al. [141] suggested that because the tea contained compounds that block the affinity between the enzyme and phenolic compounds of the tea, long-term administration showed a low probability of causing drug-metabolizing enzymes.

Moreover, short-term administration revealed that the tea did not interfere with coagulation, meaning that the tea consumption did not alter the pharmacological effect and displayed no side effects.

2.3. Clinical trials

To test the effect of guava leaf extract, several randomized clinical trials have been conducted during the last two decades, although only two studies are available in the last decade. One of the studies consisted of evaluating the effect of guava leaf extract pills on primary dysmenorrhea disorder. For this, 197 women were divided into 4 groups, and each received a different dosage; 3 and 6 mg extract/day, 300 mg placebo/day and 1200 mg ibuprofen/day. The administration took place in 5 days during 3 consecutive cycles. The results demonstrated that 6 mg extract/day alleviated menstrual pain and could replace the use of medicaments like ibuprofen. In fact, guava leaves could be used as a broad-spectrum phyto-drug and not only as an anti-spasmodic agent [143]. Furthermore, Deguchi and Miyazaki [58] reviewed several works regarding the effect of the intake of a commercial guava leaf tea (Bansoureicha®, Yakult Honsha, Tokyo, Japan) on different pathologies of diabetes mellitus illness such as the influence on postprandial blood glucose, on insulin resistance and on hypertriglyceridemia and hypercholesterolemia. The authors concluded that the ingestion of guava leaf tea can ameliorate the symptoms of diabetes mellitus and that it could be used as an alimentotherapy.

3. Other applications

Further applications found with guava leaves are listed below: firstly, to prepare gelatin beads with marine-fish gelatin for various applications such as medicine, and the food and pharmaceutical industries [144]. Secondly, Giri et al. [145] suggested guava leaves as supplementary feed for the fish species *Labeo rohita*, due to the immune-stimulatory effect. The same conclusion was reached by Fawole et al. [146] in *L. rohita*. Thirdly, Gobi et al. [147] reported that guava leaf powder, mixed

with a commercial diet, strengthened the immunological response of *Oreochromis mossambicus*, and recommended the leaves as feed complement in aquaculture.

4. Conclusions

Traditional claims generally require experimental research to establish their effectiveness. In this regard, ethnomedicine applications of *Psidium guajava* L. leaves have been verified by several researchers over the last decade against many disorders, demonstrating its potential in the treatment of the most common worldwide diseases. In addition, the effects of the leaves have been related to individual compounds such as quercetin, catechin, vescalagin, gallic acid, peltatoside, hyperoside, isoquercitrin, and guaijaverin.

Future prospects should be aimed at investigating the biodiversity of guava and/or the purification of the different compounds present in guava leaves in order to obtain functional ingredients for further uses as alternative agents in natural therapeutic approaches.

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Author Contributions

Elixabet Díaz-de-Cerio contributed to the literature review, manuscript redaction, Vito Verardo and Ana María Gómez-Caravaca contributed to the conception of the idea and framework writing; Alberto Fernández-Gutiérrez and Antonio Segura-Carretero supervised the progress of work.

Conflicts of interest

The authors declare no competing financial interest.

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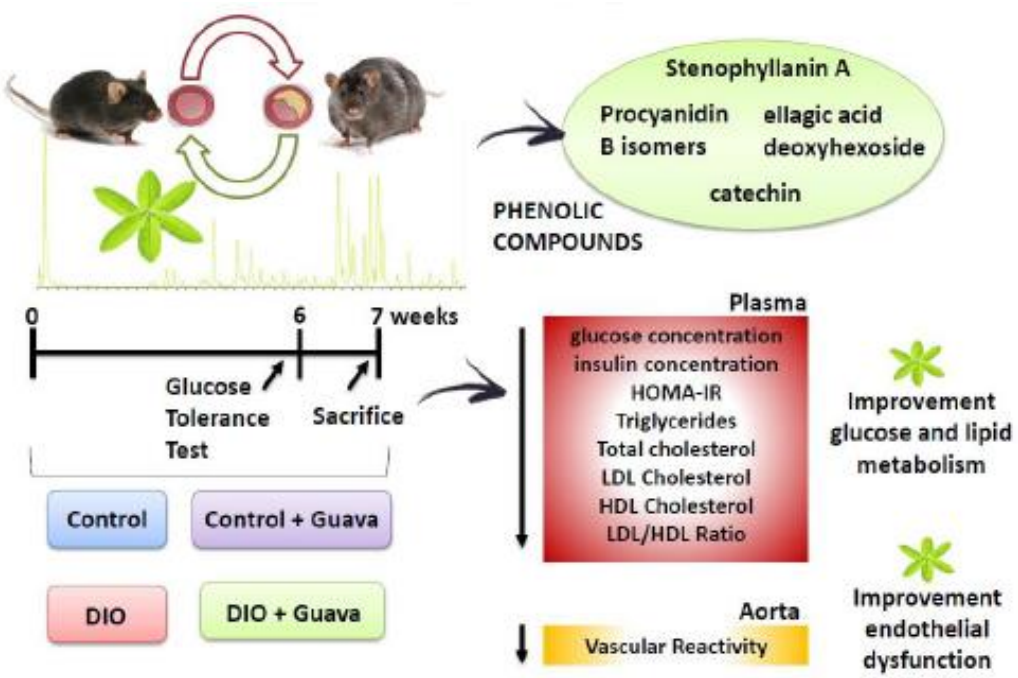
CHAPTER 7



The hypoglycemic effects of guava leaf (*Psidium guajava* L.) extract are associated with improving endothelial dysfunction in mice with diet-induced obesity

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Accepted Manuscript

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Abstract

Obesity is associated with a low-grade inflammatory status that affects vascular function. Previous studies have reported the beneficial effects of *Psidium guajava* L. (guava) on diabetes. Here we evaluate the how guava leaf extract at the dose of 5 mg/kg), affects vascular dysfunction in obese mice fed a high-fat diet for 7 weeks. Extract intake did not alter weight over time, although it reduced glycemia and insulin resistance, improving the serum lipid profile in obese mice. Additionally, guava leaf extract reversed the endothelial dysfunction found in obese mice in terms of endothelium- and NO (nitric oxide)-dependent vasodilatation induced by acetylcholine in aortic rings. In conclusion, the beneficial effects of guava leaf extract in obese mice were associated with improved vascular functions altered by obesity, probably due to its phenolic content.

Keywords: diet-induced obesity; endothelial dysfunction; guava leaf extract; mouse; flavonol; flavan-3-ol.

1. Introduction

Diabetes mellitus (DM), one of the most common metabolic disorders worldwide, is increasing. In 2013, 382 million adults worldwide had diabetes, and 592 million are projected to be affected by 2035 (Guariguata et al., 2013). DM is a leading cause of mortality, morbidity, and health-system costs in the world, mainly because of metabolic and cardiovascular complications (Creager, Lüscher, Cosentino, & Beckman, 2003). In addition, metabolic syndrome has different components, such as abdominal obesity, impaired glucose metabolism, dyslipidemia, and hypertension, which synergistically increase the risk of cardiovascular disease as well as diabetes, this being clearly involved in premature mortality. Pre-diabetes is considered an underlying etiology of metabolic syndrome, characterized by a combination of excess body fat and insulin resistance, and manifested by impaired fasting glucose and/or impaired glucose tolerance, thus resulting in hyperglycemia (Grundy, 2012). The primary target of hyperglycemia appears to be the endothelial cells, which may induce endothelial dysfunction and accelerated atherosclerosis. These processes are associated with the development of a vascular inflammatory response, with the involvement of several mediators, including reactive oxygen metabolites, chemokines, and pro-inflammatory cytokines, which are clearly responsible for the cardiovascular complications that are the leading cause of morbidity and mortality associated with diabetes (Herder, Dalmas, Böni-Schnetzler, & Donath, 2015).

Several epidemiological studies and dietary interventions in human subjects have shown that high phenolic intake from different sources, such as grape, tea, cocoa or extra virgin olive oil, may be associated with a reduced risk of cardiovascular disease (Deka & Vita, 2011; Estruch, Ros, Salas-Salvadó, & Al, 2013; Hooper et al., 2012; Tomé-Carneiro et al., 2013). Different biological actions attributed to these phenolic compounds would support their potential cardiovascular protective effects, including improved vasodilation (Perez et al., 2014), lower blood pressure (Jiménez, Duarte, & Perez-Vizcaino, 2012), reduced insulin resistance (Dragan, Andrica, Serban,

& Timar, 2015), and stronger immune responses and antioxidant defense system (Katz, Doughty, & Ali, 2011).

Psidium guajava L. is a small tree native to Mexico that has been widely used in traditional medicine for the treatment of diverse diseases, including hypertension, inflammation, pain, and diabetes, (Gutiérrez, Mitchell, & Solis, 2008). With respect to its anti-diabetic properties, the beneficial effects of different guava leaf extracts have been reported in experimental models of type I or type II diabetes (Deguchi & Miyazaki, 2010; Eidenberger, Selg, & Krennhuber, 2013; Guo et al., 2013; Khan, R, Rajendran, Bai, & Sorimuthu, 2013; Mathur, Dutta, Velpandian, & Mathur, 2015; Soman, Rauf, Indira, & Rajamanickam, 2010). All these studies have revealed their ability to detain the rise in postprandial blood glucose, to ameliorate hyperglycemia, hypertriglycemia, and hypercholesterolemia, as well as to improve both hyperinsulinemia and insulin resistance. However, little attention has been paid to the impact that guava leaf extract administration may have on endothelial dysfunction that occurs in a diabetic status. The aim of the present study was to evaluate the effects of a phenolic enriched extract of *P guajava* L. leaf on endothelial dysfunction induced by a high-fat diet (HFD) in mice. Notably, the phenolic profile of Spanish guava leaves has recently been reported with an assessment of the concentration of different flavonoids. Among these, flavonols and flavan-3-ols were the major subclasses found in the Andalusian guava leaves (Díaz-de-Cerio, Gómez-Caravaca, Verardo, Fernández-Gutiérrez, & Segura-Carretero, 2016; Díaz-de-Cerio, Verardo, Gómez-Caravaca, Fernández-Gutiérrez, & Segura-Carretero, 2016), which can contribute to the potential beneficial effects that the guava leaf extract may exert in obese mice, given the biological properties attributed to flavonoids under diabetic conditions (Testa, Bonfigli, Genovese, De Nigris, & Ceriello, 2016).

2. Materials and Methods

2.1 Chemicals and plant material

Double-deionized water with conductivity lower than 18.2 MΩ was equipped with a Milli-Q system (Millipore, Bedford, MA, USA). Methanol and acetonitrile LC-MS "optima" grade were purchased from Fisher Scientific (Leicestershire, UK). Acetic acid and the standards: gallic acid, catechin, ellagic acid, naringenin, quercetin, and rutin were all from Sigma-Aldrich (Steinheim, Germany). Ethanol was obtained from Panreac (Barcelona, Spain).

Deep-green leaves of *P. guajava* L. var. *pyrifera* harvested in Motril (Spain) (36°44′43″N 3°31′14″O) were collected in February 2015. The environmental conditions were: mean max/min temperatures of 23/8ºC, precipitation of 0-0.8 mm, and saturated light duration from 9.45 to 10.40 h per day.

2.2 Extraction of phenolic compounds from guava leaves

Phenolic compounds were extracted using the procedure previously reported (Díaz-de-Cerio, Gómez-Caravaca, et al., 2016). Briefly, guava leaves were extracted with 15 ml of ethanol: water 80:20 (v/v) (x3) using a Branson B3510 for 10 min at room temperature. Then, samples were centrifuged for 15 min at 6000 rpm to remove solids. The extractions were run in triplicate (n=3), and were dried in a Savan SC250EXP SpeedVac (Thermo Scientific, Leicestershire, UK) for the *in vivo* studies.

2.3 HPLC-TOF-MS analysis

Chromatographic analyses of 0.2 g of the phenolic fraction of guava leaves were performed in an Agilent 1200 series Rapid Resolution Liquid Chromatograph (Agilent Technologies, CA, USA) equipped with a vacuum degasser, autosampler, a thermostatically controlled column compartment, a binary pump, coupled to a DAD detector. Separation was carried out at 25°C with a Poroshell 120 EC-C18 analytical column (4.6 mm × 100 mm, particle size 2.7 μm) from Agilent Technologies. The

mobile phases used were water with acetic acid (1%) (phase A) and acetonitrile (phase B), and the gradient elution was applied as follows: 0 min, 0.8% B; 2.5 min, 0.8% B; 5.5 min, 6.8% B; 11 min, 14.4% B; 17 min, 24% B; 22 min, 40% B; 26 min, 100% B, 30 min, 100% B; 32 min, 0.8% B; and 34 min, 0.8% B. The sample volume injected was 5 µL and the flow rate used was 0.8 mL/min.

The HPLC system was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The effluent from the HPLC column was split using a T-type phase separator before placing it in the mass spectrometer (split ratio 1:3). The HPLC-MS was conducted following the conditions reported previously (Díaz-de-Cerio, Gómez-Caravaca, et al., 2016).

The quantification was carried out with Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany), the analysis was run in triplicate (n=3) and the results expressed as µg compound/100 mg extract.

2.3.1 Standard solutions

Phenolic standards of interest such as gallic acid, catechin, ellagic acid, naringenin, quercetin, and rutin were used to quantify phenolic compounds in guava leaf extracts. The standard stock solutions were prepared from the quantification limit (LOQ) to 250 mg/L in methanol, except for ellagic acid, which was solved in water.

Calibration curves showed good linearity (R^2 > 0.99) between different concentrations for all the standards. Linear range and limits of detection (LOD) and quantification (LOQ) were determined as corresponding to 3 and 10 times, respectively, the standard deviation of the background noise; values ranged from 0.002 to 0.03 mg/L for LOD and from 0.005 to 0.099 mg/L for LOQ.

2.4 In vivo studies

2.4.1. Animals and experimental groups

This study abided by the 'Guide for the Care and Use of Laboratory Animals' of the National Institute of Health and the protocols approved by the Ethic Committee of Laboratory Animals of the University of Granada (Spain) (Ref. No. 94-CEEA-OH 2015). Male C57BL/6J mice were obtained from Janvier (St Berthevin Cedex, France), and maintained at a constant temperature (24 ± 1ºC), with a 12-hour dark/light cycle. For diet-induced obesity experiments, 5-week-old mice were divided into 4 groups (n=10): control, control-treated, obese, and obese-treated. Treated mice received daily by oral gavage the extract of guava leaves at a dose of 5 mg/kg, dissolved in 0.2 mL of water solution. Control mice received a standard chow diet (13% calories from fat, 20% calories from protein, 67% calories from carbohydrates; Global diet 2014, Harlan Laboratories, Barcelona, Spain), whereas obese mice were fed a Western-type high-fat diet (HFD) in which 60% of its caloric content were derived from fat (37% saturated, 47% monosaturated, 16% polyunsaturated; Purified diet 230 HF, Scientific Animal Food & Engineering, Augy, France). The extract treatment was followed for 7 weeks, and animal body weight as well as food and water intake were controlled regularly. At the end of the experiment, after the mice had been killed, the descending thoracic aorta was removed and used for vascular reactivity studies.

2.4.2. Glucose tolerance test

One week before the mice were killed, a glucose-tolerance test was performed on mice that were food deprived for 18 h. They received a 50% glucose solution in water at a dose of 2 g/kg body weight by i.p. injection, and blood was collected from the tail vein before glucose administration and 15, 30, 45, 60, 90, and 120 min after.

2.4.3. Plasma Analyses

At the end of the treatment, mice were killed under isoflurane anesthesia. Blood samples were refrigerated on ice and centrifuged for 20 min at 5000 g at 4ºC,

and the plasma frozen at −70°C. Plasma glucose, triglycerides, LDL, HDL, and total cholesterol concentrations were measured by colorimetric methods using Spinreact kits (Spinreact, S.A., Spain). Plasma-insulin concentration was quantified using a mouse insulin ELISA kit (Alpco Diagnosis, Salem, USA). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the formula: fasting glucose (mM) x fasting insulin (μU/mL)/22.5.

2.4.4. Vascular reactivity studies

Descending thoracic aortic rings were dissected from the mice and suspended in a wire myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) for isometric tension measurement as previously described (Toral et al., 2015). The concentration-relaxation response curves to acetylcholine (10^{-9} M-10^{-6} M) in the absence or in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) were performed in intact rings precontracted by phenylephrine. The relaxant responses to sodium nitroprusside (10^{-9}M-10^{-6} M) were studied in the dark in endothelium-denuded vessels precontracted by phenylephrine (10^{-6} M). Relaxant responses to acetylcholine and sodium nitroprusside were expressed as a percentage of precontraction.

2.5 Statistical analysis

All results were normally distributed and they are expressed as the mean ± SEM. Differences among means were tested for statistical significance using a one-way analysis of variance (ANOVA), followed by a multiple-comparison Bonferroni test, when necessary. All statistical analyses were carried out with the GraphPad 6.0 software (GraphPad Software Inc., La Jolla, CA, USA), with statistical significance set at $p < 0.05$.

3. Results and Discussion

3.1 Quantitative analysis of phenolic compounds in guava leaves

The phenolic compounds under study were identified as previously (Díaz-de-Cerio, Gómez-Caravaca, et al., 2016) according to their mass spectra. Each compound was quantified by comparing the area of the peak with resulting calibration curves with the corresponding standard. When commercial standards were not available, compounds with similar structure were used for quantification. Catechin standard was used to quantify flavan-3-ol derivatives, quercetin and rutin for aglicones and glicosylated flavonols, respectively. Also, gallic acid was used for simple phenolic acids, and ellagic acid for its derivatives. Finally, naringenin was used for its quantification.

Regarding the aforementioned identification and quantification, several differences noted. Compound 5 was not detected (n.d.) and compounds 8, 11, 14, 18, 30, 31, 33, 34, 36-38, 40-42, and 48 were under LOQ (Table 1).

Table 1. Phenolic compounds in guava leaf extract (value = X ± SD)

Compound	rt (min)	Molecular Formula	*m/z* exp	Concentration (µg compound/ 100mg extract)
HHDP glucose Isomer 1	2.0	C20H18O14	481.0640	4.5 ± 0.3
HHDP glucose Isomer 2	2.1	C20H18O14	481.0638	23.4 ± 0.5
HHDP glucose Isomer 3	2.4	C20H18O14	481.0639	12.3 ± 0.8
Prodelphinidin B Isomer	3.5	C30H26O14	609.1276	5.6 ± 0.2
Pedunculagin/ Casuariin Isomer	6.5	C34H24O22	783.0699	3.2 ± 0.1
Prodelphinidin Dimer Isomer	7.0	C30H26O13	593.1311	9.3 ± 0.2
Vescalagin/castalagin Isomer	7.2	C41H26O26	933.0649	<LOQ
Gallocatechin	7.4	C15H14O7	305.0698	32.6 ± 0.6
Prodelphinidin Dimer Isomer	7.8	C30H26O13	593.1316	25.7 ± 0.4
Geraniin Isomer	9.2	C41H28O27	951.0749	<LOQ
Pedunculagin/ Casuariin Isomer	9.2	C34H24O22	783.0699	8.5 ± 0.2
Uralenneoside	9.3	C12H14O8	285.0624	21.5 ± 0.1
Geraniin Isomer	9.5	C41H28O27	951.0752	<LOQ
Procyanidin B Isomer	9.9	C30H26O12	577.1367	100.4 ± 0.4
Procyanidin B Isomer	10.1	C30H26O13	577.1367	12.2 ± 0.2
Galloyl(epi)catechin-(epi)gallocatechin	10.2	C37H30O17	745.1420	0.042 ± 0.003

Compound	rt (min)	Molecular Formula	*m/z* exp	Concentration (µg compound/ 100mg extract)
Tellimagrandin I Isomer	10.5	C34H26O22	785.0851	<LOQ
Pterocarinin A	10.9	C46H36O30	1067.1220	7.6 ± 0.3
Pterocarinin A	11.1	C46H36O30	1067.1220	21.2 ± 0.3
Stenophyllanin A	11.1	C56H40O31	1207.1495	96 ± 1
Procyanidin trimer Isomer 1	11.1	C45H38O18	865.1998	5.52 ± 0.03
Catechin	11.1	C15H14O6	289.0727	85 ± 1
Procyanidin tetramer	11.2	C60H50O24	1153.2612	0.666 ± 0.002
Procyanidin trimer Isomer 1	11.3	C45H38O18	865.1998	0.126 ± 0.001
Guavin A	11.4	C56H40O32	1223.1423	20.5 ± 0.3
Guavin A	11.8	C56H40O32	1223.1423	2.25 ± 0.01
Casuarinin/ Casuarictin Isomer	11.7	C41H28O26	935.0810	8.3 ± 0.3
Galloyl(epi)catechin-(epi)gallocatechin	12.0	C37H30O17	745.1420	2.92 ± 0.04
Galloyl-(epi)catechin trimer Isomer 1	12.1	C52H42O22	1017.2097	<LOQ
Procyanidin pentamer	12.2	C75H62O30	1441.3234	<LOQ
Gallocatechin	12.3	C15H14O7	305.0702	27.7 ± 0.1
Tellimagrandin I Isomer	12.4	C34H26O22	785.0855	<LOQ
Vescalagin	12.6	C41H26O26	933.0649	<LOQ
Stenophyllanin A Isomer	12.9	C56H40O31	1207.1472	25.0 ± 0.8
Galloyl-(epi)catechin trimer Isomer 2	13.2	C52H42O22	1017.2097	<LOQ
Myricetin hexoside Isomer	13.3	C21H20O13	479.0836	<LOQ
Stachyuranin A	13.3	C56H42O32	1225.1587	<LOQ
Procyanidin gallate Isomer	13.5	C37H30O16	729.1476	22.2 ± 0.5
Vescalagin/castalagin Isomer	13.7	C41H26O26	933.0645	<LOQ
Myricetin hexoside Isomer	13.8	C21H20O13	479.0835	<LOQ
Myricetin -arabinoside/ xylopyranoside Isomer	14.1	C20H18O12	449.0728	<LOQ
Myricetin -arabinoside/ xylopyranoside Isomer	14.3	C20H18O12	449.0726	4.47 ± 0.01
Procyanidin gallate Isomer	14.5	C37H30O16	729.6356	0.79 ± 0.04
Myricetin -arabinoside/ xylopyranoside Isomer	15.1	C20H18O12	449.0726	4.1 ± 0.1
Myricetin hexoside Isomer	15.1	C21H20O13	479.0839	9.8 ± 0.1
Myricetin hexoside Isomer	15.3	C21H20O13	479.0841	0.231 ± 0.001
Ellagic acid	15.5	C14H6O8	300.9996	<LOQ
Myricetin -arabinoside/ xylopyranoside Isomer	15.7	C20H18O12	449.0743	3.3 ± 0.1
Quercetin -galloylhexoside Isomer	15.7	C28H24O16	615.1008	1.9 ± 0.1
Ellagic acid deoxyhexoside	16.0	C20H16O12	447.0578	10.0 ± 0.5
Quercetin -galloylhexoside Isomer	16.1	C28H24O16	615.0999	1.0 ± 0.2
Myricetin -arabinoside/ xylopyranoside Isomer	16.3	C20H18O12	449.0736	3.2 ± 0.1
Morin	16.3	C15H10O7	301.0362	156 ± 1
Myricetin -arabinoside/ xylopyranoside Isomer	16.5	C20H18O12	449.0735	8.8 ± 0.1
Hyperin	16.8	C21H20O12	463.0895	80.7 ± 0.4

Compound	rt (min)	Molecular Formula	*m/z* exp	Concentration (µg compound/ 100mg extract)
Quercetin glucoronide	16.9	C21H18O13	477.0659	18.4 ± 0.4
Procyanidin gallate Isomer	17.0	C37H30O16	729.1476	0.601 ± 0.003
Isoquercitrin	17.1	C21H20O12	463.0893	48.7 ± 0.7
Reynoutrin	17.6	C20H18O11	433.0792	56.0 ± 0.2
Guajaverin	17.9	C20H18O11	433.0795	130 ± 2
Guavinoside A	18.1	C26H24O13	543.1159	16.6 ± 0.8
Avicularin	18.3	C20H18O11	433.0803	144 ± 2
Quercitrin	18.5	C21H20O11	447.0947	61.2 ± 0.6
Myrciaphenone B	19.3	C21H22O13	481.0999	20.7 ± 0.1
Guavinoside C	19.8	C27H22O15	585.0898	39.6 ± 0.8
Guavinoside B	20.6	C28H28O13	571.1470	7.66 ± 0.04
Guavinoside B Isomer	20.9	C28H28O13	571.1470	11.8 ± 0.3
Guavinoside A Isomer	20.9	C26H24O13	543.1159	3.709 ± 0.001
2,6-dihydroxy-3-methyl-4-O-(6″-O-galloyl-β-D-glucopyranosyl)-benzophenone	22.1	C27H26O13	557.1318	41.8 ± 0.5
Guavin B	22.3	C33H26O17	693.1110	3.74 ± 0.04
Quercetin	22.3	C15H10O7	301.0358	0.988 ± 0.001
Naringenin	26.8	C15H12O5	271.0622	8.5 ± 0.1
Total (µg/g extract)				1484 ± 7

The sum of phenolic compounds in the extract used for the *in vivo* studies was 1484 ± 7 µg/100 mg extract. Individual compounds could be assembled by their structure into different families. The extract was rich in flavonols, which represented 49.2% of the total phenolic content. The major compounds of this family, and of guava leaf extract, were the isomers reynoutrin, guajaverin, and avicularin, which accounted for 21.4% of the total phenolic content, followed by morin, which represented 10.1%, and hyperin and isoquercitrin isomers, representing 8.4%. Similar amounts of gallic/ellagic acid derivatives (29.3%) and flavan-3-ols (21.5%) were found. The main compounds from these families are ellagic acid deoxyhexoside and stenophyllanin A, which represented the 8 and 6.2%, and procyanidin B isomers and catechin, representing 7.3 and 5.5%, respectively. According to our previous work (Díaz-de-Cerio et al., 2016), flavonols and flavan-3-ols represented the main phenolic compounds in guava leaf extract. Morin, quercitrin, guajaverin, procyanidin B, and hyperin represented the 41.2% of the sum of phenolic compounds.

3.2 Effect of guava leaf extract on diet-induced obesity in mice

Mice fed HFD showed a higher gradual increase in the body weight over the 7-week period in comparison with the mice receiving the low-fat control diet, these differences being statistically significant from the first day after HFD feeding, and remaining so until the end of the study (Figure 1). However, the administration of the extract from guava leaves showed no significant impact over time on mouse body-weight, independently of the diet considered, i.e. control diet or HFD (Figure 1). The absence of any significant effect of the extract on body-weight gain contrasts with a previous study in which an extract from guava leaves counteracted the body-weight increase in rats submitted to high fructose intake, although those assays were performed with higher doses of extract (50- and 100-fold) than that used in the present study (5 mg/kg) (Mathur et al., 2015).

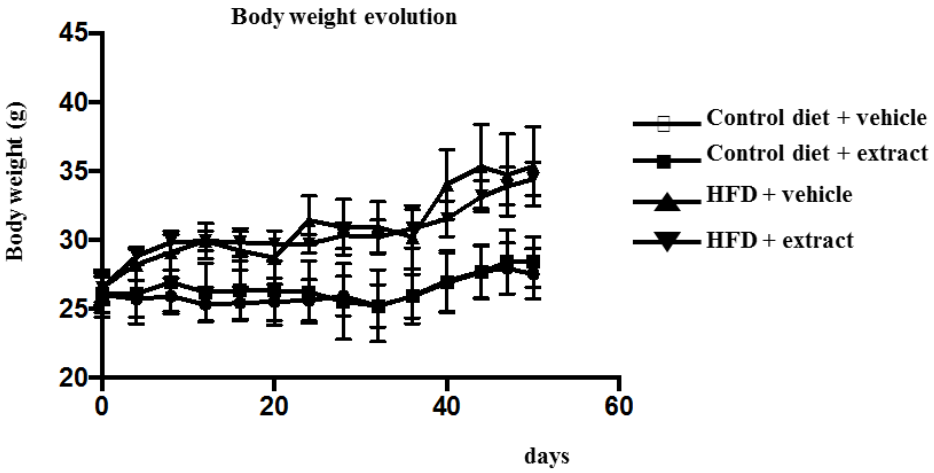

Figure 1. Effects of guava leaf extract on body-weight time course in control and HFD-fed mice. Values are expressed as mean ± SEM (n=10).

Nevertheless, although the administration of the extract failed to reduce body weight in HFD mice, it had a positive impact when the plasmatic markers of metabolic functions were evaluated. Thus, the glucose-tolerance test revealed that the area within the curve was significantly higher in HFD fed mice than in those receiving control diet 120 min after the oral glucose challenge (Figure 2). However, the administration of the

extract to obese mice resulted in a significant reduction in the values of the area under the curve in comparison with the corresponding control group (Figure 2).

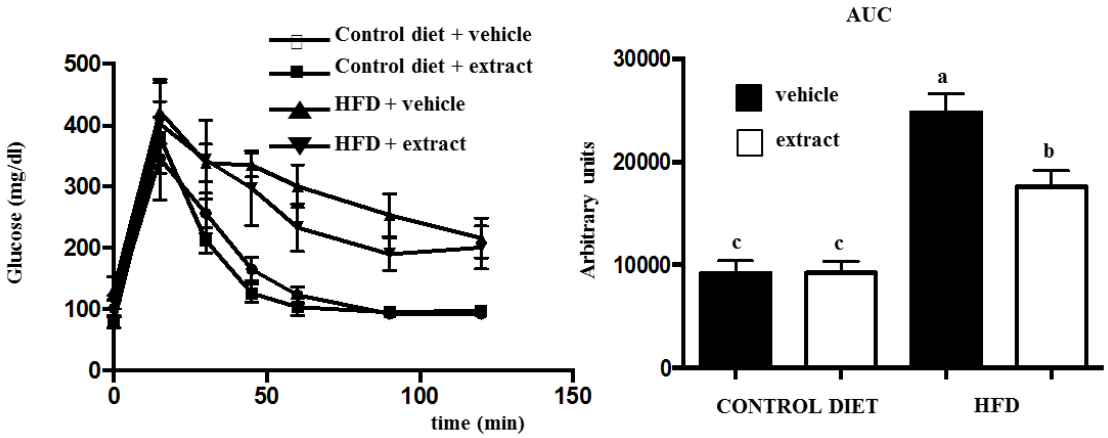

Figure 2. Effects of guava leaf extract on plasma glucose concentration after the glucose tolerance test in control and HFD-fed mice. Values are expressed as mean ± SEM (n=10). Groups with different letters statistically differ ($p < 0.05$). AUC, area under the curve

This beneficial effect on glucose metabolism was corroborated when fasted glycemia and HOMA-IR were assayed, since the extract significantly reduced the higher values found in HFD fed control mice but did not differ from those of the mice fed with low-fat control diet (Figure 3). In fact, previous studies performed either in humans or in diabetic animal models have also reported that guava leaf extracts improve diabetes symptoms such as hyperglycemia and insulin resistance (Deguchi & Miyazaki, 2010; Shen, Cheng, & Wu, 2008). Different mechanisms have been proposed to be involved in these beneficial effects on glucose metabolism, such as the stimulation of glucose uptake and utilization by the liver through the increase of different hepatic enzyme activities involved in glucose metabolism, including hexokinase, phosphofructokinase and glucose-6-phosphate dehydrogenase (Cheng, Shen, & Wu, 2009; Shen et al., 2008). It has also been speculated that the rise of postprandial blood-glucose due to alpha-glucosidase inhibition is suppressed by guava leaf extract.

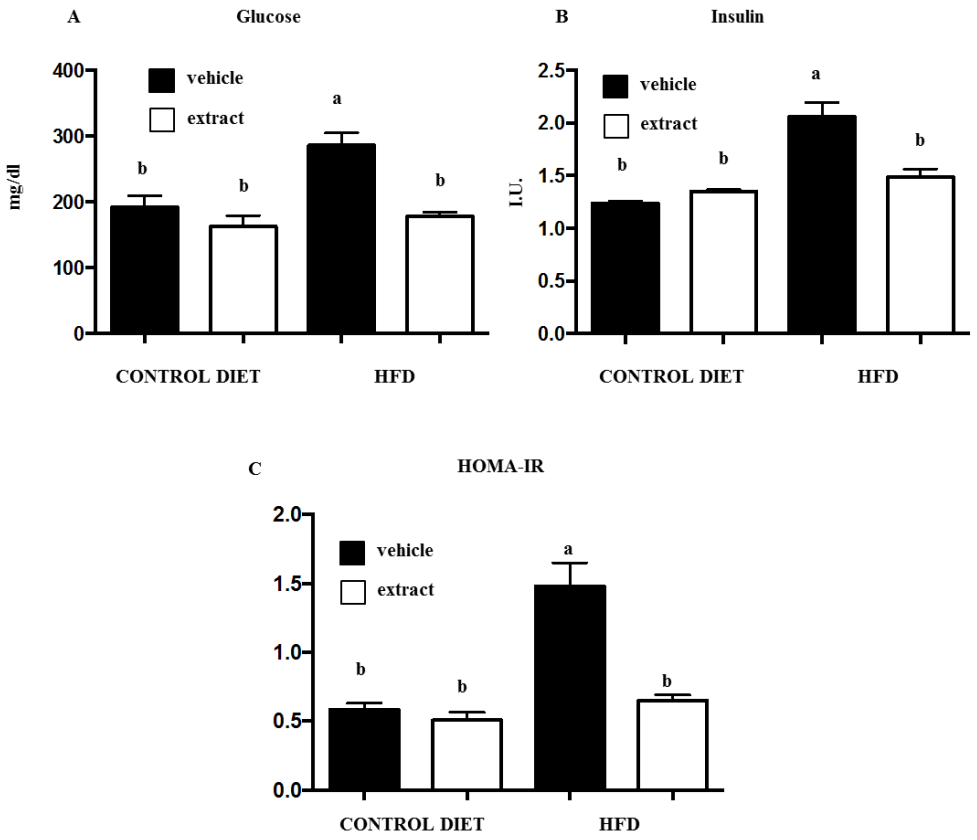

Figure 3. Effects of guava leaf extract on plasma-glucose concentration (A), plasma-insulin concentration (B) and HOMA-IR (C) in control and HFD-fed mice. Values are expressed as mean ± SEM (n=10). Groups with different letter statistically differ ($p < 0.05$).

Similarly, the present study reveals that this extract also positively influenced serum-lipid parameters in the obese mice. Thus, in comparison with the mice receiving the low-fat standard diet, the HFD control mice had hypertriglycemia and hypercholesterolemia, associated with higher levels of both low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol, and the concomitant increase in the LDL:HDL ratio (Figures 4 and 5). The administration of guava leaf extract to obese mice significantly ameliorated all these serum lipid markers, and, except for LDL-cholesterol, no statistical differences were observed in treated obese mice when compared with the non-obese groups (Figures 4 and 5). The ability of the guava leaf extract to improve the serum lipid profile in type 2 diabetes has been reported both in humans and in experimental models (Deguchi & Miyazaki,

2010). These effects can be closely related to the improvement in insulin resistance that guava leaf extract can exert, as demonstrated in the present study.

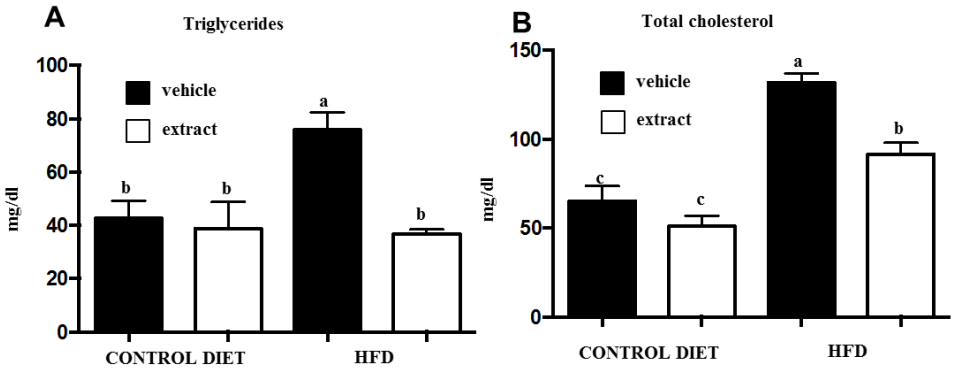

Figure 4. Effects of guava leaf extract on plasma triacylglycerols (A), total plasma cholesterol (B) in control and HFD-fed mice. Values are expressed as mean ± SEM (n=10). Groups with different letters statistically differ ($p < 0.05$).

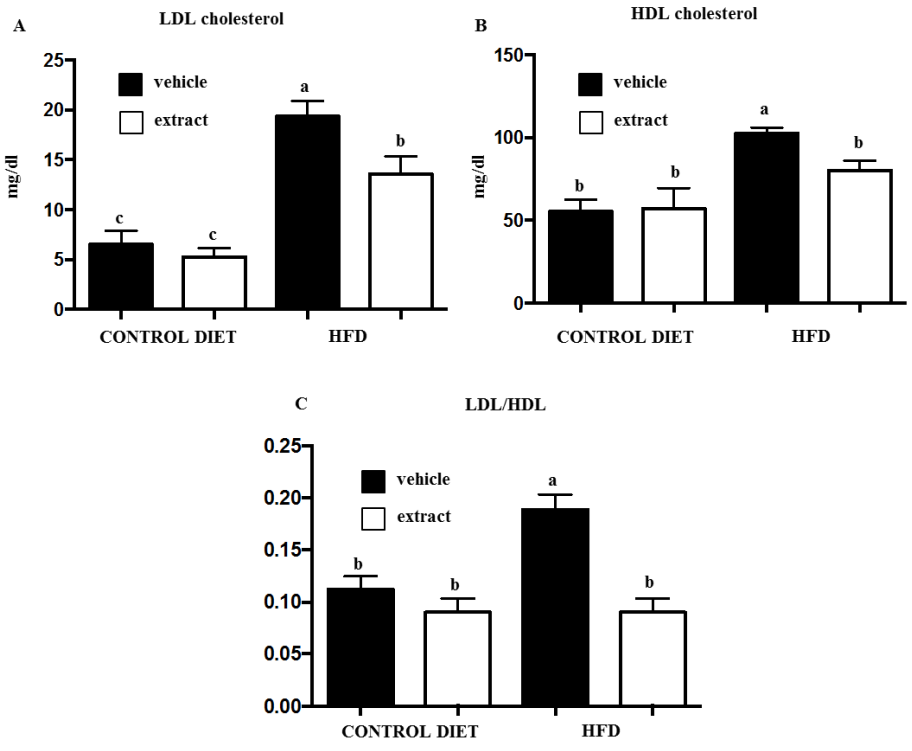

Figure 5. Effects of guava leaf extract on plasma LDL-cholesterol (A), HDL-cholesterol (B) and LDL:HDL ratio (C) in control and HFD-fed mice. Values are expressed as mean ± SEM (n=10). Groups with different letter statistically differ ($p < 0.05$).

Notably, in addition to the metabolic effects shown by this extract, which have been previously described elsewhere, the present study has demonstrated for the first time that chronic oral administration of guava leaf extract improved endothelial dysfunction in HFD fed mice. Aortas from obese mice significantly displayed a reduction in the endothelium-dependent vasodilator responses to acetylcholine (which is considered as an index of endothelial function), when compared to aortas from the control group fed the low-fat diet (Figure 6A). Thus, when the concentration-response curves were evaluated, HFD induced a change in the maximal relaxant response, the maximal relaxation being 38 ± 9% and 65 ± 5% in the HFD and control groups, respectively ($p < 0.05$), without showing any significant changes in the concentration of acetylcholine required for half-maximal relaxation (-logIC50 values were 7.34 ± 0.11 and 7.59 ± 0.15 in the HFD and control groups, respectively, $p > 0.05$). The administration of guava leaf extract to HFD-fed mice significantly increased the maximal relaxation induced by acetylcholine (64 ± 7%, $p < 0.01$ vs. HFD control group), displaying no statistical differences compared with the value found in control mice ($p > 0.05$) (Figure 6A). Subsequent experiments revealed that the relaxation with acetylcholine was almost fully suppressed in all experimental groups by the nitric synthase inhibitor L-NAME (Figure 6B), thus revealing that, in these vessels, the acetylcholine-induced relaxation in both control and HFD groups depended almost completely on endothelium-derived nitric oxide (NO). Finally, the impairment in the response to endothelial-derived NO is probably due to lower bioavailability of NO and not to a defect in the NO signaling in vascular smooth muscle. This was confirmed by the observation that nitroprusside, which directly activates soluble guanylyl cyclase in vascular smooth muscle and reproduces the effects of endogenous NO, exerts similar vasodilator effects in all experimental groups (Figure 6C). Consequently, the administration of the guava leaf extract to obese mice prevented the altered responses to acetylcholine observed in the aortae from these mice, indicating a protective role on agonist-induced NO bioactivity. It is well reported that obesity impairs the vascular endothelial function by inducing changes in blood pressure, glucose levels, and lipid

metabolism, which in turn generates a subclinical systemic inflammation (Avogaro & De Kreutzenberg, 2005; Hotamisligil, 2006).

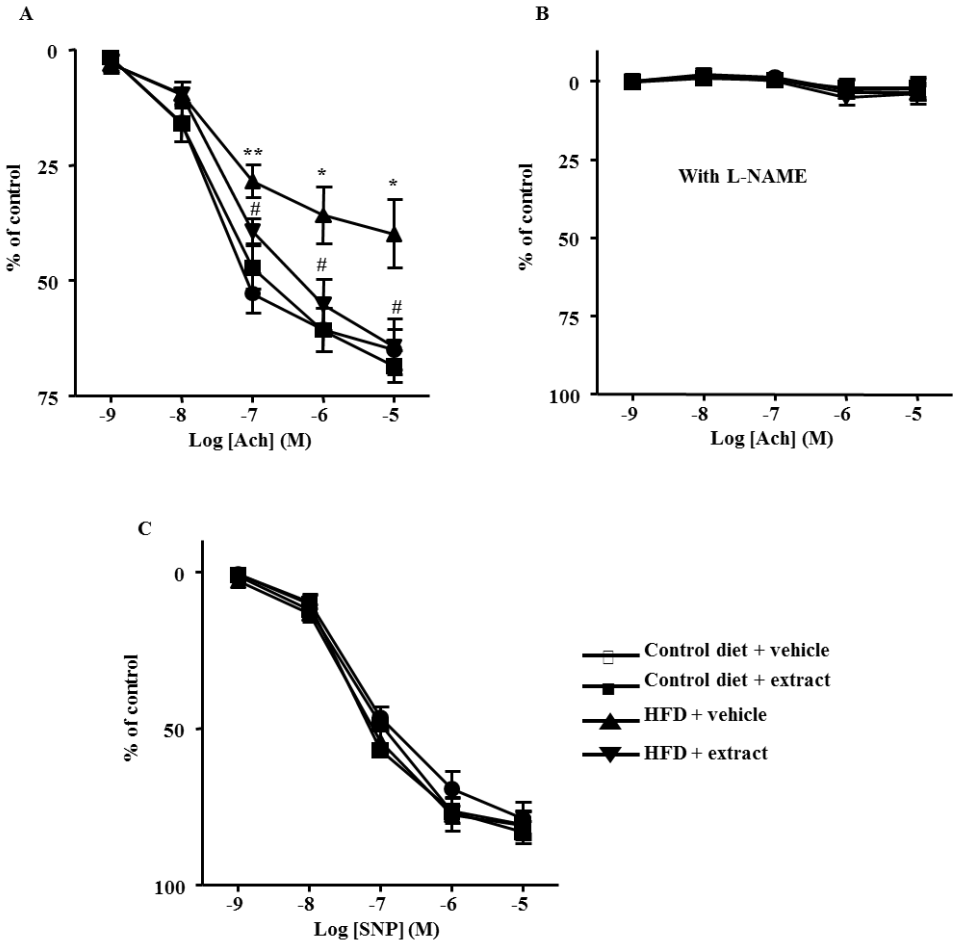

Figure 6. Effects of guava leaf extract on endothelial function. Vascular relaxant responses induced by acetylcholine (ACh) (A, B) and by sodium nitroprusside (SNP) (C) in aortae pre-contracted by phenylephrine in the absence (A, C) and presence (B) of L-NAME (10^{-4}M) in rings from all experimental groups. Values are expressed as mean ± SEM (n= 8-9 rings from different mice). * and ** indicate ($p < 0.05$) and ($p < 0.01$), respectively, compared with Control diet + vehicle group. # and ## indicate ($p < 0.05$) and ($p < 0.01$), respectively, compared with HFD + vehicle group

Furthermore, regarding the literature available, some individual compounds, also found in guava leaves, have been related to anti-diabetic activity, both *in vivo* and *in vitro*, and with the involvement of different action mechanisms, such as quercetin and naringenin (Vinayagam & Xu, 2015), catechin and geraniin (Chinchansure,

Korwar, Kulkarni, & Joshi, 2015), tellimagrandin I and casuarictin (Yoshida, Amakura, & Yoshimura, 2010), and procyanidin oligomers (Pinent et al., 2004). However, in addition to their beneficial impact on glucose and lipid metabolism, different phenolic compounds have been reported to exert direct vasodilator effects that can also account for the improvement of the vascular function observed in the present study, which could be achieved at the low plasma concentrations that would be found after the oral administration of this extract (Andriambeloson et al., 1997; Duarte et al., 2004; Fratantonio et al., 2015; López-Sepúlveda et al., 2011; Sanchez et al., 2007).

4. Conclusions

The present study suggests that the improvement in the glucose and lipid metabolism exerted by guava leaf extract in obese mice can have a positive impact on vascular dysfunction associated with obesity, thus preventing the development of atherosclerosis, and the subsequent cardiovascular events. Moreover, the presence of 72 phenolic compounds (identified by HPLC-DAD-ESI-TOF-MS) in guava leaf extract could justify the aforementioned beneficial effects. The characterization performed in the present study reveals that this extract is a good source of phenolic compounds (57 were successfully quantified). Briefly, different flavan-3-ols, flavonols, and ellagic acid derivatives have been detected and some of which have been proposed to display anti-diabetic properties.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgment

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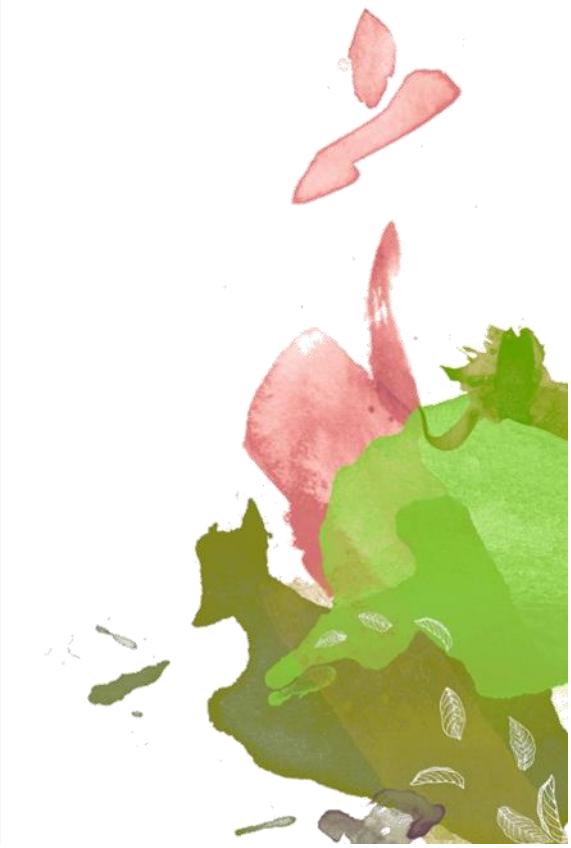
Annex



This work was carried out in collaboration with theresearch group "Advanced NMR methods and metal-based catalysts" (FQM-376) from the University of Almeria directed by Dr. Ignacio Fernández de las Nieves, and it is currently in progress. Despite this fact, it has been included in the thesis sinceit has been carried out during this period, and it is related with future studies.

Metabolite fingerprinting of Spanish guava leaves by Nuclear Magnetic Resonance (NMR) spectroscopy

1. Introduction

During the last decades, nuclear magnetic resonance (NMR) spectroscopy has gained popularity in metabolomic analysis in plants due to its unambiguous and detailed structural characterization capabilities compared to other analytical tools. The fact of being a non-destructive technique, its high reproducibility, thermal stability, combined with a simple sample preparation, make NMR one of the best options for metabolite profiling [1,2]. The main drawback is its low sensitivity (low-μM at 600 MHz), compared with mass spectrometry, which can be enhanced due to the increase of the magnetic fields frequencies (available up to 950 MHz) and the use of cryogenic probes (nM) [3].

The structural information provided by NMR detection is usually done by one and two dimension experiments. Actually, the great occurrence and abundance in nature (99.985%), makes the 1-D 1H NMR spectrum the most common one experiment. Its major problem is water suppression, which can be overcome with the combination of on-resonance and pulse sequence techniques. Concerning the 2-D experiments both, homo- and hetero-nuclear, are particularly useful for the elucidation and/or confirmation of the target metabolites [4].

For these reasons, NMR has been employed as a fingerprinting tool useful in quality control of natural products [1,2]. The metabolome of a plant matrix comprises

primary and secondary metabolites, which are characteristic of the environmental conditions and the origin where the plant grows [5]. For example, the complete profile of a whole extract achieved through NMR usually comprises from 20 to 40 compounds of different polarities. Therefore, it could become a prior step to the discrimination between plants [6].

Psidium guajava L. (guava) is a tropical tree which can be adapted to a wide range of climate conditions. Therefore, it could be cultivated in tropical and sub-tropical areas, like in the south of Spain. The leaves have demonstrated to possess beneficial effects against different diseases due to its phenolic profile composition [7]. In fact, Spanish guava leaves have been recently exhibited anti-diabetic properties, partly, due to their phenolic composition [8]. However, is it well established that this tree has different responses to environmental conditions, resulting in the accumulation of different phenolic compounds, such as quercetin and myricetin during the different seasons of the year [9]. In addition, it has been demonstrated that metabolites such as anthocyanidins and proanthocyanidins varies with the oxidative stage of guava leaf [10,11]. This variation could also affect its bioactivity, so it is important to evaluate the whole metabolic profile of guava leaves extracts in a fast and simple manner. Thus, this work is aimed to characterize for the first time the major metabolites contained in the Spanish guava leaf by NMR.

2. Material and methods

2.1 Plant material and sample preparation

Guava leaves were previously extracted, identified and characterized in Chapter 7. The extraction was optimized during the thesis and is presented in Chapter 2. For the NMR analyses, the freeze-dried extract (50 mg) was mixed with 0.65 mL of a solution based on CH_3OH-d_4 and KH_2PO_4 buffer in D_2O (pH 7.0) containing the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP, 0.01%$_{ww}$) and NaN_3 (90 μM) in a ratio (1:1). The resulting mixture was shaken in a Vortex (for 20 min at

1000 rpm) and centrifuged (for 10 min at 12500 rpm) at room temperature. Finally, the supernatant was transferred into an oven-dried 5 mm NMR tube for the analysis.

2.2 NMR spectroscopy

NMR spectra were acquired in a Bruker Avance III HD 600 MHz spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 24 SampleCase™ sampler and a quadruple QCI-P CryoProbe™. One- and two-dimensional homo- and hetero-nuclear experiments (^{1}H-^{1}H-NOESY, ^{1}H-^{1}H-TOCSY and ^{1}H-^{1}H-COSY, ^{1}H-^{13}C-HSQC and ^{1}H-^{13}C-HMBC) were recorded. All the measurements were performed at 293 ± 0.1 K, without rotation and using 8 dummy scans. Standard ^{1}H NMR spectrum was achieved using a NOESY pre-saturation pulse sequence (Bruker 1D noesygppr1d) with water suppression via irradiation of the water frequency during the recycle and mixing time delays. The spectra were automatically phased, baseline-corrected, and calibrated to the TSP signal at 0.0 ppm. The t_1 time was set to 4us and the mixing time (d8) to 100 ms. The spectrometer transmitter was locked to D_2O frequency using a mixture H_2O−D_2O (9:1). Acquisition and processing of spectra were carried out with TOPSPIN software (version 3.1). Signal assignment was accomplished with the aid of Chenomx NMR library (Edmonton, Canada) and Colmar database (Ohio State University) via the comparison of the chemical shifts and splitting patterns of the compounds.

3. Results and discussion

The evaluation of the composition of a primary extract through the use of NMR, is a hard task due to the complexity of the matrix that has many overlapped signals coming from many metabolites. One should remember that through NMR all the ^{1}H nuclei are observed since it does not depend on any ionization process, so all the analytes that are soluble at observed at the same time. Usually, in ^{1}H-NMR spectra could be distinguished three main regions corresponding to aliphatic compounds ($0 < \delta \leq 3$ ppm), carbohydrates ($3 < \delta \leq 5.5$ ppm), and aromatic structures ($6 < \delta \leq 9$ ppm) where phenolic compounds could be found. Different kinds of NMR experiments were

performed in order to elucidate the fingerprint of guava leaf extract. As shown in Figure 1, the ^{1}H NOESY NMR spectrum of guava leaves contains a vast number of overlapping signals in the two first regions. This specific sequence allows the suppression of the water signal (δ_H 4.7 ppm) with almost no perturbation on the rest of the spectrum, what is extremely important in order to characterize metabolites close to the suppressed region. The set of homo- and heteronuclear experiments usually acquired are ^{1}H-^{1}H-COSY, ^{1}H-^{1}H-TOCSY, ^{1}H-^{13}C-HSQC, and ^{1}H-^{13}C-HMBC.

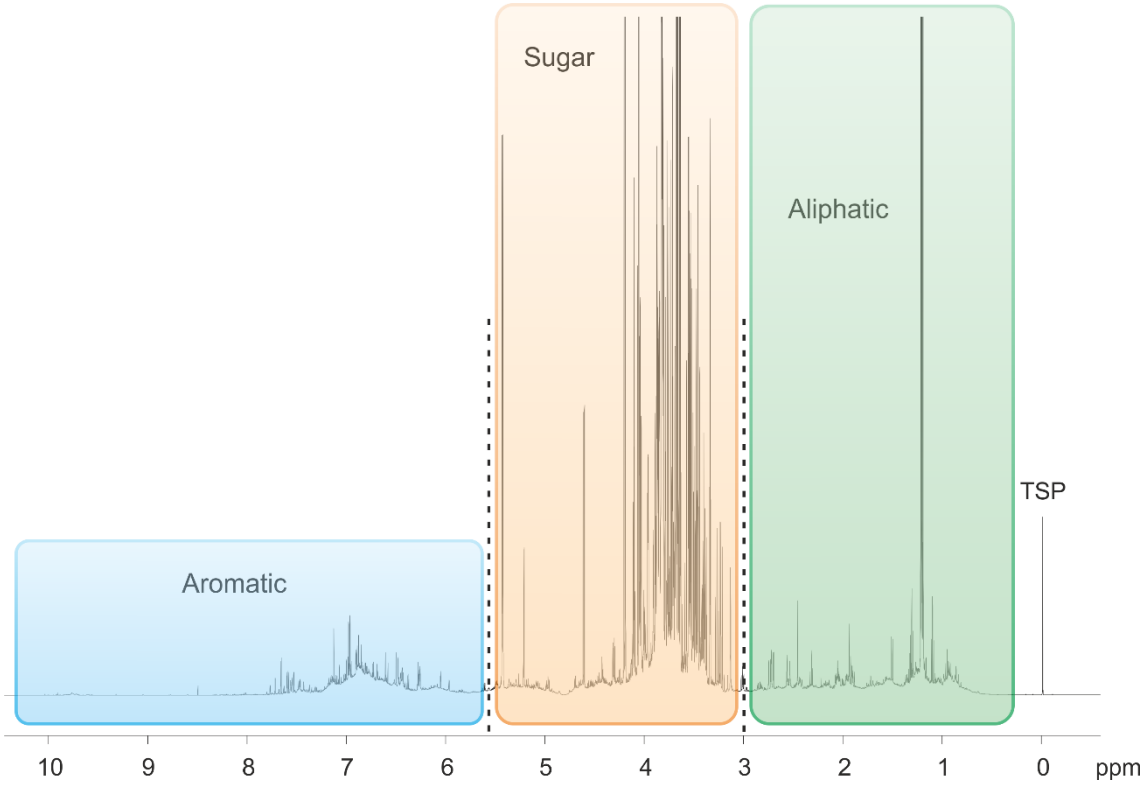


Figure 1. ^{1}H NOESY NMR spectrum (600 MHz) with water suppression of a MeOD-D_2O guava leaf extract.

As an example, we will describe in the following lines the analysis of sucrose in order to show the potential of these methods towards structural characterization. In this sense, we first located the anomeric proton of the sucrose (H-1 of D-glucopyranosyl ring, see figure 2) that was identified with the help of ^{1}H-^{1}H-TOCSY, ^{1}H-^{13}C-HSQC and ^{1}H-^{13}C-HMBC spectra and confirmed using Chenomx NMR library [12].

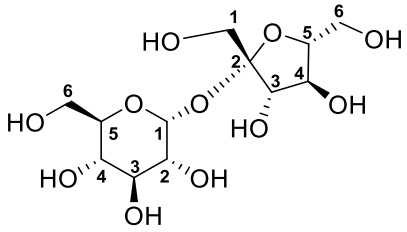


Figure 2. Chemical structure of sucrose.

The ^{1}H-^{1}H TOCSY NMR spectrum displays correlations between protons located in the same spin system. Therefore, the anomeric proton H-1 in the six-membered ring should present a correlation with the rest of protons on the same ring. ^{1}H-^{1}H TOCSY spectrum (Figure 3) confirmed the presence of the D-glucopyranosyl ring since it showed how the anomeric proton was correlated with all protons contained in the D-glucopyranosyl ring with the following chemical shifts δ_H 3.45, 3.53, 3.77 and 3.85 ppm. As expected, ^{1}H-^{1}H TOCSY experiments do not offer correlations out of the spin system, and therefore there are no correlation with the five-membered ring contained in the sucrose.

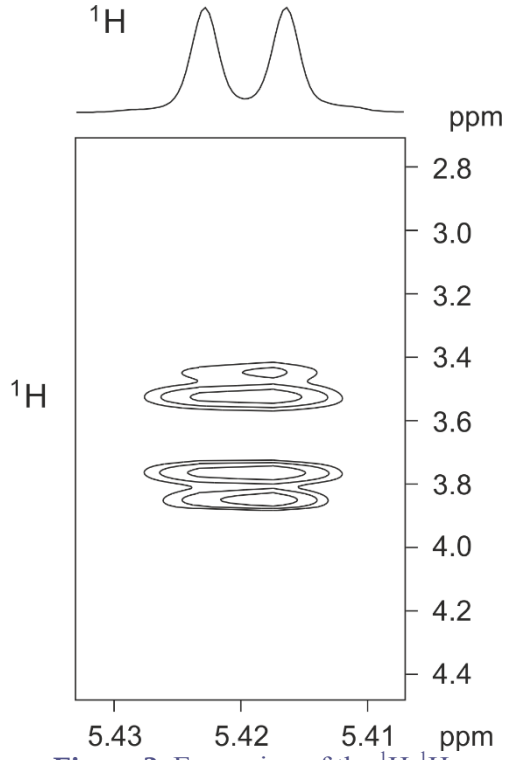


Figure 3. Expansion of the ^{1}H-^{1}H-TOCSY (600 MHz) spectrum of guava leaf extract.

The ^{1}H-^{13}C-HMBC NMR spectrum provides correlations between carbons and protons not only belonging to the same spin system, but also to different spin systems. We usually observed cross peaks between proton and carbons coupled with $^{2}J_{CH}$ and $^{3}J_{CH}$. Thus, the ^{1}H-^{13}C-HMBC NMR spectrum (Figure 4) was explored in order to get information beyond the glycosidic linkage. It shows for the anomeric proton a three bond ($^{3}J_{CH}$) correlation with the anomeric carbon (C-2) of the D-fructofuranoside ring at δ_H 104 ppm, among other interactions at lower frequency.

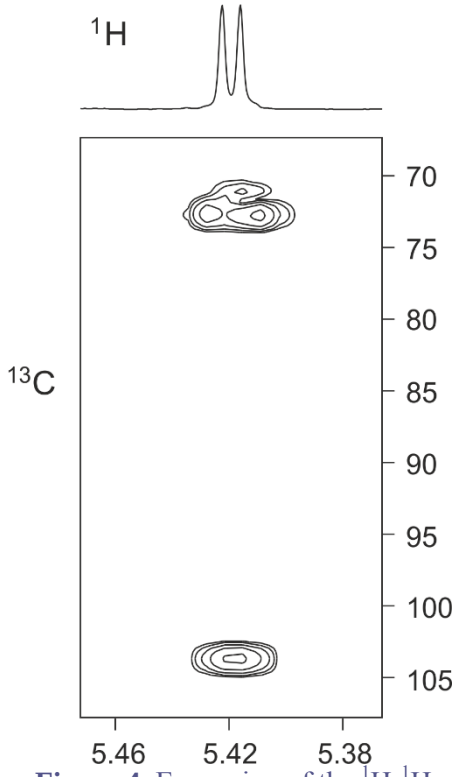


Figure 4. Expansion of the ^{1}H-^{1}H-HMBC (600 MHz) spectrum of guava leaf extract.

Finally, the presence of sucrose was confirmed comparing the ^{1}H NOESY NMR spectrum obtained from guava leaf extract with the ^{1}H NMR spectrum of a sucrose standard found with the help of the Chenomx NMR library (Figure 5).

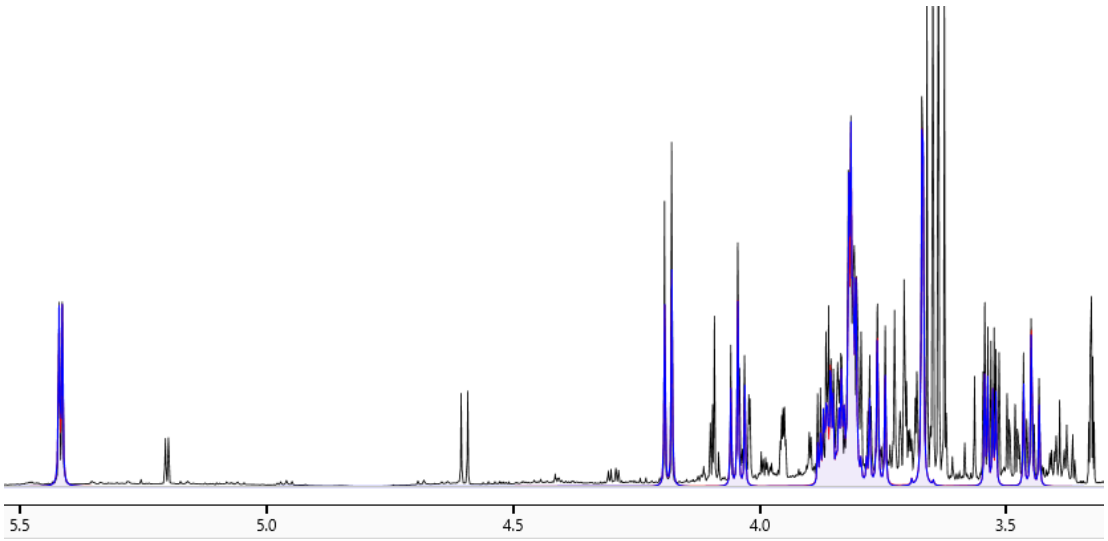


Figure 5. ^{1}H NMR overlapped spectra of guava leaf extract (black line) and sucrose standard (blue line) obtained by Chenomx NMR library.

The identification of the rest of metabolites presented in the extract is still in progress and it will not be included in this thesis.

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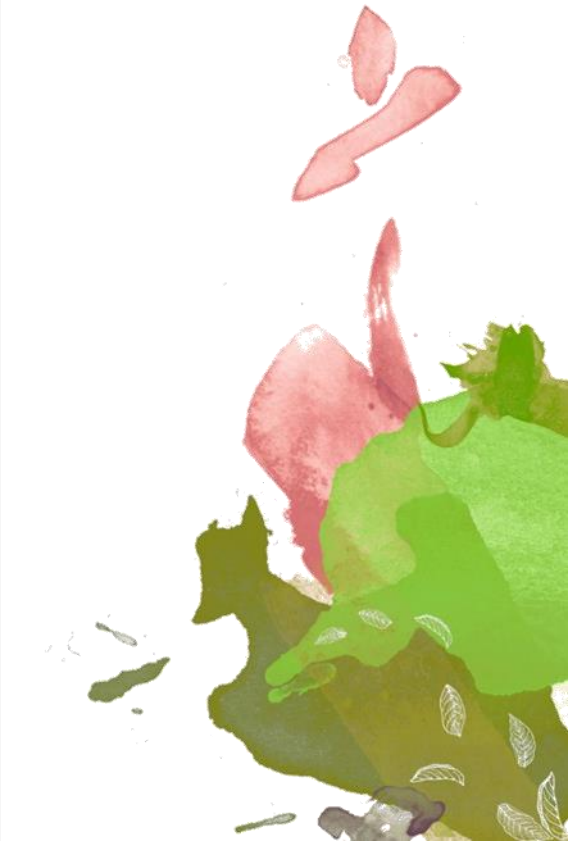
CONCLUSIONS/ CONCLUSIONES



- ✓ HPLC-ESI-Q-MS platform allowed the identification and quantification of 48 polar compounds in guava leaves extracts (aqueous-ultrasound and infusion extracts). The main families of compounds found in the leaves are flavonols flavan-3-ols, and gallic and ellagic acid derivatives, which accounted a 50, 30, and 20%, respectively. In less amount, benzophenones (4%) and, finally, only a flavanone was found. Antioxidant capacity assays (FRAP and ABTS) reported positive correlations $R > 0.98$ $P < 0.001$ with the total amount of polar compounds, and also for single compounds. According to the results, the aqueous bath-ultrasound assisted extraction provided better recovery of the target compounds than the infusion (between 3 and 4-fold higher). Regarding the optimization of infusion time, 5-minute infusion is recommended for guava leaves culinary uses, due to a high recovery of phenolic compounds.

- ✓ HPLC-DAD-ESI-QTOF-MS platform confirmed the presence of 60 polar compounds and permitted the tentative identification of 12 new phenolic compounds in *P. guajava* L. leaves. The optimization of the solvent extraction with *pyrifera* var. provided better results, especially for flavonols and flavan-3-ols, with the hydroethanolic mixture (80:20 (v/v)). Due to the high correlation noticed among the quantification of total phenolic compounds, FRAP, and ABTS assays, *P. guajava* L. var. *pyrifera* could be considered a better source of phenolic compounds than *pomifera* var. These varieties were tested for the first time by FRAP and ABTS assays.

- ✓ HPLC-DAD-ESI-QTOF-MS platform permitted to assess the influence of three different oxidative states of guava leaves var. *pyrifera* on their phenolic content. 73 compounds were determined employing the negative ionization mode, whereas in the positive ionization mode, a cyanidin-glucoside was identified for the first time. Concerning the quantification results, contrary data were obtained based on the ionization mode and the oxidative state. Briefly, low oxidative state reported great amounts of the main families of compounds previously

identified, while the cyanidin-glucoside content was large at high oxidative state. Furthermore, a vast number of phenolic compounds identified in guava leaves are described to possess anti-diabetic properties. Thus, depending upon the target compound, low or high oxidatives states might be chosen.

- ✓ NP-HPLC-FLD-ESI-Q-MS platform permitted the identification and quantification of the monomers, several oligomers (DP 2-13) and polymeric PAs. BBD demonstrated to be adequate for the optimization of PAs extraction from *P. guajava* leaves var. *pyrifera*. The influence of the factors (acetone/water ratio (% (v/v)), temperature of the ultrasound bath, time of extraction, and acetic acid percentage) was significant in the response of the sum of PAs. The optimized conditions were 50% acetone/water (v/v), 48ºC, 30 min, and 0% acetic acid (v/v). At these extraction conditions, significative differences ($p < 0.05$) were found among the three oxidative states of the leaves (low, medium, and high). Briefly, SPAs increased when the oxidative state decreased. In view of the results, guava leaves var. *pyrifera* at low oxidative state display to be a potential source of PAs with low degree of polymerization. Besides, an exhaustive characterization of the PAs profile has been reported for the first time.

- ✓ HPLC-ESI-QqQ-MS allowed a more accurate quantification of the phenolic compounds in guava leaves. BBD comfirm to be suitable for the extraction of phenolic compounds by sonotrode-ultrasound assisted extraction. A considerable influence of time of extraction, ethanol/water ratio, and US power was found in the response of sum of phenolic compounds, flavonols and flavan-3-ols, and antioxidant capacity (DPPH and ABTS assays). The best process conditions were 40 min, 60% ethanol/water (v/v), and 200 W. *Pyrifera* variety exhibited greater sum of phenolic compounds than *pomifera* var. ($p < 0.05$), 49.7 ± 0.3 and 46.2 ± 0.1 mg/g leaf d.w., respectively. Also, *pyrifera* var.

reported higher quantity of flavonols and flavan-3-ols (25 and 15%, respectively).

- ✓ Over the last decade, ethnomedical uses of *P. guajava* leaves have been verified by several investigations, *in vitro* as well as *in vivo*, over the last decade against many disorders. The potential of guava leaves has been associated to the terpenoids and phenolic compounds present in them. Also, the treatment with some individual compounds such as quercetin, catechin, vescalagin, gallic acid, peltatoside, hyperoside, isoquercitrin, and guaijaverin has demonstrated to possess beneficial effects towards the most common worldwide diseases.

- ✓ A crude extract of guava leaves var. *pyrifera* was tested in obese mice fed with high-fat diet. The leaves showed an enhancement in the glucose and lipid metabolim in obese mice. Besides, the leaves might prevent the development of atherosclerosis and the following cardiovascular events since it was found a reduction of vascular dysfunction. This health benefit is due to a lower bioavailability of nitric oxide-dependent vasodilatation. This effect was partly connected to the presence of 57 phenolic compounds, which some of them have displayed anti-diabetic properties elsewhere.

- ✓ La plataforma HPLC-ESI-Q-MS permitió la identificación y cuantificación de 48 compuestos polares en extractos de hoja de guayabo, tanto en las infusiones como en un extracto acuoso obtenido por extracción en baño de ultrasonidos. Las principales familias de compuestos encontradas en las hojas fueron flavonoles, flavan-3-oles, y derivados del ácido gálico y elágico, los cuales representaron un 50, un 30 y un 29%, respectivamente. En menor cantidad, se encontraron las benzofenonas (4%), y por último, una única flavanona. Los ensayos de capacidad antioxidante (FRAP y ABTS) presentaron correlación positiva ($R > 0.98$; $P > 0.001$) con la cantidad total de compuestos polares,

además de con cada uno de los compuestos. De acuerdo con los resultados, la extracción acuosa mediante baño de ultrasonidos produjo una mejor recuperación de los compuestos de interés, de 3 a 4 veces mayor, respecto a la infusión. En lo relativo a la optimización del tiempo de infusión, se recomienda 5 minutos de infusión para su uso culinario, ya que presentó un mayor contenido fenólico.

- ✓ La plataforma HPLC-DAD-ESI-QTOF-MS confirmó la presencia de 60 compuestos polares y permitió la identificación tentativa de 12 nuevos compuestos fenólicos en la hojas de *P. guajava*. La optimización del disolvente de extracción con la variedad *pyrifera*, originó mejores resultados con la mezcla hidroalcohólica (80:20 (v/v)), especialmente para el contenido de flavonoles y flavan-3-oles. Debido a la alta correlación encontrada entre la cuantificación total de los compuestos, y los ensayos FRAP y ABTS, se concluyó que la variedad *pyrifera* puede ser considerada, respecto a la variedad *pomifera*, una mejor fuente de compuestos fenólicos. Por último, estas variedades fueron comparadas por primera vez mediante los ensayo FRAP y ABTS.

- ✓ La plataforma HPLC-DAD-ESI-QTOF-MS permitió evaluar la influencia de tres estados oxidativos de la hoja de guayabo (var. *pyrifera*) en su contenido fenólico. A través del modo de ionización negativo, se determinaron 73 compuestos, mientras que a partir del modo de ionización positivo, se identificó por primera vez una cianidina-glucósido. De acuerdo con los resultados de cuantificación, basándose en el modo de ionización utilizado y en el estado oxidativo de las hojas (alto, medio, y bajo), se obtuvieron datos inversamente proporcionales. En resumen, las hojas con un estado de oxidación más bajo dieron lugar a un mayor contenido en las principales familias de compuestos previamente identificados, mientras que el contenido de la cianidina-glucósido fue más elevado para la hoja de un estado oxidativo más alto. Además, debido a varias referencias bibliográficas, un alto número de compuestos identificados en

las hojas han demostrado tener propiedades anti-diabéticas. Por ello, en función del compuesto de interés, se deberá elegir entre los estados de oxidación alto y bajo.

- ✓ La plataforma NP-HPLC-FLD-ESI-Q-MS hizo posible la identificación y cuantificación de monómeros, varios oligómeros (grado de polimerización 2-13) y los polímeros de PAs. El diseño Box-Behnken demostró ser adecuado para la optimización de la extracción de PAs de las hojas de *P.guajava* var. *pyrifera*. Los factores (porcentaje acetona/agua (% (v/v)), temperatura, tiempo de extracción en baño de ultrasonidos y porcentaje de ácido acético) influyeron significativamente ($p < 0.05$) en la respuesta de la suma de PAs. Las condiciones óptimas (50% acetona/agua (v/v), 48ºC, 30 minutos, y 0% ácido acético (v/v)) fueron empleadas en la extracción de los compuestos de las hojas a diferentes estados oxidativos (alto, medio, y bajo), encontrándose diferencias significativas entre los mismos ($p < 0.05$). En resumen, la suma de PAs aumentó al disminuir el estado oxidativo. En vista de los resultados, se puede decir que las hojas de guayabo var. *pyrifera* con un estado de oxidación bajo han demostrado ser una fuente potencial de PAs de bajo grado de polimerización. Además, se muestra por primera vez la caracterización exhaustiva de PAs en las hojas de guayabo.

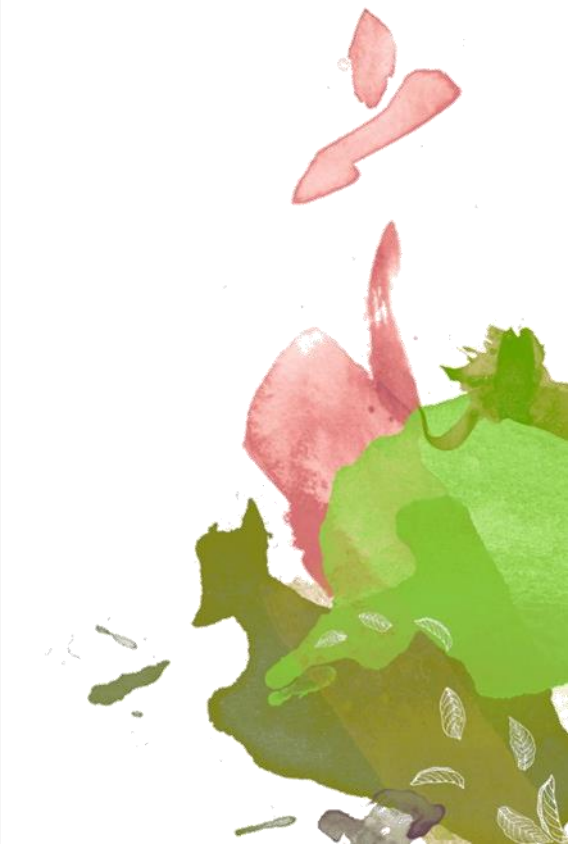
- ✓ La plataforma HPLC-ESI-QqQ-MS permitió una cuantificación más precisa de los compuestos fenólicos presentes en la hoja de guayabo. Además, se confirmó la idoneidad del diseño Box-Behnken para la extracción de dichos compuestos mediante la extracción asistida por sonda de ultrasonidos. El efecto de los factores (tiempo de extracción, porcentaje etanol/agua (% (v/v)) y potencia del aparato) en la respuesta de la suma de los compuestos fenólicos, en flavonoles, flavan-3-oles, y en los ensayos de capacidad antioxidante (DPPH y ABTS) fue considerable. Las mejores condiciones de extracción fueron: 40 minutos, 60% etanol/agua (v/v) y 200W. La suma de compuestos fenólicos fue mayor en la

variedad *pyrifera* que en la *pomifera*. Además, la presencia de flavonoles y flavan-3-oles, en dicha variedad, fue más elevado (25 y 15%, respectivamente).

- ✓ En la última década, los usos tradicionales de las hojas de *P. guajava* han sido verificados mediante investigaciones, *in vitro* e *in vivo*, frente a diversas enfermedades. El potencial de las hojas ha sido asociado a su contenido fenólico. Además, gracias al tratamiento con varios compuestos individuales (quercetina, catequina, vescalagina, ácido gálico, peltatosido, hiperósido, isoquercitrina, y guaijaverina), las hojas han demostrado poseer efectos beneficiosos frente a las enfermedades con mayor incidencia a nivel mundial.

- ✓ Se ha probado positivamente un extracto de hoja de guayabo variedad *pyrifera* en un modelo de obesidad en ratones, inducido mediante una dieta rica en grasa. Las hojas mostraron una mejora en el metabolismo de glucosa y de lípidos de dichos ratones. Además, las hojas pueden prevenir el desarrollo de la aterosclerosis y afecciones cardiovasculares derivadas de ella, ya que el extracto disminuyó la disfunción vascular. Este efecto sobre la salud es debido a una menor biodisponibilidad del vasodilatador óxido nítrico. Este efecto, está en parte asociado a la presencia de 57 compuestos fenólicos en las hojas, algunos de los cuales has demostrado poseer propiedades anti-diabéticas.

Appendix



Box-Behnken design

BBD consisted on three-level incomplete factorial designs, in which the number of experimental points (N) is given by $N = 2k(k-1)+C_0$, where k is number of factors under study and C_0 is the number of central points. For example, to study the influence of three factors in a response variable, two factors are adjusted to a full two-level design (2^k) (± 1, ± 1), whereas the third factor is arranged at middle level (0). So, in this design only 12 runs plus replicates at the center points are required, in comparison with 27 runs of a full factorial design (3^3)[a,b]. As is shown in Figure a each experimental run lie on the surface of a sphere centered at the origin of the coordinate system, and tangential to the midpoint of each edge of the cube. Indeed, this design is indicated when an intermediate response is expected[a].

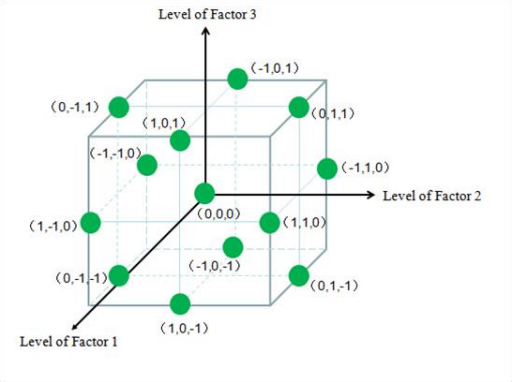


Figure a. Schematic view of BBD experimental runs

BBD is also suitable for high number of factors; in these cases, the experimental runs are set into orthogonal blocks, were the effects themselves are orthogonal to the block effects, being useful when these last are likely to be large[a,b].

[a] Ferreira, S. L. C.; Bruns, R. E.; Ferreira, H. S.; Matos, G. D.; David, J. M.; Brandão, G. C.; da Silva, E. G. P.; Portugal, L. A.; dos Reis, P. S.; Souza, A. S.; dos Santos, W. N. L. Box-Behnken design: an alternative for the optimization of analytical methods. *Anal. Chim. Acta* **2007**, *597*, 179–186.

[b] Ferreira, S. L. C.; Bruns, R. E.; da Silva, E. G. P.; dos Santos, W. N. L.; Quintella, C. M.; David, J. M.; Bittencourt de Andrade, J.; Breitkreitz, M. C.; Sales Fontes Jardim, I. C.; Barros Neto, B. Statistical designs and response surface techniques for the optimization of chromatographic systems. *J. Chromatogr. A* **2007**, *1158*, 2–14.

Once the model is run on the selected response variable, data are adjusted to a second-order polynomial equation (1)[a-c]:

$$\Upsilon = \beta_0 + \sum_{i=1}^{k} \beta_i \chi_i + \sum_{i=1}^{k} \beta_{ii} \chi_{ii}^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ii} \chi_i \chi_j$$

Equation 1. Model equation

where Y represents the response variable. X_i and X_j are the independent factors affecting the response, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the model (intercept, linear, quadratic and interaction term).

The evaluation of the model is carried out by the method of least squares. Therefore, the validation is generally done by analysis of variance, studying the goodness in prediction (R^2 and Q^2) based on the variation in the regression and the residuals, the pure error and the lack of fit[b,c].

Finally, the equation is usually represented by 2D-contour and/or 3D-surface plots to allow model visualization and to calculate the critical point. Most desirable surfaces are presented in Figure b. However, other saddle or plateau surfaces can also be found[c,d].

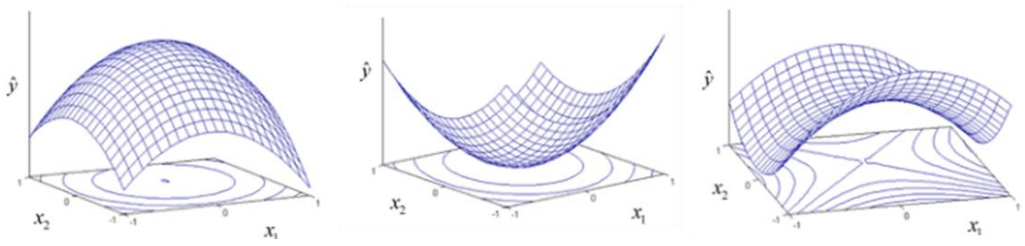


Figure b. Examples of response surface plots: maximum (left), minimum (middle), saddle point (right)

[c] Baş, D.; Boyacı, İ. H. Modeling and optimization I: usability of response surface methodology. *J. Food Eng.* **2007**, *78*, 836–845.

[d] Hibbert, D. B. Experimental design in chromatography: a tutorial review. *J. Chromatogr. B* **2012**, *910*, 2–13.