UNIVERSIDAD AUTONOMA DE AGUASCALIENTES

Centro de Ciencias Básicas
Doctorado en Ciencias Biológicas (Modalidad Directa)

Tesis:

Extracción, Purificación y Caracterización Química y Funcional de Compuestos Polifenólicos de la Hoja de *Psidium guajava* L.

Presenta:

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Para obtener el Grado de Doctora en: Ciencias Biológicas

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Aguascalientes, Ags, a 16 de enero del 2025

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TITULO: Extracción, Purificación y Caracterización Química y Funcional de Compuestos Polifenólicos de la Hoja de *Psidium guajava* L.

IMPACTO SOCIAL (señalar el impacto logrado): La extracción, purificación y caracterización química y funcional de compuestos polifenólicos de la hoja de *Psidium guajava* L., brinda una alternativa para el manejo de contaminantes microbianos. Ya que existe la posibilidad de usar este extracto como bacteriostático en superficies y como bactericida para tratamientos en la piel. El impacto social que esto tiene es importante para el tratamiento de enfermedades emergentes y evitar la resistencia a antibióticos que en la actualidad es un problema que preocupa a la sociedad en su conjunto.

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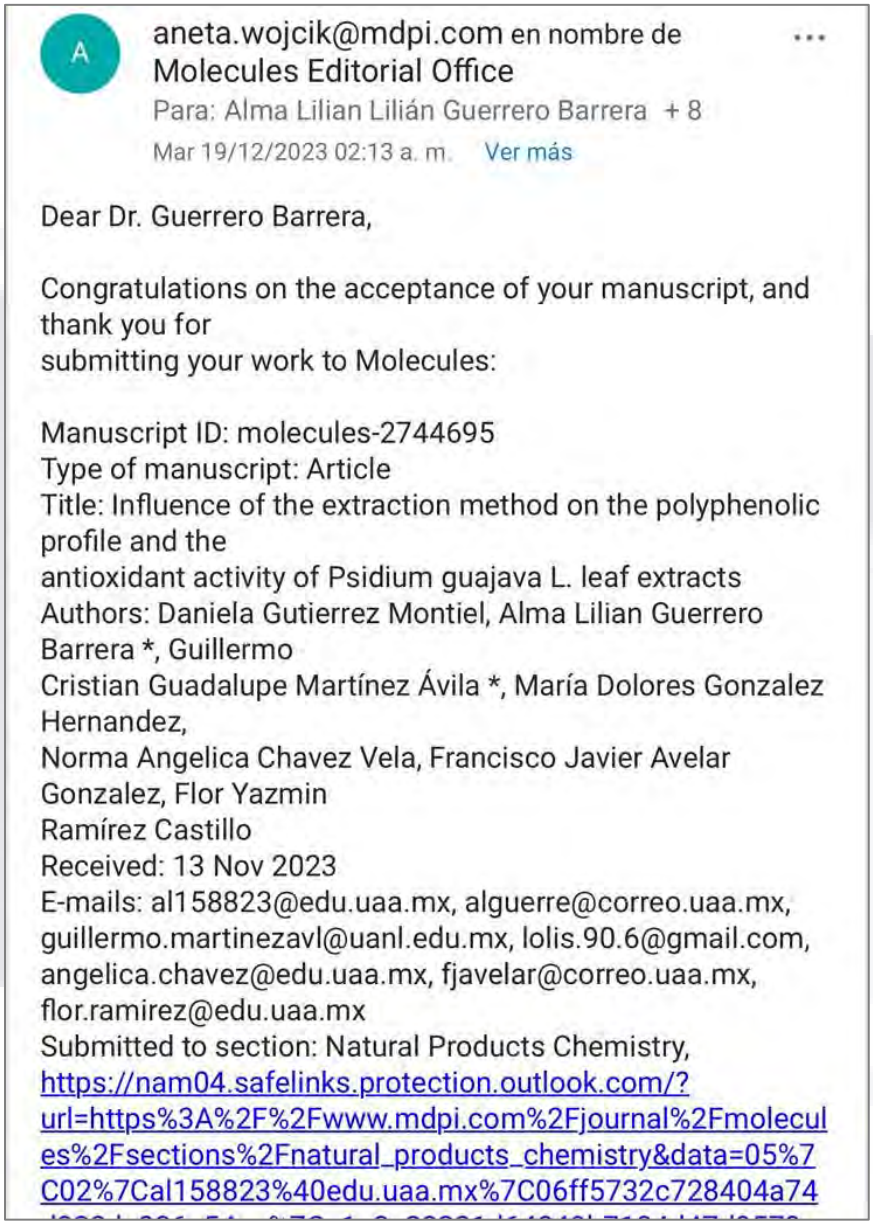

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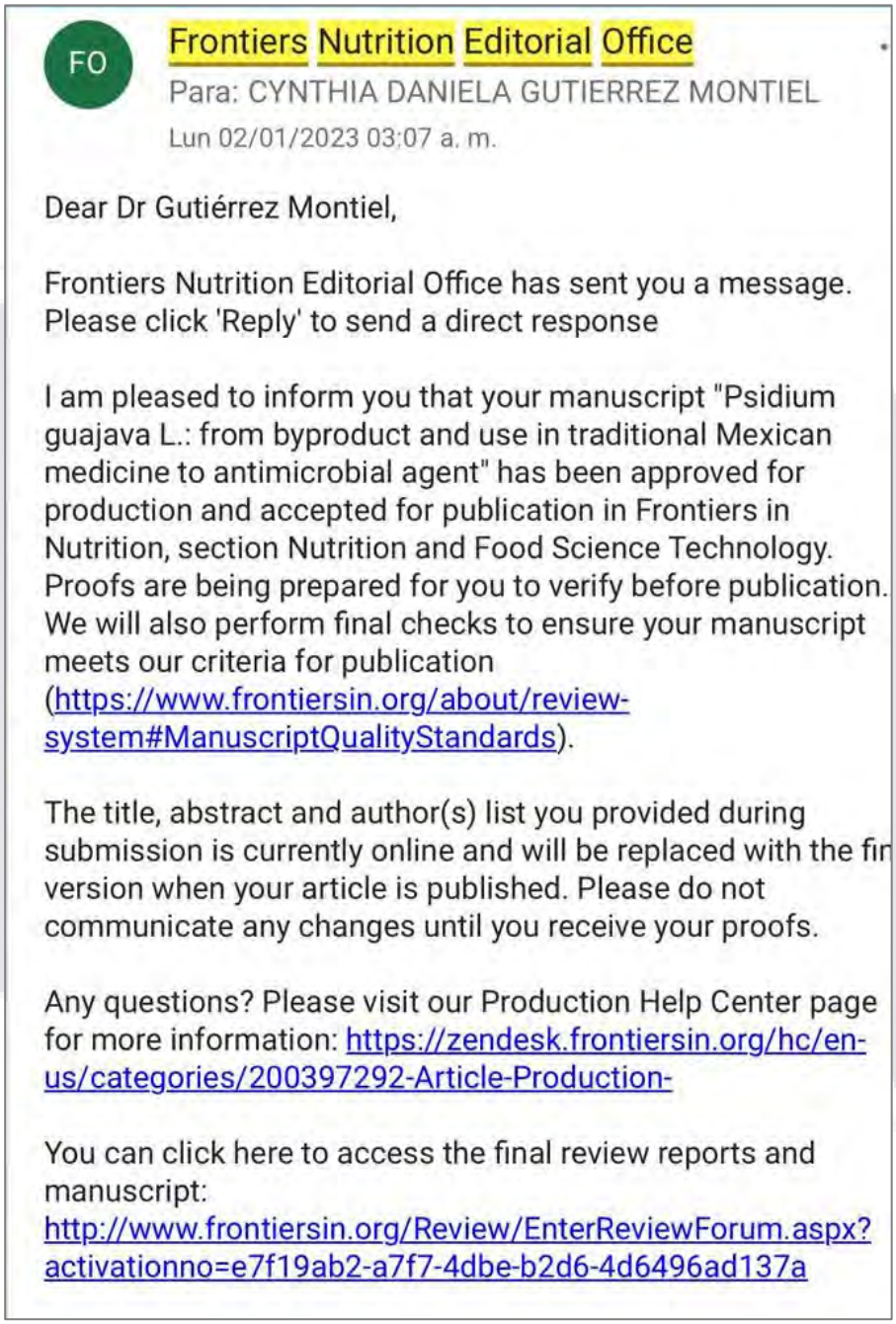

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Acronyms

IC_{50}- concentration needed to inhibit 50% of radicals

LC_{50}- concentration at which 50% mortality is observed compared to the control group.

CLSM- Confocal Laser Scanning Microscopy.

DMEM-Dulbecco's Modified Eagle Medium.

FBS- fetal bovine serum.

FTIR- Fourier Transform Infrared Spectroscopy.

GAE- Gallic Acid Equivalents.

GIIs- Growth Inhibitory Indices.

GLE- crude guava leaf extract.

GLEP- purified polyphenols from guava leaf extract.

IPEC-1- porcine intestinal epithelial cells.

LDH- enzyme lactate dehydrogenase.

LOEC- the lowest concentration with observed effect.

MBC- Minimum Bactericidal Concentration.

MIC-Minimum Inhibitory Concentration.

NOEC- the highest concentration at which no mortality was observed.

OD-Optical Density.

PoCo-83- porcine colonic epithelial cells.

RPMI-Roswell Park Memorial Institute Medium.

UAE- Ultrasound Assisted Extraction.

UPLC-MS-Ultra Performance Liquid Chromatography Mass Spectrometry

Vero-76- african green monkey kidney epithelial cells.

WST-1-(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt).

XDR- Extremely Drug-Resistant.

Resumen

Psidium guajava L. es un arbusto nativo de América perteneciente a la familia Myrtacea. Su importancia económica radica en su fruto, la guayaba, una baya de pulpa firma y numerosas semillas que es consumida a nivel mundial y de la cual México es uno de los productores más importantes. Las hojas del guayabo son un residuo agroindustrial no valorizado y altamente disponible en países productores de guayaba. Lamentablemente, terminan siendo incineradas o en vertederos a pesar de su amplio uso en la medicina tradicional y, que en los últimos años se ha reportado que pueden ejercer diferentes bioactividades gracias a su alto contenido en fitoquímicos. En el presente estudio se evalúan tres tipos diferentes de extracción de hojas de guayaba: maceración, extracción asistida por ultrasonido y Soxhlet para determinar que método es el más conveniente para obtener un extracto bioactivo. Posteriormente, se caracteriza la composición de los extractos y se analiza su actividad antioxidante, así como sus efectos antimicrobianos y antibiopelícula *in-vitro.*

Palabras clave: *Psidium guajava* L., fitoquímicos, antioxidantes, antimicrobianos, hojas de guayaba.

Abstract

Psidium guajava L. is a native American shrub belonging to the Myrtacea family. Its economic importance lies in its fruit, the guava, a berry with firm pulp and numerous seeds that is consumed worldwide and which Mexico is one of the most important producers. Guava leaves are an unvalued agro-industrial waste and are highly available in guava-producing countries. Unfortunately, they end up being incinerated or in landfills despite their extended use in traditional medicine and their high content of phytochemicals that can provide them with different bioactivities. In the present study, three different extraction methods are evaluated: maceration, ultrasound-assisted extraction and Soxhlet to determine which method is the most suitable for obtaining a bioactive extract from guava leaves. Subsequently, the composition of the extracts is characterized and their antioxidant, antimicrobial and antibiofilm activity is analyzed *in-vitro.*

Keywords: *Psidium guajava* L., phytochemicals, antioxidants, antimicrobials, guava leaves.

Introduction

1.1 *Psidium guajava* L.

Psidium guajava L. is a native shrub from America that can grow in tropical environments worldwide [1]. Its economic importance lies mainly in its fruit, the guava, which is cultivated and marketed mainly by India, Thailand, Brazil and Mexico [2].

In Mexico, guava can be found in the wild in many parts of the country, which is why it is considered the main center of diversity of this species [3]. Michoacán, Aguascalientes and Zacatecas are the main guava-producing states in the country [4]. Commercially, the most important variety is the Media China; however, in Aguascalientes outstanding guava varieties were identified and registered (Calvillo Siglo XXI, Merita, Huejucar, Caxcana and Hidrozac) with high productive potential and fruit quality [3].

1.1.1 Taxonomy

Table 1. Presents the scientific classification of the plant *Psidium guajava* L.

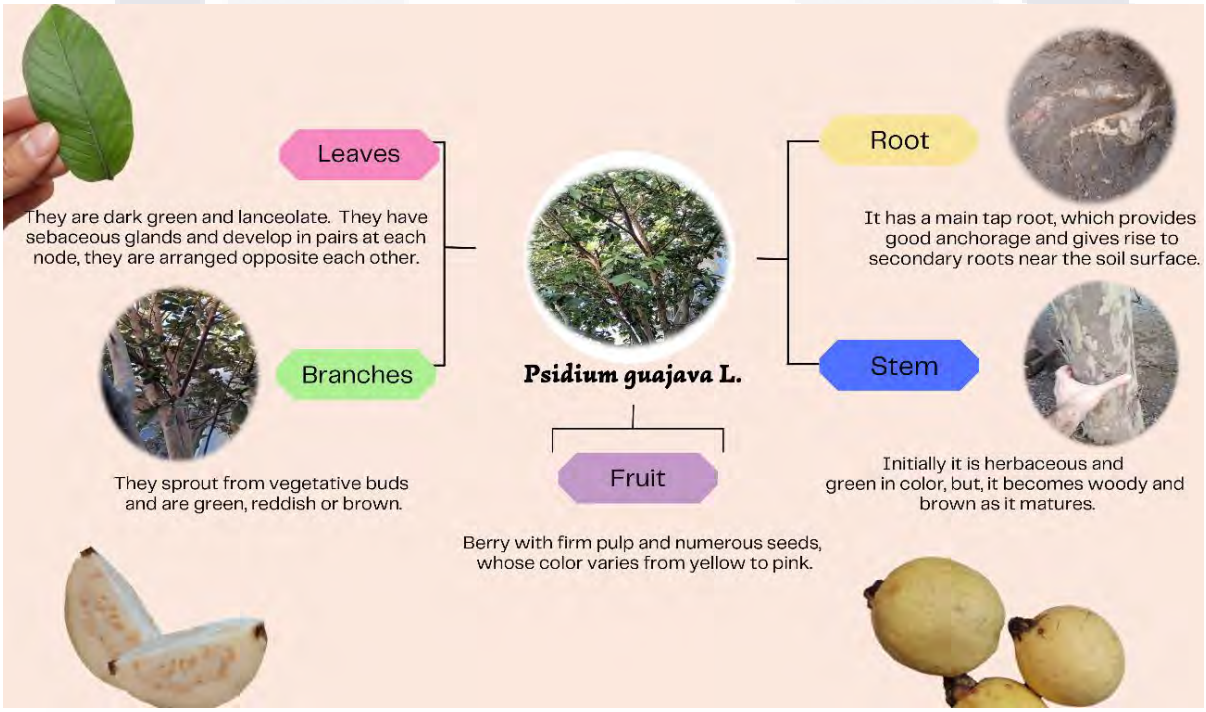
Taxonomy of guava [5].	
Kingdom	Plantae
Division	Spermatophyta
Subdivision	Angiosperms
Class	Dicotyledon
Order	Myrtales
Suborder	Myrtineae
Family	Myrtaceae
Genus	*Psidium*
Species	*guajava* L.

In general, guava belongs to the Myrtaceae family, characterized by having very aromatic fruits and flowers with long and visible stamens [5]; in addition, it is known for having numerous species with high antioxidant activity [6]. It is considered one of the largest families in the world since it has about 144 genera and approx. 3800 species of wide distribution in America [7]. On the other hand, the *Psidium* genus has approximately 150 species of shrubs, of which *Psidium guajava* L. is the best known and most widely distributed worldwide [8].

1.1.2 Morphology

The guava tree can reach up to 7 meters in height and 25 cm in diameter at the trunk when it reaches maturity. Its bark is smooth, thin and copper-brown, while its leaves are green, evergreen, leathery and short-petioled. This plant also has white flowers with 4 to 5 petals and numerous stamens, characteristic of the Myrtaceae family [9]. Its fruit, the guava, is a juicy berry with numerous seeds and a slightly acidic taste. Its shape and color depend on the variety, so we can find guavas that are yellow, or bright pink, and whose shape can be round or ovoid [10]. The morphological characteristics of the guava are illustrated in Figure 1.

Figure 1. Morphological characteristics of *Psidium guajava* L. [9,10]



Source: Own elaboration.

1.1.3 Plant growth

Psidium guajava L. behaves as a semi-deciduous tree, because, after the harvest is finished it experiences a phenomenon of exhaustion or lethargy with the presence of yellowing and fall of most of the leaves, which lasts during the dry period, restarting the growth of new branches and the regrowth of buds with the beginning of the rainy season. The duration in

days of the guava development cycle evaluating 16 phenophases, from the winter bud to the ripening of fruits, is 284 days, likewise, the flowering phase to fruit ripening lasts 129 days [11].

The flowering of guava originates from axillary buds of the new shoots that emerge from mature shoots, six or more months old; the floral buds appear in the second and third basal node of the new shoots, and mature shoots, the flowers appear in the last nodes, when growth has decreased [12].

Guava cultivation requires an optimum annual temperature of around 25±5°C, good light intensity since good solar radiation induces high levels of sugars and ascorbic acid; precipitation of 600 mm/year, but it accepts precipitation of up to 2000 mm/year, and relative humidity of 75 to 80% [11].

1.2 Traditional uses

All parts of the shrub have been used to treat different conditions (Figure 2). For example, the leaves and fruit are used to treat respiratory and digestive problems. Likewise, the seeds of the fruit and leaves have been used as an antispasmodic, anti-inflammatory and even in the control of hypertension and diabetes [13].

Its uses vary depending on the geographic location. It has been reported that in Trinidad, Fiji, China and different parts of Latin America, guava leaves are used to treat diarrhea and stomach pain. On the other hand, in Uruguay, they are used to make vaginal and uterine washes, especially in cases of leucorrhea. In the Cook Islands, guava leaves are used in cases of sores, cuts, sprains and boils; while in India they are used together with the root to treat rheumatism, convulsions and fevers [14].

Thanks to scientific research, new applications of guava have been discovered in diseases of the immune and endocrine systems, as well as in cancer and diabetes. In addition, studies have reported that guava leaf extracts can be used to improve skin problems such as acne and hyperpigmentation [15]. However, the most studied application is the antimicrobial effect that the different extracts of the plant can have, especially due to the serious crisis of

microbial resistance. These revelations have even led to the production of nanoparticles with guava extracts [16].

Figure 2. Traditional uses *of Psidium guajava* L. in the world and new therapeutic applications discovered thanks to scientific research [10].

Source: Own elaboration.

1.2.1 Traditional uses in Mexico

In Mexico, herbal medicine is an ancient practice that has been used to this day and has great cultural and economic importance. However, in most cases the active ingredients that provide the beneficial effect are unknown and, therefore, it is a field of study of high scientific interest [17]. Guava leaves have been used for medicinal purposes in Mexico since ancient times, its presence in historical documents on indigenous herbal medicine has been constant for at least 500 years [18].

This shrub was called by the ancient Mexicans as "*xalxócotl*", a Nahuatl word that refers to a fruit that has a "hard and acidic shell (*xocotl*) and a sandy texture (*xalli*), due to its abundant seeds [18]. The guava can be called in different ways depending on the Mexican state. For example, in Chiapas, this plant is usually called pata, pocscuy, potok, pox or sumbadam; in

Michoacán it is known as enendi, in Nayarit as caaru, in Morelos as coloc or jaljocote pichi and in Veracruz as asiwit, cuympatan or pitchcuy [19].

The uses of *P. guajava* L. in the country are as diverse as its names, however, its most common medicinal use is to treat stomach pain. Diarrhea, dysentery, fever, and cough are treated with infusions of guava and its leaves; in the case of skin problems, the leaves are cooked and applied locally. The infusion of guava buds and leaves are used as a de-wormer and guava tea is used to cure scares, using it to give short baths [19].

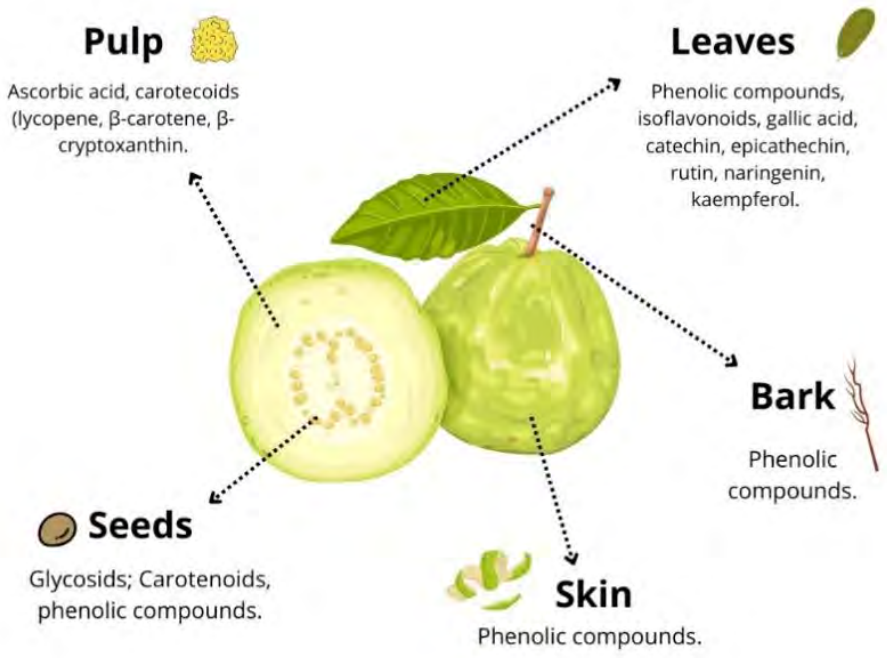
The medicinal use of guava can also vary slightly according to the geographical location in the country, for example, while in southern Veracruz it has been reported that the plant is used to treat diarrhea [20], in the Huasteca Potosina it is also used to treat herpes, wounds, toothache, gastritis and rashes [20]. On the other hand, in Guerrero, the infusion of guava leaves is used to cure cough, fever, flu and stomach pain [21].

1.3. Phytochemical composition

Psidium guajava L. has a very diverse chemical composition that includes compounds such as tannins, phenols, flavonoids, saponins, carbohydrates, alkaloids, sterols and terpenoids [22]. It is important to consider that the type and abundance of phytochemicals may vary depending on the microclimate and soil conditions [23] but also on plant tissue and seasonal changes [24]. Some compounds found in different parts of the plant are shown in Figure 3.

The most analyzed part of this shrub is undoubtedly its leaves, given its frequent use as a medicinal remedy. Shabbir *et al*. [24] reported the approximate composition of guava leaves: 82.47% moisture, 3.64% ash, 0.62% fat, 18.53% protein, 12.74% carbohydrates, 103 mg of ascorbic acid (vitamin C), and 1,717 mg of total phenolic compounds [mg of gallic acid equivalents (GAE)/g]. It should be noted that Shabbir *et al*. [24] also observed a higher concentration of phenolic compounds in the leaves than in the seeds and fruits of guava.

Figure 3. Compounds found in different parts of *P. guajava* L. [13]



Source: Own elaboration.

Guava leaves are also a rich source of vitamins and minerals, such as calcium, potassium, sodium, magnesium, iron, sulfur, vitamin B, and C [25]. Even, Thomas *et al*. [26] mentioned that the leaves have a higher concentration of vitamin B (14.80 mg/100 g), calcium (1,660 mg/100 g), magnesium (440 mg/100 g), phosphorus (360 mg/100 g), and iron (13.50 mg/100 g) compared to the fruits, however, the fruit is richer in vitamin C (228.3 mg/100 g) and potassium (417 mg/100 g).

Guava leaves are well known for their rich composition of phenolic compounds, which play an important role in managing various metabolic and physiological activities in the human body [25]. Díaz-de-Cerio, *et al*. [27] identified more than 70 phenolic compounds in guava leaves by HPLC-DAD-QTOF-MS using hydro-alcoholic extracts obtained by sonication.

1.4 Guava leaves

1.4.1 Agro-industrial residue

Mexico is a key producer of guava in the international market, with an export volume that increased more than 150% in less than 10 years, going from 4,306 tons of guava exported in 2009, to 10,850 tons exported in 2018 [28]. Unfortunately, the Mexican agricultural sector presents serious post-harvest problems, generating enormous losses of fruit during the different stages of the production chain. Guava is one of the most affected fruits with a waste of more than 50% of its national production [29].

Industrial processing of guava generates a heterogeneous mix of peels, seeds and pulp, which can represent up to 30% of the total mass. Guava leaves are other by-product obtained, mainly during the fruit harvest [30]. During guava processing, approximately 80 kg of waste is produced per metric ton of fresh fruit [31]. Unfortunately, and in most cases, the waste generated ends up in landfills or incinerated, increasing the environmental burden and the total cost of production due to the handling and transportation required by this waste [10].

Therefore, guava leaves are a highly available agro-industrial residue whose valorization is an opportunity for Mexico and all guava-producing countries. Its high content of phytochemicals and its widespread use in traditional medicine give a hint of its great potential.

1.4.2 Bioactivity

Table 2 presents some compounds found in different leaf extracts of *Psidium guajava* L. and the properties attributed to them, including antioxidant, antimicrobial, anti-inflammatory and antitumor properties.

Table 2. Bioactivity of different compounds found in *Psidium guajava* L. extracts.

Compound	Classification	Activity	Reference
Quercetin [32]	Phenolic compound	Antioxidant, anti-inflammatory and anti-allergy.	[33]
Gallic acid [32]	Phenolic compound	Antibacterial, anti-fungal, antiviral, anti-inflammatory, antioxidant, anticarcinogenic, anti-diabetic.	[33]
β-Caryophyllene [34]	Sesquiterpene	Local anesthetic, anticarcinogenic, antioxidant, antibiotic, anti-inflammatory, neuroprotective anxiolytic, antidepressant and anti-alcoholism.	[34]
Copaene [34]	Sesquiterpene	Anti-inflammatory and *in- vitro* anti-tumor activity.	[35]
Limonene [34]	Terpene	Anti-inflammatory and *in-vitro* anti-tumor activity.	[35]
Catechin [32]	Phenolic compound	Antioxidant, prevention or reduction of skin damage, activation of collagen synthesis and inhibition of the production of matrix metalloproteinase enzymes. Anti-microbial, anti-viral and anti-inflammatory.	[36]
Ellagic acid [32]	Phenolic compound	Anti-inflammatory, anti-microbial, antioxidant.	[37]
Humulene [38]	Sesquiterperne	Anti-tumor, anti-microbial and anti-inflammatory. Great gastroprotective, cicatrizing, analgesic and antioxidant potentials.	[39]
Nerolidol [40]	Sesquiterperne	Antioxidant, anti-inflammatory and anti-microbial. It also enhances skin penetration and permeation.	[41]
α-Pinene [38]	Terpene	Anticarcinogenic, anti-inflammatory and anti-allergy.	[42]
Eucalyptol [34]	Terpene	Insecticide, anti-fungal, anti-microbial, anti-inflammatory and gastroprotective effect.	[43]

1.4.3. Antimicrobial activity

The mechanism by which plant extracts exhibit antimicrobial activity is still unclear, mainly because phytochemicals have highly variable structures, generating multiple possible modes of action. Furthermore, extracts contain a complex mixture of compounds whose interaction may influence the mechanism [44]. Therefore, the mode of action depends on the type of extract or essential oil and the microorganism used [45].

However, it has been shown that phenolic compounds can interact with bacterial cell walls, causing their rupture and the release of cellular components. Furthermore, it has been reported that Gram-negative bacteria are more resistant to phenolic compounds, probably

due to the presence of an outer membrane and enzymes in the periplasmic space that can damage molecules entering the bacteria [44].

Similarly, the mode of action of terpenes, also an important component of guava extracts, remains largely unknown; however, it has been observed that most terpenoids can inhibit two processes essential for microbial survival: oxygen consumption and oxidative phosphorylation. Thus, the interaction of terpenes leads to an alteration in cellular respiration which then causes the uncoupling of oxidative phosphorylation in the microbe [46].

On the other hand, the proposed mode of action of β-caryophyllene, a bicyclic sesquiterpene considered by multiple authors as the main component of guava leaf extract [34,47,48], is through altering the bacterial membrane permeability and causing non-selective pore formation. This induces intracellular content leakage leading to damage and loss of the membrane integrity and may eventually lead to cell death [49].

Table 3. shows some studies in which different guava leaf extracts exhibit antimicrobial activity against microorganisms of clinical interest.

Table 3. Antimicrobial activity of *Psidium guajava* L. leaves.

Microorganism	Extract	Results	Reference
Streptococcus salivarius	Essential oil	MIC (µg/ml) = 400	[50]
Streptococcus mutans	Essential oil	MIC (µg/ml) = 200	[50]
Streptococcus mitis	Essential oil	MIC (µg/ml) = 200	[50]
Streptococcus sanguinis	Essential oil	MIC (µg/ml) = 400	[50]
Streptococcus sobrinus	Essential oil	MIC (µg/ml) = 100	[50]
Propionibacterium acnes	Essential oil	MIC (µg/ml) = 321	[51]
Staphylococcus epidermidis.	Essential oil	MIC (µg/ml) = 486	[51]
Staphylococcus aureus	Essential oil	MIC (µg/ml) = 6.75	[38]
Escherichia coli	Methanolic extract	MIC (µg/ml) = 40,000±0.5 MBC (µg/ml) = 80,000±0.1	[52]
	Ethyl acetate extract	MIC (µg/ml) = 40,000±0.0 MBC (µg/ml) = 80,000±0.1	
Bacillus cereus	Methanolic extract	MIC (µg/ml) = 40,000±0.1 MBC (µg/ml) = 40,000±0.4	[52]

Microorganism	Extract	Results	Reference
	Ethyl acetate extract	MIC (µg/ml) = 40,000±0.2 MBC (µg/ml) = 80,000±0.3	
Pseudomonas aeruginosa	Methanolic extract	MIC (µg/ml) = 40,000±0.7 MBC (µg/ml) = 80,000±0.0	[52]
	Ethyl acetate extract	MIC (µg/ml) = 40,000±0.1 MBC (µg/ml) = 80,000±0.1	

1.4.4 Antibiofilm activity

Bacteria can live in a planktonic state or form biofilms, architectural elements of the bacterial community embedded in self-produced extracellular polymeric substances that adhere to inert or biological surfaces [53]. The physiological adaptation of bacteria in most natural environments is mainly manifested through biofilm formation and thus they play an important role in the environmental persistence of bacteria [54]. Furthermore, 99% of bacteria in the environment are in biofilms while only 1% are in planktonic form [55].

Biofilm formation can limit or inhibit the activity of antimicrobials by different mechanisms, for example, extracellular polymeric substances are a physical barrier that protects bacteria from environmental aggressions such as antibiotics and ultraviolet light, causing a slow and difficult penetration of antimicrobials into the matrix [56]. It has also been reported that the protein expression profile in biofilms is very different and more diverse than in planktonic cells, which can drive bacterial resistance [57]. Therefore, finding substances that can inhibit biofilm formation and growth is of special interest, especially if they are substances of natural origin, such as phytochemicals, which can be substituted for synthetic drugs without presenting significant side effects [53].

Unfortunately, recent studies on the anti-biofilm activity of *P. guajava* L. are not numerous and focus on the effect on *Staphylococcus aureus*. Therefore, further evaluations of the effect of the extracts on multiple bacteria are necessary to establish a spectrum of their activity. For the moment, the results obtained in recently published articles and the possible mechanism of action proposed by the authors are presented in Table 4.

Table 4. Potential antibiofilm activity of different *Psidium guajava* L. extracts.

Microorganism	Extract	Results	Possible mechanism of action	Reference
Staphylococcus aureus clinical isolates and ATCC 25923	Benzyl isocyanate isolated from the leaves of *Psidium guajava* L.	MBIC (µg/ml) = 440 – 870 MBEC (µg/ml) = 1000 - 2100	Benzyl isocyanate induces the production of poor-quality extracellular polymeric substances and can inhibit major biofilm regulatory molecules of *S. aureus.*	[54]
Staphylococcus aureus	Pulp methanolic extract (agro-industrial waste).	MBEC (µg/ml) = 250	The presence of polyphenols and compounds like L-5-Propylthiomethylhydantoin, that has bacteriostatic activity.	[58]
Staphylococcus aureus	Petroleum ether guava leaves extract.	Dose: 1000 µg/ml Percentage of biofilm inhibition: 25.2 ± 0.53%	Flavonoids prevent the correct transmission of signals, leading to shutdown of *quorum sensing*. In addition, terpenoids alter the fatty acid composition of the cell membrane, which causes the hydrophobicity of cells leads to biofilm eradication.	[59]
		Dose: 2000 µg/ml Percentage of biofilm inhibition: 62.9 ± 0.48%		
	Ethanolic guava leaves extract.	Dose: 1000 µg/ml Percentage of biofilm inhibition: 76.83 ± 0.56%		
		Dose: 2000 µg/ml Percentage of biofilm inhibition: 80.0 %±0.86		

The lack of antibiofilm evaluations with extracts from another part of the plant, since those published focus mainly on the leaves and their derivatives, shows that there is still much to discover on this topic and a large field of study opens for those interested in this area.

1.4.5 Antioxidant activity

Essential oils and plant extracts contain organic compounds with double bonds and hydroxyl groups in their structure, which can donate hydrogen, thereby inhibiting free radicals and minimizing oxidative stress [60]. These compounds are known as antioxidants, as they can oxidize themselves before or instead of other molecules, stopping chain reactions before vital molecules are damaged [61].

Polyphenolic compounds such as gallic acid, ellagic acid, ferulic acid, quercetin, kaempferol and catechin are mainly responsible for the antioxidant activity of guava leaves [62]. Polyphenolic compounds are among the most abundant groups of phytochemicals in nature, and they have very varied structures but are characterized by the presence of aromatic rings and hydroxyl groups [63]. Polyphenolic compounds are recognized as antioxidants for their ability to donate hydrogen atoms and/or electrons to free radicals, breaking the oxidation chain [64]. This ability has become a topic of great importance considering that oxidative stress has been associated with many diseases, such as cancer, hypertension, diabetes mellitus, atherosclerosis and neurological disorders [64]. It should be noted that oxidation is not a problem specific to humans, it also appears in food, for example, the oxidation of lipids and proteins, which is a serious problem as it can destroy essential nutrients, bad odors and even the generation of toxic compounds in food systems [65].

Table 5 presents different studies on the antioxidant activity of guava leaves. The results are very variable, even when comparing studies with the same methods. This may be because the phytochemical content and, therefore, its antioxidant activity can be affected by different factors, including extraction conditions (technique, temperature, solvent, time, etc.), climatic conditions and soil quality [23,66,67]. This is why it is important to characterize and evaluate the activity of the plant material you want to work with.

Table 5. Antioxidant activity of guava leaves.

Extract	Method	Results	Reference
Essential oil	DPPH Folin-Ciocalteu	IC_{50} = 460.37 ± 1.33 µg/ml 495.93 ± 7.88 mg of GAE/g	[68]
Methanolic and aqueous extracts	ABTS Folin-Ciocalteu	IC_{50} = 152 ±12 -78 ±4 µg/ml 10 - 28 µg of GAE/g	[69]
Methanolic extract	ABTS DPPH	IC_{50} = 24.37 ± 3.85 µg/ml IC_{50} = 20.83 ± 3.15 µg/ml	[70]
Methanolic and aqueous extracts	ABTS DPPH	IC_{50} = 5.79 ± 0.87 -8.26 ± 1.06µg/ml IC_{50} = 40.53 ±1.56 - 53.83±1.89 µg/ml	[71]

* DPPH: (2,2-diphenyl-1-picrylhydrazyl); ABTS: (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)).

2. Methodology

2.1. Plant material

The collection of guava leaves was carried out manually and randomly from different specimens free of pesticides in Aguascalientes, Mexico. The plant sample was transferred in a botanical press to the laboratory where only green leaves, without damage by insects or pests, were selected. Subsequently, the leaves were thoroughly washed with distilled water to remove traces of dust and other contaminants and dried at 40°C for 72 hours [24,72]. Finally, the sample was pulverized with an electric processor and the obtained powder was stored at room temperature in an airtight container protected from light [73].

2.2. Extraction of phytochemicals

Three different extraction techniques were tested: Soxhlet, maceration, and ultrasound-assisted extraction (UAE). In all cases, a solid-liquid ratio of 1:20 was used (5 grams of plant sample per 100 ml of solvent). It was decided to use two different solvents: methanol since multiple articles[74–76] and previous work from our laboratory have been reported to have better extractive power; and distilled water, as a green alternative. The continuous extraction by Soxhlet was carried out during 7 siphons [77], while the maceration lasted 8 days and two temperatures were evaluated: 25°C and 37°C [78–80]. The ultrasound-assisted extraction lasted 40 minutes and 2 temperatures were evaluated: 23°C and 30°C [81–84]. The extracts obtained by maceration and UAE were centrifuged (5000 rpm for 17 minutes) and filtered (0.2 µm) to eliminate guava leaf particles [27].

2.3. Solvent elimination

The aqueous extracts were subjected to low temperatures (-48°C) and vacuum for 5 days in a Labconco lyophilizer to remove distilled water [85]. On the other hand, the methanolic extracts were subjected to 50°C in an oven to eliminate the solvent[86]. In all cases, a green to reddish brown powder was obtained, which was stored in an Eppendorf tube at room temperature and protected from light until use.

2.4. Purification of polyphenolic compounds

Phenolic compounds from guava leaves were purified with the commercial adsorbent Amberlite XAD-16. Briefly, the lyophilized aqueous extracts were solubilized in distilled water while the methanolic extracts were solubilized in 80% methanol and the alcohol was removed by rotary evaporator, to obtain the water solubilized extract. Subsequently, 20 ml of the extracts were added to a column packed with amberlite XAD-16 as a stationary phase and distilled water was added to eliminate sugars and other compounds present in the extract, and finally, the polyphenolic compounds were eluted with absolute ethanol. The solvent was removed in an oven at 50°C for 24 hours and the crystals obtained were kept protected from light at room temperature [86,87].

The yield of polyphenols per gram of plant material was determined as follows:

$$\textbf{Yield} = \frac{\text{Milligrams of phenolic compounds obtained}}{\text{Grams of plant material used for extraction}}$$

2.5. Antioxidant capacity tests

The antioxidant capacity of both the crude guava leaf extracts and their purified polyphenolic compounds was analyzed. Three different assays were carried out: Folin-Ciocalteu, ABTS and FRAP.

The reducing power was determined by the Folin-Ciocalteu reagent test in microplate [88]. Briefly, 25 µL of Folin-Ciocalteu reagent and 25 µL of sodium carbonate (75 g/L) were added to 25 µL of properly diluted sample (1:4 v/v) for the reaction to occur under alkaline conditions. The obtained mixture was homogenized and incubated at 40°C for 30 minutes. Subsequently, 200 µL of distilled water were added and the absorbance at 750 nm was recorded. Results were reported as the average of three replicates in gallic acid equivalents expressed in micrograms per milliliter (GAE µg/ml) according to the calibration curve prepared with the same standard.

The $ABTS^{\bullet+}$ radical scavenging capacity assay was carried out in a microplate according to the methodology proposed by Hernández *et al*. [87]. Briefly, a solution of ABTS (7 mM) and one of potassium persulfate (2.45 mM) were mixed (2:1) and allowed to rest for 12 h at room temperature, then it was adjusted with absolute ethanol until reaching an absorbance of (0.7 ± 0.002 nm). Subsequently, 5 µL of each test sample and calibration curve were pipetted in triplicate into the microplate and 95 µL of the adjusted $ABTS^{\bullet+}$ solution was added to them. After 1 minute the absorbance at 734 nm was measured. Results were expressed as percentage inhibition of $ABTS^{\bullet+}$ radicals, according to the Trolox calibration curve or as IC_{50} (sample concentration needed to inhibit 50% of radicals).

The iron-reducing antioxidant power (FRAP) was determined in a microplate according to the methodology reported by Bautista-Hernández *et al.* [89] with slight modifications. Briefly, 5 µl of the samples to be analyzed were mixed with 12 µl of phosphate buffer (pH 7) and 22 µl of 1% potassium ferrocyanide, and then incubated at 50°C for 20 minutes. Next, 12 µl of 10% trichloroacetic acid, 45 µl of distilled water and 10 µl of ferric chloride were added to finally read the absorbance at 700 nm. Results were reported as µg gallic acid equivalent per milliliter (GAE µg/ml).

2.6. Extracts preparation for antimicrobial tests

The methanolic extracts obtained by Soxhlet were placed in an oven at 50 °C to remove the solvent, then resolubilized in distilled water at a concentration of 100 mg/mL (stock solution) [86,88]. Finally, they were filtered with 0.2 µm membranes [27] and stored protected from light at 4°C until use [90]. These extracts are referred to as “crude” throughout the document. On the other hand, 10 mg/ml stock solutions of purified polyphenols were prepared by dissolving the obtained crystals in 5% DMSO and subsequently filtering the solutions with 0.2 µm membranes to finally store them under the conditions mentioned above [86]. These extracts are referred to as “purified” throughout the document.

2.7. Microorganisms and culture media

Five microorganisms from the Cellular and Tissue Biology laboratory were used: clinical isolates of *Escherichia coli* and *Actinobacillus pleuropneumoniae*, *Enterococcus faecalis*

ATCC 29212*, Staphylococcus epidermidis* ATCC 12228 and *Cutibacterium acnes* CDBB-B-1909. *E. coli* was cultured in Luria Bertan (LB) medium (BD Bioxon, Le Pont de Claix, France), while the rest of the bacteria were cultured in brain heart infusion broth (BHI) (BD Bioxon, Le Pont de Claix, France).; in the case of *A. pleuropneumoniae* the medium was supplemented with NAD and for *C. acnes* with defibrillated blood. In addition, four clinical isolates phenotypically characterized as *Staphylococcus aureus*, *Pseudomonas aeuriginosa*, *Enterococcus fecalis* and *Acinetobacter baumannii*, were obtained from the Microbiology Laboratory of the Autonomous University of Aguascalientes and cultured in BHI medium. Analyses with *Salmonella enterica* serovar Typhimurium strain X4232 were performed during the research stay at the University of Saskatchewan, and the microorganism was cultured in LB medium.

Finally, a total of ten clinical isolates previously phenotypically characterized as extremely drug-resistant (XDR) *A. baumannii* isolated from patients with nosocomial infections were used. The microorganisms were donated by the Hospital Centenario Miguel Hidalgo, Aguascalientes, Mexico. The cultures were collected anonymously, and the study protocol was approved by the Ethics Committee of the Hospital Centenario Miguel Hidalgo on January 16, 2023, with the assigned number CEI-CI/008/23. All *A. baumannii* isolates were cultured in a MacConkey medium (BD Bioxon, Le Pont de Claix, France).

Antimicrobial activity assessments were performed using the Mueller-Hinton medium (BD Bioxon, Le Pont de Claix, France).

2.8. Antimicrobial activity of guava leaf extract against XDR *A. baumannii* and potential antimicrobial synergic effects with gentamicin

The antimicrobial activity of the guava leaf extract was analyzed using the agar diffusion technique. Briefly, XDR *A. baumannii* clinical isolates were grown on MacConkey agar for 24 hours at 37°C and subsequently used to standardize to 0.5 McFarland 0.85% NaCl solutions to have a final inoculum of 1.5×10^8 CFU/ml [91]. Mueller-Hinton agar (30 ml) was inoculated by the pour plate technique using 1 ml of the standardized NaCl solutions [92]. Once the agar was solidified, 5 mm diameter wells were made with the help of a sterile pipette tip, and 50 µl of the solution to be tested was added to each well [93,94]. The crude

guava leaf extract was used at 100 mg/ml concentration, and the purified one at 5 mg/ml. The controls were gentamicin (16 µg/ml), sterile distilled water and sterile 5% DMSO aqueous solution. Afterwards, to assess the possible synergistic effect of the extracts with gentamicin, the extracts were combined with 16 µg/ml of the antibiotic. The plates were incubated at 37°C for 24 hours. The clear zones were identified around the wells, corresponding to the antimicrobial activity, and the inhibition halos were measured [91]. The assay was performed in triplicate.

The results are reported as the average of the zone diameter of inhibition (ZDI), the percentage increase of the ZDI, and the growth inhibitory indices (GIIs) which allow us to corroborate the synergistic activity of the combination of the guava leaf extract with the antibiotic.

The GIIs and the percentage of increase were calculated as follows:

$$\textbf{Increase of the ZDI}\ (\%) = \frac{ZDI\ \textit{in}\ \text{combination} - ZDI\ \text{of the extract in single action}}{ZDI\ \text{of the extract in single action}} \times 100$$

$$\textbf{GIIs} = \frac{ZDI\ \text{in combination}}{ZDI\ \text{of the two agents in single action}}\ [95{,}96]$$

The effect was considered synergistic if the value of GIIs > 0.5, additive if GIIs = 0.5, or antagonistic GIIs < 0.5 [95,96].

2.9. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined in 96-well microplates (Costar® 3370, Corning, NY, USA) by performing two-fold serial dilutions of the guava leaf extract, in triplicate. Extract concentrations ranged between 6.25 to 50 mg/ml with crude extracts or between 0.625 to 5 mg/ml with purified extracts. Furthermore, in the evaluation of the activity of the guava leaf extract against the clinical isolates of XDR *A. baumannii* the extract was analyzed alone and

in combination with gentamicin (16 µg/ml). The inoculum was prepared from a 24-hour culture and the final concentration of microorganisms in the wells was 5×10^7 CFU/ml. A growth control and blanks containing medium and extract without inoculum (at each concentration studied) were included for each strain. The microplate was incubated at 37°C for 24 hours and subsequently agar plates were inoculated (in triplicate) to count the CFU. The optical density (595 nm) was measured using a spectrophotometer (Benchmark plus Microplate Reader, BIO-RAD). The MIC was defined as the lowest concentration of the extract necessary to inhibit bacterial growth (where turbidity is not present); while MBC is the lowest concentration of the extract that killed 99% of the bacteria, without showing growth on agar plates [97,98].

2.10. Effect of guava leaf extract on bacterial adhesion

The effect of *Psidium guajava* L. extract on biofilm formation was examined in 96-well microplates (Costar® 3370, Corning, NY, USA). The bacteria *Staphylococcus epidermidis* ATCC 12228*, Enterococcus faecalis* ATCC 29212 and a clinical isolate of *Enterococcus faecalis* obtained from a vaginal exudate (donated by the Microbiology Laboratory of the Autonomous University of Aguascalientes) were selected for this trial because they had previously been characterized as biofilm formers and because they are strains of clinical importance with an increasing number of reports of nosocomial infections. Briefly, overnight cultures (BHI) were diluted 1:100 in BHI + 0.8% glycerol, then 100 µL were aliquoted in triplicate in a sterile 96-well microtiter plate. Subsequently, 100 µl of guava leaf crude extract were added at different concentrations (6.25 to 50 mg/ml). Distilled water was used as a negative control and medium with extract or water (at each concentration studied) was used as blank. The plate was incubated for 24 hours at 37°C and then it was stained with crystal violet [99,100] in the following way: first, the culture medium with the planktonic cells was discarded using a multichannel pipette, then two washes were performed with distilled water, leaving the plate to dry at 37°C for approx. 30 minutes. Finally, 150 µl of crystal violet was added and left to act for 2 minutes before removing it by immersing the plate in a tub of water. The plate was able to dry completely before 150 µl of 30% acetic acid was added. The optical density (OD) was read at 595 nm (Benchmark plus Microplate Reader, BIO-RAD) [101].

The inhibition percentage was estimated as follows:

$$\textbf{Percentage inhibition} = \frac{\mathrm{OD}_{\text{Negative control}} - \mathrm{OD}_{\text{Experimental}}}{\mathrm{OD}_{\text{Negative control}}} \text{ x } 100[99]$$

2.11. Confocal Laser Scanning Microscopy (CLSM)

To further confirm the results of bacterial adhesion on crystal violet assay, the effect of guava leaf extract on the biofilm formation was analyzed by confocal laser scanning microscopy (CLSM). For biofilm formation on slides, the glass was placed in a Petri dish along with BHI nutrient medium + 0.8% glycerol and sub-inhibitory concentrations of guava leaf extract. For *S. epidermidis*, a concentration of 6.25 mg/ml was used. For the two strains of *E. faecalis* 25 mg/ml was selected. The plates were incubated for 24 hours and subsequently stained according to the supplier's instructions. Briefly, the slides were fixed to the burner, exposed to 80% ethanol for 15 min, and exposed to 200 µl of commercial dye FilmTracer FM 1-43 (Invitrogen, Eugene, OR), capable of staining the extracellular matrix. The samples were incubated for 30 minutes protected from light at room temperature and rinsed gently with distilled water to remove excess dye. Finally, the coverslips were mounted with an anti-fade (ProLong Gold, Invitrogen, Eugene, OR) and observed using a confocal laser scanning microscopy (CLSM; LMS 700 ZEISS; Carl Ziess Microscopy, Jena, Germany) and a EC PlnN 40X 1.3 NA Oil DICIII objective. Images were acquired using Zen Black 2012 (black edition) software (ZEISS).

2.12. FTIR

The polyphenolic compounds of the methanolic extracts obtained by Soxhlet, maceration at 25 °C and ultrasound at 30 °C were analyzed using Fourier transform infrared spectroscopy (Agilent Technologies, Santa Clara, CA, USA, FTIR model Cary 630 coupled to a zinc selenide crystal (ZnSe) ATR). The obtained powder after the purification process was deposited on the surface of the reader and secured using the equipped press. The spectra were acquired at a range of 4000–600 cm^{-1} across 32 scans with a resolution of 2 cm^{-1} [102]

2.13. UPLC-MS

The analysis was carried out with an Acquity UPLC system (Waters, Milford, MA, USA) consisting of an auto-sampler, a binary pump equipped with a 10 µL Loop (partial Loop injection mode) and a BEH PHENYL column (2.1 mm × 100 mm, 1.7 µm; WATERS, Waxford, Ireland). The solvents used were (A) water + 0.1% (*v*/*v*) formic acid and (B) acetonitrile + 0.1% (*v*/*v*) formic acid at a constant flow rate of $0.3\ ml \cdot min^{-1}$ The elution gradient (for 113 min) was 100% A, gradually decreasing until reaching 10% A and 90% B, to move from normal conditions (100% A) one minute later to re-equilibrate the column. MS detection was performed on a Q-ToF quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF™, Waters, Milford, MA, USA) equipped with an electrospray ionization source (ESI). The sample acquisition mode was in negative ionic polarity, analysis mode in sensitivity and normal dynamic range, in a mass range of 50 to 1200 Da, sweep conditions of $0.5\ s^{-1}$, a centroid data format, a collision energy of 6 V and a cone voltage of 40 V.

2.14. Cell culture

We worked with five different cell lines during the research stay in the University of Saskatchewan with the Costa Lab: porcine colonic epithelial cells (PoCo-83) kindly obtained from the research of Kaiser, *et al*. [103] porcine intestinal epithelial cells (IPEC-1), African green monkey kidney epithelial cells (Vero-76), kindly obtained from the Misra lab [104], alveolar porcine macrophages 3D4/31 and B cells (Murtaugh001). In all cases, three or more passages were performed to establish the cell culture before seeding them in 96-well plates and performing viability tests. Vero-76 cells were cultured in DMEM/F12 medium (1:1) (Gibco, Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-strep), similarly, IPEC-1 cells were grown in the same medium, but supplemented with 5% FBS, 1% Pen-strep and 1.6% HEPES. On the other hand, PoCo-83 cells were cultured in DMEM high glucose, HEPES modification medium (Sigma-Aldrich) supplemented with 10% FBS, 4 mM L -glutamine, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng /ml sodium selenite, 1% Pen-strep and 50 µg/ml gentamicin. Finally, the macrophages were cultured in RPMI 1640 medium (ATCC modification) (Gibco, Fisher Scientific) supplemented with 5% FBS, 1% Pen-strep and 0.1% gentamicin, and B cells in RPMI 1640 medium (1x)

(Gibco, Fisher Scientific) supplemented with 5% FBS, 1 mM sodium pyruvate, 10 mM HEPES, 50 µg/ml gentamicin and 1% Pen-strep. WST-1 and LDH assays were performed in the respective culture media of each cell line.

In addition, one cell line from the Cellular and Tissue Biology Laboratory of the Autonomous University of Aguascalientes was used: cells isolated from human lung tissue ATCC 549, which were seeded in high glucose DMEM medium with 1% of antibiotics (Pen-strep) and 1% of glutamine.

2.15. WST-1 assay

The WST-1 assay (Sigma-Aldrich) was performed according to the supplier's instructions [105]. Briefly, cells were seeded at a concentration of 5×10^4 cells per well in a 96-well microplate and exposed to different concentrations of guava leaf extract (3-10 mg/ml), using gentamicin (16 µg/ml) as a positive control and cells only with culture media as a growth control. The microplate was incubated for 24 hours (37 °C and 5% CO_2). After 24 h, the medium was replaced with 100 µl of fresh media [106,107], in the case of B cells (suspension cells), the microplate was centrifuged at 1000 rpm for 3 minutes to be able to remove the medium without losing the cells. Then, 10 µl of the WST-1 reagent was added per well and the plate was incubated for four more hours. Finally, the plate was shaken for one minute and the absorbance was measured at a wavelength of 440 nm with an ELISA reader (Varioskan Lux, Thermofisher).

Results are presented as optical density or as % viability:

$$\%\ \textbf{Cell viability} = \frac{\text{Absorbance of treatment} - \text{absorbance of medium}}{\text{Absorbance of control cell} - \text{absorbance of medium}}\ \text{x}\ 100 \text{ [106]}$$

2.16. LDH assay

The CyQUANT™ LDH Cytotoxicity Assay (Invitrogen, Thermo Fisher Scientific) was performed according to the supplier's instructions [108], to corroborate the results obtained in the WST-1 assay. Briefly, cells were seeded at a concentration of 5×10^4 cells per well in a 96-well microplate and exposed to different concentrations of guava leaf extract (3-10

mg/ml), using the same controls as in the WST-1 assay. The microplate was incubated for 24 hours (37 °C and 5% CO_2). After 24 hours, 10 µl of the lysis buffer was added to the triplicate designated as Maximum LDH activity, and the plate was incubated for 45 min, then, 50 µl of the medium of each sample was transferred to a new microplate and 50 µl of the reaction mixture was added to each well, the plate was incubated for 30 more minutes. Finally, 50 µl of the stop solution was added to each sample and the optical density was read at 490 and 680 nm. Results were expressed as the percentage of LDH released, the percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate (Maximum LDH activity) and that in the culture medium [109].

2.17. Effect of guava leaf extract on A549 lung cells

Once the culture was established, the cells were seeded in a 24-well microplate at a concentration of 5×10^4 cells per well and incubated until they reached confluence. Thereafter, the cells were exposed to crude extract (100 mg/ml) and purified extract (5 mg/ml) for 24 hours. Finally, morphological changes and the percentage of viable cells were evaluated using the commercial dye trypan blue and the Amscope MU-500 digital microscope camera. Sterile distilled water and a 5% aqueous DMSO solution were used as negative controls.

The experiment was performed in triplicate, and the percentages of viable cells were calculated as follows [110]:

$$\textbf{Viable cells}\ (\%) = \frac{\text{total number of viable cells}}{\text{total number of cells}} \times 100$$

2.18. Toxicological evaluation in rotifers

In the present project, it was decided to evaluate the acute toxicity of the *Psidium guajava* L. leaf extract on *Lecane papuana* and *Paramecium caudatum* as a first approach to the potential effect that it can have on aquatic ecosystems. To do so, the *rotifer Lecane papupana* was cultured in EPA medium with the addition of *Nannochloropsis oculata* marina adapted to fresh water; and *Paramecium caudatum* was cultured in wheat infusion.

Subsequently, the acute toxicity test was carried out; briefly, 10 neonates were exposed for 24 hours to different concentrations of guava leaf extract (0 - 15 mg/ml for the rotifer and 0-5 mg/ml for the ciliate) and finally, the live and dead organisms were counted. The results are expressed as LC50, the concentration at which 50% mortality is observed compared to the control group; LOEC, the lowest observed effect concentration and NOEC, the highest concentration where no mortality was observed [111].

3. Results

3.1 Purification of polyphenolic compounds

Table 6 shows the different extraction methods used, and the yields of polyphenolic compounds obtained by each of them, which ranged from 16 to 45 $mg \cdot g^{-1}$. The highest values were observed with the extraction methos of Soxhlet using methanol as extracting solvent (44 $mg \cdot g^{-1}$) and ultrasound-assisted extraction at 30°C with distilled water (45 $mg \cdot g^{-1}$). On the other hand, all other extraction methods had a yield around 20 $mg \cdot g^{-1}$. approximately.

Table 6. Extraction yield of purified polyphenolic compounds.
Chemistry and Biochemistry Laboratory,Faculty of Agronomy, Autonomous University of Nuevo León.
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Extraction method				Extraction yield
Technique	Solvent	Temperature (°C)	Time (min)	mg of phenolic compounds/ g of guava leaf
Soxhlet	Methanol	65	5	44
Soxhlet	Distilled water	100	5	24
Maceration	Methanol	37	192	21
Maceration	Methanol	25	192	22
Ultrasound	Methanol	30	0.66	18
Ultrasound	Methanol	23	0.66	19
Ultrasound	Distilled water	30	0.66	45

Source: Own elaboration.

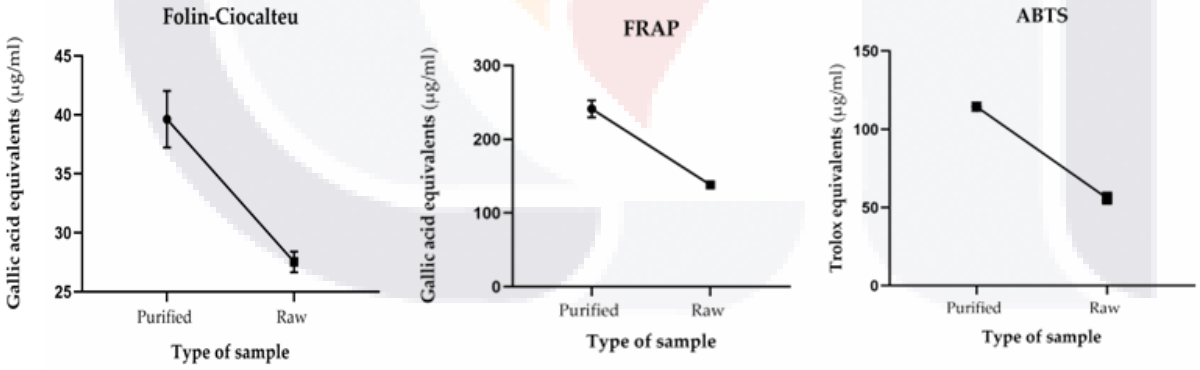
Multiple authors have reported different yields of total polyphenolic compounds in guava leaves. For example, Farag, *et al*. [112]observed a total content of 59.267 ± 0.348 mg GAE (Gallic Acid Equivalent) $\cdot g^{-1}$; Sowmya *et al*. [113] reported a content of 41.33 ± 0.92 mg GAE$\cdot g^{-1}$ and 37.60±0.26 mg GAE$\cdot g^{-1}$ in two guava varieties; and Laily, *et al.* [114] published a content of 101.93 mg GAE$\cdot g^{-1}$. Although these concentrations may be higher than those obtained in this study, it is important to mention that, in the works mentioned, a purification process for polyphenolic compounds was not carried out, so the total content may be overestimated, especially considering that, the Folin-Ciocalteu assay was used for the determination. Even if this assay is well established and widely used, it should be noted that

since it is based on a redox reaction, compounds other than phenols, for example, reducing sugars and ascorbic acid, can also reduce the Folin-Ciocalteu reagent [115]. It is worth mentioning that the yield is not directly related to the phenolic composition of the samples or to their antioxidant activity; therefore, a greater number of polyphenolic compounds does not always mean better antioxidant activity [116].

3.2 Antioxidant activity

The antioxidant activity was evaluated using three different assays: Folin-Ciocalteu and FRAP (Ferric Reducing Antioxidant Power), both to analyze the reducing capacity of the samples, and ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) to analyze the ability to inhibit the radical cation $ABTS^{\bullet+}$. Preliminarily, the three tests (Folin-Ciocalteu, FRAP and $ABTS^{\bullet+}$) were carried out with both, crude and purified solutions of the guava leaf extracts at 0.250 $mg \cdot ml^{-1}$ and it was observed that in all the tests the samples with purified polyphenolics had a higher antioxidant activity in comparison with the raw samples, as shown in Figure 4.

Figure 4. Mean and SEM of crude and purified samples in different antioxidant capacity assays. Chemistry and Biochemistry Laboratory,Faculty of Agronomy, Autonomous University of Nuevo León. January-June 2022



Source: Own elaboration.

This behavior is especially interesting given that Folin-Ciolcalteu and FRAP are not specific tests for polyphenols, since they can be reduced by other agents such as reducing sugars, amino acids, and ascorbic acid [117] which could be present in raw samples [118]. For this reason, it is important to carry out a purification process, since it allows us to eliminate inert

and undesirable components that can interfere with the study and/or that have limited antioxidant activity that represses the activity of polyphenols [119].

The determination of the IC_{50} (the minimum extract concentration at which 50% of the free radicals are inhibited) values was carried out with the ABTS$^{\bullet+}$ assay. The results are within the range from 78 to 152 μg·ml^{-1}, as shown in Table 7. It is worth mentioning that the lowest values of IC_{50} indicate a greater capacity to inhibit free radicals of the samples, which in the present study were exhibited by the methanolic extracts obtained by maceration and ultrasound (87 to 78 μg·ml^{-1}).

Table 7. IC_{50} values according to the extraction method obtained in the ABTS•+ assay.

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Extraction Method	IC_{50} (μg·ml^{-1})
Soxhlet, Methanol, 65°C, 5 hours.	119 ± 6^{B}
Soxhlet, Distilled water, 100°C, 5 hours.	137 ± 12^{AB}
Maceration, Methanol, 37°C, 192 hours.	89 ± 3^{C}
Maceration, Methanol, 25°C, 192 hours.	87 ± 6^{C}
Ultrasound, Methanol, 30°C, 0.66 hours.	78 ± 4^{C}
Ultrasound, Methanol, 23°C, 0.66 hours.	78 ± 4^{C}
Ultrasound, Distilled water, 30°C, 0.66 hours.	123 ± 4^{B}
Ultrasound, Distilled water, 23°C, 0.66 hours.	152 ± 12^{A}

Source: Own elaboration.

Other studies with guava leaves have reported lower IC_{50} values: 24.37 ± 3.85 μg·ml^{-1} [70], 31.19 ± 5.01 to 72.31 ± 3.57 μg·ml^{-1} [120], 3.23 ± 0.24 to 8.26 ± 1.06 μg·ml^{-1} [71]. This may be because the polyphenol content and therefore its antioxidant activity may be affected by different factors, including the extraction conditions (technique, temperature, solvent, time, etc.), climatic conditions and soil quality [23,66,121]. Even so, guava leaf collected in Aguascalientes, Mexico presented relevant antioxidant activity compared to other plant extracts, characterized by having different functional properties such as avocado leaf extracts (IC_{50}=269.56 ± 6.52 to 442.72 ± 9.62 μg·ml^{-1}) [86], rosemary leaf extracts (IC_{50}=70 ± 4.67 μg·ml^{-1}) [122], and oriental ebony leaf extracts (IC_{50}= 108.7 μg·ml^{-1}) [123].

The FRAP and Folin-Ciocalteu assays were performed with the purified guava leaf extracts at their respective concentration of IC_{50} (Table 7). In both tests, it was observed that the extract with the highest reducing capacity was the one obtained by the Soxhlet extraction method using methanol as extracting solvent, while the other extracts exhibited a similar reducing capacity, as can be seen in Figures 5 and 6.

Figure 5. Folin-Ciocalteu reagent reducing capacity according to the extraction method.
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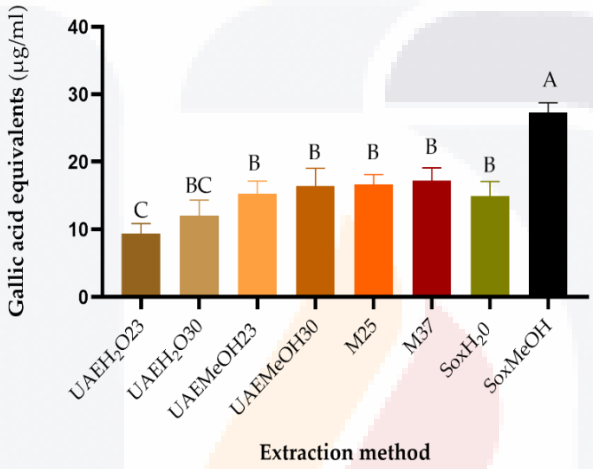
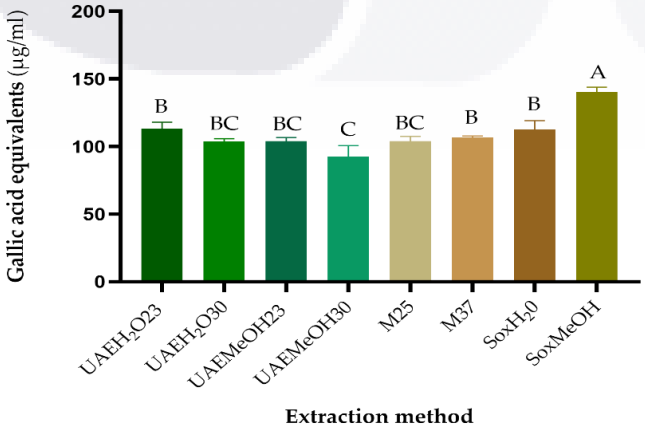


***SoxMeOH: Soxhlet, methanol, 65°C, 5 hours.; SoxH_2O: Soxhlet, distilled water, 100°C, 5 hours.; M37: Maceration, methanol, 37°C, 192 hours.; M25: Maceration, methanol, 25°C, 192 hours.; UAEMeOH30: Ultrasound, methanol, 30°C, 0.66 hours.; UAEMeOH23: Ultrasound, methanol, 23°C, 0.66 hours.; UAEH_2O30: Ultrasound, distilled water, 30°C, 0.66 hours.; UAEH_2O23: Ultrasound, distilled water, 23°C, 0.66 hours. Methods that do not share a letter are significantly different (p<0.05).
Source: Own elaboration.

Figure 6. Iron reducing capacity according to the extraction method.
Chemistry and Biochemistry Laboratory,Faculty of Agronomy, Autonomous University of Nuevo León
January – June 2022



***SoxMeOH: Soxhlet, methanol, 65°C, 5 hours.; SoxH_2O: Soxhlet, distilled water, 100°C, 5 hours.; M37: Maceration, methanol, 37°C, 192 hours; M25: Maceration, methanol, 25°C, 192 hours; UAEMeOH30: Ultrasound, methanol, 30°C, 0.66 hours; UAEMeOH23: Ultrasound, methanol, 23°C, 0.66 hours; UAEH_2O30: Ultrasound, distilled water, 30°C,0.66 hours; UAEH_2O23: Ultrasound, distilled water, 23°C, 0.66 hours. Methods that do not share a letter are significantly different ($p<0.05$).
Source: Own elaboration.

In recent years, the search for natural antioxidants has gained importance given the need to replace fossil-derived resources, as well as to avoid the use of synthetic antioxidants, which can be cytotoxic and carcinogenic [124,125]. The different guava leaf polyphenolic extracts studied exhibited good antioxidant activity, properties useful in a wide variety of applications, such as in the food [126], cosmetic [127,128] and pharmaceutical industry[129], so they can be a low-cost and natural origin alternative. For example, the methanolic extract obtained by Soxhlet, on account of its significant reducing capacity, may be a good candidate for the green synthesis of nanoparticles [130,131], while the methanolic extracts obtained by maceration and ultrasound-assisted extraction may be good candidates as nutraceuticals or cosmetics given their ability to inhibit free radicals [132]. Previous studies on toxicity, stability, bioavailability as well as a solvent removal process are necessary before the extract can have an industrial application.

3.3 Antimicrobial activity of guava leaf extract against XDR *A. baumannii* and potential antimicrobial synergic effects with gentamicin

The antimicrobial activity of the crude extract (GLE) and purified polyphenols (GLEP) are presented in Tables 8 and 9. Guava leaf crude extract exhibited antimicrobial activity against all clinical isolates of *A. baumannii* (Table 8), and a synergistic effect with gentamicin was recorded in all cases, with increases in the inhibition diameters from 2.77% to 40.74%. On the other hand, gentamicin by itself did not affect the growth of the strains. The inhibition diameters observed in combination with gentamicin are between 12 mm and 13 mm, when they normally oscillated between 9 mm and 10.67 mm with the crude extract.

Table 8. Evaluation of the antimicrobial activity of guava leaf crude extract against XDR *A. baumannii* by the agar diffusion technique.

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Isolate ID	Susceptibility to guava leaf extract	ZDI GLE 100 mg/ml	ZDI GLE 100 mg/ml + GEN 16 µg/ml	ZDI GEN 16 µg/ml	ZDI Negative control (distilled water)	Percentage (%) of increase of the ZDI	GIIs	Type of effect
A1	Yes	9 ± 0	12.6 ± 0.577	0	0	40.74	1.4	Synergistic
A2	Yes	9 ± 0	12.6 ± 0.577	0	0	40.74	1.4	Synergistic
A3	Yes	9.33 ± 0.58	13 ± 0	0	0	39.28	1.39	Synergistic
A4	Yes	9.66 ± 0.57	12.66 ± 0.57	0	0	31.03	1.31	Synergistic
A6	Yes	10 ± 0	12 ± 0	0	0	20	1.2	Synergistic
A25	Yes	9.33 ± 0.58	12 ± 0	0	0	28.57	1.28	Synergistic
A26	Yes	10.33 ± 0.57	13 ± 0	0	0	25.8	1.25	Synergistic
A27	Yes	10.33 ± 0.58	12.66 ± 0.58	0	0	22.58	1.22	Synergistic
A34	Yes	10.67 ± 0.58	13 ± 0	0	0	21.87	1.21	Synergistic
A38	Yes	12 ± 0	12.33 ± 0.57	0	0	2.77	1.02	Synergistic
***E. coli* ATCC 25922**	No	0	14.33 ± 0.58	15 ± 0	0	100	0.95	Synergistic

***ZDI= zone of inhibition diameters; GLE= guava leaf crude extract; GEN= gentamicin; GIIs= growth inhibitory indices
Source: Own elaboration.

Similarly, purified polyphenols also had antimicrobial activity against all clinical isolates (Table 9), but surprisingly, at a 20-fold lower concentration (5 mg/ml), the zone of inhibition diameter (ZDI) was equal or greater than those observed with the crude extract, specifically, diameters from 10.66 mm to 11.66 mm using the purified extract alone and from 13.66 mm to 17.33 mm in combination with gentamicin were recorded. Therefore, a synergistic effect was also obtained with increases from 20.58 to 48.57%.

Table 9. Evaluation of the antimicrobial activity of the purified guava leaf extract against XDR A. *baumannii* by the agar diffusion technique.
Cellular and Tissue Biology Laboratory, Autonomous University of Aguascalientes.
January – June 2023

Isolate ID	Susceptibility to guava leaf extract	ZDI GLEP 5 mg/ml	ZDI GLEP 5 mg/ml + GEN 16 µg/ml	ZDI GEN 16 µg/ml	ZDI Negative control (DMSO)	Percentage (%) of increase of the ZDI	GIIs	Type of effect
A1	Yes	11± 2	14.66 ± 0.577	0	0	33.33	1.33	Synergistic
A2	Yes	10.66 ± 1.15	14 ± 0	0	0	31.25	1.31	Synergistic

Isolate ID	Susceptibility to guava leaf extract	ZDI GLEP 5 mg/ml	ZDI GLEP 5 mg/ml + GEN 16 µg/ml	ZDI GEN 16 µg/ml	ZDI Negative control (DMSO)	Percentage (%) of increase of the ZDI	GIIs	Type of effect
A3	Yes	11.33 ± 0.58	15 ± 1	0	0	32.35	1.32	Synergistic
A4	Yes	10.66 ± 0.6	15 ± 0	0	0	40.62	1.4	Synergistic
A6	Yes	11 ± 0	15.33 ± 0.58	0	0	39.39	1.39	Synergistic
A25	Yes	11 ± 1	15 ± 0	0	0	36.36	1.36	Synergistic
A26	Yes	11 ± 0	15.5 ± 0.70	0	0	40.9	1.4	Synergistic
A27	Yes	11 ± 1	14 ± 0	0	0	27.27	1.27	Synergistic
A34	Yes	11.66 ± 0.58	17.33 ± 0.58	0	0	48.57	1.48	Synergistic
A38	Yes	11.33 ± 1.2	13.66 ± 0.57	0	0	20.58	1.2	Synergistic
***E. coli* ATCC 25922**	Yes	12 ± 0	15 ± 0	15 ± 0	0	25	1.25	Synergistic

*** ZDI= zone of inhibition diameters; GLEP= purified polyphenolic compounds from guava leaves; GEN= gentamicin; GIIs= growth inhibitory indices.

Source: Own elaboration.

The polyphenols purification allowed considerably to reduce the concentration of the extract and, at the same time, to improve the antimicrobial activity. In addition, gentamicin did not affect any of the clinical isolates; however, in combination with the plant extract, it had a synergistic effect.

After confirming the antimicrobial activity of the extracts using antibiograms, we determined the minimum inhibitory and minimum bactericidal concentrations. We only used the purified extract because it had better activity. We evaluated it alone and in combination with gentamicin (16 µg/ml). Unfortunately, minimum bactericidal concentrations (MBC) could not be settled in the range of concentrations evaluated (0.6 – 5 mg/ml). However, two minimum inhibitory concentrations (MIC) were determined with purified extract alone, specifically, for the clinical isolates A38 and A26 at a concentration of 5 mg/ml. When combined with gentamicin, MICs for seven of the ten isolates evaluated were determined (Table 10). It should be noted that in all *A. baumannii* strains, a decrease in microbial growth was observed, either with the purified extract in single action or in combination with the antibiotic. In addition, in this assay, it was also observed that the presence of gentamicin improved the antimicrobial activity. This observation underscores the effectiveness of the purified extract.

Table 10. MIC and MBC of purified guava leaf extract against clinical isolates of *Acinetobacter baumannii*.
Cellular and Tissue Biology Laboratory, Autonomous University of Aguascalientes.
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	Without gentamicin		With gentamicin (16 µg/ml)	
Isolate ID	**MIC**	**MBC**	**MIC**	**MBC**
A1	> 5 mg/ml	>5 mg/ml	> 5 mg/ml	> 5 mg/ml
A2			**5 mg/ml**	
A3			> 5 mg/ml	
A4			**5 mg/ml**	
A6			> 5 mg/ml	
A25			**5 mg/ml**	
A26	**5 mg/ml**		**5 mg/ml**	
A27	> 5 mg/ml		**2.5 mg/ml**	
A34			**5 mg/ml**	
A38	**5 mg/ml**		**5 mg/ml**	

*** The MICs are highlighted in bold.

Source: Own elaboration.

Studies on the antimicrobial activity of guava leaves in *A. baumannii* are scarce, among them, Díaz *et al*., [133] evaluated the effect of different concentrations of ethanolic leaf extract of *P. guajava* L. in *A. baumannii* ATCC 19606, obtaining the best result at 125 mg/ml with an inhibition diameter of 16.78 mm, while at 75 mg/ml they registered a diameter of 10.33 mm. On the other hand, Saleh *et al.,* [134] analyzed the effect of five leaf extracts of *Psidium guajava* L. (100 mg/ml) obtained with different solvents in a clinical isolate of *A. baumannii*, the inhibition diameters ranged from 12 to 19 mm and the best result was obtained using methanol as solvent, which was also selected as extraction solvent in this work. The inhibition diameters in our study using guava leaf extract in single action ranged between 9 and 12 mm with 100 mg/ml of crude extract and between 10.66 and 11.66 mm with 5 mg/ml of purified extract. The results are within the range, and in the case of the purified extract, it was possible to have similar ZDI values with a lower concentration than those reported in other studies and our assay using crude extract, highlighting that this may be a possible strategy to improve the antimicrobial activity of plant extracts.

Notably, the antimicrobial activity can be improved through different approaches, among them, the use of delivery systems such as nanoemulsions, encapsulations, micellar and liposomal nanocarriers, and nanoparticles [131,135–137]. For example, Zhang *et al*., [138] nanoencapsulated essential oil from guava leaves in chitosan and reported increased antimicrobial activity against MDR *Klebsiella pneumoniae*. Similarly, Rakmai *et al.,* [139] reported improvements in the activity of guava leaf oil against *Staphylococcus aureus* and *E. coli* after being encapsulated in hydroxypropyl-β-cyclodextrin. Another strategy that has gained importance due to its ease and low cost and which this study has focused, is the synergistic interaction between phytochemicals and antibiotics. Some of the advantages of this one are the possible restoration of an existing drug or the reduction of the dose of new synthetic antibiotics [140].

Regarding the determination of MIC and MBC, with the purified extract as a solitary agent, MIC could only be determined for strains A26 and A38 with a 5 mg/ml concentration in both cases. On the other hand, with the combination with 16 µg/ml of gentamicin, MIC of 5 mg/ml could be detected for isolates A2, A4, A25, A26, A34 and A38. In the case of A27, a MIC of 2.5 mg/ml was recorded, the lowest in our study. However, no MBC could be determined. Other authors have reported different MIC values against clinical isolates of *A. baumannii*. These include values ranging from 116.7 to 8.2 mg/ml for different leaf extracts of *Psidium guajava* L. [134], concentrations of 25 and 50 mg/ml for the methanolic extract of *Hibiscus sabdariffa* L., and minimum bactericidal concentrations (MBC) of 50 and 100 mg/ml [141], MIC of 8.67 ± 1.93 mg/ml and MBC of 9.24 ± 1.95 mg/ml for the essential oil of rhizomes of *Zingiber cassumunar* Roxb against an XDR *A. baumannii* [142]. The variation in these values is due to numerous factors, including the type of plant material used, the climatic and soil conditions, the extraction method (temperature, solvent, time), and differences in methodology [143,144]. The minimum inhibitory concentrations (MICs) established in the present study are lower than those reported by other authors, even when considering other plant species and strains that are not extensively drug-resistant (XDR). Although it did not exhibit bactericidal effects at the concentrations used, it did lead to a significant decrease in microbial growth in all cases, even without gentamicin, denoting its potential as an antimicrobial agent.

3.4 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in different microorganisms of clinical importance.

The antimicrobial effects of crude guava leaf extract were evaluated on ten different microorganisms. It was observed that the presence of extract decreased the growth of the clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Actinobacillus pleuropneumoniae*, *Enterococcus faecalis* and also, *Enterococcus faecalis* ATCC 29212, however, it did not generate total growth inhibition at the concentrations used (6.25 to 50 mg/ml), as can be seen in Figure 7. Therefore, in the future, concentrations higher than 50 mg/ml or strategies to improve activity, such as phenol purification or synergy with other molecules should be used to determine the MIC and MBC for these bacteria strains. On the other hand, *Cutibacterium acnes* CDBB 1909, *Staphylococcus epidermidis* ATCC 12228 and the clinical isolates of *Staphylococcus aureus* and *Acinetobacter baumannii* were sensitive to guava leaf extract, presenting a total inhibition of their growth.

It should be noted that the bacteria that were susceptible to guava leaf extract are mostly gram-positive, except for the clinical isolate of *A. baumannii*. In contrast, the bacteria that only had partial inhibition of their growth are mostly gram-negative, with the exception of the *E. faecalis* strains. This phenomenon has already been observed before and it has been reported that gram-negative bacteria are probably more resistant to phenolic compounds due to the presence of the outer membrane and enzymes in the periplasmic space that can damage the molecules that enter the bacterial cell [10]. The minimum inhibitory and minimum bactericidal concentrations are summarized in Table 11.

Other authors have reported different MIC and MBC values with *P. guajava* L. extract, for example, Festus, *et al*.[52] reported a MIC of 40 mg/ml and an MBC of 80 mg/ml for *Escherichia coli* and *Pseudomonas aeruginosa*. On the other hand, Soliman *et al*. [38] reported a MIC of 6.75 µg/ml for *S. aureus* and Pandey, *et al*. [51] reported a MIC of 321 and 486 µg/ml for *C. acnes* and *S. epidermidis*, respectively. These changes are due, as mentioned in the previous section, to differences in the strain used, extraction conditions as well as methodological differences.

Figure 7.- MIC and MBC determination of **a)** Clinical isolate of *A. pleuropneumoniae* **b)** Clinical isolate of *E. coli* **c)** Clinical isolate of *P. aeruginosa* **d)** Clinical isolate of *E. faecalis* **e)** *E. faecalis* ATCC 29212 **f)** *Cutibacterium acnes* CDBB 1909 **g)** Clinical isolate of *A. baumannii* **h)** Clinical isolate of *S. aureus* **i)** *S. epidermidis* ATCC 12228 **j)** *Salmonella enterica* serovar Typhimurium strain X4232.

Cellular and Tissue Biology Laboratory, Autonomous University of Aguascalientes.
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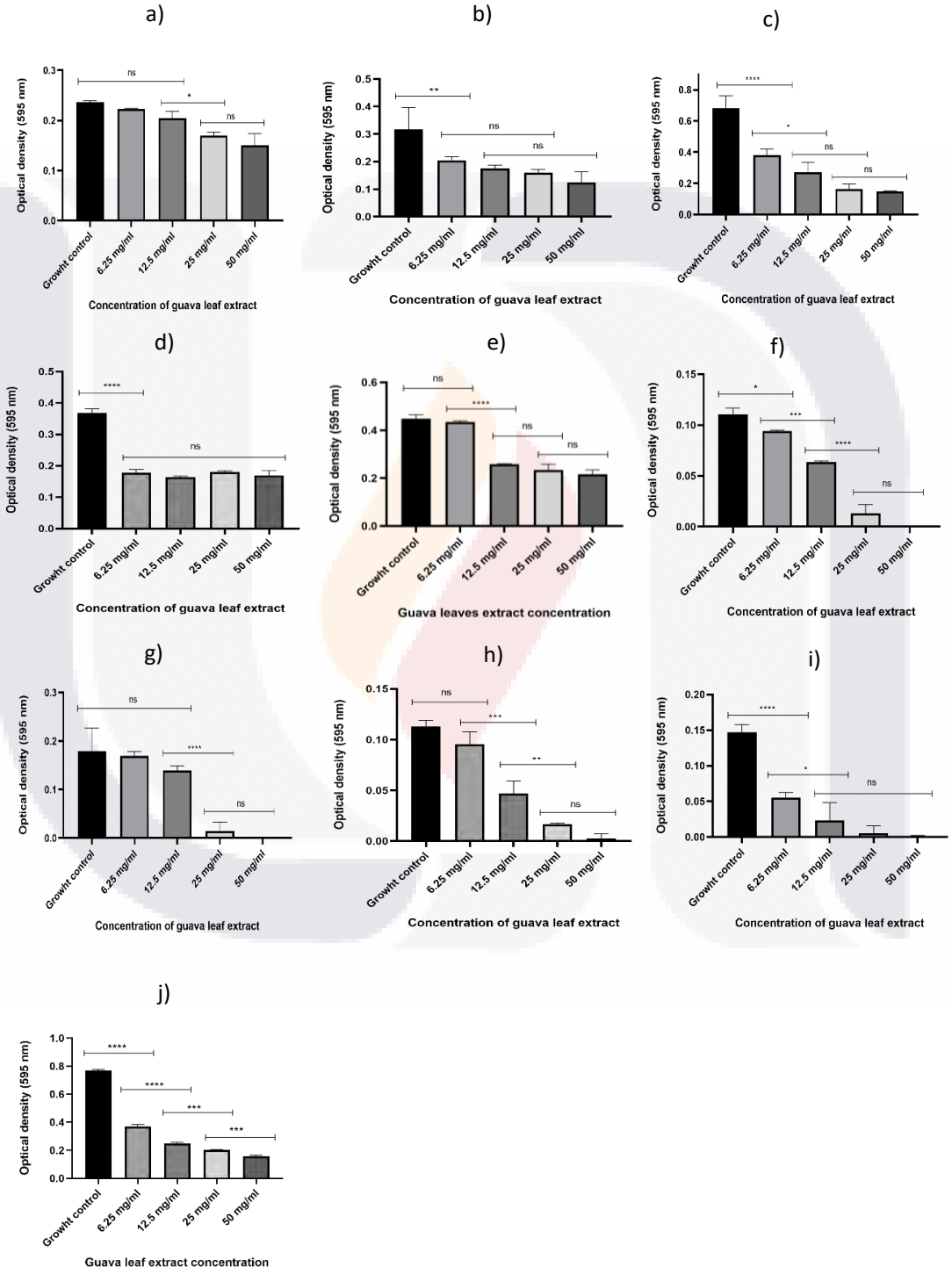

Source: Own elaboration.

Table 11. MIC and MBC of crude guava leaf extract against different pathogenic strains.

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Strain	Minimum Inhibitory Concentration (MIC)	Minimum Bactericidal Concentration (MBC)
***Enterococcus faecalis* ATCC 29212**	> 50 mg/ml	> 50 mg/ml
***Staphylococcus epidermidis* ATCC 12228**	25 mg/ml	50 mg/ml
***Escherichia coli* (clinical isolate)**	> 50 mg/ml	> 50 mg/ml
***Acinetobacter baumannii* (clinical isolate)**	25 mg/ml	50 mg/ml
***Cutibacterium acnes* CDBB 1909**	25 mg/ml	50 mg/ml
***Pseudomonas aeruginosa* (clinical isolate)**	> 50 mg/ml	> 50 mg/ml
***Enterococcus faecalis* (clinical isolate)**	> 50 mg/ml	> 50 mg/ml
***Staphylococcus aureus* (clinical isolate)**	25 mg/ml	50 mg/ml
***Actinobacillus pleuropneumoniae 719* (clinical isolate)**	> 50 mg/ml	> 50 mg/ml
***Salmonella enterica serovar Typhimurium* X4232**	> 50 mg/ml	> 50 mg/ml

Source: Own elaboration.

Although it was not possible to determine a MIC and MBC for the ten microorganisms tested at the concentrations evaluated, it was possible to see that the extract has an impact on the growth of most of them, which can be improved, as we saw previously, through different strategies, such as the purification of polyphenols and synergy with other molecules. In addition, most of the strains evaluated are clinical isolates, which better represent the microorganisms we face every day.

3.5 Effect of guava leaf extract on bacterial adhesion

The ability of bacteria to adhere to biotic or abiotic surfaces allows them to colonize spaces that we are frequently in contact such as railings, catheters and handles, and even allows them to colonize our body tissue, thus facilitating the formation of biofilms and the establishment of an infection [56,145]. Bacterial adhesion has been recognized as the first stage before biofilm development and a key step in pathogenesis. Therefore, adhesion becomes an important step to eliminate pathogens before a biofilm is organized and well-structured [146].

In the present study, the anti-adhesion activity of guava leaf crude extract at different concentrations was analyzed against *E. faecalis* ATCC 29212, *E. faecalis* (clinical isolate) and *S. epidermidis* ATCC 12228 in a microplate. The results are presented in Figure 8.

Figure 8. Percentage of inhibition of bacterial adhesion of two strains of *E. faecalis* and one of *S. epidermidis* at different concentrations of crude guava leaf extract. Methods that do not share a letter are significantly different ($p < 0.05$).
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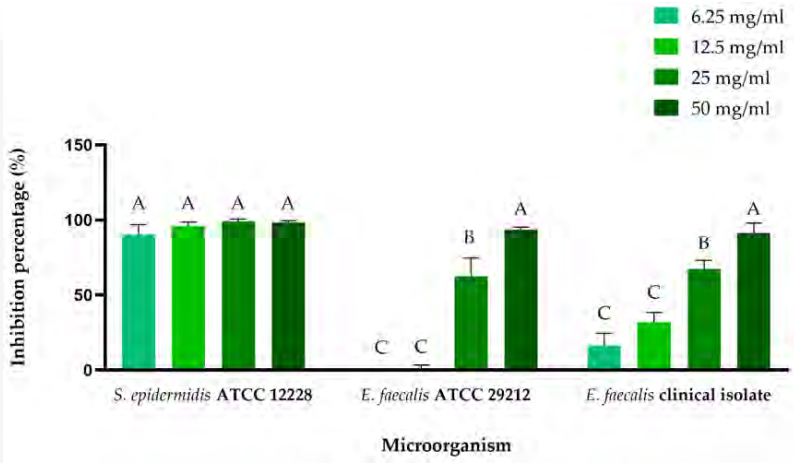

Source: Own elaboration.

As shown in Figure 8, each bacterium behaves differently towards the guava leaf extract. *S. epidermidis* had an almost total inhibition of adhesion in all the concentrations used. It should be noted that in the last two concentrations (25 and 50 mg/ml), we reached the minimum inhibitory and bactericidal concentration, so we can no longer attribute this to an anti-adhesion effect but rather to an antimicrobial one, as bacterial growth is inhibited. At a concentration of 6.25 mg/ml, the lowest used in this study, an inhibition of almost 100% was observed. Therefore, the extract not only affected the growth of the organism but also its adhesion capacity.

In the case of *E. faecalis* ATCC 29212, a phenomenon that was not seen in the other two bacteria occurred, greater biofilm formation was obtained at the two lowest concentrations (6.25 and 12.5 mg/ml) compared to the negative control; this may be due since some microorganisms under stress conditions, such as the presence of phytochemicals, favor the formation of biofilms as a survival mechanism [147,148]. This same phenomenon was reported by Negreiros *et al.* [149]who also observed that the presence of subinhibitory concentrations of *Baccharis psiadioides* essential oil favored the

formation of biofilms of a clinical isolate of *E. faecalis*; and by Suhartono, *et a*l. [150] who reported that low and high concentrations of neem leaf extract induced biofilm formation of a strain of *E. faecalis*. However, in our case, from a guava leaf extract concentration of 25 mg/ml, there is an inhibition of more than 50% of adhesion and at 50 mg/ml an inhibition of almost 100%. Finally, the vaginal clinical isolate of *E. faecalis* has a gradual and concentration-dependent inhibition of adhesion. Although the growth of both strains of *E. faecalis* was not completely inhibited by the guava leaf extract, their adhesion and, therefore, their capacity for biofilm formation was strongly affected, with an inhibition of almost 100% using a concentration of 50 mg/ml of extract.

Although multiple articles report the antimicrobial activity of *Psidium guajava* L. extracts against *E.* faecalis [151–153], and *S. epidermidis* [154,155], to our knowledge, there are still no reports of anti-adhesion or antibiofilm activity against these microorganisms. Moreover, relevant inhibition percentages were obtained when compared with those obtained by other plant extracts, for example, Negreiros *et al*. [149] reported a percentage of biofilm formation inhibition of 17.1% and 75.8% for *E. faecalis* ATCC 29212 and *S. epidermidis* ATCC 35984, respectively, using the MIC concentration of *Baccharis psiadioides* essential oil. Suhartono, *et al*. [150] reported a value of 36.85% for a strain of *E. faecalis* using a concentration of 12.5% of neem leaf extract; Linda, *et al*. [156] reported an inhibition percentage of 55.78 ± 3.68 % using a concentration of 80% of the *Moringaoleifera* extract; Martínez-Chamás, *et al*. [157] reported a value of 66.56 ± 0.43 for a *S. epidermidis* isolate with a treatment of 1/8× MIC of *Fabiana densa* extract; and Di- Lodovico, *et al*. [158]obtained an inhibition percentage of 86.96% for *S. epidermidis* ATCC 12228 at the minimum inhibitory concentration of hop extract.

3.6 Confocal Laser Scanning Microscopy (CLSM)

To corroborate that there is an inhibition of adhesion and therefore, a prevention of biofilm development, the formation of biofilms on slides was evaluated in the presence of sub-inhibitory concentrations of guava leaf extract using the commercial Film-tracer dye that allows biofilms to be observed in the confocal microscope by staining the extracellular matrix (Figures 9-11).

As shown in Figure 9, the presence of guava leaf extract (6.25 mg/ml) affected the formation of biofilms of *S. epidermidis* ATCC 12228 since multiple bacterial aggregates were observed (Figure 9B) and areas where the adhesion and formation of biofilms decreased considerably compared to the growth control (Figure 9A), reducing the thickness of the biofilm from 16 µm to 5 µm (Figure 9C). A similar effect was observed in the two strains of *E. faecalis.* In the presence of guava leaf extract (25 mg/ml), aggregates formed (Figures 10B and 11B), which had a thickness greater than that of the biofilms formed in the growth control, as is the case of *E. faecalis* ATCC 29212 (Figure 10B), where an aggregate thickness of 14 µm is reported. In contrast, the thickness of the biofilm formed in the growth control was 9 µm. However, there were also numerous areas where biofilm formation was reduced, with biofilm thickness decreasing from 9 µm to 4.5 µm for the ATCC strain and from 16 µm to 4 µm for the clinical isolate.

It should be noted that, in the three microorganisms studied, the same phenomenon was observed: the formation of aggregates of bacterial cells in the presence of guava leaf extract compared to the growth control (Figures 9B, 10B, and 11B). This has already been reported by other authors, including Wolinsky *et al*. [159], who observed that incubation of oral *streptococci* with neem stick extract resulted in microscopically observable bacterial aggregation and reducing the ability of some of them to colonize the surface of teeth. Lee, *et al*. [160], reported that the presence of soy extract increased bacterial agglutination, decreasing the adhesion of bacteria, including *Streptococcus mutans*, to an orthodontic wire; and Wang *et al*. [161], who observed that oolong and pu-erh tea extracts influenced streptococcal autoaggregation, resulting in a reduction of adhesion.

Although auto-aggregation is an important and advantageous physical interaction for biofilm development, when excessively large aggregates and cell groups form, they are particularly susceptible to physical or chemical detachment that can lead to their subsequent removal [161,162]. This may explain and corroborate what was observed in the microplate, since the aggregates that could have formed were perhaps easily eliminated in the washes along with the planktonic cells; and in the slide test case, the aggregates were not eliminated since the first step was heat fixation. However, it has also been reported that bacteria autoaggregation is a protective measure against external stress, so it may represent a risk, since it could protect the bacteria, for example, from the host's immune system or antibiotics [163].

Therefore, more studies are needed to evaluate the viability and biofilm formation capacity of these aggregates and the risk they could represent. Although we do not know if the flocculating effect of the guava leaf extract could be a problem later if the aggregates are left on the surface in question, its presence can be considered a good time to remove the microorganisms given their reduced adhesion to the surface [164]. Furthermore, it would be interesting to analyze the possible synergy with other antimicrobials and its potential as a disinfectant.

Figure 9. Biofilm formation of *S. epidermidis* ATCC 12228 on slides after 24 hours of incubation at 37°C. In the slide that was subjected to the presence of crude guava leaf extract (6.25 mg/ml), bacterial aggregates were observed (B) and areas where the adhesion and formation of bacterial biofilms decreased considerably (C) compared to the growth control (A).

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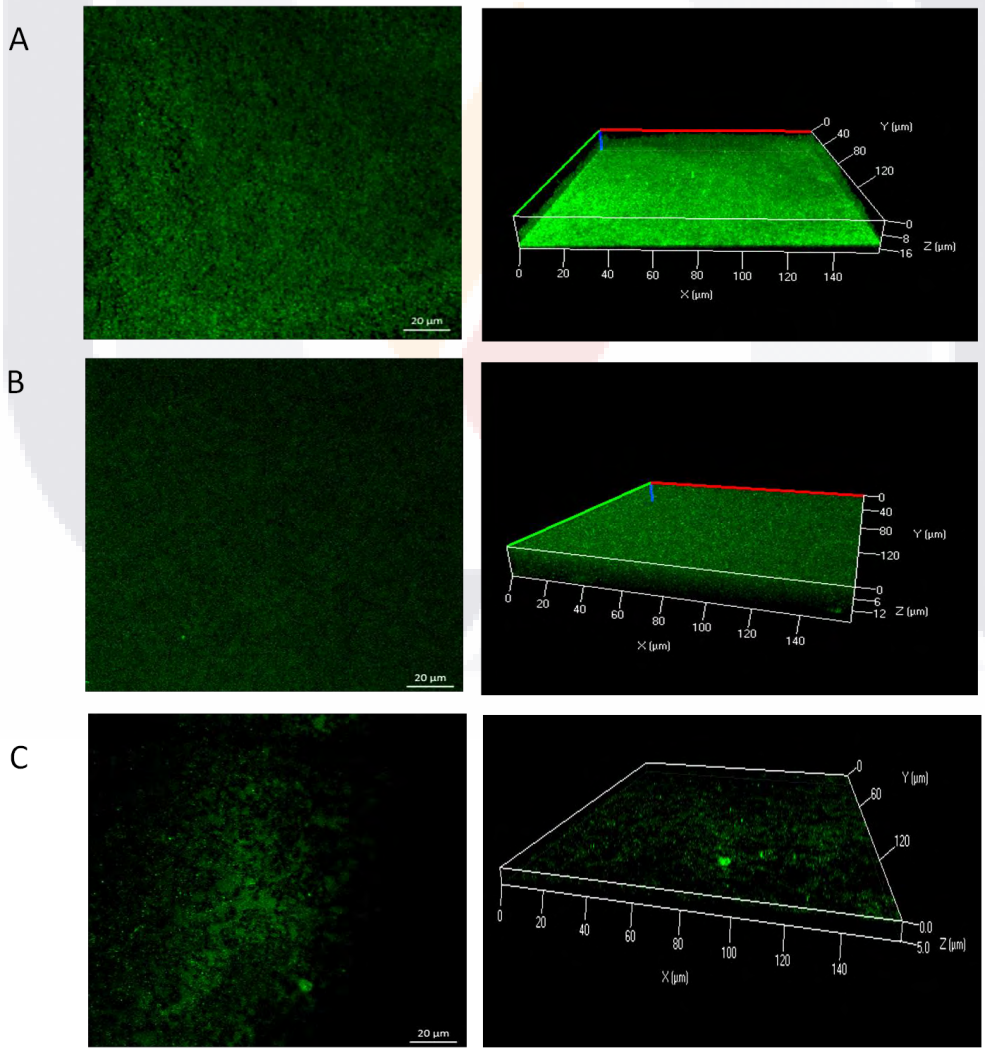

Source: Own elaboration.

Figure 10. Biofilm formation of *E. faecalis* ATCC 29212 on slides after 24 hours of incubation at 37°C. In the slide that was subjected to the presence of crude guava leaf extract (25 mg/ml), areas where the adhesion and formation of bacterial biofilms decreased considerably (C) were observed. Moreover, bacterial aggregates (B) had an even greater thickness compared to biofilms formed in the growth control (A) were also observed.

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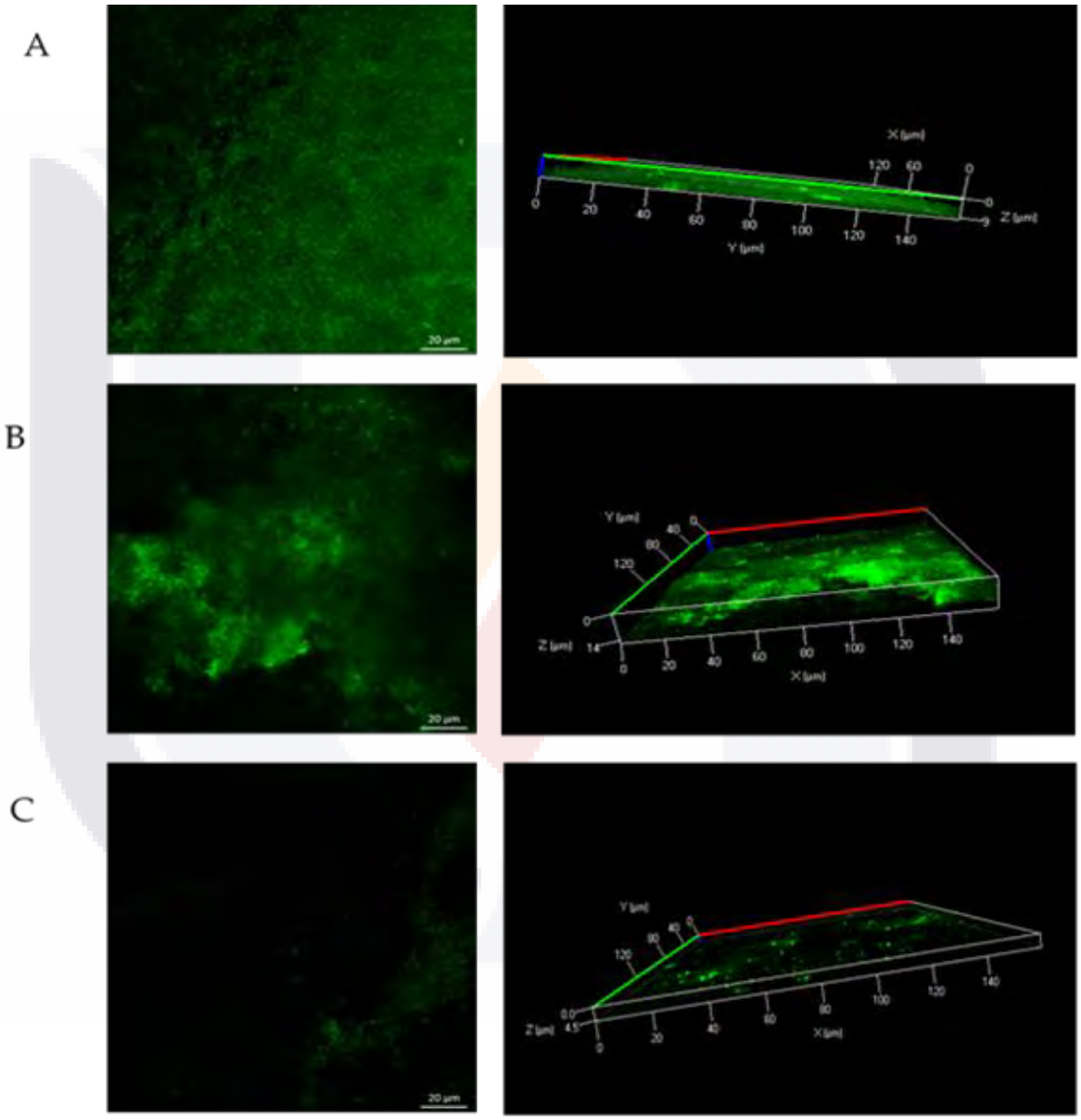

Source: Own elaboration.

Figure 11. Biofilm formation of a clinical isolate of *E. faecalis* on slides after 24 hours of incubation at 37°C. In the slide that was subjected to the presence of crude guava leaf extract (25 mg/ml), bacterial aggregates (B) and areas of little bacterial presence with slight biofilm formation (C) were observed compared to the growth control (A).

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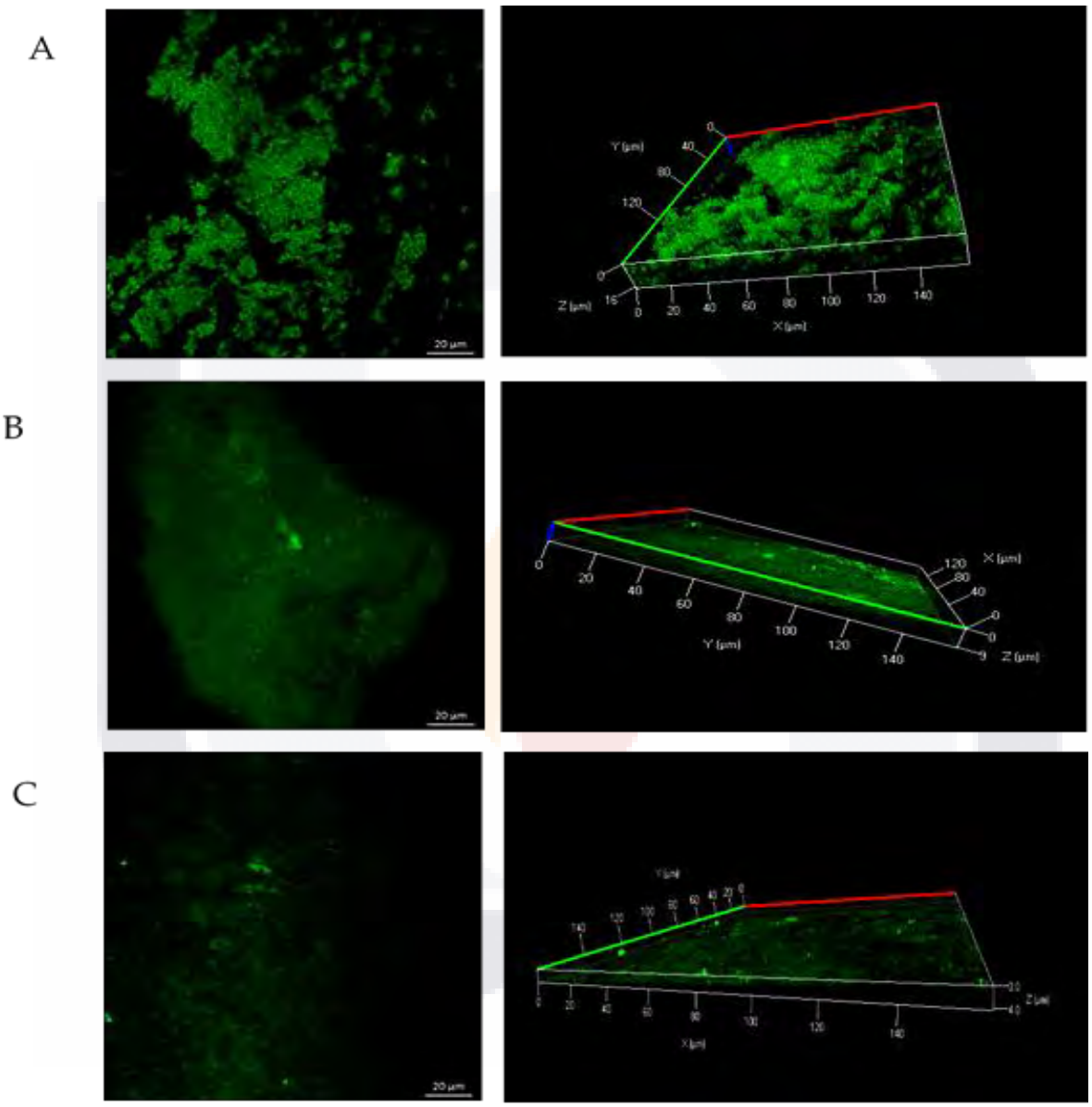
January – June 2023



Source: Own elaboration.

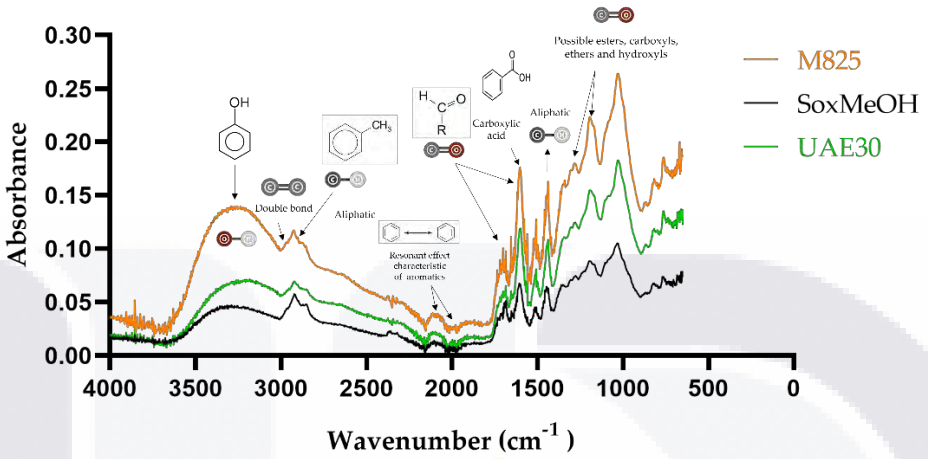
3.7 FTIR

The dried purified methanolic extracts obtained by three different methods (Soxhlet, maceration at 25°C and ultrasound-assisted extraction at 30°C) were analyzed by Fourier transform infrared spectroscopy (FTIR). It should be noted that, as the samples analyzed are purified extracts, the peaks of the spectra correspond to the different functional groups present in the polyphenolic compounds.

In Figure 12, from 3000 to 3600 cm^{-1} the broad and strong band corresponds to the stretching vibrations of the O–H bond, which indicates the presence of functional groups such as hydroxyls. In addition, the presence of O–H groups is confirmed since there is vibration between 1600 and 1300 cm^{-1}, 1200 and 1000 $cm^{-1}$and 800 and 600 cm^{-1}. The presence of peaks from 3000 to 2900 cm^{-1}corresponds to the stretching of the C–H bond, characteristic of aliphatic functional groups. On the other hand, from 2200 to 1950 cm^{-1}we can observe two small vibrations caused by the resonance effect of aromatic compounds. The presence of the carbonyl functional group is confirmed since peaks are observed from 1850 to 1650 cm^{-1} corresponding to the stretching of the C–O bond. The intense and well-defined peak between 1650 and 1600 cm^{-1} indicates the presence of carboxylic acids, while the two peaks around 1615 and 1495 cm^{-1} indicate the presence of double bounds for the vibration of the C–C bond, confirming the presence of aromatic compounds. Finally, from 1500 to 600 cm^{-1}corresponds to the fingerprint area, which is specific and unique; the bands present from 1000 to 1300 $cm^{-1}$are due to vibrations of the C–O bonds found in esters, carboxyls, ethers, and hydroxyls.

Other studies on the phytochemicals from guava leaf extracts show similar spectra. Lok *et al.* [165] analyzed guava leaf extracts obtained with three different solvents: distilled water, ethanol and n-hexane; and they also observed the stretching bands of the C–H and O-H bonds; on the other hand, Nagpal, *et al*. [166] and Lahlou, *et al*. [167] also reported the vibration of the functional groups C=O, O–H, C–O and C–H.

Figure 12. FTIR analysis of the methanolic extracts of guava leaves.
Chemistry and Biochemistry Laboratory, Faculty of Agronomy, Autonomous University of Nuevo Leon.
August – December 2022



** M825: maceration at 25°C, SoxMeOH: Soxhlet at 65°C, UAE30: ultrasound-assisted extraction at 30°C.
Source: Own elaboration.

3.8 UPLC-MS

The purified methanolic extract obtained by Soxhlet was selected for analysis by UPLC-MS given its outstanding reducing and antimicrobial activity. The tentative identification of the phytochemicals is presented in Table 12.

In the extract, 13 compounds were identified, among them, quercetin, catechin, kaempferol, avicularin, guavinoside B and C, all of them with different beneficial activities for the human health; *e.g.* anti-inflammatory, antimicrobial and antitumor activity [168–176]. Therefore, guava leaves are a rich source of polyphenolic compounds and a potential nutraceutical. On the other hand, there were also compounds that could not be identified, giving rise to new investigations for the identification, characterization, and *in-vitro* evaluation of the activity of these phytochemicals.

Table 12. Tentative identification of phenolic compounds in *Psidium guajava* L. leaf extracts, with the Extraction Method: Soxhlet with methanol.
Chemistry and Biochemistry Laboratory, Faculty of Agronomy, Autonomous University of Nuevo Leon.
August – December 2022

#	Tentative Identity	Tr (min)	m/z exp	m/z calculated	Molecular Formula	Fragments	Ref.
1	Not identified	0.749	249.0305	248.034588	$C_{19}H_5O$	113.0318, 181.0322, 207.0358	
2	Vescalagin	7.71	933.2043	933.07178	$C_{41}H_{26}O_{26}$	466.1265, 179.0356, 289.1466	[27]
3	Catechin	9.587	289.147	289.079587	$C_{15}H_{14}O_6$	179.0359, 207.0354, 287.1305, 245.1503	[27]
4	Not identified	18.88	603.1823	602.181312	$C_{40}H_{27}O_6$	179.0356, 207.0351, 235.9931, 257.02	
5	Casuarinin/ Casuarictin Isomer	19.637	935.2216	935.08743	$C_{41}H_{28}O_{26}$	467.1334, 145.9832, 385.2003, 478.1260	[27]
6	Not identified	33.071	381.0783	380.076847	$C_{24}H_{13}O_5$	379.0626, 301.1106, 299.0956	
7	Not identified	33.475	381.0775	380.076847	$C_{24}H_{13}O_5$	299.0951, 301.110, 302.1134, 379.0617, 271.098	
8	Quercetin glucuronide	34.788	477.1651	477.075289	$C_{21}H_{18}O_{13}$	463.1837, 299.0954, 301.1106	[27]
9	Reynoutrin	37.414	433.1703	433.08546	$C_{20}H_{18}O_{11}$	431.1533, 181.0318, 235.9926, 300.1021, 415.2863	[27]
10	Guajaverin	40.242	433.1713	433.08546	$C_{20}H_{18}O_{11}$	431.1543, 300.1037, 301.1096, 391.9761	[27]
11	Avicularin	40.798	433.1707	433.08546	$C_{20}H_{18}O_{11}$	431.1534, 300.1033, 302.1121	[27]
12	Myrciaphenone B	48.222	481.1956	481.106589	$C_{21}H_{22}O_{13}$	479.1792, 417.1714, 365.9648, 257.0243, 235.9927, 239.9696, 207.0348, 181.0316,179.0352	[27]
13	Guavinoside C	57.06	585.1982	585.096419	$C_{27}H_{22}O_{15}$	583.1833, 304.9899, 285.9811, 235.9928, 257.0249, 352.9331	[27]
14	Not identified	63.473	551.2103	550.207527	$C_{34}H_{31}O_7$	541.1789, 343.1274, 328.1021,	
15	Guavinoside B	64.887	571.2532	571.15354	$C_{28}H_{28}O_{13}$	569.2360, 481.2711, 257.0239	[27]
16	Not identified	78.22	711.5146	710.401989	$C_{30}H_{63}O_{18}$	701.4839, 549.4456, 503.4370	
17	Not identified	82.967	695.5202	694.51974	$C_{51}H_{67}O$	685.4873, 533.4503, 487.4390	
18	Luteolin 7-O-malonyl-glucoside	86.2	533.4523	533.101504	$C_{24}H_{22}O_{14}$	487.4415, 488.445, 523.4203, 501.4174	[177]
19	Kaempferol 3-O-(6"-malonyl-glucoside)	86.907	533.4519	533.101504	$C_{24}H_{22}O_{14}$	487.4413, 488.4441, 523.4199	[177]
20	Chrysoeriol 7-O-(6"-malonyl-glucoside)	87.816	547.4318	547.117154	$C_{25}H_{24}O_{14}$	501.4212, 502.4242, 427.0584, 533,4493	[177]

Source: Own elaboration.

3.9 WST-1 and LDH assays

Nowadays, there are a wide variety of commercial assays to evaluate whether compounds of interest have effects on cell proliferation or show cytotoxic effects [178]; one of the most frequently used, and therefore, one of the most important, is MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) whose principle is that the metabolic activity of viable cells is constant, so an increase or decrease in the number of viable cells is linearly related to mitochondrial activity, specifically, this assay is based on the reduction of tetrazolium salts by mitochondrial dehydrogenases to form formazan violet crystals, which can be measured spectrophotometrically [179]. Unfortunately, it has been reported that in the case of the evaluation of plant extracts, the MTT can give false positives since the reagent can be reduced by compounds with antioxidant activity, even in the absence of living cells [180]. The WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay has been proposed as a more reliable and accurate alternative for the evaluation of the metabolic activity of cell lines in the presence of natural products; like the MTT assay, the WST-1 assay is based on the reduction of tetrazolium salts, but they are negatively charged, so they cannot enter the cell and are reduced extracellularly through electron transport, which gives the advantage that cell solubilization with organic solvents does not need to be carried out, making the test simpler, easier and faster [181,182]. In the present study we evaluated the effect of the presence of crude guava leaf extract on different cell lines (PoCo-83, IPEC-1, B-cells, Vero-76 and Macrophages) using the WST-1 assay, the results are presented in Figure and Table 13.

Table 13. Cell viability (%) of different cell lines in the presence of crude guava leaf extract using the WST-1 assay.
Costa Laboratory, University of Saskatchewan.
August – December 2023

Guava leaf extract concentrations	Cell lines				
	IPEC-1	***PoCo-83***	***Vero-76***	***B-cells***	***Macrophages***
Growth control	100 ± 0 ^{A}	100 ± 0 ^{A}	100 ± 0 ^{A}	100 ± 0 ^{F}	100 ± 0 ^{CD}
Gentamicin (32 ug/ml)	110 ± 11.44 ^{A}	98.24 ± 8.08 ^{A}	89.37 ± 4.11 ^{ABC}	107.58 ± 3.36 ^{F}	94.38 ± 4.79 ^{D}
3 mg/ml	107.62 ± 11.89 ^{A}	77.18 ± 15.71 ^{B}	90.94 ± 4.68 ^{ABC}	165.47 ± 13.16 ^{E}	111.43 ± 6.91 ^{BCD}
4 mg/ml	79.51 ± 5.05 ^{B}	71.36 ± 8.90 ^{B}	86.30 ± 3.71 ^{BC}	206.95 ± 13.26 ^{DE}	113.39 ± 5.90 ^{ABCD}
5 mg/ml	61.39 ± 4.18 ^{C}	51.14 ± 3.56 ^{C}	84.23 ± 4.82 ^{BC}	226.26 ± 18.69 ^{D}	116.90 ± 6.90 ^{ABC}

Guava leaf extract concentrations	Cell lines				
	IPEC-1	*PoCo-83*	*Vero-76*	*B-cells*	*Macrophages*
6 mg/ml	37.77 ± 5.28 ^{D}	34.72 ± 5.76 ^{D}	95.75 ± 4.80 ^{AB}	281.92 ± 26.29 ^{C}	113.63 ± 10.88 ^{ABCD}
8 mg/ml	16.77 ± 4.01 ^{E}	25.84 ± 4.48 ^{D}	80.92 ± 5.09 ^{C}	400.21 ± 26.29 ^{B}	127.58 ± 19.97 ^{AB}
10 mg/ml	5.91 ± 2.08 ^{E}	19.76 ± 2.47 ^{D}	51.55 ± 9.43 ^{D}	488.59 ± 47.87 ^{A}	132.39 ± 6.16 ^{A}

Source: Own elaboration.

Figure 13. Optical density according to the crude guava leaf extract concentration obtained with the WST1 assay in different cell lines. **A**) IPEC-1 cells, **B**) PoCo-83 cells, **C**) Macrophages 3D4/31, **D**) Vero-76 cells, **E**) B-cells.
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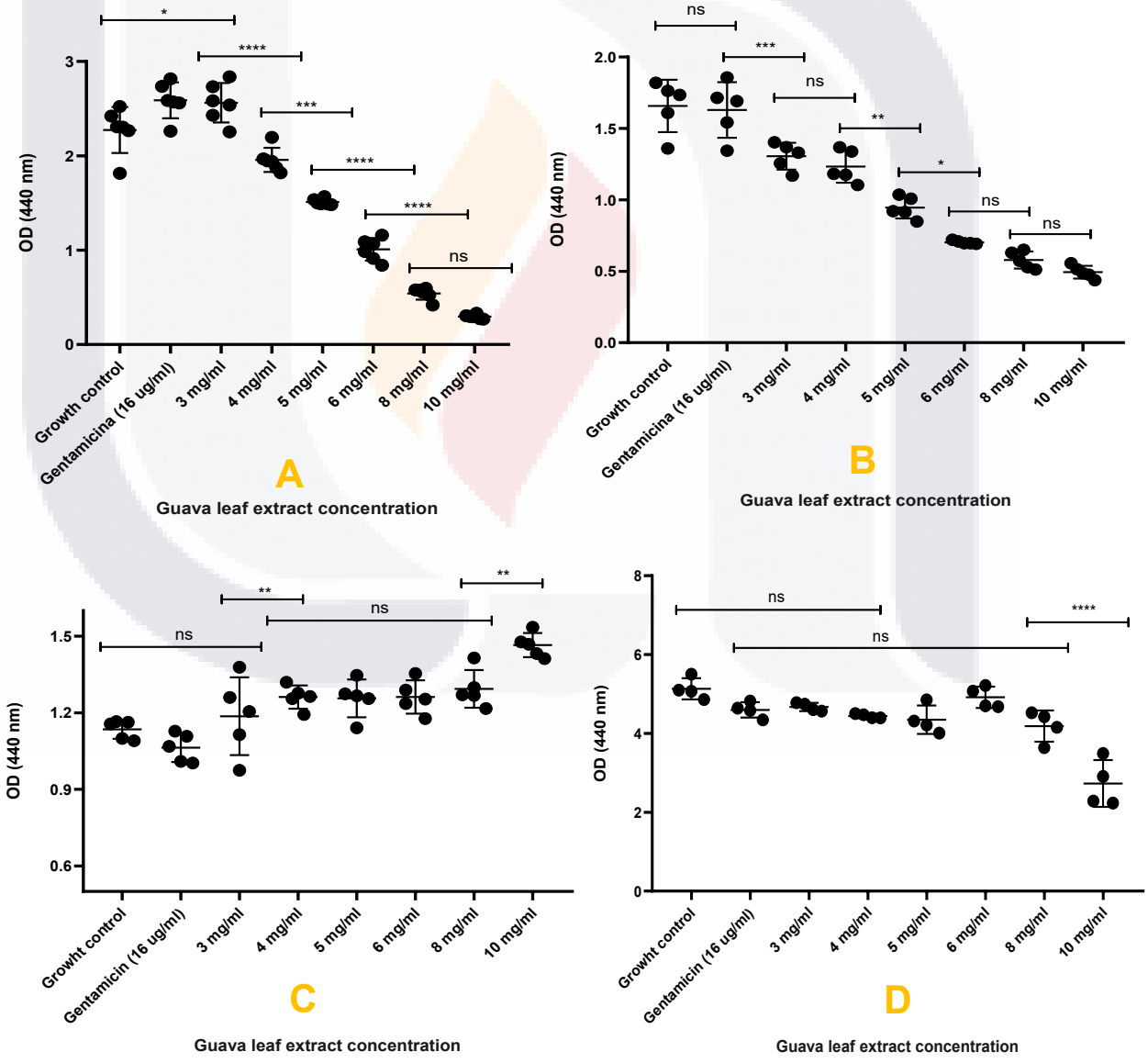

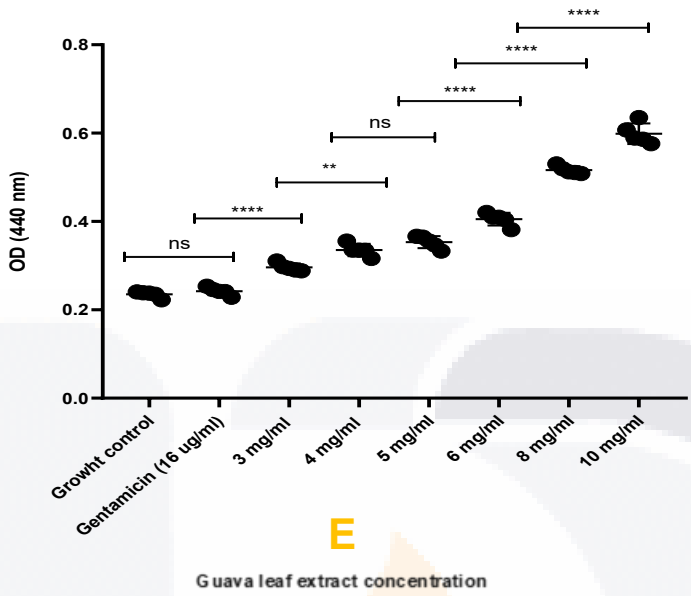

Source: Own elaboration.

All cell lines evaluated behaved differently against guava leaf extract; on one hand, pig intestine (IPEC-1) and colon (PoCo-83) epithelial cells decreased their optical density (Figure 13 A and B) and viability (Table 13) according to the concentration of plant extract. IPEC-1 cells were the most affected, obtaining a viability of 5.91± 2.08% in the concentration of 10 mg/ml of *P. guajava* L. crude leaf extract, while the PoCo-83 cells exhibited a viability of 19.76± 2.47% at the same concentration. On the other hand, there were no significant differences in the viability and optical density of African green monkey kidney epithelial cells (Vero-76) in the presence of different concentrations of guava leaf extract (3-6 mg/ml), only for the highest concentrations (8 and 10 mg/ml) where viability decreased to 80.92 ± 5.09 and 51.55 ± 9.43% respectively, while in the lower concentrations it ranged between 84.23 ± 4.82 and 95.75 ± 4.80%. Finally, a tendency for leukocytes to increase their metabolic activity, and, therefore, the optical density and viability according to the concentration of plant extract is reported, with B cells being the ones in which a more drastic increase was seen, while in the macrophages the increase was gradual. It should be noted that this increase may be since the metabolic activity of immune cells can be altered in response to activation induced by antigens (in this case the plant extract), as well as by their cell cycle, given that immune cells increase in size after activation [183]. Therefore, viability values higher than 100% do not always mean that the viability of the cells has improved or

increased, and supplementary tests must be carried out to verify that the extract does not have cytotoxic effects, however, we can see that the plant extract activates these cells of the immune system. It has been reported that polyphenols, abundant compounds in guava leaf extract [69], can act as immunostimulants, strengthening the immune system and preventing infectious diseases, moreover, it has been observed that the aqueous leaf extract of *P. guajava* L. has immunostimulant activity in human lymphocytes [184]. Nevertheless, more studies are necessary to corroborate this information and rule out possible undesired effects, such as an excessive inflammatory response.

Different authors have used the WST-1 assay to evaluate cell viability in the presence of guava leaf extract, Chen *et al*. [185] reported cytotoxic effects of the acetonic and ethanolic extract in clone 9 liver cells (epithelial cell isolated from the liver of a rat) at concentrations higher than 100 µg/mL; Han, *et al*. [186] observed that ethyl acetate extract had no cytotoxic effects on RBL-2H3 cells (basophilic leukemia cell line) at concentrations ranging between 0.1 and 50 µg/ml, similarly, Han, *et al*. [187] reported that the ethyl acetate extract had no negative effects on HaCaT cells (immortalized human keratinocytes) using the same concentrations. Likewise, several studies using the MTT assay are available, Levy, *et al.* [188] reported dose-dependent cytotoxic effects in Kasumi-1 cancer cells at concentrations of 100-500 ug/ml; Jayakumari, *et al.* [189] observed that the cell viability of Vero cells was greater than 80% when exposing them to different concentrations (3.125 - 400 µg/ml) of the rich fraction in tannins from the guava leaf extract, agreeing with the results obtained in the present study with Vero 76 cells, since the viability was greater than 80% even in much higher concentrations of guava leaf extract (3-8 mg/ml). On the other hand, Sang Bong, *et al*. [190] reported a 30-70% reduction in the viability of HT-29 cells (human colon cancer cells) dependent on the dose (50 -250 µg/ml).

Another procedure to evaluate potential cytotoxicity is the LDH assay, a simple, reliable and widely used method; it is based on the measurement of the activity of the cytoplasmic enzyme lactate dehydrogenase in the extracellular medium. If there is damage to the cell membrane, the LDH enzyme will be found in the cell culture supernatants and will be measured by the commercial kits[191]. The LDH assay was performed to corroborate the results obtained with the WST-1 kit, and the information obtained is presented in Figure and Table 14.

Figure 14. Percentages of LDH enzyme release in the supernatant of different cell cultures in the presence of crude guava leaf extract. **A**) IPEC-1 cells, B) PoCo-83 cells, **C**) Macrophages 3D4/31, **D**) Vero-76 cells, **E**) B-cells.
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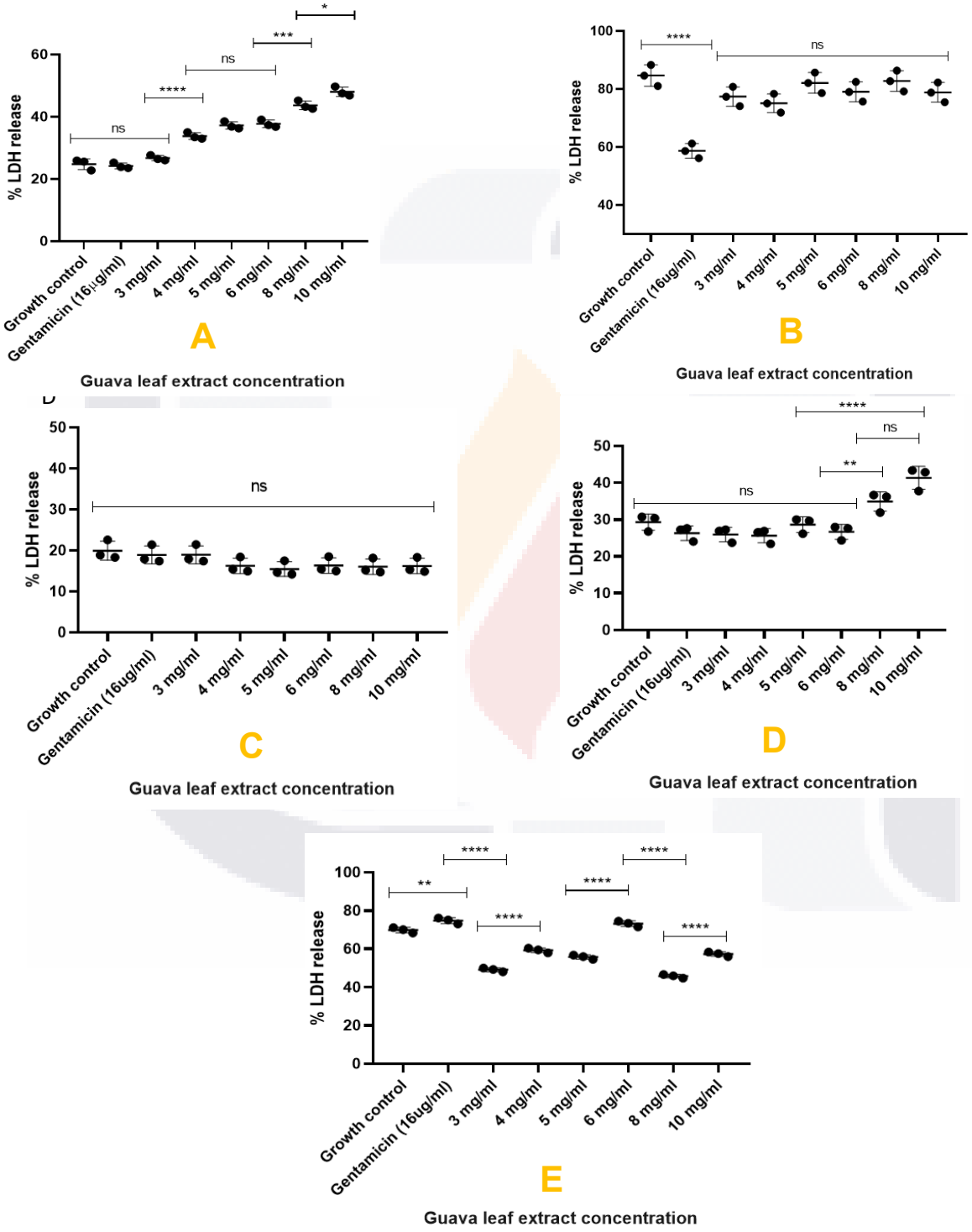

Source: Own elaboration.

Table 14. %LDH release by different cell lines in presence of different concentrations of guava leaf extract.
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	Vero-76	PoCo-83	IPEC-1	Macrcophages	B-cells
Growth control	29.31 ± 2.20	84-64 ± 3.65	24.76 ± 1.72	19.91 ± 2.31	69.85 ± 1.44
Gentamicine	26.31 ± 1.97	58.64 ± 2.53	24.19 ± 0.93	18.90 ± 2.19	74.78 ± 1.54
3 mg/ml	25.97 ± 1.95	77.39 ± 3.34	26.71 ± 0.84	18.93 ± 2.19	49.12 ± 1.01
4 mg/ml	25.62 ± 1.92	75.08 ± 3.24	33.80 ± 1.07	16.24 ± 1.88	59.29 ± 1.22
5mg/ml	28.63 ± 2.15	82.10 ± 3.54	37.21 ± 1.18	15.44 ± 1.79	55.8 ± 1.15
6 mg/ml	26.66 ± 2.00	79.02 ± 3.41	37.74 ± 1.19	16.30 ± 1.89	73.16 ± 1.51
8 mg/ml	34.93 ± 2.62	82.74 ± 3.57	43.68 ± 1.38	16.02 ± 1.85	45.78 ± 0.94
10 mg/ml	41.34 ± 3.10	78.83 ± 3.40	48.02 ± 1.52	16.18 ± 1.87	57.31 ± 1.18

Source: Own elaboration.

As we can see in Figure 14, a higher release of the LDH enzyme in Vero-76 cells in comparation with the presented in the growth control was only reported at the two highest concentrations, which is consistent with what was observed in the WST-1 assay, where the metabolic activity decreased in the presence of a concentration of 8 and 10 mg/ml of extract. In the case of colon cells (PoCo-83), a different behavior was observed, since the percentage of LDH enzyme release reported in all concentrations of guava leaf extract (75.08 ± 3.24 – 82.10 ± 3.54) was very close to that of the growth control (84.64 ± 3.65) (Figure 14B). It should be noted that the percentage of LDH release in the control is very high, more than 80%. This may be due to the number of cell passages used (>34), since this specific cell line is not immortal, but is called "pre-crisis cultures with a long shelf life". On the other hand, in the intestinal cells (IPEC-1) a greater release of LDH enzyme is observed depending on the concentration of plant extract, agreeing with what was observed in the WST-1 test where metabolic activity decreases as a function of concentration, reaffirming that the intestinal cells were very sensitive to guava leaf extract. In the case of macrophages, no significant differences were observed in LDH release at any of the concentrations compared to the control, strengthening the theory that guava leaf extract stimulated the metabolic activity of these immune cells. Finally, LDH enzyme release in B cells in the presence of guava leaf extract was lower compared to the growth control and the positive control with gentamicin, so apparently, guava leaf extract did not affect either the metabolic activity or the membrane stability of the lymphocytes evaluated.

By using different cell lines, we have a more complete and comprehensive view of the *in-vitro* cytotoxicity data, which is highly beneficial for the subsequent design and planning of *in-vivo* experiments, saving time, resources, and animals [192]. The epithelial cells of the colon and intestine are a physical, biochemical, and dynamic barrier that limits the passage of nutrients, compounds, and microorganisms to the internal compartments of the body, maintaining homeostasis, therefore, they play an important role in our health and whose dysfunction has been related to different pathologies [193–195]. On the other hand, kidney cells from the African green monkey (Vero cell lines) are recommended to study chemical toxicity *in-vitro* and are even considered a model to evaluate general cytotoxicity in mammalian cells [196,197]. Finally, the immune system is an important and vulnerable target for xenobiotics, therefore, the evaluation of immunotoxic and immunomodulatory effects is an important component of toxicity studies [198]. Cell culture is one of the techniques to evaluate immunotoxicity *in-vitro*, where the most used cell lines are macrophages, natural killer cells and B lymphocytes. There are different approaches for the detection of immunotoxicity induced by xenobiotics: cell viability and apoptosis, indicators of oxidative stress, and dysfunctions in cytokine secretion or phagocytosis [199], in this study we evaluate cell viability through the WST-1 and the LDH assay and we report that the presence of crude guava leaf extract in concentrations ranging between 3-10 mg/ml generate changes in the metabolic activity of 5 different cell lines compared to the growth control.

It is worth highlighting that the effective concentrations for the crude extract to exhibit antimicrobial activity are higher than those at which it has been recorded that it generates effects on cell viability and metabolic activity. For example, in our research, MICs and MBCs ranging between 25 and 50 mg/ml for microorganisms such as *Staphylococcus epidermidis* ATCC 12228, *Cutibacterium acnes* CDBB 1909 and a clinical isolate of *Acinetobacter baumannii* were determined; however, at concentrations lower than 10 mg/ml, effects are already recorded on several cell lines *in-vitro*. Therefore, it is necessary to explore different strategies to reduce its potential cytotoxicity.

3.10 Effect of guava leaf extract on A549 lung cells.

Additionally, the morphological changes and the percentage of viable lung cancer cells (ATCC A549) were evaluated after exposure to guava leaf extract. Specifically, A549 cells were exposed to crude and purified guava leaf extract at 100 mg/ml and 5 mg/ml, respectively. After 24 hours, morphological changes were observed, and the percentage of viable cells was determined with the commercial dye trypan blue. The results are presented in Figure and Table 15.

The presence of crude and purified guava leaf extract caused morphological changes in lung cells (Figure 15). In both cases, the cells lost their epithelial morphology and became rounded shape, which is related to cell death [200]. Moreover, when cells were exposed to purified polyphenols, a significant number of exosomes, vesicles involved in intracellular communication and numerous physiological and pathological conditions, including exposure to acute stressors, were observed [201]. On the other hand, the percentage of viable cells decreased to 38% with crude extract and more drastically to 11.3% with purified extract, even though the latter was used at a lower concentration (Table 15).

Table 15. Percentage of viable A549 cells after 24 hours of exposure to *Psidium guajava* L. leaf extracts.
Cellular and Tissue Biology Laboratory, Autonomous University of Aguascalientes.
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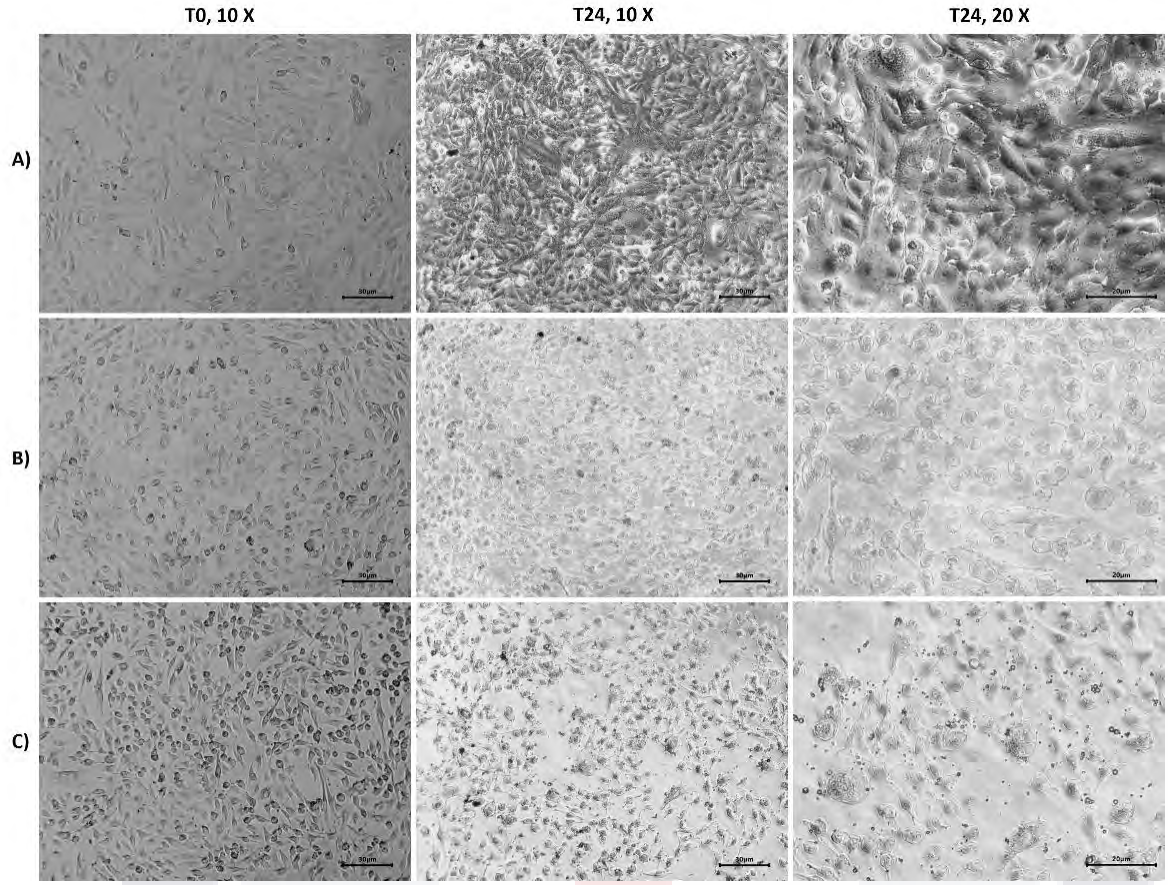
Experimental group	Viable cells (%)
Growth control	90.0
Crude extract, 100 mg/ml	38.0
Negative control of the crude extract	90.9
Purified extract, 5 mg/ml	11.3
Negative control of the purified extract	87.8

Source: Own elaboration.

Figure 15. Morphological changes registered in A549 cells after 24 hours of exposure to guava leaf extracts. **A**) Growth control; **B**) crude extract at 100 mg/ml, and **C**) purified extract at 5 mg/ml.
Cellular and Tissue Biology Laboratory, Autonomous University of Aguascalientes.
January – June 2024



*** T0 = time zero; T24= after 24 hours of exposure.
Source: Own elaboration.

Therefore, the purification of polyphenolic compounds from guava leaf extract improved the antimicrobial activity but also increased the cytotoxicity of the extract. This is not surprising, given that while dietary polyphenols are considered safe and have been attributed to beneficial effects, there is evidence that they can also have deleterious effects at certain concentrations, especially among vulnerable populations [202]. Furthermore, this study observed a loss of epithelial morphology and the appearance of typical features of apoptosis such as cell shrinkage, rounded shape and the presence of apoptotic vacuoles. Similar findings were reported by other researchers including Hsu [203], who exposed A549 cells to *Typhonium blumei* extract, and Cheng [204], who exposed them to different extracts of *Bupleurum scorzonerifolium*. Consequently, it is fundamental for further research to study the potential toxicity of guava leaf extract.

Natural extracts are generally considered safe due to their natural origin. Nonetheless, they can also exert cytotoxic effects, especially if they are used indiscriminately [205]. Although there are multiple new studies evaluating the safety of guava leaf extract in different *in-vitro* and *in-vivo* models [206–210], the results are variable, and more information is needed to ensure its safe use.

3.11 Toxicological evaluation in rotifers.

Interest in plant-based products such as extracts, tinctures and essential oils has increased considerably in recent years, mainly due to the growing recognition of their bioactivity. Therefore, their use is currently widespread in a variety of industries that produce and use large quantities of these substances, and, consequently, they have the potential to reach the environment [211]. Unfortunately, the potential effects that these products may have on ecosystems are largely unknown, so it is important to evaluate the toxic effects of plant products and their derivatives towards non-target organisms [211]

Rotifers are cosmopolitan, aquatic or semi-aquatic microscopic invertebrates that have been used as models in the detection of chemical substances and environmental samples. To do so, they must be abundant, native and/or representative of the ecosystem and ecological importance [212]. The *Lecane* genus is one of the largest representatives among freshwater rotifers in Mexican territory, occupying up to 45% of rotifer species, having a presence in the central and southern states of Mexico, and it also has a wide distribution and can be found from the United States to Brazil [213]. On the other hand, protozoa are often used as bioindicators of chemical contamination, especially in aqueous environments, and, among protozoa, *Paramecium caudatum* is one of the most used ciliate models for laboratory research [214].

In our project, it was decided to evaluate the acute toxicity of the crude leaf extract of *Psidium guajava* L. on *Lecane papuana* and *Paramecium caudatum* as a first approach to the potential effect that it may have on aquatic ecosystems. The results are presented in Figure 16, where LC50 is the concentration at which 50% mortality is observed compared to the control group, LOEC is the lowest concentration with observed effect and NOEC is the

highest concentration at which no mortality was observed. Although there are no studies that have used the same model organisms to test the acute toxicity of guava leaf extract, there are studies with other invertebrates, especially with *Artemia salina*, a crustacean widely used in tests to determine the toxicity of natural products [215]. Among them, Bautista, *et al*. [216] determined an LC50 of 0.929 mg/ml for the aqueous extract of guava leaves; Lee, *et al*. [217] published an LC50 value of 1.0009 mg/ml for the essential oil of *P. guajava* L. bark. For our part, an LC50 value equal to 1.27 mg/ml was reported for *Paramecium caudatum*, and 3.38 mg/ml for *Lecane papuana*. It should be noted that *P. caudatum* was more sensitive to the presence of guava leaf phytochemicals since its LC50 value is lower.

Figure 16. Acute toxicity test of *Psidium guajava* L. leaf extract on two invertebrates microorganisms. LC50 is the concentration at which 50% mortality is observed compared to the control group, LOEC is the lowest concentration with observed effect and NOEC is the highest concentration where no mortality was observed.

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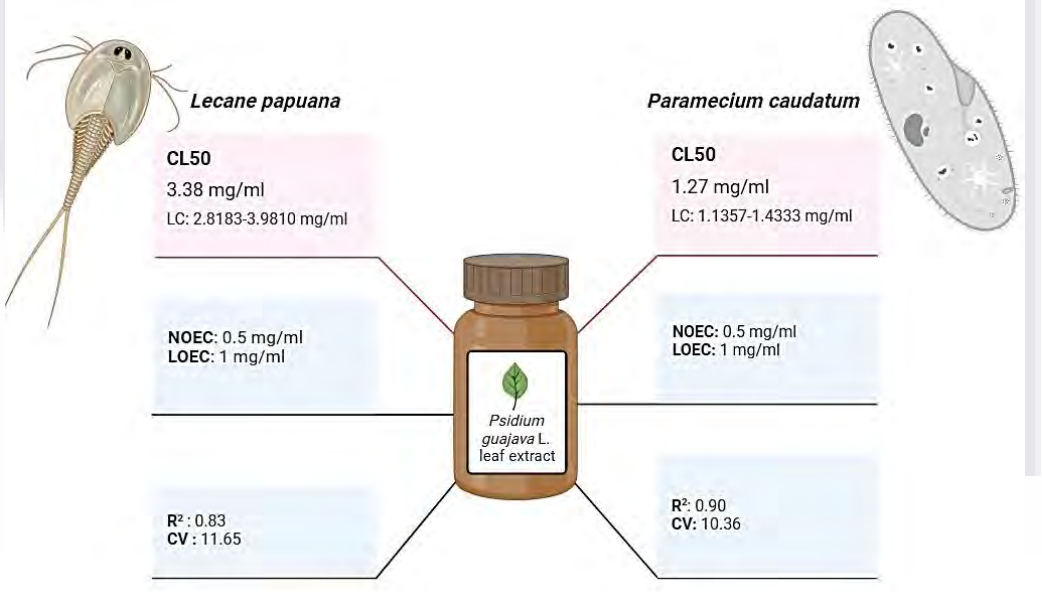

***Figures are illustrative and do not represent the actual morphology of the microorganisms.

Source: Own elaboration.

4. Project Justification

After the COVID-19 pandemic, the current crisis we are facing is microbial resistance. The excessive use of antibiotics in different areas such as medicine, agriculture and aquaculture has caused these compounds to be found increasingly in the environment, exerting selective pressure on microorganisms and accelerating the resistance process [218].

Unfortunately, the WHO [219] reported that recently approved antibiotics and products in clinical development are not enough to address the problem of the increasing emergence of multidrug-resistant strains. Therefore, there is an urgent need for new and better antimicrobials, where compounds of natural origin have gained importance in recent times due to their great structural diversity, cost-effectiveness, environmental friendliness and lower risk of development of resistance by microorganisms [220].

The leaves of *Psidium guajava* L. are an agro-industrial residue of the guava harvest that is highly available and not valued despite its great richness in polyphenolic compounds and its wide use in traditional medicine, not only in Mexico, but throughout the world. In addition, in recent years, different studies have reported its outstanding bioactivities, especially as antioxidants and antimicrobials, however, little is known about its activity on gram-negative bacteria, since it is said that they are less susceptible to phytochemicals due to their double membrane, and its anti-biofilm activity has been very little explored.

Furthermore, it is important to mention that the potential activity of any plant material depends on many factors, such as weather and soil conditions, and the extraction method (solvent, temperature, technique, time) [23,67,69]. Therefore, it is important to characterize the plant or part of the plant with which you wish to work and establish the best method for the extraction of phytochemicals.

In this study, the polyphenolic compounds of guava leaves collected in Aguascalientes, Mexico, one of the main guava-producing states in the country, are characterized. In addition, their antioxidant and antimicrobial activity is evaluated *in-vitro*. This, with the aim of valorising and expanding its medicinal use, as well as contributing to the search for sustainable antimicrobial alternatives.

5. Objectives

5.1 General Objective

To extract, purify and characterize the polyphenols from the leaf extracts of *Psidium guajava* L. obtained by different extraction techniques and to evaluate their antioxidant and antimicrobial activity *in-vitro.*

5.2. Specific objectives

1. Standardize and define the protocols for: collection and preparation of biological material, polyphenol extraction processes and their conservation.
2. Extract polyphenols from guava leaves using three different techniques: Soxhlet, ultrasound-assisted extraction and maceration.
3. Purify the polyphenolic compounds present in the extracts and evaluate their antioxidant properties *in-vitro.*
4. Analyze the phytochemical composition of the polyphenolic extracts by UPLC-MS and FTIR.
5. Evaluate *in-vitro* the antimicrobial and anti-biofilm activity of polyphenolic extracts of guava leaf.

Chapter 1.

molecules

MDPI

Article

Influence of the Extraction Method on the Polyphenolic Profile and the Antioxidant Activity of *Psidium guajava* L. Leaf Extracts

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Article

Influence of the extraction method on the polyphenolic profile and the antioxidant activity of *Psidium guajava* L. leaf extracts

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Abstract:

The leaves of *Psidium guajava* L. are an agro-industrial by-product with an outstanding content of polyphenolic compounds; however, there are many factors which can affect the phytochemical profile when valuing this type of plant material, such as temperatures and extraction times involving in the extraction methods applied. In this sense, this study

analyzed the impact of different extraction methods (Soxhlet, maceration and ultrasound-assisted extraction) on the phytochemical profile (FTIR and UPLC-MS) and the antioxidant activity (ABTS, FRAP and Folin-Ciocalteu) of guava leaf extracts. A yield of phenolic compounds per gram of guava leaf was obtained within the range of 16 to 45 mg/g; on the other hand, the IC_{50}values determined with the ABTS assay ranged between 78 ± 4 to 152 ± 12 µg/ml. The methanolic extract obtained by Soxhlet was the one with the best reducing power, both in the FRAP assay and in the Folin-Ciocalteu assay. Finally, bioactive compounds such as quercetin, kaempferol and avicularin were identified in the guava leaf extract. It was concluded that the purification of polyphenolics compounds improves the antioxidant capacity, and that the extraction method greatly influences the phytochemical profile and activity of the extracts.

Keywords:

Psidium guajava L., polyphenolics, antioxidants, characterization, phytochemicals, guava leaves, UPLC-MS.

Introduction

Currently, there has been a growing interest from the scientific community in phytochemicals, bioactive compounds of plant origin, non-nutritive, which have beneficial properties for health, as well as great antioxidant power, which makes them potential nutraceuticals, products that are able to prevent diseases and thereby increasing its value for the society [1].

One of the most abundant groups of phytochemicals are polyphenolic compounds, which are secondary plant metabolites having highly varied structures but are characterized by the presence of aromatic rings and hydroxyl groups [2]. Polyphenolic compounds are recognized as antioxidants for their ability to donate hydrogen atoms and/or electrons to free radicals, breaking the oxidation chain [3]. This ability has become a topic of great importance considering that oxidative stress has been associated with many diseases, such as cancer, hypertension, diabetes mellitus, atherosclerosis, and neurological disorders [3].

Oxidative stress occurs when there is no balance between pro-oxidant species and antioxidants [4]. It should be noted that oxidation is not a specific problem for humans, it also appears in food, *e.g.* the oxidation of lipids and proteins, which is a serious problem, as it can cause the destruction of essential nutrients, bad odors and even the generation of toxic compounds in food systems [5].

Downstream processing involves essential steps in discovering bioactive compounds from raw plant materials, including the extraction methods. Different factors such as solvent type, temperature, time, and the solid-liquid ratio affect the extraction efficiency, so they must be carefully selected, considering the technique to be used [6]. The most popular techniques are maceration, infusion, and continuous hot extraction, such as Soxhlet; however, alternative methods such as ultrasonic or microwave-assisted extraction and supercritical fluid extractions have become available, and have gained interest because they are faster and, above all, respectful of the environment by reducing solvent and energy consumption [7].

Each extraction technique has its own advantages and disadvantages, in the case of Soxhlet extraction, it allows to extract a large quantity and variety of compounds in a relatively short time; furthermore, the solvent can be reused and the filtration and/or centrifugation process for separation the plant material can be avoided. However, generally, high temperatures are used, and, consequently, degradation of phytochemicals, including polyphenolic compounds, may occur [6]. Therefore, in this study we evaluated other options which did not require high temperatures: maceration due to its simplicity and wide use [8] and ultrasound-assisted extraction that facilitates the breaking of plant cells, increasing the contact area between plant material and solvent, decreasing heat requirements [6]. It is important to highlight that the extraction of polyphenolic compounds is a challenge since they can be unstable and the biological activity can be damaged and/or lost by high temperatures, presence of oxygen and light, therefore, the choice of the extraction technique and all its parameters is key to have good yields and at the same time maintain the integrity of the compounds [9].

In this sense, *Psidium guajava* L. is a native American shrub of great economic importance for its fruit, the guava, a berry with firm pulp and numerous seeds that is marketed worldwide. The harvest *P. guajava* generates a large number of leaves as by-product, which are rich in polyphenolic compounds, also it has been reported that they have antimicrobial and antioxidant activity [10]. Unfortunately, about 1.3 billion tons of agricultural by-products, including leaves, seeds, and skins are wasted each year [2], and are landfilled or incinerated even though they may contain large amounts of bioactive compounds, these practices only increase the environmental load and the total cost of production [11,12]. In the specific case of guava, approximately 80 kg this by-product per metric ton of fresh fruit is produced during guava processing [13].

The valorization of guava leaves is an opportunity for producing countries such as Mexico, India, and China, where large amounts of leaves are available [8]. However, for its use it is necessary to consider that, depending on the geographical location, weather conditions, the presence of different pathogens, the variety, etc. changes can occur in the phytochemical composition of the plant organs [14,15], so it is extremely important to consider the parts of the plant material to work.

In the present study, the impact of different extraction methods on the antioxidant activity and the phytochemical profile of guava leaf extracts was evaluated, considering two conventional techniques and a green alternative. In addition, to our best knowledge, this is the first report in which the polyphenolic compounds of the guava leaf are purified with amberlite XAD-16, achieving improvements in their bioactivity.

2. Results

The leaves of *P. guajava* L. are recognized for their high and diverse content of polyphenolic compounds [16-18]; as well as for its wide use in traditional medicine to treat conditions such as stomach pains, wounds, cavities, and cough [10]. In recent decades, evidence has indicated the beneficial properties of polyphenols, for example, their antioxidant, antimicrobial, and anti-inflammatory activity [3,19]. The extraction of polyphenolic compounds from agro-industrial residues is a viable option to value this type of waste, minimizing environmental damage and generating value-added products, such as natural

antioxidants in functional foods or for the development of dietetic supplements [19]. This section presents the tentative identification of the polyphenolic compounds present in the guava leaf extracts as well as their antioxidant activity, discussing the differences observed according to the extraction method used, which consists of a given technique, temperature, and time.

2.1. Purification of polyphenolic compounds

Table 1 shows the different extraction methods used and the yields of polyphenolic compounds obtained by each of them, which ranged from 16 to 45 $mg \cdot g^{-1}$. The highest values were observed with the extraction methos of Soxhlet using methanol as extracting solvent (44 $mg \cdot g^{-1}$) and ultrasound-assisted extraction at 30°C with distilled water (45 $mg \cdot g^{-1}$). On the other hand, all other extraction methods had a yield around 20 $mg \cdot g^{-1}$ approximately.

Table 1. Extraction yield of purified polyphenolic compounds

Extraction method				Extraction yield
Technique	**Solvent**	**Temperature (°C)**	**Time (min)**	**mg of phenolic compounds/ g of guava leaf**
Soxhlet	Methanol	65	5	44
Soxhlet	Distilled water	100	5	24
Maceration	Methanol	37	192	21
Maceration	Methanol	25	192	22
Ultrasound	Methanol	30	0.66	18
Ultrasound	Methanol	23	0.66	19
Ultrasound	Distilled water	30	0.66	45

Multiple authors have reported different yields of total polyphenolic compounds in guava leaves. For example, Farag, *et al*. [20] observed a total content of 59.267 ± 0.348 mg GAE (Gallic Acid Equivalent) $\cdot g^{-1}$; Sowmya *et al*. [21] reported a content of 41.33 ± 0.92 mg GAE$\cdot g^{-1}$ and 37.60±0.26 mg GAE$\cdot g^{-1}$ in two guava varieties; and Laily, *et al*. [22] published a content of 101.93 mg GAE$\cdot g^{-1}$. Although these concentrations may be higher than those obtained in this study, it is important to mention that, in the works mentioned, a purification process for polyphenolic compounds was not carried out, so the total content may be

overestimated, especially considering that, the Folin-Ciocalteu assay was used for the determination. Even if this assay is well established and widely used, it should be noted that since it is based on a redox reaction, compounds other than phenols, for example, reducing sugars and ascorbic acid, can also reduce the Folin-Ciocalteu reagent [23]. It is worth mentioning that the yield is not directly related to the phenolic composition of the samples or to their antioxidant activity; therefore, a greater number of polyphenolic compounds does not always mean better antioxidant activity [24].

2.2. Antioxidant activity

The antioxidant activity was evaluated using three different assays: Folin-Ciocalteu and FRAP (Ferric Reducing Antioxidant Power), both to analyze the reducing capacity of the samples, and ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) to analyze the ability to inhibit the radical cation $ABTS^{\bullet+}$.

Preliminarily, the three tests (Folin-Ciocalteu, FRAP and $ABTS^{\bullet+}$) were carried out with both, crude and purified solutions of the guava leaf extracts at 0.250 $mg \cdot mL^{-1}$ and it was observed that in all the tests the samples with purified polyphenolics had a higher antioxidant activity in comparison with the raw samples, as shown in Figure 1.

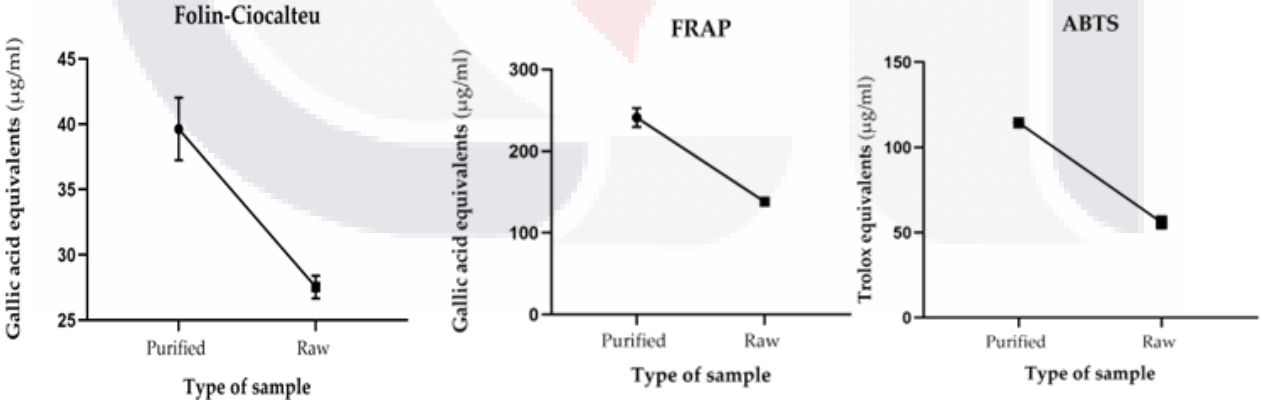

Figure 1. Mean and SEM of crude and purified samples in different antioxidant capacity assays.

This behavior is especially interesting given that Folin-Ciolcalteu and FRAP are not specific tests for polyphenols, since they can be reduced by other agents such as reducing sugars, amino acids, and ascorbic acid [25] which could be present in raw samples [26]. For this reason, it is important to carry out a purification process, since it allows us to eliminate inert and undesirable components that can interfere with the study and/or that have limited antioxidant activity that represses the activity of polyphenols [27].

The determination of the IC_{50} (the minimum extract concentration at which 50% of the free radicals are inhibited) values were carried out with the $ABTS^{\bullet+}$ assay. The results are within the range from 78 to 152 μg·mL^{-1}, as shown in Table 2. It is worth mentioning that the lowest values of IC_{50} indicate a greater capacity to inhibit free radicals of the samples, which in the present study were exhibited by the methanolic extracts obtained by maceration and ultrasound (87 to 78 μg·mL^{-1}).

Other studies with guava leaves have reported lower IC_{50} values: 24.37 ± 3.85 μg·mL^{-1}[28], 31.19 ± 5.01 to 72.31 ± 3.57 μg·mL^{-1} [29], 3.23 ± 0.24 to 8.26 ± 1.06 μg·mL^{-1} [30]. This may be since the polyphenol content and therefore its antioxidant activity may be affected by different factors, including the extraction conditions (technique, temperature, solvent, time, etc.), climatic conditions and soil quality [14,15,31]. Even so, guava leaf collected in Aguascalientes, Mexico presented relevant antioxidant activity compared to other plant extracts characterized by having different functional properties such as avocado leaf extracts (IC_{50}=269.56 ± 6.52 to 442.72 ± 9.62 μg·mL^{-1}) [32], rosemary leaf extracts (IC_{50}=70 ± 4.67 μg·mL^{-1}) [33], and oriental ebony leaf extracts (IC_{50}= 108.7 μg·mL^{-1}) [34].

Similarly, it has been observed that guava leaves have a concentration of polyphenolic compounds and an antioxidant capacity higher than other parts of the bush such as seeds, fruit, and bark [30,35] consequently, the present study shows that guava leaf extracts can be considered as an alternative for obtaining bioactive compounds with multiple applications in industry.

Table 2. IC_{50} values according to the extraction method obtained in the ABTS$^{\bullet+}$ assay

Extraction Method	IC_{50} (µg·mL^{-1})
Soxhlet, Methanol, 65°C, 5 hours.	119 ± 6^{B}
Soxhlet, Distilled water, 100°C, 5 hours.	137 ± 12^{AB}
Maceration, Methanol, 37°C, 192 hours.	89 ± 3^{C}
Maceration, Methanol, 25°C, 192 hours.	87 ± 6^{C}
Ultrasound, Methanol, 30°C, 0.66 hours.	78 ± 4^{C}
Ultrasound, Methanol, 23°C, 0.66 hours.	78 ± 4^{C}
Ultrasound, Distilled water, 30°C, 0.66 hours.	123 ± 4^{B}
Ultrasound, Distilled water, 23°C, 0.66 hours.	152 ± 12^{A}

**Different letters mean significant ($p < 0.05$) differences between the extraction methods.

The FRAP and Folin-Ciocalteu assays were performed with the purified guava leaf extracts at their respective concentration of IC_{50} (Table 1). In both tests it was observed that the extract with the highest reducing capacity was the one obtained by Soxhlet extraction method using methanol as extracting solvent, while the other extracts exhibited a similar reducing capacity, as can be seen in Figure 2 and 3.

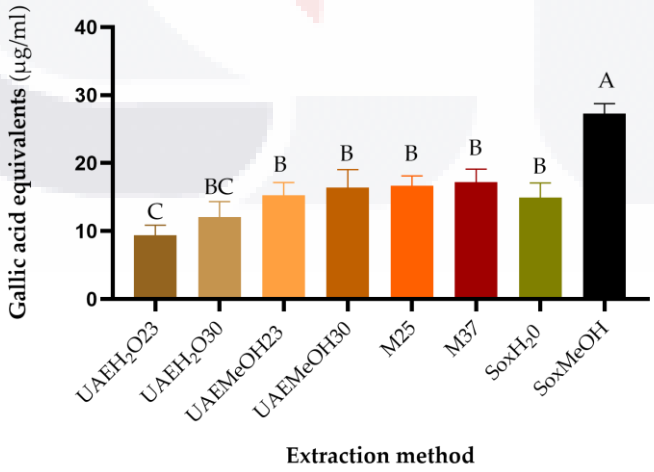

Figure 2. Folin-Ciocalteu reagent reducing capacity according to the extraction method. SoxMeOH: Soxhlet, methanol, 65°C, 5 hours.; SoxH_2O: Soxhlet, distilled water, 100°C, 5

hours.; M37: Maceration, methanol, 37°C, 192 hours.; M25: Maceration, methanol, 25°C, 192 hours.; UAEMeOH30: Ultrasound, methanol, 30°C, 0.66 hours.; UAEMeOH23: Ultrasound, methanol, 23°C, 0.66 hours.; UAEH_2O30: Ultrasound, distilled water, 30°C, 0.66 hours.; UAEH_2O23: Ultrasound, distilled water, 23°C, 0.66 hours. Methods that do not share a letter are significantly different ($p<0.05$).

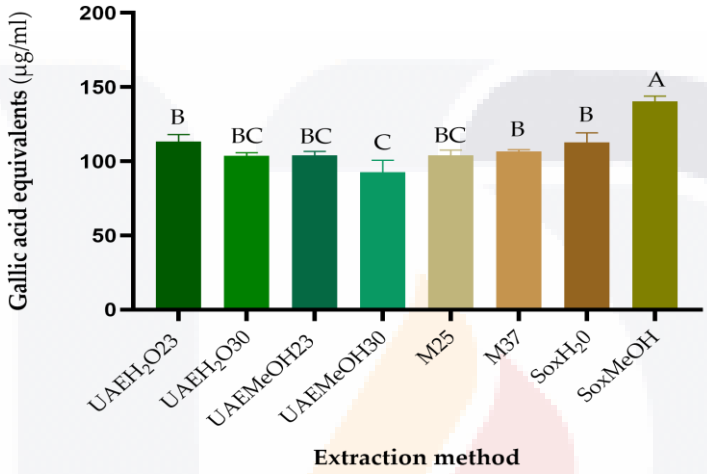

Figure 3. Iron reducing capacity according to the extraction method. SoxMeOH: Soxhlet, methanol, 65°C, 5 hours.; SoxH_2O: Soxhlet, distilled water, 100°C, 5 hours.; M37: Maceration, methanol, 37°C, 192 hours; M25: Maceration, methanol, 25°C, 192 hours; UAEMeOH30: Ultrasound, methanol, 30°C, 0.66 hours; UAEMeOH23: Ultrasound, methanol, 23°C, 0.66 hours; UAEH_2O30: Ultrasound, distilled water, 30°C, 0.66 hours; UAEH_2O23: Ultrasound, distilled water, 23°C, 0.66 hours. Methods that do not share a letter are significantly different ($p<0.05$).

Temperature is an important factor for the extraction of phytochemicals [8]; increases in temperature can result in improvements in extraction thanks to better diffusion and solubilization [36,37], however, it could also generate degradation of compounds and decrease antioxidant activity [38,39]. In our case, the extraction method with Soxhlet is the one that used the highest temperature (65°C), and this favored the yield of polyphenolic compounds and antioxidant activity, specifically the reducing activity.

In recent years, the search for natural antioxidants has gained importance in view of the need to replace fossil-derived resources, as well as to avoid the use of synthetic antioxidants, which

can be cytotoxic and carcinogenic [40,41]. The different guava leaf polyphenolic extracts studied exhibited good antioxidant activity, property useful in a wide variety of applications, such as in the food [42], cosmetic [43,44] and pharmaceutical industry [45], so they can be a low-cost and natural origin alternative. For example, the methanolic extract obtained by Soxhlet, on account of its significant reducing capacity, may be a good candidate for the green synthesis of nanoparticles [46,47], while the methanolic extracts obtained by maceration and ultrasound-assisted extraction may be good candidates as nutraceuticals or cosmetics given their ability to inhibit free radicals [48]. Obviously, previous studies on toxicity, stability, bioavailability as well as a solvent removal process are necessary before the extract can have an industrial application.

2.3. Characterization by FTIR

The dried purified methanolic extracts obtained by three different methods (Soxhlet, maceration at 25°C and ultrasound-assisted extraction at 30°C) were selected due to their notable results in antioxidant activity and yield in polyphenolic compounds for analysis by Fourier transform infrared spectroscopy (FTIR). It should be noted that, as the samples analyzed are purified extracts, the peaks of the spectra correspond to the different functional groups present in the polyphenolic compounds.

In the Figure 4, from 3000 to 3600 cm^{-1} the broad and strong band corresponds to the stretching vibrations of the O–H bond, which indicates the presence of functional groups such as hydroxyls. In addition, the presence of O–H groups is confirmed since there is vibration between 1600 and 1300 cm^{-1}, 1200 and 1000 $cm^{-1}$and 800 and 600 cm^{-1}. The presence of peaks from 3000 to 2900 cm^{-1}corresponds to the stretching of the C–H bond, characteristic of aliphatic functional groups. On the other hand, from 2200 to 1950 cm^{-1}we can observe two small vibrations caused by the resonance effect of aromatic compounds. The presence of the carbonyl functional group is confirmed since peaks are observed from 1850 to 1650 cm^{-1} corresponding to the stretching of the C–O bond. The intense and well-defined peak between 1650 and 1600 cm^{-1} indicates the presence of carboxylic acids, while the two peaks around 1615 and 1495 cm^{-1} indicate the presence of double bounds for the vibration of the C–C bond, confirming the presence of aromatic compounds. Finally, from 1500 to 600 cm^{-1}corresponds to the fingerprint area, which is specific and unique; the bands present from

1000 to 1300 $cm^{-1}$are due to vibrations of the C–O bonds found in esters, carboxyls, ethers, and hydroxyls.

Other studies on the phytochemicals from guava leaf extracts show similar spectra. Lok *et al*. [49] analyzed guava leaf extracts obtained with three different solvents: distilled water, ethanol and n-hexane; and they also observed the stretching bands of the C–H and O-H bonds; on the other hand, Nagpal, *et al*. [50] and Lahlou, *et al*. [51] also reported the vibration of the functional groups C=O, O–H, C–O and C–H.

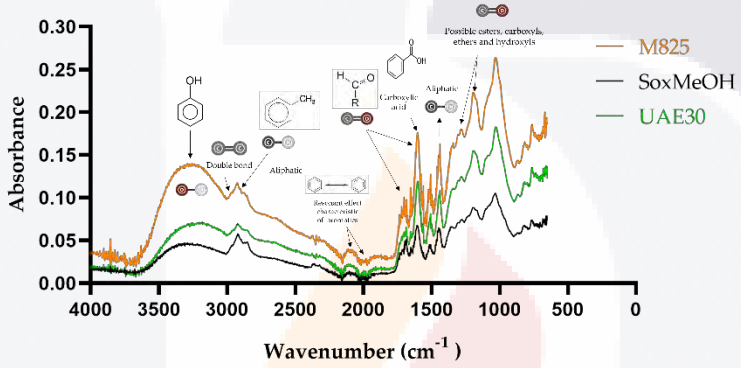

Figure 4. FTIR analysis of the methanolic extracts of guava leaves. ** M825: maceration at 25°C, SoxMeOH: Soxhlet at 65°C, UAE30: ultrasound-assisted extraction at 30°C.

2.4. UPLC-MS

The purified methanolic extract obtained by Soxhlet was selected for analysis by UPLC-MS given its outstanding reducing activity and yield of polyphenolic compounds, in comparison with the other two techniques evaluated. The tentative identification of the phytochemicals is presented in Table 3.

In the extract, 13 compounds were identified, among them, quercetin, considered the most active and powerful antioxidant of guava leaves [52], catechin, kaempferol, avicularin, guavinoside B and C, all of them with different beneficial activities for the human health; *e.g.* anti-inflammatory, antimicrobial and antitumor activity [53-61]. Therefore, guava leaves are a rich source of polyphenolic compounds and a potential nutraceutical. On the other hand, there were also compounds that could not be identified, giving rise to new investigations for

the identification, characterization, and *in-vitro* evaluation of the activity of these phytochemicals.

For additional information, see Supplementary Material 1.

#	Tentative Identity	Tr (min)	m/z exp	m/z calculated	Molecular Formula	Fragments	Reference
1	Not identified	0.749	249.0305	248.034588	$C_{19}H_5O$	113.0318, 181.0322, 207.0358	
2	Vescalagin	7.71	933.2043	933.07178	$C_{41}H_{26}O_{26}$	466.1265, 179.0356, 289.1466	[17]
3	Catechin	9.587	289.147	289.079587	$C_{15}H_{14}O_6$	179.0359, 207.0354, 287.1305, 245.1503	[17]
4	Not identified	18.88	603.1823	602.181312	$C_{40}H_{27}O_6$	179.0356, 207.0351, 235.9931, 257.02	
5	Casuarinin/ Casuarictin Isomer	19.637	935.2216	935.08743	$C_{41}H_{28}O_{26}$	467.1334, 145.9832, 385.2003, 478.1260	[17]
6	Not identified	33.071	381.0783	380.076847	$C_{24}H_{13}O_5$	379.0626, 301.1106, 299.0956	
7	Not identified	33.475	381.0775	380.076847	$C_{24}H_{13}O_5$	299.0951, 301.110, 302.1134, 379.0617, 271.098	
8	Quercetin glucuronide	34.788	477.1651	477.075289	$C_{21}H_{18}O_{13}$	463.1837, 299.0954, 301.1106	[17]
9	Reynoutrin	37.414	433.1703	433.08546	$C_{20}H_{18}O_{11}$	431.1533, 181.0318, 235.9926, 300.1021, 415.2863	[17]

#	Tentative Identity	Tr (min)	m/z exp	m/z calculated	Molecular Formula	Fragments	Reference
10	Guajaverin	40.242	433.1713	433.08546	$C_{20}H_{18}O_{11}$	431.1543, 300.1037, 301.1096, 391.9761	[17]
11	Avicularin	40.798	433.1707	433.08546	$C_{20}H_{18}O_{11}$	431.1534, 300.1033, 302.1121	[17]
12	Myrciaphenone B	48.222	481.1956	481.106589	$C_{21}H_{22}O_{13}$	479.1792, 417.1714, 365.9648, 257.0243, 235.9927, 239.9696, 207.0348, 181.0316,179.0352	[17]
13	Guavinoside C	57.06	585.1982	585.096419	$C_{27}H_{22}O_{15}$	583.1833, 304.9899, 285.9811, 235.9928, 257.0249, 352.9331	[17]
14	Not identified	63.473	551.2103	550.207527	$C_{34}H_{31}O_{7}$	541.1789, 343.1274, 328.1021,	
15	Guavinoside B	64.887	571.2532	571.15354	$C_{28}H_{28}O_{13}$	569.2360, 481.2711, 257.0239	[17]
16	Not identified	78.22	711.5146	710.401989	$C_{30}H_{63}O_{18}$	701.4839, 549.4456, 503.4370	
17	Not identified	82.967	695.5202	694.51974	$C_{51}H_{67}O$	685.4873, 533.4503, 487.4390	
18	Luteolin 7-O-malonyl-glucoside	86.2	533.4523	533.101504	$C_{24}H_{22}O_{14}$	487.4415, 488.445, 523.4203, 501.4174	[62]
19	Kaempferol 3-O-(6"-malonyl-glucoside)	86.907	533.4519	533.101504	$C_{24}H_{22}O_{14}$	487.4413, 488.4441, 523.4199	[62]

#	Tentative Identity	Tr (min)	m/z exp	m/z calculated	Molecular Formula	Fragments	Reference
20	Chrysoeriol 7-O-(6"-malonyl-glucoside)	87.816	547.4318	547.117154	$C_{25}H_{24}O_{14}$	501.4212, 502.4242, 427.0584, 533,4493	[62]

3. Materials and Methods

3.1 Plant material

The collection of guava leaves was carried out manually and randomly from different specimens free of pesticides in Aguascalientes, Mexico in November 2021. The plant sample was transferred in a botanical press to the laboratory where only green leaves, without damage by insects or pests, were selected. Subsequently, the leaves were thoroughly washed with distilled water to remove traces of dust and other contaminants and dried at 40°C for 72 hours [35,63]. Finally, the sample was pulverized with an electric processor and the obtained powder was stored at room temperature in an airtight container protected from light [64].

3.2. Extraction of phytochemicals

Three different extraction techniques were tested: Soxhlet, maceration, and ultrasound-assisted extraction (UAE). In all cases, a solid-liquid ratio of 1:20 was used (5 grams of plant sample per 100 ml of solvent). It was decided to use two different solvents: methanol since in multiple articles [65-67] and previous work from our laboratory has been reported to have better extractive power; and distilled water, as a green alternative. The continuous extraction by Soxhlet was carried out during 7 siphons [68], while the maceration lasted 8 days and two temperatures were evaluated: 25°C and 37°C [69-71]. The ultrasound-assisted extraction lasted 40 minutes and 2 temperatures were evaluated: 23°C and 30°C [72-75]. The extracts obtained by maceration and UAE were centrifuged (5000 rpm for 17 minutes) and filtered (0.2 µm) to eliminate guava leaf particles [17].

3.3. Solvent elimination

The aqueous extracts were subjected to low temperatures (-48°C) and vacuum for 5 days in a Labconco lyophilizer to remove distilled water [76]. On the other hand, the methanolic extracts were subjected to 50°C in an oven to eliminate the solvent [32]. In all cases, a green to reddish brown powder was obtained, which was stored in an Eppendorf tube at room temperature protected from light until use.

3.4. Purification of polyphenolic compounds

Phenolic compounds from guava leaves were purified with the commercial adsorbent Amberlite XAD-16. Briefly, the lyophilized aqueous extracts were solubilized in distilled water while the methanolic extracts were solubilized in 80% methanol and the alcohol was removed by rotary evaporator, to obtain the water solubilized extract. Subsequently, 20 ml of the extracts were added to a column packed with amberlite XAD-16 as a stationary phase and distilled water was added to eliminate sugars and other compounds present in the extract, and finally the polyphenolic compounds were eluted with absolute ethanol. The solvent was removed in an oven at 50°C for 24 hours and the crystals obtained were kept protected from light at room temperature [32,77].

The yield of polyphenols per gram of plant material was determined as follows:

$$\boldsymbol{Yield} = \frac{Milligrams\ of\ phenolic\ compounds\ obtained}{Grams\ of\ plant\ material\ used\ for\ extraction}$$

3.5. Antioxidant capacity tests

The antioxidant capacity of both the crude guava leaf extracts and their purified polyphenolic compounds was analyzed. Three different assays were carried out in microplates: Folin-Ciocalteu, ABTS, and FRAP.

The reducing power was determined by adding 25 µL of Folin-Ciocalteu reagent and 25 µL of sodium carbonate (75 g/L) to 25 µL of properly diluted sample (1:4 v/v). The obtained mixture was homogenized and incubated at 40°C for 30 minutes. After that, 200 µL of

distilled H_2O were added, and the absorbance at 750 nm was recorded [78]. Results were expressed as gallic acid equivalents in micrograms per milliliter (GAE µg/mL) using the calibration curve prepared with the same standard.

The $ABTS^{\bullet+}$ radical scavenging capacity assay was carried out according to the methodology proposed by Hernández *et al.* [77]. Briefly, a solution of ABTS (7 mM) and one of potassium persulfate (2.45 mM) were mixed (2:1) and allowed to rest for 12 h at room temperature; then, it was adjusted with absolute ethanol until reaching an absorbance of 0.7 nm. Subsequently, five microliters of each test sample and calibration curve were pipetted in triplicate into the microplate, and 95 µL of the adjusted $ABTS^{\bullet+}$ solution was added to them. After 1 minute the absorbance at 734 nm was measured. According to the Trolox calibration curve, results were expressed as percentage inhibition of $ABTS^{\bullet+}$ radicals or as IC_{50} (sample concentration needed to inhibit 50% of radicals).

The iron-reducing antioxidant power (FRAP) was determined by mixing 5 µl of the samples to be analyzed with 12 µl of phosphate buffer (pH 7) and 22 µl of 1% potassium ferrocyanide, and then incubated at 50°C for 20 minutes. Then, 12 µl of 10% trichloroacetic acid, 45 µl of distilled water, and 10 µl of ferric chloride were added to read the absorbance at 700 nm [79]. Results were reported as µg gallic acid equivalent per milliliter (GAE µg/mL).

Initially, as a preliminary test, antioxidant capacity tests were carried out with solutions of the extracts (both crude and purified) at 0.250 $mg \cdot mL^{-1}$ to see their behavior. Subsequently, the IC_{50} of the purified extracts were determined with the $ABTS^{\bullet+}$ assay, and finally, the FRAP and Folin-Ciocalteu assays were performed with the purified extracts at their determined IC_{50}.

Statistical analysis was performed using Excel, MiniTab and GraphPad Prism 8.0.1 software.

3.6. FTIR (Fourier Transform Infrared Spectroscopy).

The polyphenolic compounds of the methanolic extracts obtained by Soxhlet, maceration at 25°C and by ultrasound at 30°C were analyzed by Fourier transform infrared spectroscopy

(Agilent Technologies, FTIR model Cary 630 coupled to a zinc selenide crystal (ZnSe) ATR). The obtained powder after the purification process was deposited on the surface of the reader and secured by means of the equipped press. The spectra were acquired at a range of 4000–600 cm^{-1} through 32 scans, with a resolution of 2 cm^{-1} [80].

3.7. UPLC-MS

The analysis was carried out with an Acquity UPLC system (Waters, Milford, MA, USA) consisting of an auto-sampler, a binary pump equipped with a 10 µl Loop (partial Loop injection mode) and a BEH PHENYL column (2.1 mm × 100 mm, 1.7 µm; WATERS, Waxford, Ireland). The solvents used were (A) water + 0.1% (v/v) formic acid and (B) acetonitrile + 0.1% (v/v) formic acid at a constant flow rate of 0.3 $mL \cdot min^{-1}$. The elution gradient (for 113 min) was 100% A, gradually decreasing until reaching 10% A and 90% B, to move from normal conditions (100% A) one minute later to re-equilibrate the column. MS detection was performed on a Q-ToF quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF™, Waters, Milford, MA, USA) equipped with an electrospray ionization source (ESI). The sample acquisition mode was in negative ionic polarity, analysis mode in sensitivity and normal dynamic range; in a mass range of 50 to 1200 Da, sweep conditions of 0.5 s^{-1}, centroid data format and with a collision energy of 6 V and a cone voltage of 40 V.

4. Conclusions

The present study shows that guava leaves are a rich resource in polyphenolic compounds, which antioxidant activity can be of great interest in the pharmaceutical and food industry, so guava-producing countries can value this agro-industrial by-product.

About 13 compounds were identified in the analyzed guava leaf extract, including quercetin, catechin, hyperin, and guajaverin. Of the three methods studied, Soxhlet is postulated as an advantageous option for the recovery of phenolic compounds, given that it does not require a significant amount of time, no processes are needed to separate the plant material from the solvent, the solvent can be reused, and the extract obtained exhibits outstanding reducing activity and yield in phenolic compounds compared to maceration and ultrasound-

assisted extraction. In addition, different commercially available systems allow the Soxhlet extraction method to be scaled and even automated for industrial applications. Furthermore, the purification of phenolic compounds generated improvements in the antioxidant activity in the three assays carried out (FRAP, Folin-Ciocalteu and $ABTS^{\bullet+}$) in our study, exposing that this process may be a way to increase the bioactivity of these phytochemicals.

It should be noted that, for its future industrial application, it will be necessary to face different challenges, especially to improve the long-term stability of polyphenolic compounds and increase their bioavailability; this can be achieved, for example, through encapsulation. In addition, more studies are needed on its toxicity, *in-vivo* activity, and safety.

Author Contributions:

Conceptualization, DGM, GCGMA and ALGB; methodology, DGM, GCGMA and MDGH.; writing—original draft preparation, DGM, GCGMA and ALGB; formal analysis, DGM, MDGH, GCGMA; investigation, DGM, FRC, NACV, FJAC, resources, DGM, ALGB, GCGMA; writing—review and editing, DGM, NACV, FJAG and FRC; supervision, ALGB, GCGMA, NACV, FJAG. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 2.

Guava Leaf Extract Exhibits Antimicrobial Activity in Extensively Drug-Resistant (XDR) *Acinetobacter baumannii*

Article

Guava Leaf Extract Exhibits Antimicrobial Activity in Extensively Drug-Resistant (XDR) *Acinetobacter baumannii*

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Abstract:

Currently, a global health crisis is caused by the microbial resistance, in which *Acinetobacter baumannii* plays a crucial role, being considered the highest priority microorganism by the World Health Organization (WHO) for discovering new antibiotics. As a result, phytochemicals have emerged as a potential alternative to combat resistant strains

since they can exert antimicrobial activity through various mechanisms and, at the same time, represent a more natural and safe option. This study analyzes the antimicrobial effects of guava leaf extract in ten clinical isolates of extensively drug resistant (XDR) *A. baumannii*, using the agar diffusion technique and the microdilution method to determine the minimum inhibitory concentrations (MIC). Additionally, the possible improvement of the antimicrobial activity after the purification of the polyphenolic compounds and the potential synergy with the antibiotic gentamicin are examined in this research. Moreover, the effect of the plant extract in the cell line A549 derived from lung tissue was also evaluated. The extract exhibited antimicrobial activity against all the strains studied, and the purification of polyphenols along with the combination with gentamicin, improved the extract activity. The presence of the plant extract induced morphological changes in the lung cells after 24 hours of exposure. Therefore, *Psidium guajava* L. leaf extract is a potential antimicrobial agent.

Keywords:

Psidium guajava L.; *Acinetobacter baumannii*; extensively drug-resistant; antimicrobial activity; guava leaf extract, phytochemicals.

1. Introduction

Acinetobacter baumannii is a Gram-negative opportunistic pathogenic bacterium that has become a major concern for healthcare workers as it has been strongly associated with nosocomial infections leading to prolonged hospital stays, high morbidity, and mortality, particularly amongst hospitalized patients in the intensive care unit (ICU) [1,2]. Although it is found primarily in hospital settings, this microorganism has been isolated from a wide variety of environmental samples, including soil, food, animals, and humans [3]. Unfortunately, *A. baumannii* strains have been reported to have developed resistance to most clinically significant antibiotics, including colistin, considered the last resort option for treating carbapenem-resistant, Gram-negative bacterial infections [4,5]. Therefore, it is not surprising that *A. baumannii* ranks first among microorganisms considered as critical priority for developing new antibiotics by the World Health Organization [6].

In recent years, new antibiotics have emerged to treat multidrug-resistant (MDR) strains of *A. baumannii*, including cefiderocol, the first siderophore cephalosporin approved and designed for the treatment of Gram-negative pathogens resistant to carbapenems [7], and ceftazidime-avibactam, a combination of a third-generation cephalosporin with a new beta-lactamase inhibitor [8]. However, they have been linked to adverse effects and are costly. Therefore, their availability is limited, especially for patients with medical complications and people living in developing countries [9–12]. XDR strains are a type of MDR organisms which are resistant to almost all approved antimicrobial agents except to at least one agent in all but two or fewer antimicrobial categories, thus, bacterial isolates should be tested against all or nearly all the antimicrobial agents within the antimicrobial categories [13]. In recent years, the reports of XDR *A. baumannii* have been increased. This bacterium is a severe concern in healthcare settings since may cause nosocomial bacteremia and ventilator associated pneumonia (VAP) with high morbidity and mortality [14].

Amid this global crisis, phytochemicals have outstood as a potential alternative, which are also more natural, safer, cheaper, and with fewer side effects than synthetic antibiotics [15]. In addition, these secondary metabolites exert their antimicrobial activity through different mechanisms, including destabilization of the cell wall, inhibition of protein synthesis, hindering quorum-sensing, and DNA damage; thus, they have a lower risk of generating resistance [15,16]. Among phytochemicals, polyphenolic compounds have succeeded not only because they are the most abundant secondary metabolites, but because they exert antibacterial activity against a large number of bacteria and fungi, as well as for its notable antioxidant activity [17–19]. These activities are deeply related to chemical groups grafted on the phenolic core, which expand the diversity and intensity of biological activities of phenolics and, at the same time, offers opportunities to synthesize new compounds [20].

One strategy in the use of phytochemicals as potential antimicrobial agents is their combination with antibiotics which can result in a synergistic effect. A positive interaction created when two agents are combined that results in an inhibitory effect greater than the sum of their individual effects [21]. This method generates multiple advantages over conventional drug discovery methods, since the objective is to restore an existing drug to a state of significantly reduced resistance, so that clinical use can reach more quickly and with a lower development cost. Other advantages of synergistic interactions are increased

efficiency, reduced side effects, increased stability and bioavailability, and the need for lower doses compared to synthetic alternatives [22].

Psidium guajava L. is a native American shrub belonging to the Myrtaceae family. Its economic importance lies in its fruit, the guava, a berry with firm pulp and numerous seeds whose world production is around 2.3 million tons per year. This fruit can be found in tropical and subtropical climate regions around the world [23]. *P. guajava* L. has traditionally been used as a medicinal plant to treat different ailments including gastrointestinal problems, cavities, coughs, and wounds. In recent years it has been reported that extracts from different parts of this plant exhibit antimicrobial effects against many pathogenic strains such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa* and *Bacillus cereus* [24].

This study aims to determine whether the leaves of *Psidium guajava* L., a highly available and unvalued agro-industrial residue, have antimicrobial activity against different clinical isolates of XDR *Acinetobacter baumannii*, and whether the purification of polyphenols and the combination with the aminoglycoside antibiotic gentamicin have a positive effect on microbial inhibition. Furthermore, since *A. baumannii* can cause a range of lung tissue damage, the possible effects of the plant extract were also evaluated in a cell line derived from human lung tissue (ATCC A549).

2. Results

2.1. Evaluation of antimicrobial activity using the agar diffusion technique.

The antimicrobial activity of the crude extract (GLE) and purified polyphenols (GLEP) are presented in Tables 1 and 2. Guava leaf crude extract exhibited antimicrobial activity against all clinical isolates of *A. baumannii* (Table 1), and a synergistic effect with gentamicin was recorded in all cases, with increases in the inhibition diameters from 2.77% to 40.74%. On the other hand, gentamicin by itself did not affect the growth of the strains. The inhibition diameters observed in combination with gentamicin are between 12 mm and 13 mm, when they normally oscillated between 9 mm and 10.67 mm with the crude extract.

Table 1. Evaluation of the antimicrobial activity of guava leaf crude extract against XDR *A. baumannii* by the agar diffusion technique.

Isolate ID	Susceptibility to guava leaf extract	[a]ZDI GLE 100 mg/ml	ZDI [b]GLE 100 mg/ml + GEN 16 µg/ml	ZDI [c]GEN 16 µg/ml	ZDI Negative control (distilled water)	Percentage (%) of increase of the ZDI	GIIs[d]	Type of effect
A1	Yes	9 ± 0	12.6 ± 0.577	0	0	40.74	1.4	Synergistic
A2	Yes	9 ± 0	12.6 ± 0.577	0	0	40.74	1.4	Synergistic
A3	Yes	9.33 ± 0.58	13 ± 0	0	0	39.28	1.39	Synergistic
A4	Yes	9.66 ± 0.57	12.66 ± 0.57	0	0	31.03	1.31	Synergistic
A6	Yes	10 ± 0	12 ± 0	0	0	20	1.2	Synergistic
A25	Yes	9.33 ± 0.58	12 ± 0	0	0	28.57	1.28	Synergistic
A26	Yes	10.33 ± 0.57	13 ± 0	0	0	25.8	1.25	Synergistic
A27	Yes	10.33 ± 0.58	12.66 ± 0.58	0	0	22.58	1.22	Synergistic
A34	Yes	10.67 ± 0.58	13 ± 0	0	0	21.87	1.21	Synergistic
A38	Yes	12 ± 0	12.33 ± 0.57	0	0	2.77	1.02	Synergistic
***E. coli* ATCC 25922**	No	0	14.33 ± 0.58	15 ± 0	0	100	0.95	Synergistic
***P. aeruginosa* ATCC 27853**	Yes	29.67 ± 1.53	30.33 ± 1.53	14.33 ± 0.58	0	2.24	0.68	Synergistic

[a]ZDI = zone of inhibition diameters, [b]GLE = guava leaf crude extract; [c]GEN = gentamicin; [d]GIIs = growth inhibitory indices.

Similarly, purified polyphenols also had antimicrobial activity against all clinical isolates (Table 2), but surprisingly, at a 20-fold lower concentration (5 mg/ml). The diameters of the zone of inhibition (ZDI) were larger for nearly all tested strains compared to the crude extracts, except for the A38 strain, where the ZDI values were similar on crude and purified polyphenols (Tables 1 and 2). Furthermore, the diameters ranged from 10.66 mm to 11.66 mm for purified extract alone, and from 13.66 mm to 17.33 mm when combined with gentamicin. Therefore, a synergistic effect was also obtained with increases in the ZDI values from 20.58 to 48.57%.

Table 2. Evaluation of the antimicrobial activity of the purified guava leaf extract against XDR *A. baumannii* by the agar diffusion technique.

Isolate ID	Susceptibility to guava leaf extract	ZDI[a] GLEP 5 mg/ml	ZDI GLEP[b] 5 mg/ml + GEN 16 µg/ml	ZDI GEN[c] 16 µg/ml	ZDI Negative control (DMSO)	Percentage (%) of increase of the ZDI	GIIs[d]	Type of effect
A1	Yes	11± 2	14.66 ± 0.577	0	0	33.33	1.33	Synergistic
A2	Yes	10.66 ± 1.15	14 ± 0	0	0	31.25	1.31	Synergistic
A3	Yes	11.33 ± 0.58	15 ± 1	0	0	32.35	1.32	Synergistic
A4	Yes	10.66 ± 0.6	15 ± 0	0	0	40.62	1.4	Synergistic
A6	Yes	11 ± 0	15.33 ± 0.58	0	0	39.39	1.39	Synergistic
A25	Yes	11 ± 1	15 ± 0	0	0	36.36	1.36	Synergistic
A26	Yes	11 ± 0	15.5 ± 0.70	0	0	40.9	1.4	Synergistic
A27	Yes	11 ± 1	14 ± 0	0	0	27.27	1.27	Synergistic
A34	Yes	11.66 ± 0.58	17.33 ± 0.58	0	0	48.57	1.48	Synergistic
A38	Yes	11.33 ± 1.2	13.66 ± 0.57	0	0	20.58	1.2	Synergistic
***E. coli* ATCC 25922**	Yes	12 ± 0	15 ± 0	15 ± 0	0	25	1.25	Synergistic
***P. aeruginosa* ATCC 27853**	Yes	35 ± 1	36 ± 1	14.33 ± 0.58	0	2.85	0.72	Synergistic

[a]ZDI = zone of inhibition diameters, [b]GLEP = purified polyphenolic compounds from guava leaves; [c]GEN = gentamicin; [d]GIIs = growth inhibitory indices.

Figure 1 shows how the inhibition diameters and, subsequently, the growth inhibition of the *A. baumannii* isolates increased with the purification of the polyphenols and with the presence of gentamicin. Therefore, the polyphenols purification allowed considerably to reduce the concentration of the extract and, at the same time, to improve the antimicrobial activity. In addition, gentamicin did not affect any of the clinical isolates; however, in combination with the plant extract, it had a synergistic effect.

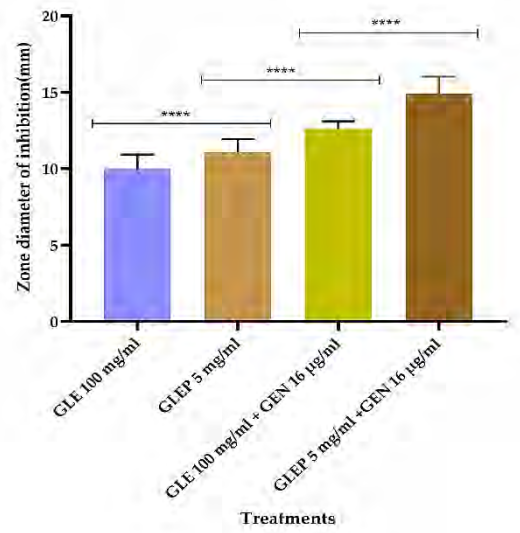


Figure 1. Inhibition of the growth of *Acinetobacter baumannii* clinical isolates by guava leaf extracts and gentamicin.

***GLE= guava leaf crude extract, GLEP= purified polyphenolic compounds from guava leaves, GEN= gentamicin, **** $p < 0.0001$.

2.2. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

After confirming the antimicrobial activity of the extracts using antibiograms, we determined the minimum inhibitory concentration and minimum bactericidal concentration. We only used the purified extract because it had better activity. We evaluated it alone and in combination with gentamicin (16 µg/ml).

Unfortunately, minimum bactericidal concentrations (MBC) could not be settled in the range of concentrations evaluated. However, two minimum inhibitory concentrations (MIC) were determined with purified extract alone, specifically, for the clinical isolates A38 and A26 at a concentration of 5 mg/ml. When combined with gentamicin, MICs for seven of the ten isolates evaluated were determined (Table 3). All *A. baumannii* strains exhibited a decrease in microbial growth, whether treated with the purified extract alone or in combination with the antibiotic. In addition, in this assay it was also observed that the presence of gentamicin improved the antimicrobial activity. This observation underscores the effectiveness of the purified extract. Detailed results on the MIC determination for each isolate can be found in the supplementary materials of this article.

Table 3. MIC and MBC exhibited by purified guava leaf extract against clinical isolates of XDR *Acinetobacter baumannii*.

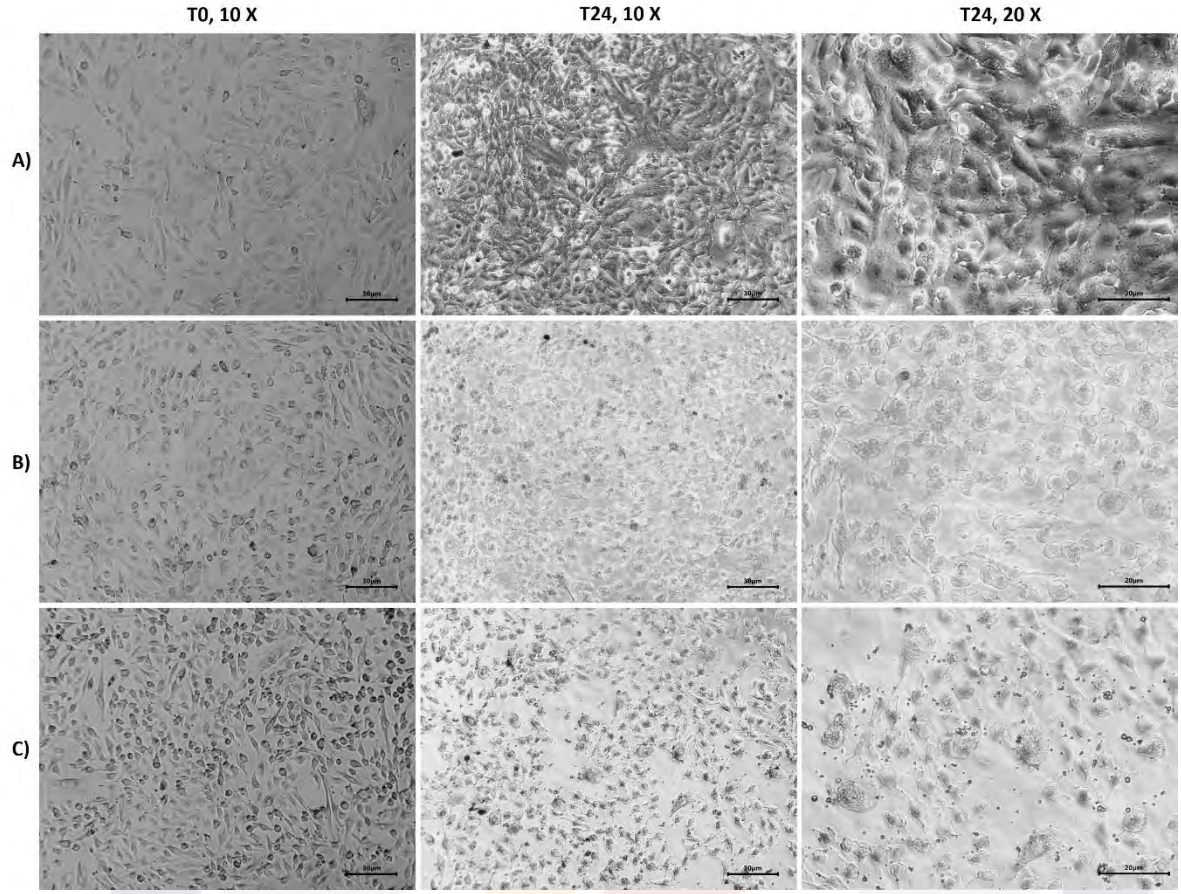
	Without gentamicin		With gentamicin (16 µg/ml)	
Isolate ID	**MIC**	**MBC**	**MIC**	**MBC**
A1	> 5 mg/ml	>5 mg/ml	> 5 mg/ml	> 5 mg/ml
A2			**5 mg/ml**	
A3			> 5 mg/ml	
A4			**5 mg/ml**	
A6			> 5 mg/ml	
A25			**5 mg/ml**	
A26	**5 mg/ml**		**5 mg/ml**	
A27	> 5 mg/ml		**2.5 mg/ml**	
A34			**5 mg/ml**	
A38	**5 mg/ml**		**5 mg/ml**	

The MICs are highlighted in bold.

2.3. Effect of guava leaf extract on the A549 cell line.

A549 cells were exposed to crude and purified guava leaf extract at 100 mg/mL and 5 mg/ml, respectively. After 24 hours, morphological changes were observed, and the percentage of viable cells was determined with the commercial dye trypan blue. The results are presented in Figure 2 and Table 4.

Figure 2. Morphological changes registered in A549 cells after 24 hours of exposure to guava leaf extracts. A) Growth control; B) crude extract at 100 mg/ml, and C) purified extract at 5 mg/ml.



T0 = time zero; T24= after 24 hours of exposure.

Table 4. Percentage of viable A549 cells after 24 hours of exposure to *Psidium guajava* L. leaf extracts.

Experimental group	Viable cells (%)
Growth control	90.0
Crude extract, 100 mg/ml	38.0
Negative control of the crude extract	90.9
Purified extract, 5 mg/ml	11.3
Negative control of the purified extract	87.8

The presence of raw and purified guava leaf extract caused morphological changes in lung cells (Figure 2). In both cases, the cells lost their epithelial morphology and became rounded shape, which is related to cell death [25]. Moreover, when cells were exposed to purified polyphenols, a significant number of exosomes, vesicles involved in intracellular communication, and numerous physiological and pathological conditions, including

exposure to acute stressors, were observed [26]. On the other hand, the percentage of viable cells decreased to 38% with crude extract and more drastically to 11.3% with purified extract, even though the latter was used at a lower concentration (Table 4).

3. Discussion

Antimicrobial resistance is one of the main threats to public health. This global concern has not been controlled or slowed down despite the measures taken to avoid the irresponsible use of antibiotics [27]. Certainly, there is an urgent need for new and better antimicrobials. Multiple articles have reported that *Psidium guajava* L. leaf extracts have antimicrobial activity [28–35]. Regardless, many of the strains tested are ATCC, which do not represent the bacteria that cause diseases in daily life [36].

The ability to form biofilms, to resist desiccation, and the presence of virulence factors such as surface adhesins and secretion systems are some of the qualities that allow *A. baumannii* to thrive in different environments and make it difficult to eradicate [37]. Along with, *A. baumannii* can acquire antimicrobial resistance through different mechanisms: by disrupting the antibiotic target site, controlling the passage of antibiotics across membranes, and by enzymatic neutralization of antibiotics [38]. In addition, one of this bacterium best weapon is its remarkable genetic plasticity, which facilitates rapid genetic mutations and rearrangements, as well as the integration of foreign sequences [38]. Specifically, XDR *A. baumannii* can produce β-lactamases, flow pumps, MDR proteins, modify penicillin-binding proteins and decrease porin permeability [39].

Although *A. baumannii* has a wide variety of strategies to acquire and exhibit resistance, the phytochemicals present in guava leaf extract may also act through multiple mechanisms, including cell membrane destabilization, inhibition of biofilm formation, hindering of quorum-sensing, uncoupling of oxidative phosphorylation, inhibition of nucleic acid synthesis, alteration of intracellular pH, suppression of toxins and virulence factors, and inhibition of important enzymes such as ATPase [24,40]. This arsenal of such varied mechanisms is probably the key that allows plant extracts to be effective in the fight against resistant strains.

Studies on the antimicrobial activity of guava leaves in *A. baumannii* are scarce, among them, Bernabe-Díaz *et al*., [41] evaluated the effect of different concentrations of ethanolic leaf extract of *P. guajava* L. in *A. baumannii* ATCC 19606, obtaining the best result at 125 mg/ml with an inhibition diameter of 16.78 mm, while at 75 mg/ml they registered a diameter of 10.33 mm. On the other hand, Saleh *et al*., [42] analyzed the effect of five leaf extracts of *Psidium guajava* L. (100 mg/ml) obtained with different solvents in a clinical isolate of *A. baumannii*, the inhibition diameters ranged from 12 to 19 mm and the best result was obtained using methanol as solvent, which was also selected as extraction solvent in this work. The inhibition diameters in our study using guava leaf extract in single action ranged between 9 and 12 mm with 100 mg/ml of crude extract and between 10.66 and 11.66 mm with 5 mg/ml of purified extract. The results are within the range, and in the case of the purified extract, it was possible to have similar ZDI values with a lower concentration of those reported in other studies and in our own assay using crude extract, highlighting that this may be a possible strategy to improve the antimicrobial activity of plant extracts.

The improvement in antimicrobial activity through the purification of polyphenols may result from removing phytochemical compounds with limited or no antibacterial activity, which reduces the effectiveness of the crude extract [43,44]. It is important to mention that the purification process was not specific; a large mixture of polyphenols was obtained, each with different structures and modes of action. Among the potential antimicrobial mechanisms of polyphenols are their ability to interact with different compounds involved in microbial metabolism, damage to the structure and formation of the bacterial surface through the accumulation of hydroxyl groups in the lipid layers and the suppression of microbial virulence factors such as biofilm formation and toxin production [24,45]. Figure 3 illustrates the potential mechanisms of action of guava leaf extract.

Notably, the antimicrobial activity can be improved through different approaches, among them, the use of delivery systems such as nanoemulsions, encapsulations, micellar and liposomal nanocarriers, and nanoparticles [46-49]. For example, Zhang *et al*., [50] nanoencapsulated essential oil from guava leaves in chitosan and reported increased antimicrobial activity against MDR *Klebsiella pneumoniae*. Similarly, Rakmai *et al*., [51] reported improvements in the activity of guava leaf oil against *Staphylococcus aureus* and *E. coli* after being encapsulated in hydroxypropyl-β-cyclodextrin. Another strategy that has

gained importance due to its ease and low cost and which this study has focused, is the synergistic interaction between phytochemicals and antibiotics. Some of advantages of this one are the possible restoration of an existing drug or the reduction of the dose of new synthetic antibiotics [39].

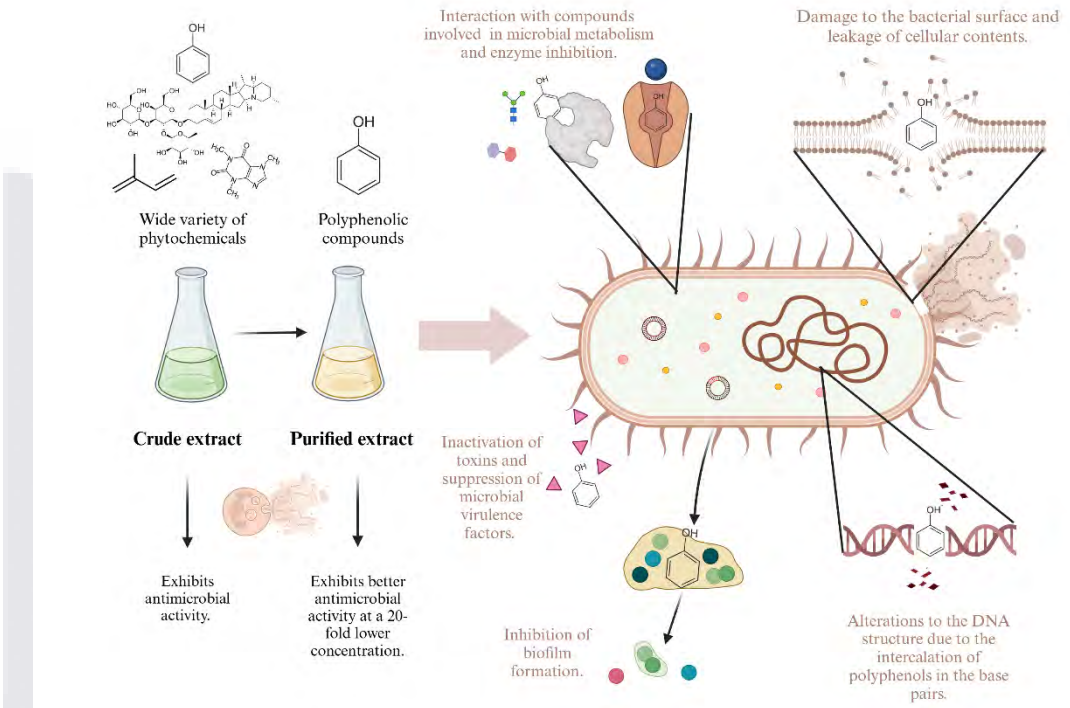


Figure 3. Potential mechanisms of action by which guava leaf polyphenols exhibit antimicrobial activity. Adapted and modified from: Lobiuc, *et al*., [45].

Aminoglycosides, including gentamicin were commonly used during the 1970s. Nowadays, they remain as drugs of choice for treatment of *Acinetobacter* infections. Nevertheless, resistance to aminoglycosides has increased in the recent years, including most of the first-line antibiotics [52,53]. Gentamicin was selected in this study since it is a widely known and used drug with very low cost compared to the latest generation antibiotics, whose effectiveness needs to be restored [54]. Fortunately, the synergy of guava leaf extract with gentamicin was observed in the agar diffusion assay, resulting in increases in the inhibition diameters up to 40.47% with crude extract and 48.57% with purified polyphenols. Similar results were obtained determining the minimum inhibitory concentration (MIC) since the presence of gentamicin allowed MICs to be determined for seven of the ten clinical isolates evaluated, while with the extract as single agent, MICs can only be determined in two strains.

Likewise, Phatthalung *et al*., [55] reported synergy between guava leaf extract and novobiocin against *A. baumannii* ATCC 19606, reinforcing the idea that guava leaf extract can be a valuable ally in the fight against this pathogenic microorganism and the combination with antibiotics is a feasible strategy.

Regarding the determination of MIC and MBC, with the purified extract as a solitary agent, MIC could only be determined for strains A26 and A38 with a 5 mg/ml concentration in both cases. On the other hand, with the combination with 16 µg/ml of gentamicin, MIC of 5 mg/ml could be detected for isolates A2, A4, A25, A26, A34 and A38. In the case of A27, a MIC of 2.5 mg/ml was recorded, the lowest in our study. However, no MBC could be determined. Different MIC values have been reported by other authors against clinical isolates of *A. baumannii*. These include values ranging from 116.7 to 8.2 mg/ml for different leaf extracts of *Psidium guajava* L. [42], concentrations of 25 and 50 mg/ml for the methanolic extract of *Hibiscus sabdariffa* L., and minimum bactericidal concentrations (MBC) of 50 and 100 mg/ml [56], MIC of 8.67 ± 1.93 mg/ml and MBC of 9.24 ± 1.95 mg/ml for the essential oil of rhizomes of *Zingiber cassumunar* Roxb against an XDR *A. baumannii* [57]. The variation in these values is due to numerous factors, including the type of plant material used, the climatic and soil conditions, the extraction method (temperature, solvent, time), and differences in methodology [58,59]. The minimum inhibitory concentration (MICs) established in the present study are lower than those reported by other authors, even when considering other plant species and strains that are not extensively drug-resistant (XDR). Although it did not exhibit bactericidal effects at the concentrations used, it did lead to a significant decrease in microbial growth in all cases, even without gentamicin, denoting its potential as an antimicrobial agent.

It is crucial to establish the safety and confirm that guava leaf extract does not have any harmful effects to reach its application in the future. As a first approach, we decided to assess the viability and morphology of human lung cells line ATCC A549 in the presence of plant extract. After 24 hours of exposure to both the crude and purified extracts, we observed morphological changes and a decrease in the percentage of viable cells. The purified extract had a more significant effect, reducing the percentage of viable cells by almost 90% at a concentration of 5 mg/ml, while the crude extract, at a concentration of 100 mg/ml, reduced it by 62%. Therefore, the purification of polyphenolic compounds from guava leaf extract

improved the antimicrobial activity but also increased the cytotoxicity of the extract. This is not surprising, given that while dietary polyphenols are considered safe and have been attributed to beneficial effects, there is evidence that they can also have deleterious effects at certain concentrations, especially among vulnerable populations [60]. Furthermore, this study observed a loss of epithelial morphology and the appearance of typical features of apoptosis such as cell shrinkage, rounded shape and the presence of apoptotic vacuoles. Similar findings were reported by other researchers including Hsu [61], who exposed A549 cells to *Typhonium blumei* extract, and Cheng [62], who exposed them to different extracts of *Bupleurum scorzonerifolium*. Consequently, it is fundamental for further research to study the potential toxicity of guava leaf extract. Natural extracts are generally considered safe due to their natural origin. Nonetheless, they can also exert cytotoxic effects, especially if they are used indiscriminately [63]. Although there are multiple new studies evaluating the safety of guava leaf extract in different *in-vitro* and *in-vivo* models [64–68], the results are variable, and more information is needed to ensure its safe use.

4. Materials and Methods

4.1 Plant material

The collection of guava leaves was carried out manually and randomly from different pesticide-free specimens in Aguascalientes, Mexico, in July 2023. Only green and healthy leaves were selected and thoroughly washed with distilled water to eliminate traces of dust and other contaminants. They were subsequently dried at 40°C for 72 hours [69,70] and pulverized with an electric processor to finally store the powder obtained at room temperature in an airtight container protected from light until use [71].

4.2 Extraction of phytochemicals from guava leaves

The plant extract was obtained using the Soxhlet technique during seven siphons [72] with a solid-liquid ratio of 1:20 (5 grams of plant material in 100 ml of solvent). Methanol was selected as the solvent since preliminary tests in our laboratory showed that it has a better extractive power, which coincides with what has been published in other studies [73–75]. The obtained extract was diluted with distilled water to obtain an 80% methanol solution and

then subjected to 50°C in an oven to eliminate the solvent and preserve the aqueous fraction adjusted to a concentration of 100 mg/ml (stock solution). The obtained extract was diluted with distilled water to obtain an 80% methanol solution and then subjected to 50°C in an oven to eliminate the solvent and preserve the aqueous fraction adjusted to a concentration of 100 mg/ml (stock solution). Finally, it was filtered with 0.2 µm membranes [76] and stored protected from light at 4°C until use [77]. Details on the characterization of the composition of the guava leaf extract used in this study were previously published [44].

4.3 Purification of Polyphenolic Compounds

Phenolic compounds from guava leaves were purified with the commercial adsorbent Amberlite XAD-16 (Sigma-Aldrich, Saint Louis, MO, USA). Briefly, the extracts were added to a column packed with the adsorbent as a stationary phase, and distilled water was added to eliminate sugars and other compounds present in the extract. Finally, the polyphenolic compounds were eluted with absolute ethanol [78,79]. The solvent was removed in an oven at 50 °C for 24 h, and the crystals obtained were solubilized in 5% DMSO aqueous solutions at a concentration of 10 mg/ml (stock solution) and preserved protected from light at 4°C [77].

4.4 Microorganisms and culture media

A total of ten clinical isolates previously phenotypically characterized as XDR *A. baumannii* isolated from patients with nosocomial infections were used. The microorganisms were donated by the Hospital Centenario Miguel Hidalgo, Aguascalientes, Mexico, a tertiary care institution for the population without health insurance. Antimicrobial susceptibility profiles of the clinical strains of *A. baumannii* are described in Supplementary Table S2. In addition, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains.

All *A. baumannii* strains were cultured in MacConkey agar (BD-Bioxon, Cuautitlan Izcalli, Mexico) while *E. coli* and *P. aeruginosa* were cultured in brain heart infusion medium (BD-Bioxon, Le Pont de Claix, France). Moreover, all antimicrobial evaluations were carried out in Mueller-Hinton culture (BD-Bioxon, Le Pont de Claix, France).

4.5 Antimicrobial activity of guava leaf extract and potential antimicrobial synergic effects with gentamicin.

The antimicrobial activity of the guava leaf extract was analyzed using the agar diffusion technique. Briefly, XDR *A. baumannii* clinical isolates were grown on MacConkey agar for 24 hours at 37°C. Then, 0.85% NaCl solutions (Baker, Mexico) were standardized to 0.5 McFarland for a final inoculum of 1.5×10^8 CFU/mL [80]. Mueller-Hinton agar (30 ml) was inoculated by the pour plate technique using 1 ml of the standardized inoculum solution [81]. Once the agar was solidified, 5 mm diameter wells were hole made with the help of a sterile pipette tip, and 50 µl of the solution to be tested was added to each well [77,82]. The crude guava leaf extract was used at 100 mg/ml, and the purified extract at 5 mg/ml. Controls were gentamicin (16 µg/ml), sterile distilled water and sterile 5% DMSO aqueous solution. Afterward, to assess the possible synergistic effect of the extracts with gentamicin, the extracts were combined with 16 µg/ml of the antibiotic. The plates were incubated at 37°C for 24 hours. After this period, the clear zones (or zones of inhibition) were identified around the wells, corresponding to the antimicrobial activity, and the inhibition halos were measured [80]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains [83] All assays were performed in triplicate.

The results are reported as the average of the zone diameter of inhibition (ZDI), the percentage increase of the ZDI, and the growth inhibitory indices (GIIs) which allow us to corroborate the synergistic activity of the combination of the guava leaf extract with the antibiotic. The Growth Inhibition Index (GII) is used to compare the inhibitory effects of a combination of two antimicrobial agents or compounds against the effects of each agent individually, allowing for an assessment of their type of interaction such as synergy, in terms of ZDI values. The GIIs and the percentage of increase were calculated using the following formula [84-89]:

$$\textbf{Increase of the ZDI}\ (\%) = \frac{ZDI\ i\text{n combination} - ZDI\ \text{of the extract in single action}}{ZDI\ \text{of the extract in single action}} \times 100$$

$$\textbf{GIIs} = \frac{ZDI\ \text{in combination}}{ZDI\ \text{of the two agents in single action}}$$ Haga clic o pulse aquí para escribir texto.

The effect was considered synergistic if the value of GIIs > 0.5, additive if GIIs = 0.5, or antagonistic GIIs < 0.5. Specifically, the agents that are being evaluated are the GLE or GLEP with gentamicin [84,85].

4.6 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined in 96-well microplates (Costar® 3370, Corning, NY, USA) by performing two-fold serial dilutions of the guava leaf extract in triplicate. Only the purified extract was used with concentrations that ranged between 0.625 and 5 mg/ml and were analyzed alone or in combination with gentamicin (16 µg/ml). Inoculums were prepared from 24-h cultures by standardizing Mueller-Hinton broths to 0.5 McFarland, which corresponds to a bacterial load of 1.5×10^8 CFU/mL. The purified extract was first diluted to the highest concentration to be tested, and then serial two-fold dilutions were made. The 96-well plates were prepared by dispensing 50 µl of the standardized inoculum into each well, and 50 µl of the extract at different concentration with or without gentamicin. The final volume of each well was 100 µl, and the final concentration of microorganism was 5×10^7 CFU/ml. A growth control and blanks containing Mueller-Hinton medium and extract without inoculum (at each concentration tested) were included for each strain. The microplate was incubated at 37°C for 24 hours and subsequently Mueller-Hinton agar plates were inoculated in triplicate to count the CFU. The optical density (595 nm) of the microplate was measured using a spectrophotometer (Benchmark plus Microplate Reader, BIO-RAD). The MIC was defined as the lowest concentration of the extract necessary to inhibit bacterial growth (where turbidity is not present), while MBC is the lowest concentration of the extract that eliminate 99% of the bacteria without showing growth on agar plates [90–92].

4.7 Effect of guava leaf extract on A549 lung cells.

The human lung cancer A549 / CCL-185 ™ cell line was obtained from the ATCC (American Type Culture Collection). The cells were cultured in high glucose DMEM (Dulbecco's Modified Eagle Medium, Gibco, NY, USA) with 5% fetal bovine serum, 1% penicillin-

streptomycin and amphotericin, and 1% GlutaMAXTM (Gibco, NY, USA). Cells were incubated at 37°C, in a 5% CO_2 environment. Once the culture was established, cells were seeded in a 24-well microplate at a concentration of 5×10^4 cells per well and incubated until they reached confluence. Thereafter, the cells were exposed to crude extract (100 mg/ml) and purified extract (5 mg/ml) for 24 hours. Finally, morphological changes and the percentage of viable cells were evaluated using the commercial dye trypan blue and digital microscope camera (AmScope MU-500, USA, 2016). Sterile distilled water and a 5% aqueous DMSO solution were used as negative controls. The experiment was performed in triplicate, and the percentages of viable cells were calculated using the following formula [93]:

$$\textbf{Viable cells}\ (\%) = \frac{\text{total number of viable cells}}{\text{total number of cells}} \times 100$$

4.8 Statistical analysis.

Statistical analysis was performed with Prism (GraphPad, Boston, Massachusetts USA, v. 8.0.1). Data were analyzed using one-way ANOVA and Tukey post-hoc test (alpha=0.05); results are presented as the mean ± SD.

5. Conclusions

The leaf extract of *Psidium guajava* L. is a potential antimicrobial agent that exhibit activity even against strains that are extremely resistant to antibiotics. Purification of polyphenols improved the extract's activity. When combined with gentamicin, an antibiotic that by itself had no effect against the strains evaluated, the results show a synergistic effect. However, the presence of the plant extract caused morphological changes in ATCC A549 lung cells after 24 hours of exposure, decreasing the percentage of viable cells. Therefore, for future application, multiple challenges need to be overcome, including further studying in its safety and in finding a way to reduce its toxicity, evaluating its long-term stability, as well as studying its bioavailability. To our knowledge, this is the first report of the antimicrobial activity of guava leaf extract against XDR *A. baumannii.*

Author Contributions: Conceptualization, D.G.M., F.Y.R.C. and A.L.G.B.; methodology, D.G.M., F.Y.R.C, F.G.G. and E.V.P; writing—original draft preparation, D.G.M., F.Y.R.C. and A.L.G.B.; formal analysis, D.G.M., F.Y.R.C. and A.L.G.B.; investigation, D.G.M., F.Y.R.C., I.G.O.G., F.G.G., N.A.C.V., E.V.P. and F.J.A.G.; resources, D.G.M., A.L.G.B.; writing—review and editing, D.G.M., F.Y.R.C., F.G.G., I.G.O.G., N.A.C.V., F.J.A.G., J.M.A.G., M.O.C, and M.G.G.; supervision, A.L.G.B., N.A.C.V., M.O.C., and F.J.A.G. Finally, J.M.A.G. and M.G.G. provided the *A. baumannii* XDR strains for our study. All authors have read and agreed to the published version of the present manuscript.

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Chapter 3.

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Article

Psidium guajava L.*:* from byproduct and use in traditional Mexican medicine to antimicrobial agent.

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Keywords:

Guava, extracts, antimicrobial, phenolic compounds, biofilm, by-products recovery.

Abstract

Mexico is one of the largest guava producers in the world, so it has access to a huge amount of waste and byproducts obtained after the industrial processing of the fruit. This review discusses the potential recovery of this residue for its application as an antimicrobial agent, considering the phytochemical composition, the bioactivity reported *in-vivo* and *in-vitro*, and the toxicology of the plant. Nowadays there is a growing demand for more natural and safer products, so the use of guava extracts is an interesting initiative, especially due to its

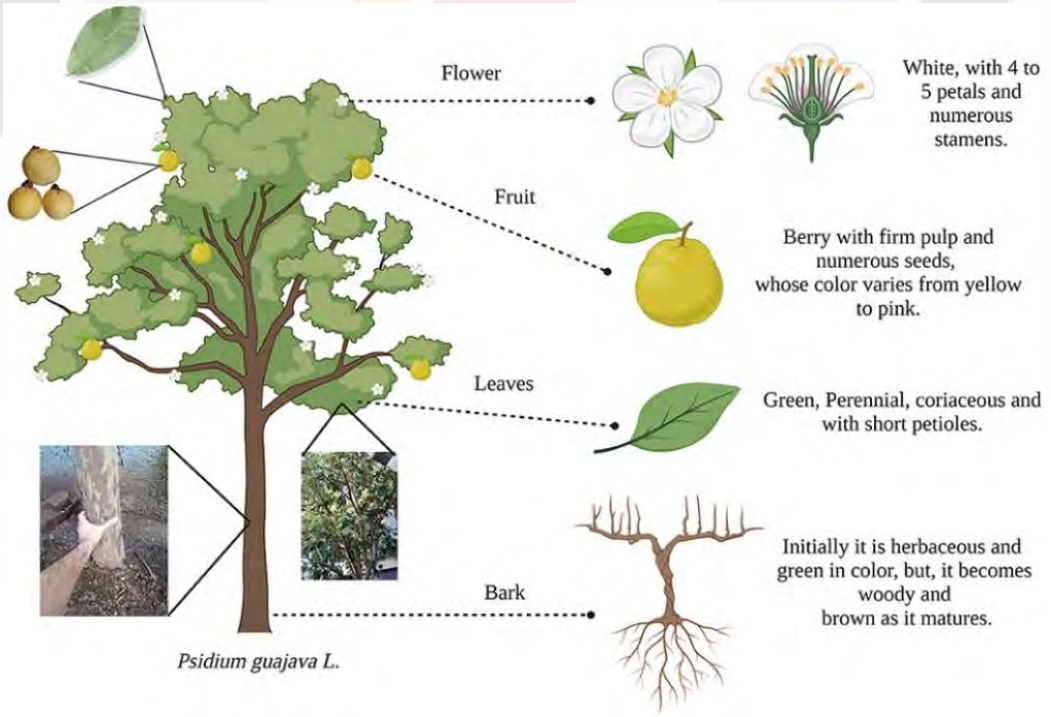
availability in the country, its wide variety of traditional uses, and its phytochemical profile. This review highlights the importance and potential of this plant in today's world.

1. Introduction

Psidium guajava L. is a native American shrub that can grow in tropical environments around the world (Rajan, *et al*., 2019). It can reach up to 7 meters in height and 25 cm in diameter in the trunk when it reaches maturity. Its bark is smooth, thin and coppery brown, while its leaves are green, perennial, coriaceous and with short petioles. This plant also has white flowers with 4 to 5 petals and numerous stamens, characteristic of the *Myrtaceae* family (Hussain, *et al*., 2021).

However, its economic importance lies mainly in its fruit, the guava, a juicy berry with numerous seeds and a slightly acid taste. Its shape and color depend on the variety, so we can find guavas from yellow to bright pink and round, ovoid or pear-shaped (Hussain, *et al.,* 2021). The morphological characteristics of *P. guajava* L. are illustrated in Figure 1.

Figure 1. Morphological characteristics of *Psidium guajava* L.



The guava belongs in the *Myrtaceae* family, known for having numerous species with high antioxidant activity (Bouchoukh, *et al*., 2019). On the other hand, the *Psidium* genus has approximately 150 species of shrubs, of which *Psidium guajava* L. is the best known and distributed worldwide (Mendes-Pereira, *et al*., 2016). Regarding its phenology, this shrub flowers mainly in spring and fruiting can appear throughout the year, mostly during the summer months (Landrum, *et al*., 2021).

Guava worldwide production is estimated to be around 40 million tons. Even if fresh guava trade is limited internationally, the marketing of a wide variety of products derived from its fruit such as preserves, jams, jellies and syrups is becoming more common (Angulo-López, *et al*., 2021). The highest production of guava can be found in India, Thailand, Brazil, and Mexico (Sánchez, *et al.* 2021).

Mexico is a key guava producer in the international market, whose export volume increased more than 150% in less than 10 years, from 4,306 tons of guava exported in 2009, to 10,850 tons exported in 2018 (Landrum, *et al*., 2021). Unfortunately, the Mexican agricultural sector presents serious post-harvest problems, generating enormous fruit losses during the different stages of the production chain. Guava is one of the most affected fruits with a waste of more than 50% of its national production (González-Arias, *et al*., 2021).

Specifically, in the industrial processing of guava, a heterogeneous mixture of peels, seeds and pulp is generated, and it that can represent up to 30% of the total mass. Guava leaves and bark are other byproducts that are obtained, mainly during the harvest of the fruit (Kuila, *et al*., 2020). Approximately 80 kg of waste per metric ton of fresh fruit is produced during guava processing (Lim, *et al*., 2018). Unfortunately, and in most cases, the waste generated is thrown into landfills or is incinerated, increasing the environmental load and the total cost of production due to the handling and transportation that these residues require (Kuila, *et al*., 2020; Khalid, *et al*., 2022).

The aim of this updated review is to expose the potential of *Psidium guajava* L. as an antimicrobial agent to promote the valorization of guava agro-industrial waste and byproducts in Mexico.

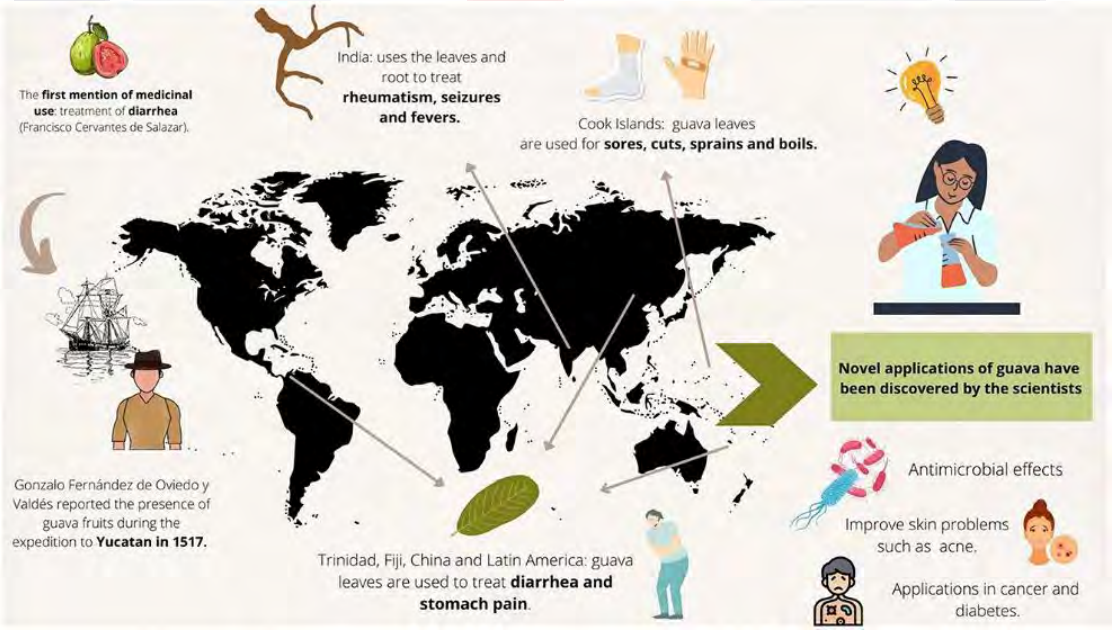
2. Review methodology

The search engines Google scholar, Elsevier, Springer, MDPI and Science Direct were used to find research articles and reviews related to *Psidium guajava L*. Documents from 2000 to 2022 in Spanish and English were included, focusing in the most recent articles. Articles published before the 2000s were excluded. Book chapters, official sites of the Mexican government and Mexican companies were also consulted. The keywords used were "*Psidium guajava* L.", "traditional uses", "phytochemistry", "biological activities", "toxicology", "*in-vivo* evaluation" and "antimicrobial effects" "diabetes".

3. Traditional uses

Psidium guajava L. has been traditionally used as a medicinal plant in different places around the world. Its applications are highly diverse and range from the treatment of gastrointestinal diseases such as vomiting and simple diarrhea to the treatment of wounds, caries, and cough (Gutiérrez, *et al*., 2018; Alonso-Castro, *et al*., 2012; Juárez-Vázquez, *et al*., 2013; Omayio, *et al*., 2019) as we can see in Figure 2.

Figure 2. Traditional uses of guava around the world and new perspectives and applications.



All parts of the bush have been used to treat different disorders (Borah, *et al*., 2019). For example, the leaves and fruit are used to treat respiratory and digestive problems, while the seeds and the leaves have been used as an antispasmodic, anti-inflammatory and even in the control of hypertension and diabetes (Barbalho, *et al*., 2012).

Its use also varies depending on geographical location. It has been reported that in Trinidad, Fiji, China and distinct parts of Latin America, guava leaves are used to treat diarrhea and stomach pain (Gutiérrez, *et a*l., 2008). On the other hand, in Uruguay they are used to wash the vagina and uterus, especially leucorrhoea cases; in the Cook Islands, guava leaves are used for sores, cuts, sprains, and boils; while in India they are used together with the root to treat rheumatism, seizures and fevers (Gutiérrez, *et a*l., 2008). In the Amazonia this plant is used for the treatment of menstrual disorders, stomach pain and vertigo, in Cuba, for colds, dysentery, dyspepsia, diarrhea and hypertension. Finally, in Haiti, guava is used for epilepsy, diarrhea and different skin disorders (Omayio, *et al*., 2019).

Thanks to scientific research, novel applications of guava have been discovered in immune and endocrine systems diseases, as well as in cancer and diabetes. In addition, studies have reported that guava leaf extracts can be used to improve skin problems such as acne and hyperpigmentation (Díaz-de-Cerio, *et al*. 2017).

3.1. Traditional uses of Psidium *guajava L*. in Mexico

In Mexico, herbalism is an ancient practice that has been used to this day and has great cultural and economic importance. However, in most cases, the active ingredients that provide the beneficial effect are unknown and, therefore, it is a field of study of high scientific interest (Guzmán, *et al*., 2017). Guava leaves have been used for medicinal purposes in the country since very remote times, their presence in historical documents on indigenous herbalism has been constant for at least five hundred years (Rivera-Arce, *et al*., 2003).

This shrub used to be called by the ancient Mexicans as "*xalxócotl"*, a word in Nahuatl that refers to a fruit that has a "hard and acid shell (*xócotl*) and a sandy texture (*xalli*), due to its abundant seeds (Rivera-Arce, *et al*., 2003). Guava can be called in diverse ways depending on the Mexican state, for example, in Chiapas, this plant is usually called pata, pocscuy, potok, pox or sumbadam; in Michoacán it is known as enendi, in Nayarit as caaru, in Morelos

as coloc or jaljocote pichi and in Veracruz as asiwit, cuympatan or pitchcuy (Fonseca-Chávez, *et al*., 2020).

The uses of *P. guajava* L. in the country are as diverse as its names, however, its most common medicinal use is to treat stomach pain. Diarrhea, dysentery, fever, and cough are treated with infusions of guava and its leaves; in the case of skin problems, the leaves are cooked and applied locally. The infusion of guava buds and leaves are used as a de-wormer and guava tea is used to cure scares, using it to give short baths (Fonseca-Chávez, *et al*., 2020).

The medicinal use of guava can also vary slightly according to the geographical location in the country, for example, while in southern Veracruz it has been reported that the plant is used to treat diarrhea (Leonti, *et al*., 2003), in the Huasteca Potosina it is also used to treat herpes, wounds, toothache, gastritis and rashes (Alonso-Castro, *et al*., 2012). On the other hand, in Guerrero, the infusion of guava leaves is used to cure cough, fever, flu and stomach pain (Juárez-Vázquez, *et al*., 2013). Figure 3. represents and summarizes the different names and traditional uses of guava in Mexico

Figure 3. Traditional uses and names of guava in different states of Mexico**.**

4. Current importance of guava in Mexico

In Mexico there are twenty entities that harvest this fruit, with Michoacán being the main producing state, followed by Aguascalientes and Zacatecas (SIAP, 2019). In 2020, a planted area of 29,872.67 hectares was reported, from which a production of 287,273.02 tons of guava was obtained. This meant an economic spill of $1,664,607.62 billion Mexican pesos (SIACON NG, 2021). The most harvested variety of guava in the country is undoubtedly the "media-china", while the "china" and the "criolla" are produced in far less quantity (SIACON NG, 2021).

It should be noted that, as of 2008, fresh guava started being sent to the United States of America (Quintero-Ramírez, *et al*., 2019). This is interesting given that, traditionally, the export of this fruit was conducted in its dehydrated or processed versions; however, its nutritional and exotic appeal increased the interest of developed countries like United States, Canada, and Japan (Morales-Troncoso, 2009)

A wide variety of guava processed products such as candies, rolls, ates, ice cream, jams, jellies, juices, and even "guava mole", a highly seasoned sauce, can be found in Mexico. However, its importance lies not only in its alimentary use, but it also has great relevance for its cosmetic and medicinal applications. In the Mexican market we can also find multiple products that contain guava leaves, like extracts, pills, concentrates and food supplements that are presented as a natural remedy for stomach pain or as an antioxidant and anti-aging agent. The most relevant example is a drug to relieve the symptoms of colitis called *QG5*, based on a dry extract of *Psidium guajava* L. which is sold in pharmacies throughout the country. The effect of *QG5* is attribute to the presence of quercetin (Marcas.GenommaLab, 2022).

The composition of the extracts marketed in the country is not clear and, in general, their beneficial effects are attributed to what is already known for its traditional uses. Hence the importance of knowing which compounds are responsible for such effects, and thus be able to move from tradition to science. This will allow us to give a better use to the extract, obtaining a better and safer beneficial effect and we can even get to know interesting new bioactivities.

5. Phytochemical composition

Psidium guajava L. chemical composition includes compounds such as tannins, phenols, flavonoids, saponins, carbohydrates, alkaloids, sterols, and terpenoids (Millones-Gómez, *et al.* 2020). It is important to consider that the type and abundance of phytochemicals can vary depending on the microclimate and soil conditions of the habitat (Lavola, *et al*., 2017), but also depending on the plant tissue and seasonal changes (Hardage, 2009).
The most analyzed part of this shrub is undoubtedly its leaves, given its frequent use as a medicinal remedy. Shabbir *et al*., (2020) reported the approximate composition of guava leaves: 82.47% moisture, 3.64% ash, 0.62% fat, 18.53% protein, 12.74% carbohydrates, 103 mg of ascorbic acid (vitamin C) and 1717 mg of total phenolic compounds (mg of gallic acid equivalents (GAE) / g). It should be noted that Shabbir *et al*., (2020) also observed a higher concentration of phenolic compounds in the leaves than in the seeds and fruits of guava.

Guava leaves are also a rich source of vitamins and minerals, such as calcium, potassium, sodium, magnesium, iron, sulfur, vitamin B and C (Adrian, et al., 2015). Even, Thomas, *et al*., (2017) mentioned that the leaves have a higher concentration of vitamin B (14.80 mg/100 g), calcium (1660 mg/100 g), magnesium (440 mg/100 g), phosphorus (360 mg/100 g), and iron (13.50 mg/100 g) compared to the fruits, however, the fruit is richer in vitamin C (228.3 mg/100 g) and potassium (417 mg/ 100 g).

The higher concentration of calcium in guava leaves (1660 mg/100g) compared to the concentration in the fruit (18 mg/100g) may be since its transpiration rate is higher (Gilliham, et al., 2011). Similarly, there is a trend towards major magnesium allocation in transpiring organs such as leaves (440 mg/100 g) and flowers rather than roots and fruits (22 mg/100 g) (Barker, et al., 2015).

Furthermore, it is important to consider that the nutrient content varies considerably between different plant organs, as well as by the age of the tissue in question. Beyond that, the nutrient content largely depends on optimal uptake and soil quality (Mengel, et al., 2000).

The polysaccharides found in guava leaves have also had great relevance in recent years since they have been found to be beneficial for the treatment of diabetes mellitus symptoms,

as will be discussed in more depth later, and they can also be used as antioxidant additives in food (Kumar, *et al*., 2021).

Díaz-de-Cerio, *et al*., (2016) identified more than 70 phenolic compounds in guava leaves by HPLC-DAD-QTOF-MS using hydro-alcoholic extracts obtained by sonication. Table 1. shows the main compounds identified in extracts of *Psidium guajava* L. reported by different authors.

Table 1. Phytochemical composition of different extracts of *Psidium guajava* L.

Method	Extract	Location	Quantity of detected compounds	Main identified compounds	Reference
UPLC-ESI-QTOF-MS	Tannic Fraction of Dried Leves	South of Ceará, Brazil	10	Vescalagin, Catechin, Quercetin, Guavinoside C, 2,6-dihydroxy-3-methyl-4- O-(6″-O-galloyl-β-Dglucopyranosyl)-benzophenone, (1S*,5S*)-2,2-Bis(biphenyl-4-yl)-5-indian-1-yltetrahydrofuran(IV-81).	Bezerra, *et al*., 2018.
UPLC-ESI-QTOF-MS	Flavonoid Fraction of Dried Leaves	South of Ceará, Brazil	11	Catechin, Ellagic acid, Reynoutrin, Guajaverin, Myrciaphenone B, Guavinoside B, Morin.	Bezerra, *et al*., 2018.
GC-MS	Leaves essential oil	Rio Verde in Goiás, Brazil	17	Limonene, 1,8-Cineole, α-Copaene, β-Caryophyllene, α-Humulene, 4,11-Selinadiene, γ-Muurolene, Aromadendrene oxide, δ-Selinene, α-Panasinsene, *trans*-Nerolidol, β-Caryophyllene oxide, Humulene epoxide II, Longipinene epoxide, *epi*-α-Muurolol, Selin-11-en-4α-ol, α-Cadinol.	Silva, *et al*., 2018.
GC-MS	Leaves essential oil	El-Behera Governorate, Egypt	10	α -Pinene, Benzaldehyde, ρ -Cymene, Limonene, 1, 8-Cineole, β -*cis*-Ocimene, γ -Terpinene,	Soliman, *et al.,* 2016.

Method	Extract	Location	Quantity of detected compounds	Main identified compounds	Reference
				α -Terpineol, β -Caryophyllene, α -Humulene.	
GC-MS	Leaves ethyl acetate extract	Vadlamudi, India	25	Caryophyllene, α-Copaene, *cis*-Muurola-3,5-diene, Humulene, Cyclosativene, *cis*-à-Bisabolene, Spathulenol, Cubenol, Torreyol, α-Cadinol, α-Bisabolol, Isoamyllaurate, Bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl- 5,6,6-Trimethyl-5-(3-oxobut-1-enyl)-1-oxaspiro[2.5]octan-4-one.	Shaheena, *et al.,* 2019.
GC-MS	Guava seed oil	Punjab, India	33	N-Acetylethylenediamine, Cyclobutylsilane, Oxirane, Valeric acid, 2-Heptenal, D-Limonene, Decane, 2,6-Dimethyl-6-nitro-2-hepten-4-one, 2,4-Nonadienal, 4,7-dimethyl Undecane, 9-methyl-1-undecane, Cubebene, 2,4-dihydroxy-6-methyl Benzaldehyde, Caryophyllene, Caryophyllene oxide, 2,4,6-trimethyl octane, 6,9-Heptadecadiene, 2,7,10-trimethyl dodecane, n-Hexadecanoic acid, 2-Chloroethyl linoleate, Octadecanoic acid, 7-Nonenamide.	Kapoor, *et al*., 2020.
GC-MS	Methanolic pulp extract (industrialized liquid pulp with food additives like BHT)	Brazil	36	L-5-Propylthiomethylhydantoin, Ethanediamide, L-Alanine, methyl ester, Sulfur tetrafluoride, Pentanoic acid, 3-methyl-4-oxo, Isobutyl acetate, 2-Ethoxytetrahydrofuran, Furfural, 2-Azetidinone, 1-phenyl-, Ethylbenzene,	Dos Santos, *et al*., 2020.

Method	Extract	Location	Quantity of detected compounds	Main identified compounds	Reference
				Butyrolactone, 1,2-Propanediol, diacetate, Propanedioic acid, diethyl ester, 1,4-Butanediol, diacetate, Benzoic acid, Benzaldehyde, 2,4-dimethyl, 5-Hydroxymethylfurfural, 1,5-Diacetoxypentane, 5-Acetoxymethyl-2-Furaldehyde, Pentadecane, Hexadecanoic acid, ethyl ester, 7-Octadecenoic acid, methyl ester, 7-Tetradecenal.	
GC-MS	Fruit essential oil	Pakistan	38	1R- α-Pinene, Decane, D-Limonene, Eucalyptol, Globulol, Caryophyllene, Tau-cadinol, Nerolidol, Copaene, α-Cadinol, aromandendrene, Humulene, (Z,Z)-2,6-farnesol, *cis*-α-Bisabolene, β-Bisabolene, *epi*-Globulol, α-Bisabolol, α-Selinene.	Sherazi, *et al*., 2019.
GC-MS	Leaves essential oil	Pakistan	39	Caryophyllene, Nerolidol 2, Aromadendrene, *cis*-α-bisabolene, Tetracosane, Octadecane, Z,Z,Z-1,5,9,9-tetramethyl-1,4,7-cycloundecatriene, β-bisabolene, Limonene, Octacosane, δ-cadinene, 1,4-cadadiene, β-caryophyllene.	Sherazi, *et al*., 2019.
GC-MS	Flowers essential oil	Rio Verde in Goiás, Brazil	13	*trans*-β-Caryophyllene, α-Humulene, Nerolidol, β-Selinene, α-Selinene, Germacrene D, δ-Selinene, Caryophyllene oxide,	Fernandes, *et al*., 2021.

Method	Extract	Location	Quantity of detected compounds	Main identified compounds	Reference
				Spathulenol, Globulol, Cubenol, *epi*-α-Cadinol, α-Cadinol.	

The recovery of the unused flesh, peels, seeds, flowers, bark, and leaves is a great opportunity to guava producing countries, considering the variety of bioactive compounds that can be extracted (Liu, *et al*., 2018).

Nonetheless, an aspect to consider during the use of industrial byproducts is the possible presence of food additives, as is the case of the pulp extract used by Dos Santos, *et al*., (2020), which contained different additives, among them, the antioxidant BHT, which may cause allergies, eczema, rash, and angioedema, therefore BHT cannot exceed an ADI of 0.5 mg/kg (Bensid, *et al*., 2020). As the presence of these additives probably remains after the extraction and can affect the activity of the extract, it is important to determine in what concentrations are they found in the plant material and what are the possible toxicological effects that they may cause depending on the route of administration.

On the other hand, several authors have reported that the main component of the extracts or essential oils of guava leaves is β-Caryophyllene (Sherazi *et al*., 2019, De Souza, *et al*., 2021, Jassal, *et al*., 2019), which has recently gained attention due to its potential application for the treatment of various disorders such as cancer, chronic pain, and inflammation (Maffei, *et al*., 2020). In addition, many of the plant extracts where β-Caryophyllene is found have antimicrobial effects, notwithstanding the role that this compound plays in said activity is still unclear. One of the proposed modes of action is that it alters the permeability of the bacterial membrane causing the formation of non-selective pores (Moo, *et al*., 2020).

Even if the mechanism of action and the interactions between all the components of the *Psidium guajava* L. extracts is ambiguous, many researchers around the world have identify different beneficial effects of their components, for example, their anti-inflammatory, antioxidant, and antimicrobial activity. Table 2 shows some properties attributed to compounds found in the different guava extracts.

Table 2. Bioactivity of different compounds found in *Psidium guajava* L. extracts.

Compound	Classification	Activity	Reference
Quercetin	Phenolic compound	Antioxidant, anti-inflammatory and anti-allergy.	Sampath, *et al*., 2021.
Gallic acid	Phenolic compound	Antibacterial, anti-fungal, antiviral, anti-inflammatory, antioxidant, anticarcinogenic, anti-diabetic.	Sampath, *et al*., 2021.
β-Caryophyllene	Sesquiterpene	Local anesthetic, anticarcinogenic, antioxidant, antibiotic, anti-inflammatory, neuroprotective anxiolytic, antidepressant and anti-alcoholism.	Sherazi, *et al*., 2019.
Copaene	Sesquiterpene	Anti-inflammatory and *in- vitro* anti-tumor activity.	Murthy, *et al*., 2020.
Limonene	Terpene	Anti-inflammatory and *in-vitro* anti-tumor activity.	Murthy, *et al*., 2020.
Catechin	Phenolic compound	Antioxidant, prevention or reduction of skin damage, activation of collagen synthesis and inhibition of the production of matrix metalloproteinase enzymes. Anti-microbial, anti-viral and anti-inflammatory.	Bae, *et al*., 2020.
Ellagic acid	Phenolic compound	Anti-inflammatory, anti-microbial, antioxidant.	Evtyugin, *et al*., 2020.
Humulene	Sesquiterperne	Anti-tumor, anti-microbial and anti-inflammatory. Great gastroprotective, cicatrizing, analgesic and antioxidant potentials.	Mendes de Lacerda Leite, *et al*., 2021.
Nerolidol	Sesquiterperne	Antioxidant, anti-inflammatory and anti-microbial. It also enhances skin penetration and permeation.	Chen, *et al*., 2020.
α-Pinene	Terpene	Anticarcinogenic, anti-inflammatory and anti-allergy.	Salehi, *et al*., 2019.
Eucalyptol	Terpene	Insecticide, anti-fungal, anti-microbial, anti-inflamatory and gastroprotective effect.	Vijayakumar, *et al*., 2020.
Guajaverin	Phenolic compound	Anti-viral and anti-bacterial activity.	Ortega, *et al*., 2017; Prabu, *et al*., 2006.
Bisabolol	Sesquiterpene	Anti-inflammatory, antispasmodic, anti-allergic and vermifuge properties	Kamatou, *et al*., 2010.
α-Cadinol	Sesquiterpene	Anti-bacterial and anti-fungal.	Chang, et al., 2003.

Observing the properties of some of its components (table 2), it is not surprising that numerous studies have reported the potential of different extracts of *Psidium guajava* L. as an antimicrobial agent, so in this section we will dedicate ourselves to present the most relevant discoveries of recent times on this topic to create an overview of the different

applications that we can get from this plant and what needs to be done to make these benefits available to society.

6. Antimicrobial effects of *Psidium guajava L.*

We are currently facing a serious global problem regarding microbial resistance, which has been aggravated during the COVID-19 pandemic. In Mexico, it has already been reported that strains such as *Staphylococcus aureus, Klebsiella pneumoniae* and *Enterococcus faecium* increased their resistance during the pandemic. (López-Jacame, *et al.*, 2022). Consequently, there is an urgent need for new and better antimicrobials, where the use of phytochemicals has drawn the attention of many researchers around the world.

Table 3. summarizes various evaluations of the antimicrobial effect of *Psidium guajava* L. carried out in recent years. Different solvents, plant parts and tests were used. Overall, the results indicate that guava extracts have potential activity on multiple bacteria strains and on yeasts of the genus *Candida*.

Table 3. Antimicrobial effects of different extracts of *Psidium guajava* L.

Microorganism	Method	Anatomical Part	Extract	Results	Reference
Candida albicans	96-well plate broth microdilution method (the concentrations in the wells ranged from 2 to 2048 µg/mL). Standard: Fluconazole	Leaves	Tannic Fraction	IC_{50}(µg/mL) = 69.29 ± 1.89	Bezerra, *et al.*, 2018.
			Flavonoid Fraction	IC_{50}(µg/mL) = 2690.52 ± 3.18	
			Tannic + Flavonoid Fraction	IC_{50}(µg/mL) = 207.8 ± 4.28	
Candida tropicalis	96-well plate broth	Leaves	Tannic Fraction	IC_{50}(µg/mL) = 188.9 ± 2.59	Bezerra, *et al.*, 2018.

Microorganism	Method	Anatomical Part	Extract	Results	Reference
	microdilution method (the concentrations in the wells ranged from 2 to 2048 µg/mL). Standard: Fluconazole		Flavonoid Fraction	IC_{50}(µg/mL) = 3444.62 ± 3.47	
			Tannic + Flavonoid Fraction	IC_{50}(µg/mL) = 315.1 ± 4.41	
Candida krusei	96-well plate broth microdilution method (the concentrations in the wells ranged from 2 to 2048 µg/mL). Standard: Fluconazole	Leaves	Tannic Fraction	IC_{50}(µg/mL) = 261.7 ± 3.25	Bezerra, *et al.*, 2018.
			Flavonoid Fraction	IC_{50}(µg/mL) = 1199.57 ± 3.66	
			Tannic + Flavonoid Fraction	IC_{50}(µg/mL) = 115.7 ± 2.58	
Streptococcus salivarius	96-well plate broth microdilution method (the concentrations in the wells ranged from 50 to 400 µg/mL). Standard: Chlorhexidine digluconate	Fresh leaves	Essential oil	MIC (µg/mL) = 400	Silva, *et al.*, 2019.
Streptococcus mutans	96-well plate broth microdilution method (the concentrations in the wells ranged from 50 to 400 µg/mL). Standard: Chlorhexidine digluconate	Fresh leaves	Essential oil	MIC (µg/ml) = 200	Silva, *et al.*, 2019.

Microorganism	Method	Anatomical Part	Extract	Results	Reference
Streptococcus mitis	96-well plate broth microdilution method (the concentrations in the wells ranged from 50 to 400 µg/mL). Standard: Chlorhexidine digluconate	Fresh leaves	Essential oil	MIC (µg/ml) = 200	Silva, *et al*., 2019.
Streptococcus sanguinis	96-well plate broth microdilution method (the concentrations in the wells ranged from 50 to 400 µg/mL). Standard: Chlorhexidine digluconate	Fresh leaves	Essential oil	MIC (µg/ml) = 400	Silva, *et al*., 2019.
Streptococcus sobrinus	96-well plate broth microdilution method (the concentrations in the wells ranged from 50 to 400 µg/mL). Standard: Chlorhexidine digluconate	Fresh leaves	Essential oil	MIC (µg/ml) = 100	Silva, *et al*., 2019.
Propionibacterium* acnés (now *Cutibacterium acnes)	96-well plate broth microdilution method (the concentrations in the wells ranged from 20 to 2500 µg/mL) Standard: Tetracycline	Leaves	Essential oil	IC_{50}(µg/mL) = 309 MIC (µg/mL) = 321	Pandey, *et al*., 2017.
***Staphylococcus epidermidis*.**	96-well plate broth microdilution	Leaves	Essential oil	IC_{50}(µg/mL) = 416	Pandey, *et al*., 2017.

Microorganism	Method	Anatomical Part	Extract	Results	Reference
	method (the concentrations in the wells ranged from 20 to 2500 µg/mL). Standard: Tetracycline			MIC (µg/mL) = 486	
	Broth dilution method (concentrations ranging from 40,000 to 160,000 µg/ml). Recovery of medium and streaking in solid medium to determine the MBC. Standard: nutrient broth without extract	Leaves	Methanolic extract	MIC (µg/ml) = 20,000±0.3 MBC (µg/ml) = 80,000±0.2	Festus, *et al*., 2020.
			Ethyl acetate extract	MIC (µg/ml) = 20,000±0.0 MBC (µg/ml) = 40,000±0.3	
Streptococcus gordonii	Disc diffusion test and 96-well plate broth microdilution method (the concentrations in the wells ranged from 200 to 50000 µg/mL) Standard: Chlorhexidine 0.12%	Leaves	Chloroformic residue of *P. guajava L.* crude extract (50000 µg/mL)	Mean diameters of inhibition halos (mm) = 9.12 MIC (µg/mL) = 780	Millones Gómez, *et al*., 2020.
Staphylococcus aureus	Agar dilution method (concentrations ranging from 625 to 10,000 µglml).	Dried powdered plant	Ethanolic extract	MIC (µg/ml) = 1250	Hemeg, *et al*., 2020
	Agar dilution method.	Leaves	Essential oil	MIC (µg/ml) = 6.75	Soliman, *et al*., 2016

Microorganism	Method	Anatomical Part	Extract	Results	Reference
	96-well plate broth microdilution method (the concentrations in the wells ranged from 7.8 µg /mL to 1000 µg/mL) Recovery of medium and streaking in solid medium to determine the MBC. Standard: extracts were used as negative controls.	Pulp (agroindustrial waste)	Methanolic extract	MIC (µg/ml) = 31.25 MBC (µg/ml) = 62.5	Dos Santos, *et al*., 2020
Escherichia coli	Agar dilution method (concentrations ranging from 625 to 10,000 µg/ml).	Dried powdered plant	Ethanolic extract	MIC (µg/ml) = 625	Hemeg, *et al*., 2020.
	Broth dilution method (concentrations ranging from 40,000 to 160,000 µg/ml). Recovery of medium and streaking in solid medium to determine the MBC. Standard: nutrient broth without extract	Leaves	Methanolic extract	MIC (µg/ml) = 40,000±0.5 MBC (µg/ml) = 80,000±0.1	Festus, *et al*., 2020.
			Ethyl acetate extract	MIC (µg/ml) = 40,000±0.0 MBC (µg/ml) = 80,000±0.1	
Salmonella Enteritidis	Agar dilution method (concentrations ranging from 625 to 10,000 µg/ml).	Dried powdered plant	Ethanolic extract	MIC (µg/ml) = 625	Hemeg, *et al*., 2020.
Pasteurella multocida	Agar dilution method (concentrations	Dried powdered plant	Ethanolic extract	MIC (µg/ml) = 5000.00	Hemeg, *et al*., 2020.

Microorganism	Method	Anatomical Part	Extract	Results	Reference
	ranging from 625 to 10,000 µg/ml).				
Mycoplasma gallisepticum	Agar dilution method (concentrations ranging from 625 to 10,000 µg/ml).	Dried powdered plant	Ethanolic extract	MIC (µg/ml) = 1250.00	Hemeg, *et al*., 2020.
Bacillus cereus	Broth dilution method (concentrations ranging from 40,000 to 160,000 µg/ml). Recovery of medium and streaking in solid medium to determine the MBC. Standard: nutrient broth without extract.	Leaves	Methanolic extract	MIC (µg/ml) = 40,000±0.1 MBC (µg/ml) = 40,000±0.4	Festus, *et al*., 2020.
			Ethyl acetate extract	MIC (µg/ml) = 40,000±0.2 MBC (µg/ml) = 80,000±0.3	
Pseudomonas aeruginosa	Broth dilution method (concentrations ranging from 40,000 to 160,000 µg/ml). Recovery of medium and streaking in solid medium to determine the MBC. Standard: nutrient broth without extract	Leaves	Methanolic extract	MIC (µg/ml) = 40,000±0.7 MBC (µg/ml) = 80,000±0.0	Festus, *et al*., 2020.
			Ethyl acetate extract	MIC (µg/ml) = 40,000±0.1 MBC (µg/ml) = 80,000±0.1	

It should be noted that a proper comparison between studies cannot be made, even if similar methodologies were used, since each author established the MIC differently, for example, Soliman, *et al*., (2016) defined MIC as the lowest concentration that had granular appearing micro-colonies of growth instead of filamentous radiating colonies on solid agar; while for

Hemeg, *et al*., (2020) MIC was the lowest concentration of extract that resulted in no visible growth on the surface of the agar. In addition, we can also find multiple differences between the methods to measure the antimicrobial effect, for example, Dos Santos, *et al*., (2020) used a 0.1% resazurine solution, while Festus, *et al*., (2020) analyzed bacterial growth based on turbidity.

Due to all these variants, it is difficult to interpret the results in a general way and the fact that different solvents, types of extraction, and parts of the plant were used, makes everything more complex, because the results depend on a great variety of factors. The selection of controls is also a factor to improve, given that in some studies negative controls were used but not positive ones and vice-versa. In addition, the part of the plant used to perform the extraction of phytochemicals and the location where the plant material was collected should be clear and precise.

However, the highest dose used in the studies analyzed was 160 mg/ml (160,000 ug/ml) by Festus, *et al*., (2020), but the highest reported MIC was 40 mg /ml (40,000 ug/ml) for the bacteria *Pseudomonas aeruginosa, Bacillus cereus and Escherichia coli* (Festus, *et al*., 2020).

6.1. Anti-biofilm effects of guava extracts

Biofilms are architectural elements embedded in self-produced extracellular polymeric substances that adhere to inert or biological surfaces. These elements allow microorganisms to adapt to their environment and on certain occasions give them the ability to escape host defense systems and may even confer resistance to antibiotics given the difficult penetration of molecules into the matrix of extracellular polymeric substances (Dutta, *et al*., 2020).

Therefore, finding substances that can inhibit the formation and growth of biofilms is of special interest, especially if they are substances of natural origin, such as phytochemicals, which can be substitutes for synthetic drugs without presenting significant secondary effects (Millones-Gomez, *et al*., 2020).

Unfortunately, recent studies of the potential antibiofilm activity of *P. guajava* L. are not numerous and focus on the effect on *S. Aureus*. Therefore, research on multiple microorganisms is needed to evaluate the spectrum of antibiofilm activity of guava extracts. Table 4 presents a summary of the results obtained and the possible mechanism of action proposed by the authors.

Table 4. Potential antibiofilm activity of different *Psidium guajava* L. extracts.

Microorganism	Extract	Results	Possible mechanism of action	Reference
Pseudomonas aeruginosa PAO1	Flavonoid fraction of guava leaves.	Anti-*quorom sensing* activity in a *C. violaceum* CV026 biosensor bioassay.	The flavonoid fraction interferes *quorom sensing* by inhibiting the response to the autoinducer (N-acyl homoserine lactone). Compounds that probably are responsible for this effect are quercetin and quercetin-3-O-arabinoside.	Vasavi, 2014.
***Staphylococcus aureus* clinical isolates and ATCC 25923**	Benzyl isocyanate isolated from the leaves of *Psidium guajava L.*	MBIC (µg/ml) = 440 – 870 MBEC (µg/ml) = 1000 - 2100	Benzyl isocyanate induces the production of poor-quality extracellular polymeric substances and can inhibit major biofilm regulatory molecules of S. *aureus.*	Dutta, *et al*., 2020
Staphylococcus aureus	Pulp methanolic extract (agroindustrial waste).	MBEC (µg/ml) = 250	The presence of polyphenols and compounds like L-5-Propylthiomethylhydantoin, that has bacteriostatic activity.	Dos Santos, *et al*., 2020
Staphylococcus aureus	Petroleum ether guava leaves extract.	Dose: 1000 µg/ml Percentage of biofilm inhibition: 25.2 ± 0.53% Dose: 2000 µg/ml Percentage of biofilm inhibition: 62.9 ± 0.48%	Flavonoids prevent the correct transmission of signals, leading to shutdown of *quorum sensing*. In addition, terpenoids alter the fatty acid composition of the cell membrane, which	Umamaheswari, *et al*., 2020

Microorganism	Extract	Results	Possible mechanism of action	Reference
	Ethanolic guava leaves extract.	Dose: 1000 µg/ml Percentage of biofilm inhibition: 76.83 ± 0.56% Dose: 2000 µg/ml Percentage of biofilm inhibition: 80.0 %±0.86	causes the hydrophobicity of cells leads to biofilm eradication.	

The MBEC (Minimum Biofilm Eradication Concentration) reported by Dos Santos, *et al*., 2020 (250 µg/ml) is lower than those reported by Dutta, *et al*., 2020 (1000 - 2100 µg/ml). This could indicate that probably the guava leaf extract has a higher activity compared to the compound Benzyl isocyanate (isolated from guava leaves) on its own. The interaction of the components of the extract and a possible synergy may be involved in this fact, but future research is needed to clarify and verify this issue.

The antibiofilm mechanism exhibit by the phytochemicals presents in plant extracts are highly diverse, and among them are the decrease in the production of virulence factors, the inhibition of the formation of the polymeric matrix, the suppression of cell adhesion and the alteration of *quorum sensing* (Asma, *et al*., 2022).

The lack of antibiofilm evaluations with extracts from another part of the plant, since those published focus mainly on the leaves and their derivatives, shows that there is still much to discover on this topic and a large field of study opens for those interested in this area.

6.2. Antiviral effects of *Psidium guajava L.*

Nowadays, researchers have been particularly interested in the study of medicinal plants as a therapeutic option for the treatment of viral infections, based on traditional ethnomedical wisdom and taking advantage of their molecules to develop antiviral drugs (Cáceres, 2020). Different studies have revealed that *Psidium guajava L*. extracts may have antiviral activity and some of them are summarized below.

The infusion of guava leaves was able to inhibit the growth of isolated influenza A (H1N1) virus. Likewise, it was able to inhibit viral hemagglutination and sialidase activity (IC_{50}= 0.44±0.05 - 7.50±0.49) (Sriwilaijaroen, *et al*., 2012). Therefore, it appears to be effective for the control of pandemic and epidemic influenza viruses, including oseltamivir-resistant strains (Ishimine *et al*, 2021).

Sharma et al., (2021) reported that the guava leaves extract and its nanoparticles reduce/stop chikungunya virus replication in the Vero cell line. Specifically, the authors reported a viability of 84.21% of the cells treated with the guava extract, a viability of 64.40% of the cells treated with the nanoparticles of the extract, and a viability of 24.03% of the positive control (virus+cells). The authors conclude that in the absence of vaccines and antivirals, the extracts of *P. guajava L*. can be an alternative treatment.

The antiviral effect of the crude aqueous extract of 10 plants used in traditional medicine in the Philippines, including the leaves of *Psidium guajava* L., was analyzed by Vista *et al*., (2020). The authors reported that guava leaves were one of the best candidates against ZIKA virus (ZIKV) along with *M. charantia, V. negundo*, and *B. balsamifera*.

Trujillo-Correa, *et al*., (2019) reported that four compounds isolated from guava bark extracts, specifically gallic acid, quercetin, naringin and catechin, inhibited dengue virus (DENV-2) replication. Of these, catechin was the most promising compound. The authors also mention that the anti-DENV effect of the *P. guajava* L. extract could be related to the inhibition of the enzyme α-glucosidase, since it has been described as essential for the correct folding of viral glycoproteins and for the assembly of the virion.

Shin, *et al*., (2021) studied the potential of BEN815, a natural nutraceutical composed of extracts of guava leaves (*Psidium guajava L*.), green tea leaves (*Camellia sinensis*), and rose petals (*Rosa hybrida*) to treat COVID -19. The authors reported that BEN815 showed antiviral activity against SARS-CoV-2, with an IC_{50}= 34.38 µg/ml. In addition, it is mentioned that of the compounds found in the nutraceutical, EGCG (Epigalocatechin gallate) plays a key role in the antiviral effect against SARS-CoV-2.

7. Potential mechanisms of action of antimicrobial activity

The exact mechanism of antibacterial activity is still not clear, mainly because phytochemicals have highly variable structures, generating multiple possible modes of action. Moreover, plant extracts contain a complex mixture of compounds whose interaction can influence the mechanism (Efenberger-Szmechtyk, *et al*., 2021). Therefore, the mode of action depends on the type of extract or essential oil and the microorganism used (Guimarães, *et al*., 2019).

Nonetheless, it has been shown that phenolic compounds can interact with bacterial cell walls, leading to their rupture and the release of cellular components (Efenberger-Szmechtyk, *et al*., 2021), they can also suppress several microbial virulence factors (among them biofilm formation and toxin production), inhibit the synthesis of nucleic acids and the activity of enzymes (Takó, *et al*., 2020). In addition, it has been reported that gram-negative bacteria are more resistant to phenolic compounds, probably due to the presence of an outer membrane and enzymes in the periplasmic space that can damage molecules that enter the bacteria (Efenberger-Szmechtyk, *et al*., 2021).

Similarly, the mode of action of terpenes, also an important component of guava extracts, remains largely unknown, however, it has been observed that most terpenoids can inhibit two essential processes for microbial survival: oxygen uptake and oxidative phosphorylation. Thus, terpene interaction leads to alteration in cellular respiration which later causes uncoupling of oxidative phosphorylation in the microbe (Mahizan, *et al*., 2019).

On the other hand, the proposed mode of action of β-caryophyllene, a bicyclic sesquiterpene considered by multiple authors as the main component of guava leaf extract (Sherazi *et al*., 2019, De Souza, et al., 2021, Jassal, et al., 2019), is through altering the bacterial membrane permeability and causing non-selective pore formation. This induces the intracellular content leakage leading to damage and loss of the membrane integrity and may eventually lead to cell death (Moo, *et al*., 2020). Figure 4 summarizes the possible mechanisms of action for the bactericidal activity.

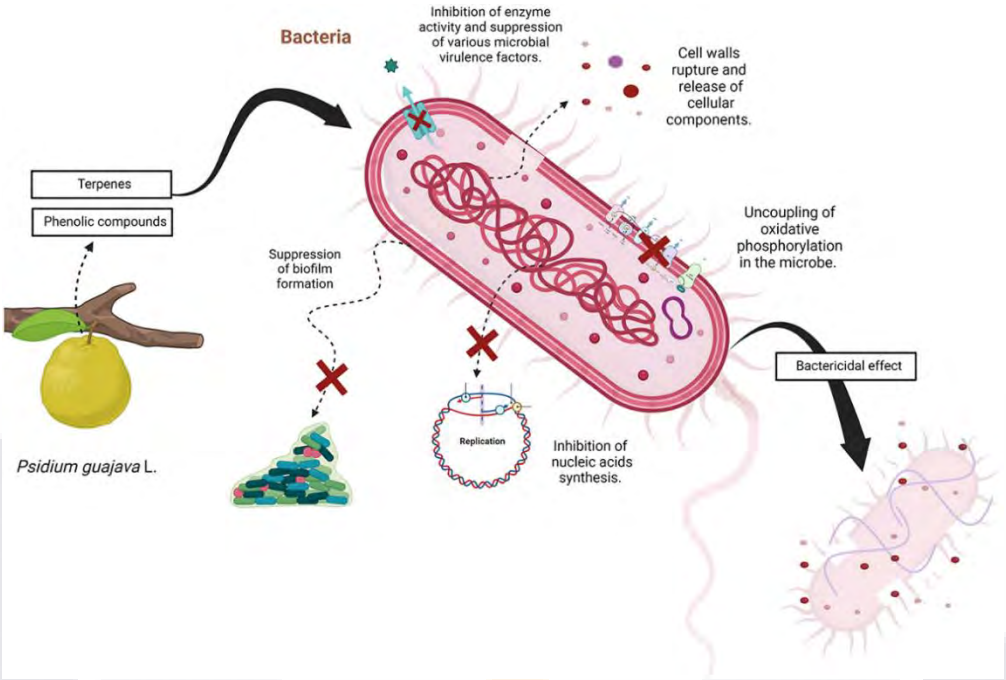


Figure 4. The possible antibacterial mechanisms of action of *Psidium guajava* L., adapted and modified from Bankour, *et al*., 2022.

Regarding the antiviral activity exerted by phenolic compounds, the mechanisms proposed are the suppression of the infection process and/or the repression of viral replication. In this regard, reported antiviral modes of action include viral DNA/protein damage and/or inhibition of viral enzymes (Rashad, *et al*., 2020).

8. Antidiabetic activity

Diabetes mellitus is a chronic disease that causes an increase in blood glucose levels, type 1 is caused by the loss of beta cells from the pancreatic islets, so the pancreas is no longer capable of producing insulin, while type 2, is caused by insulin resistance, so the body cannot effectively use the insulin it produces (Huerta-Reyes, et al. 2022).

In 2021, 13% of deaths in Mexico were due to diabetes, and of the deceased, 74.9% were not insulin dependent (INEGI, 2022). In addition, the country ranks second in Latin America with the highest prevalence of diabetes with 11.5 million cases (Quiroga-Garza, et al., 2022).

Although, in Mexico there are different treatments available such as insulin and oral hypoglycemics, a large part of the population continues to use and even prefers the use of

medicinal plants (Huerta-Reyes, et al., 2022). In addition, it should be noted that many current treatments have side effects such as gastrointestinal problems, heart failure, weight gain, edema, impaired kidney function, pancreatitis, and genital infections (Lee, et al., 2021).

Multiple hydrolytic enzymes are involved in carbohydrate digestion, including α-amylase, which hydrolyzes starch, glycogen, and many oligosaccharides into maltose, maltotriose, and oligoglycans, which are further hydrolyzed by α-glucosidase to glucose, more suitable for absorption, increasing blood glucose levels (Quan, et al., 2019; Jafet, et al., 2020). Thus, inhibition of these enzymes can decrease postprandial glucose uptake and is therefore a key therapeutic target in the management of type 2 diabetes mellitus (Jafet, et al., 2020). Table 5 presents different studies that have analyzed the inhibition of these enzymes by extracts of *P. guajava* L. and their results.

Table 5. Inhibition of α-amylase and α-glucosidase by *Psidium guajava* L. extracts.

Extract	Location	Enzyme	Results	Reference
Ethanolic leaves extract	Madagascar	α-glucosidase, from *Saccharomyces cerevisiae*	IC_{50} = 1.0 ± 0.3 µg/ml	Beidokhti, et al., 2020
Ethanolic bark extract	Madagascar	α-glucosidase, from *Saccharomyces cerevisiae*	IC_{50} =0.5±0.01 µg/mL	Beidokhti, et al., 2020
		α-amylase type VI-B, from porcine pancreas	IC_{50} =10.6±0.4 µg/mL	
Methanolic leaves extract (crude extract)	Japan	α-amylase, from *Bacillus sp.*	IC_{50} = 38.0 ± 1.6 µg/mL	Samejima, et al., 2019
		α-glucosidase from *Saccharomyces sp.*	IC_{50} = 46.0 ± 3.5 µg/mL	
Butanol-soluble fraction from methanolic leaf extract	Japan	α-amylase, from *Bacillus sp.*	IC_{50} = 21.9 ± 0.7 µg/mL	Samejima et al., 2019
		α-glucosidase from *Saccharomyces sp.*	IC_{50} = 54.2 ± 3.5 µg/mL	
Fruit Juice	Mexico	α-glucosidase	76.62% of inhibition	Jafet, et al., 2020
Leaves extract	Taiwan	α-amylase	IC_{50} = 50.5 g/mL	Liu, et al., 2014
		α-glucosidase	IC_{50} =34.6 g/mL	

As we can see in table 5. multiple studies have analyzed the effect of *Psidium guajava* L. on the inhibition of α-amylase and α-glucosidase, obtaining good *in-vitro* results; this may be due to the presence of quercetin in the extracts, a competitive inhibitor of α-glucosidase, since the hydroxyl present in the 3, 3' and 4' carbons in its structure can interact with the Asp214 and Glu276 present in the enzymatic active site (Jafet, et al., 2020). In addition, tannins have also been shown to be good inhibitors of α -glucosidase with Ki values similar to those of synthetic inhibitors such as acarbose and voglibose, which are currently used as treatment to control type 2 diabetes (Cao, et al. al., 2018). The variations in the IC_{50} values can be due to multiple factors, among them, the location and season of the year in which the biological material was collected, the extraction method, possible differences in the methodologies and in the the enzymes used, for example, Beidokhti, et al., 2020 used a porcine α-amylase, while Samejima, et al., 2019 a bacterial one.

Xu, *et al*., (2019) administered 800 mg/kg of guava leaf extract daily to diabetic rats and observed a significant reduction in total cholesterol, Low-density lipoprotein cholesterol, (LDL-C) and glycosylated serum protein (precise intermediate marker of glycemia) compared with the control group (diabetic rats that received only distilled water). Exposing that guava leaves have antihyperglycemic and antihyperlipidemic effects.

The same year, Luo, *et al*., (2019) worked with diabetic mice induced by streptozotocin and high-fat diets and reported an improvement in body weight, decrease in fasting blood glucose, total triglycerides and total cholesterol levels with the daily intragastric administration of polysaccharides isolated from guava leaves (100 and 200 mg/kg) for 4 weeks. They even observed protective effects on the kidney, since the creatine content, whose excretion is an indicator of the organ's metabolism and whose accumulation indicates impaired functioning, decreased after the administration of the polysaccharides. Similarly, Glycated serum protein levels decreased in a dose-dependent manner.

On the other hand, Eidenberger, et al., (2013) analyzed the absorption of guava leaf extract in the CaCo-2 cell line, widely used to investigate nutrient absorption, and concluded that the extract can exert its observed effect in- vitro after being administered orally. Although there are results that indicate a potential antidiabetic effect, there is still much to be

investigated, especially the activity and safety in humans, the possible mechanisms of action and the long-term effects.

9. Toxicity

Numerous health benefits of plants and their extracts have been reported, for example, as potential antioxidant, anticancer, antimicrobial, and anti-inflammatory agents. However, even though they may exhibit excellent *in-vitro* activity, we cannot take advantage of these benefits until they are proven to have no harmful side effects. The use of a plant for a certain purpose must not only be effective, but also safe for the consumer (Amos, *et al*., 2015).

In-vivo and *in-vitro* toxicology studies are conducted to determine a drug's short and long term functional and morphologic adverse effects. There is a large variety of toxicological studies, among them, the acute toxicity studies are carried out to determine the short-term adverse effects of a single dose of a drug (or multiple doses during a period of 24h) and the subacute toxicity studies, performed to evaluate the possible adverse effects of a new drug after a treatment period of 2 to 4 weeks duration (Colerangle, *et al*., 2017). Table 6 shows some of the toxicological studies carried out with guava extracts.

Table 6. Toxicological evaluation of extracts of *Psidium guajava* L.

Extract	Assay	Animal	Dose	Results	Reference
Bark methanolic extract	*In-vivo*, Acute and subacute toxicity.	Nulliparous and nonpregnant female Wistar rats (12 weeks) for the acute toxicity. Males and females (nulliparous and nonpregnant) Wistar rats (170 -245 g) for the subacute toxicity test	Oral 5000 mg/kg	Acute toxicity: no abnormality or mortality was observed.	Manekeng, *et al*., 2019
			Oral 250, 500, and 1000 mg/kg	Subacute toxicity: variations in body weight, relative weight of organs, and biochemical parameters were observed.	
Fruit ethanolic extract	*In-vivo,* Acute toxicity.	Nulliparous and nonpregnant female Swiss Webster albino mice (20–30 g, 8–12 weeks	Oral 5000 and 2000 mg/kg	Any differences in body weight, number of hepatocyte in liver, and podocyte in kidney compared with control.	Atik, *et al*., 2019

Extract	Assay	Animal	Dose	Results	Reference
Leaves aqueous extract	*In-vivo*, Acute toxicity.	Female Wistar albino rats (150–180 g, 10–12 weeks)	Oral 50, 500, and 5000 mg/kg	Administration did not cause death of any of the animals. There was no toxicity signs, or any pathological observation.	Babatola, *et al*., 2019
Leaves and bark aqueous extracts	*In-vivo*, Brine Shrimp Lethality Assay (BSLA).	Brine Shrimp *(Artemia salina)*	1000 µg/mL 500 µg/mL and 100 µg/mL	Leaves extract LC_{50} (µg/ml) = 949.13 Bark extract LC_{50} (µg/ml) = 480.14	Bautista, *et al*., 2018
Leaves extracts	*In-vivo*, Brine Shrimp Lethality Assay (BSLA).	Brine Shrimp *(Artemia salina)*	-	Methanolic extract: LC_{50} (µg/ml) = 63.81 ± 2.65 Chloroform extract: LC_{50} (µg/ml) = 41.05 ± 3.19 Hexane extract: LC_{50} (µg/ml) = 32.18 ± 0.24	Ashraf, *et al*., 2016

Manekeng, *et al*. (2019) evaluated the toxicity of orally administered *Psidium guajava* L. bark methanolic extract in Wistar rats, the authors observed no abnormalities or mortality in rats at a dose of 5000 mg/kg, however, they mention that the repeated administration of high doses (1000 mg/kg or more) could exhibit mild organ toxicity. Similarly, Atik, *et al*. (2019) in their acute toxicity test observed no side effects in Swiss Webster mice after oral treatment with 2000 and 5000 mg/kg of guava ethanolic extract.

Babatola, *et al*. (2019) evaluated the acute toxicity of aqueous extracts of guava leaves of three different varieties (white, red, and pink guava), none of the extracts caused death or generated any symptoms of pathology at concentrations of 50, 500, and 5000 mg/kg. These results are interesting because we must remember that the composition of the extracts can

vary due to many factors, among them the variety and therefore, the toxicity can also be affected; this study was not the case, but it is important to consider this fact to avoid generalizing the results.

Bautista, *et al*. (2018) conducted the Brine Shrimp Lethality Assay (BSLA), a simple and inexpensive bioassay used for evaluating the efficacy of phytochemical present in the plant extracts. This assay is based on the ability to kill a simple zoological organism on 24 hours (Sarah, *et al*., 2017). The authors evaluated the toxicity of aqueous extracts of guava bark and leaves and concluded that bark leaf extract is more toxic compared to leaf extract.

The toxicological evaluation is very important, especially considering that in many parts of the world, including Mexico, it is thought that herbal products, thanks to their natural origin, are not toxic and are used indiscriminately, nonetheless, everything depends on the dose and the consumer. Unfortunately, in recent years an increase in cases of adverse effects of herbal products has been reported (Babatola, *et al*., 2019), which reflects the need for better and more complete toxicological evaluations of plant extracts.

10. Bioavailability

Bioavailability is the ability of certain compound to reach the circulatory system and be distributed throughout the body. While bioaccessibility, another important concept, refers to the amount of compound that is available for absorption in the gastrointestinal tract (Lorenzo, *et al*., 2019).

The digestion of phenolic compounds is a complex and not fully understood process, however, today we know that the release of phenolic compounds begins with mastication (chewing) and the low pH of the stomach, which generates the disintegration of the food matrix. Subsequently, depolymerization begins, also in the stomach, where the phenolic compounds are degraded into small structures so that they can be absorbed. Uptake of small phenolic compounds, particularly aglycones, occurs by passive diffusion (lactase phloridizin hydrolase activity) or active transport by sodium-dependent glucose transporter (cytosolic β-glucosidase). Unabsorbed phenolic compounds now move to the colon, where the gut microbiota can facilitate absorption of phenolic compounds, nevertheless, the

variability within individual gut microbiota is a determinant factor to increase the bioaccessibility of phenolic compounds (Lorenzo, *et al*., 2019).

The bioavailability of phenolic compounds depends on several factors, including release from the matrix during gastrointestinal digestion, bioaccessibility, cellular uptake, metabolism, and further transport in the circulatory system. Unfortunately, most phenolic compounds are poorly absorbed in the small intestine, as they are either heavily metabolized or rapidly eliminated. In addition, polyphenols can be associated with the dietary fiber matrix, through covalent bonds or hydrophobic interactions, which means that they are not bioavailable in the human intestine (Blancas-Benitez, *et al*., 2017).

Similarly, terpenes have low water solubility and low bioavailability. Furthermore, it has been noticed that terpenes have a highly lipophilic behavior that influences their solubility in the aqueous phase of the intestinal lumen and, therefore, their bioavailability according to their incorporation into a lipid phase, either during digestion or during food processing (Niaz, *et al*., 2020).

To enable phytochemicals compounds, like polyphenols and terpenes, applications as antimicrobial agents, it is necessary to improve its bioavailability. One of the most promising strategies, which can also increase their stability, is the use of delivery systems, such as liposomes, nanoemulsions, and polymeric/biopolymeric nanoparticles (Câmara, *et al*., 2020). For example, Vasconcelos, *et al*., (2021) generated a self-emulsifying drug delivery system containing purified lycopene from red guava.

11. Clinical trials

Birdi, *et al. (*2020) conducted a clinical trial with 109 patients diagnosed with diarrhea. The authors reported that a decoction of guava leaves was a safe and simple treatment for common diarrhea. However, some trial limitations must be considered, first, the study was conducted with adults, so the safety and effectiveness in children, who are more susceptible to this pathology, remains to be proven. Moreover, since bacteriological examination of the stools was not undertaken at screening to identify causative organism, the laboratory results could not be verified clinically from the patients in this trial.

In addition, Nayak, *et al.* (2019) conducted a double blind randomized, placebo controlled clinical trial with subjects with moderate to severe chronic gingivitis. The authors evaluated the potential activity of an hydroethanolic extract of guava leaves incorporated in a mouthrinse in 56 patients aged between 18 and 40 years who were evaluated for 3 months. Although the guava mouthrinse group showed beneficial results, like reductions in plaque scores and improvement in gingival health, the results were not statistically significant. In addition, further randomized clinical trials should be carried out for an extended period to substantiate its long-term effects.

Similarly, Amaliya, *et al*. (2018) evaluated the effect of guava and vitamin C supplementation on gingivitis. The randomized clinical trial had the participation of 48 persons; however, it was also carried out in a short period of time (4-week) which did not allow to make firm conclusions.

Pongsakornpaisan, *et al*. (2019) conducted a study on the efficacy of their anti-sebum toner based on guava leaf extract with 10 volunteers. The authors observed that guava toner suppressed the sebum level in the nose and forehead area after 28 days of treatment and concluded that guava may be a good agent for cosmetic products. Although, the number of participants was small and long-term effects need to be assessed.

König, et al., (2019) reported that guava extract obtained by supercritical CO_2 extraction reduced postprandial blood glucose levels in a randomized, double-blind, parallelized clinical study conducted with 31 young and healthy participants (20 female and 11 male, ages 19-29) from the Paracelsus Medical University Salzburg. After an overnight fast, each participant underwent an oral glucose tolerance test, in which members of the control group each received a glucose solution (75 g glucose) and members of the intervention group each received a glucose solution that also contained 2.5 ml of extract. However, it should be considered that the number of participants was small and only healthy young people were considered, so the effect on adults, children, and people with chronic diseases, including diabetes, is unknown. In addition, a low concentration of the extract was used, so possible side effects at higher concentrations and after long periods of administration were not reported. An important fact also mentioned by the authors is that the effect of the extract

decreased after storage, therefore, it is necessary to consider the possible instability of the extracts over time for future applications.

Another similar clinical trial was carried out by Kumari, et al., (2016), which had 45 young and healthy participants (31 male and 14 females, 18 to 25 years). The trial was conducted for 4 months, with 6 weeks of guava supplementation. 15 subjects were supplemented with 400 g of ripe guava with peel, another 15 with 400 g guava without peel and 15 subjects were the control group, which did not receive any supplement. After supplementation with ripe guava without peel a drop in blood sugar was observed. The authors attribute this effect to the high fiber content in the guava pulp, since it delays the intestinal absorption of glucose; the presence of flavonoid glycosides that improve insulin sensitivity, as well as the inhibition of the α-glucosidase enzyme. In addition, there was also a decrease in total cholesterol and triglycerides and an increase in high-density lipoproteins (HDLc). However, supplementation with ripe guava fruits with peel gave different results, since it increased blood glucose levels, cholesterol, triglyceride, and low-density lipoprotein (LDLc) levels. According to the authors, this effect may be since the peels have a low concentration of magnesium, necessary for the activity of multiple enzymes and which favors insulin-dependent glucose absorption; however, this explanation is ambiguous, and more studies are necessary to clarify this phenomenon. More detailed clinical trials need to be conducted to establish the efficacy of the guava extracts.

12. Perspectives and conclusions.

The potential of *Psidium guajava* L. and its different extracts is evident, however, there is still much to investigate so the different benefits can have an industrial application and therefore can reach society.

First, the repertoire of strains used in the analysis of antibiofilm effects must be expanded, moreover, most of the published studies about antimicrobial effects are *in-vitro* evaluations (antibiograms, microdilution method, etc.), therefore, *in-vivo* studies should be encouraged in the coming years.

On the other hand, the literature focuses mostly on the bioactivity of guava leaves, however, if the aim is to use agro-industrial residues, it will probably be difficult to separate the leaves

from the rest of the waste (heterogeneous mixture of leaves, bark, seeds, and pulp), so it would be interesting to analyze the effect of an extract obtained from this combination. Another inconvenience that should be considered when using waste and byproducts from the agroindustry is the possible presence of food additives and pesticides.

Besides, it is important to improve the long-term stability of the extract, since in most studies the conservation of the extracts is carried out by freezing, refrigeration or drying, which would not be very viable in industrial applications. In addition, it is known that phenolic compounds have low bioavailability, so it is necessary to find a way to protect them and make them more available, for example, through encapsulation.

More detailed clinical trials need to be conducted to establish the efficacy of guava extracts, and it would be interesting to analyze their effect in the treatment of other diseases than diarrhea or oral problems, for example, flu and acne.

Finally, the possible variation in phytochemicals present in the extracts due to changes in climate, season, and other factors such as the presence of insects can cause a lack of homogeneity in the final product, which should also be considered if commercialization is sought.

The recovery and valorization of guava waste can bring economic benefits to the agroindustry and can represent an alternative to the use of traditional antimicrobials, helping the problem of microbial resistance in Mexico, which has easy access to this residue. In addition, it can reduce environmental problems caused by leaching and emission of greenhouse gases generated during the treatment of agro-industrial waste.

13. Conflict interest statement.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

14. Author Contributions.

DGM was the main writer of the review, ALGB is the main head of the laboratory, NACV and FJAG review the manuscript, and IGOG also participate in the redaction of the review.

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Chapter 4.

Bougainvillea glabra Choisy (Nyctinaginacea): review of phytochemistry and antimicrobial potential

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Article

Bougainvillea glabra Choisy (Nyctinaginacea): review of phytochemistry and antimicrobial potential.

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The Bougainvillea glabra or bougainvillea is a climbing plant native from South America belonging to the Nyctaginaceae family. The bougainvillea is recognized worldwide for its horticultural importance, due to the color of its bracts, commonly known as "flowers," made up of bracts, which are the striking parts, and the true flowers, which are white and small. Bougainvillea is widely known in traditional medicine to treat respiratory diseases such as cough, asthma, and bronchitis, gastrointestinal diseases, also for its antibacterial and insecticidal capacity. The antimicrobial potential of the involucre of this plant has not been studied, despite research showing a high phytochemical presence of secondary metabolites such as alkanes, phenols, terpenes, and betalains. This review compiles information about the traditional uses of B. glabra, its botanical description, ecological relevance, phytochemistry, antimicrobial and antibiofilm activity, such as the toxicology of bracts and flowers.

KEYWORDS

Bougainvillea glabra, bracts, traditional medicine, betalains, betacyanins, antimicrobial, phytochemistry

Introduction

Plants are part of the history of man, since antiquity they have served as a natural medicinal remedy to cure different diseases, the knowledge of these plants has been maintained from generation to generation by sorcerers, healers, or shamans (Azmir et al., 2013; Tugume and Nyakoojo, 2019).

The World Health Organization (WHO) reports that there are about 20,000 medicinal plants, which provide primary healthcare to more than 80% of the world's population (Sasidharan et al., 2011; Tugume and Nyakoojo, 2019). For this reason, phytochemistry and pharmacology have used medicinal plants to investigate new ecological and biodegradable chemical entities that function in the treatment of different pathologies due to their central structures (Yusuf and Khan, 2022). In addition, the WHO recommends and promotes the use of herbal remedies in national healthcare programs, due to their low cost, popular acceptance, and safety by causing fewer side effects (Pandey and Tripathi, 2013).

Plant tissue produces secondary metabolites, which allow them to grow, reproduce, and defend themselves in stressful environments and are the main active principles of the plant which have biological activity with a variety of properties such as antimicrobial, anti-inflammatory, antioxidant, analgesic properties, among others; obtaining these active ingredients is achieved through extracts from different parts of the plant such as its leaves, stems, flowers, and fruits (Azmir et al., 2013; Abubakar and Haque, 2020; Che et al., 2021).

Infectious diseases caused by bacteria are one of the main health problems with high morbidity and mortality worldwide (Frieri et al.,2017). This is related to the resistance of bacteria to existing antibiotics, caused by the indiscriminate use of drugs, which is why it has been decided to obtain extracts from medicinal plants as possible antimicrobial agents (Dhankhar et al., 2013; Hemeg et al., 2020).

There are hundreds of plant species traditionally used as medicinal, but their active ingredients have not been fully studied, such as B. glabra Choisy, a climbing plant native to Brazil, belonging to the Nyctaginaceae family, which inhabit in warm climates, of great

ornamental and horticultural importance, due to its striking "inflorescences", formed by the involucre, which it is made up of a set of colorful bracts and the true flower (Tcherkez, 2004; Zahidul et al., 2016; Marrelli, 2021).

B. glabra is used in traditional medicine to treat respiratory diseases such as cold, flu, cough, bronchitis, and asthma, as well as for gastrointestinal problems such as diarrhea and dysentery (Schlaepfer and García, 2017; Rodríguez-Herrera et al., 2023). Properties with antimicrobial activity are also attributed to it due to the presence of active compounds such as flavonoids, tannins, alkaloids, phenols, betacyanins, terpenoids, glycosides, and essential oils (Edwin et al., 2007; Zahidul et al., 2016).

This review provides a comprehensive overview of botany, traditional uses, ecology, toxicology, phytochemistry, antimicrobial potential, and antibiofilm of B. glabra bracts and flowers, plant organs that are widely used in traditional medicine, but little investigated.

Methodology

The research for information on B. glabra was carried out using different databases such as PubMed, Google Scholar, ResearchGate, eBook, Elsevier, as well as government and botanical pages. Information was included from 1994 to 2023.

The research was carried out using the keywords "Bougainvillea glabra", "buganvilia", later Boolean operators were used such as: bracts and flowers, antimicrobial activity or biology, traditional medicine, phytochemistry and active principles; toxicity; botany and biology.

Origin and distribution

The name of the genus Bougainvillea comes from the French naturalist and explorer Philibert Commerson, who discovered it for the first time in Rio de Janeiro, Brazil, in the year 1768, naming it in honor of his compatriot Louis Antoine de Bougainville, French explorer and navigator (Cumo, 2013; El-Sayed et al., 2021).

Bougainvillea is a plant of ornamental importance, endemic to South America. Pantropically introduced and distributed in warm regions of Mexico, Asia, Australia, the Caribbean, South Africa, the United States, and other countries (Lim, 2014).

Botanical description of Bougainvillea

They are woody or shrubby climbing plants; that present the leaf throughout the year. It has stems with thorns that help it climb; simple leaves, arranged alternately, entire, ovate to elliptic or lanceolate in shape; its flowers are small (Figure 1), tubular, appear in groups of three, white or yellow, bloom in spring and summer and even in early autumn; the flowers are surrounded by three colorful bracts, which have the consistency of paper, size and appearance of leaves; its fruit is elongated, no more than1 cm

Figure 1. Bougainvillea: involucre constituted by bract and flowers

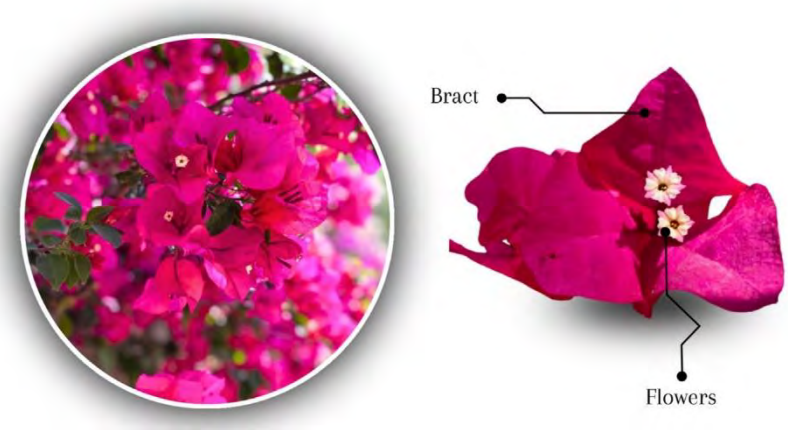


Table 1. Taxonomic classification (Saleem et al., 2021).

Kingdom	Plant
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Caryophyllidae
Order	Caryophyllales
Family	Nyctaginaceae
Gender	Bougainvillea
Species	Bougainvillea glabra Choisy

Direct sunlight allows the growth and flowering of bougainvillea, as well as acidic and well-drained soils, with a pH of 5.5–6; they are tolerant to droughts (Napoleón et al., 2013).

Taxonomy

The Bougainvillea genus belongs to the Nyctaginaceae family (Table 1), which houses around 33 genera and 400 species, from which Mexico reports 18 genera and approximately 110 species. Bougainvillea spectabilis, B. glabra and B. peruviana are the three most important horticultural species and the most studied. There are around more than 100 cultivars and hybrids that have not been studied (Gupta et al., 2009; He et al., 2020; El-Sayed et al., 2021).

Botanical description of Bougainvillea glabra

Swiss botanist Jacques Denys Choisy identified B. glabra in 1850 (Napoleón et al., 2013). It is a perennial climbing shrub 1–7 m tall n(Figure 2), with branches that have curved spines 5–15 mm long; simple leaves, dark green, somewhat glossy on the upper side, 1 cm long petiole, adaxially glabrous and abaxially pubescent, approximately 10 cm long; flowers 0.4 cm in diameter, bisexual, in a cymose inflorescence with three white to cream-colored flowers, perianth 1–2.5 cm long, slightly pubescent, with a single carpel, ovary and six to eight stamens; chartaceous bracts, ovate of 5 cm long and 1.54 cm wide, cardioid base and pointed tips, adhered to the flowers in the terminal region of the middle rib, of various colors; with small, dry, one-seeded and ribbed achene fruit. B. glabra habits warm, semi-warm, dry, semi-dry, and temperate climates (Lim, 2014; Saleem et al., 2021).

Color of the bracts of B. glabra

The color of the bracts of B. glabra is due to the presence of pigments known as betalains (Napoleón et al., 2013).

Betalains are water-soluble, vacuolar pigments, they present nitrogen with a heterocyclic ring in their structure. They are responsible for the color of the flowers, and fruits, as well as the leaves and roots of plants belonging to the Order Cariophyllales. Betalains are divided

into betacyanins (Figure 3A) that are derivatives of betanidine, through an iminium adduct of cyclodioxyphenylalanine from the cyclo- dihydroxyphenylalanine (DOPA cycle), as well as betalamic acid, which provides a red-violet color; while the condensation of betalamic acid with α-amino acids or amines produce betaxanthins (Figure 3B) that provide a yellow-orange color (Vargas-Campos et al., 2018; Devadiga and Ahipa, 2020; Sadowska and Bartosz, 2021).

Ecological importance

The coloration provided by betalains to the bracts of B. glabra favors the attraction of pollinators (Figure 4) and the dispersal of seeds (Sadowska and Bartosz, 2021). The attraction of pollinators is of utmost importance to our environment since they are responsible for 80% of the sexual reproduction of terrestrial plants, helping the functioning of ecosystems (Ghisbain et al., 2021).

Figure 2. Bougainvillea glabra morphology.

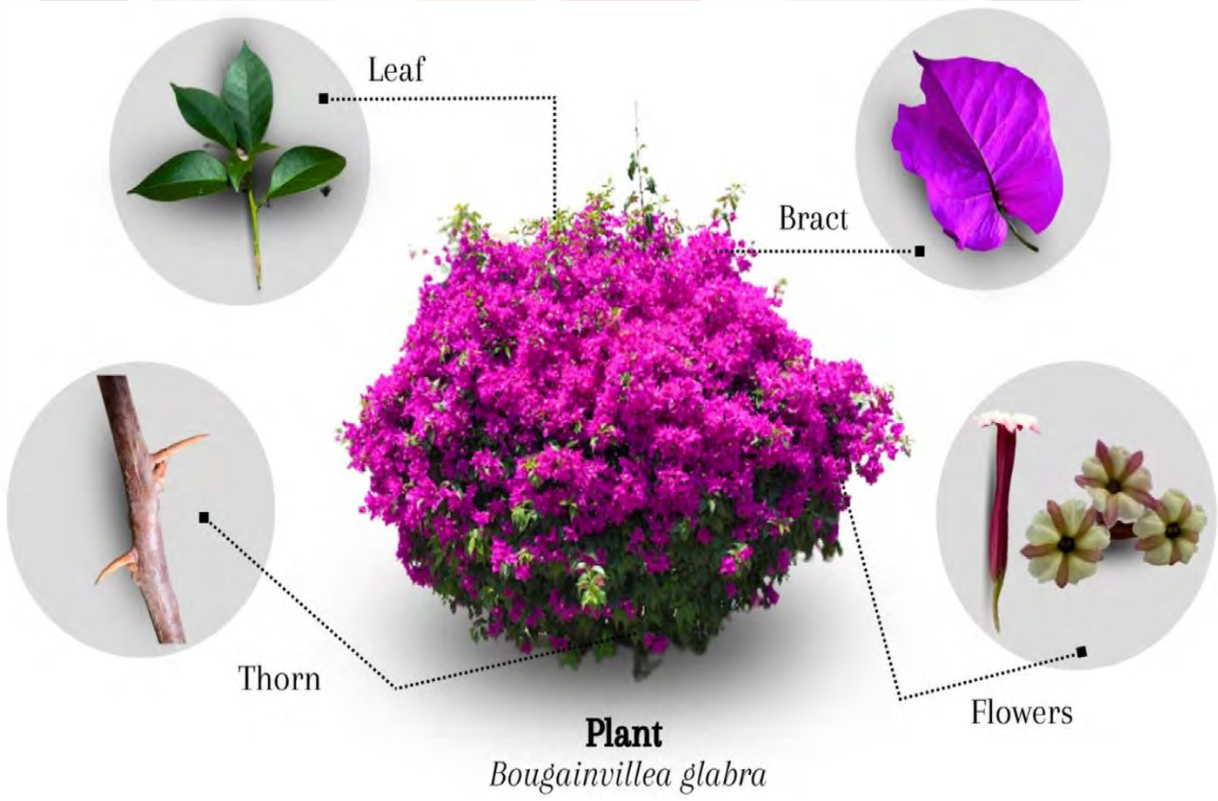
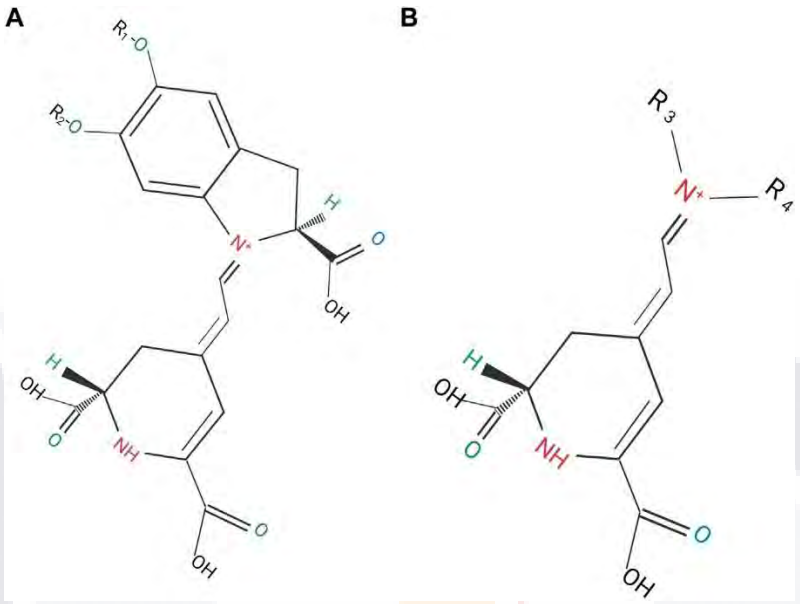


Figure 3. (A) Betacyanin structure: R1 and R2 represent Hydrogen or sugar moieties; (B) betaxanthin: R3 amino acid and R4: Hydrogen (Napoleón et al., 2013).



Plants exposed to air-polluting particles have been reported to show tissue damage, changing epidermal cells and stomata. In a study carried out in India, leaves of Bougainvillea "Mahara", Terminalia arjuna, Cassia fistula, and Plyalthia longifolia exposed to contaminating particles were collected, observing using a scanning electron microscope (SEM) that the bougainvillea did not present cuticular damage, indicating that this plant it acts as a mitigator of particulate pollution in urban and industrial areas (Kulshreshtha et al., 2009).

Therefore, the presence of plants such as B. glabra in urban areas is extremely important, not only to beautify the landscape but also to help mitigate the problem of pollution, as well as to maintain the functioning of ecosystems through pollination.

Traditional uses and importance of B. glabra

The involucre of B. glabra is widely used in traditional medicine to mainly treat respiratory diseases and different conditions (Figure 5).

Figure 4. Bougainvillea being pollinated.

In Mexico, B. glabra is popularly known by a wide variety of names such as purple bugambilia, paper flower, Santa Rita, but it is also named in indigenous languages such as shpupukuishonat (Mixtec), katsjoxhuan (Popolac) and jukua (Nahuatl) (Lim, 2014; Schlaepfer and García, 2017). The bougainvillea bracts, are commonly confused as flower petals, and these are the most used part in Mexican traditional medicine to treat respiratory conditions such as cough, asthma, flu and bronchitis through a variety of recipes; its use has also been reported to treat gastrointestinal problems such as diarrhea and dysentery; as well as to treat people who suffer from lung pain, whooping cough, drowning, urine sickness, pimples, and for cleaning wounds (Enciso-Díaz et al., 2012; Schlaepfer and García, 2017).

In Nigeria it is used to treat inflammation and as an analgesic (Ogunwande et al., 2019). In Thailand flowers are included in the daily diet to cure stomachache, and nausea (Kaisoon et al., 2012). In Mandsaur, India, bougainvillea helps reduce heartburn, treat sore throat, leucorrhea, blood vessels and hepatitis (Edwin et al., 2007; Gupta et al., 2009). To improve intestinal disorders extracts of B. glabra are used in Africa (He et al., 2020).

It has also been reported that extracts of B. glabra, popularly known as "glory of the garden", work to increase collagen production, inhibit tyrosinase and TNF activity, and are recognized as antioxidant, antimicrobial, antiviral, insecticide, larvicide, antidiabetic, antilipidemic, antihyperglycemic, hepatoprotective, antiulcer, anthelmintic, antipyretic, antifertility, and anticancer (Ogunwande et al., 2019; El-Sayed et al., 2021; Saleem et al., 2021).

Other studies have focused on bougainvillea betalains to use them as food, cosmetic, textile and pharmaceutical pigments, due to their antioxidant and non-toxic properties (Kumar et al., 2017).

There are currently some natural bougainvillea-based syrups on the market to treat respiratory tract discomfort, but generally, these products are used only as supplements since there are no scientific studies that guarantee efficacy and safety (Enciso-Díaz et al., 2012).

In addition to its ethnobotanical application, B. glabra is classified as one of the plant species of great horticultural importance worldwide, due to its ramifications and abundant colorful inflorescences that create a surprising appearance on walls, gates, or pergolas in gardens (Cumo, 2013).

Despite the great variety of traditional uses, the study of the chemical and pharmacological properties of B. glabra is limited (Saleem et al., 2019).

Figure 5. Traditional uses of B. glabra.

Phytochemistry of Bougainvillea glabra involucre

In 1970, the chemical components of the Bouganvillea genus began to be studied, using extracts from different organs of the plant (Abarca-Vargas et al., 2016). Next, the studies on the phytochemical composition (Supplementary Table S1) of the involucre of B. glabra are presented.

Due to its complexity, different techniques were used to determine the structure of the betacyanins present in the bougainvillea bracts. Using HPLC, about thirty complex patterns of betacyanins were detected. HPLC-MS-MS (Ultra-high performance liquid chromatography-MS/MS) recorded sixteen betacyanin precursor ions. In addition, nine structures were identified by HPLC- DAD (high performance liquid chromatography with a diode array detector), HPLC-MS and NMR-1D and 2D spectra, of which the latter helped to identify the betanidine fraction (Heuer et al., 1994).

In 2006, (Simon et al., 2006) using 1D and 2D nuclear magnetic spectroscopy (NMR) isolated three glycosides: momordin IIC (quinoside D), quercetin and a quercetin derivative, from an extract of the aerial part, without bracts, of B. glabra.

Using preparative ion-pair high-speed countercurrent chromatography coupled with electrospray ionization mass spectrometry (IP-HSCCC/ESI-MS-MS) six high molecular weight acyl-oligosaccharide-linked betacyanins were identified from a macerate of water, trifluoroacetic acid, and acetonitrile from bracts of a violet bougainvillea, collected in Guadalajara, Mexico (Jerz et al., 2010).

To know the phytochemical composition of four flowers used in the Thai diet: Tagetes erecta, Cosmos sulphureus, Antigonon leptopus and B. glabra; extractions with acidified methanol were performed and the compounds were identified by HPLC-DAD, where many phenolic acids and flavonoids were detected (Kaisoon et al., 2012).

Saleem et al. (2019) studied the phytochemical composition of methanol and dichloromethane extracts of B. glabra flowers using UHPLC-MS, revealing that most of the twenty-seven compounds are flavonoids and phenolic acids.

In Egypt, a study was carried out on the 'Scarlett O'Hara variety bougainvillea, where the ethyl acetate fraction of the extract of the aerial part was used: stem, leaves and flowers, to detect different groups of metabolites using ultra-performance liquid chromatography with electrospray ionization quadrupole-linear ion trap tandem mass spectrometry, performed on ESI-MS positive and negative ion (UPLC-ESI-MS/MS), where about fifty-seven phytochemicals were detected, including seven organic acids, fourteen phenolic compounds, one betacyanin, seven anthocyanins, ten flavonoids, three saponins, six tannins, four cyclic tetrapyrolic derivatives and five miscellaneous (El-Sayed et al., 2021).

Table 2. Functional groups of betacyanins detected by FTIR.

Functional group	Absorption band (cm^{-1})	References
OH	339	Kumar et al. (2017); Pérez et al. (2017)
	252	
COOO-	328	
N-H		
CH	295	
	291	
	284	
CΞ	214	
C=O	178	
	165	
C=C	145	
	141	
	82	
C-O	111	
	104	
	101	
N-H	156	
	71	
COO	151	
C-N	136	
C-OH	127	
	104	
OC-	88	

Knowing the variety of secondary metabolites present in the involucre of B. glabra, will allow us to improve its biological application in the future since it has been reported that the extracts that contain betalains present a variety of activities such as the inhibition of the growth of bacteria, as well as the yeasts and molds also prevent virus replication and have been reported to limit the growth of parasites. In the United States, betalains are patented as components of anticancer drugs due to their low cytotoxicity. On the other hand, it has been documented that they help reduce dyslipidemia, diabetes and have hepatoprotective, anti- inflammatory, neuroprotective and cardiovascular effects. These properties have been reported in clinical trials that provide safety to the use of these compounds, but they have only been studied for the genus Opuntia and red beet (Beta vulgaris), so the presence of these compounds in extracts of B. glabra opens a new opportunity to obtain and apply it (Devadiga and Ahipa, 2020; Sadowska and Bartosz, 2021).

Polyphenols are the most diverse group of secondary metabolites present in plants, more than 8,000 structures are currently known, and they are classified into phenolic acids, flavonoids, lignans, stilbenes, and tannins; to observe a variety of these compounds in extracts of B. glabra, which would benefit their study of new drugs since they have been reported to provide a wide variety of biological activities: they are natural anticancers due to their antioxidant and anti-inflammatory properties; they reduce the progress of neurodegenerative and cardiovascular diseases, they are excellent antithrombotic, antiallergic, anti-inflammatory and antimicrobial agents (Gorzynik-Debicka et al., 2018; Lobiuc et al.,2023).

In addition, it is important that when characterizing an extract, seasonal, local, and ontogenetic variations are reported when collecting the species, since this influences the phytochemical profile of the plant and therefore the pharmacological response (Enciso-Díaz et al., 2012). A challenge that is observed in the phytochemical studies of B. glabra is the correct identification of the plant organ, in addition, the choice of the color of the bract would offer a better identification between the pigments betacyanin and betaxanthin.

The presence of betacyanin in B. glabra has also been detected by Fourier-transformed infrared spectroscopy (FTIR), observing different absorption bands characteristic of the following functional groups (Table 2).

Table 3. Antimicrobial activity of bougainvillea wrapper.

Plant part	Extract	Inhibited microorganisms	Dose	Inhibition zone (mm)	References
Flower	96% ethyl alcohol.	Staphylococcus aureus	NA	9.4	Cardona et al. (2017)
		Pseudomonas aeruginosa		9.85	
		Escherichia coli		8	
Flower	95% methanol with successive extractions of n-hexane, carbon tetrachloride and water.	Staphylococcus aureus	20 mg/ mL	17–22	Zahidul et al. (2016)
		Escherichia coli		15–16	
		Bacillus cereus		12–14	
Bract	Methanol-aqueous	Bacillus subtilis	60 µL	7.4	Napoleón et al. (2013)
		Pseudomonas aeruginosa		5.3	
Flower	95% ethanol	Pseudomonas aeruginosa	NA	7	Perales and Leysa (2012)
		Escherichia coli		7	
Part area	Ethanol and water (90:10) with differential extracts of hexane, ethyl acetate, and butanol	Coccidioides immitis	NA	CIM = 500 µg/mL	Alanís-Garza et al. (2007)

Antimicrobial activity

Microorganisms are the major contributors to mortality worldwide due to infectious diseases. Currently the proliferation of diseases caused by pathogenic microorganisms is a risk factor for public health, these diseases are prevented by antibiotics, but due to their scarcity and current resistance of microorganisms, the use of phytochemical compounds from plants has been chosen for its medicinal properties due to their antimicrobial functions (Napoleón et al., 2013; Saeloh and Visutthi, 2021).

In recent years, it has been decided to scientifically investigate the use of B. glabra against bacteria and fungi that induce respiratory conditions (Table 3), based on the traditional use of involucre.

Perales et al. (Perales and Leysa, 2012) made 95% ethanolic extracts of leaves, stems, roots and flowers of B. glabra to test their antimicrobial activity against two Gram-positive bacteria Bacillus subtilis and Staphylococcus aureus, as well as two Gram-negative bacteria Escherichia coli and Pseudomonas aeruginosa using the Kirby-Bauer diffusion method, as

a positive control, amikacin 30 mcg, cephalexin 30 mcg, oxacillin 1 mcg and penicillin 10 U were used, and distilled water as a negative control. The flower extract was effective only against Gram-negative bacteria E. coli and P. aeruginosa, with a zone of inhibition of 7 mm, respectively. It is ensured that the extracts can have a greater action on Gram-negative bacteria because they do not have an external membrane (Lobiuc et al., 2023).

The antioxidant and antibacterial activity of betacyanins from B. glabra bracts was investigated by aqueous-methanol extraction. The antimicrobial activity was evaluated using the well diffusion technique against the bacteria B. subtilis, P. aeruginosa and E. coli, using Ampicillin as a control. The betacyanins showed greater antibacterial activity against B. subtilis, inhibiting a zone of 6.7–7.4 mm, against P. aeruginosa they inhibited 4.8–5.3 mm, and for E. coli only from 3.4 to 3.7 mm (Napoleón et al., 2013). The antimicrobial action of betalains, like betacyanins, has been reported to mainly affect the structure and permeability of the cell membrane (Sadowska and Bartosz, 2021).

To evaluate the antimicrobial and antioxidant activity of the B. glabra flower, (Zahidul et al., 2016) carried out a flower extraction with 95% methanol for 2 weeks at room temperature, later fractions of the extract were subjected to successive extractions of n-Hexane and carbon tetrachloride. The antimicrobial activity was evaluated against S. aureus, B. cereus, P. aeruginosa and E. coli with the disk diffusion method, counting the antibiotic Imipenem as a positive control and each solvent as a negative control. The bacterium that presented the greatest zone of inhibition was S. aureus (17–22 mm), followed by E. coli (15–16 mm), B. cereus (12–14 mm), while P. aeruginosa showed the greatest sensitivity low (0–6 mm). A preliminary phytochemical analysis of the extract demonstrated the presence of alkaloid, flavonoid, tannin, phenolic compound, reducing sugar, amino acid, and protein. The antimicrobial activity of the extract may be due to the presence of hydrophobic flavonoid that penetrate the nonpolar core of the bacterial cell membrane, or hydrophilic flavonoids that form hydrogen bonds with the polar groups of membrane lipids; furthermore, the presence of quercetin causes DNA breakage and inhibits bacterial gyrase. The presence of tannin prevents bacterial growth by chelating iron and prevents cell wall synthesis by inactivating enzymes. While phenolic acids damage the cell membrane of Gram-positive bacteria, and the cytoplasm of Gram negative ones; gallic acid alters the hydrophobicity, charge, and permeability of the membrane (Lobiuc et al., 2023). On the other hand, saponin causes the

release of proteins and enzymes and alkaloids interfere with cell division (Hemeg et al., 2020).

Table 4. Toxicology of B. glabra.

Extract	Animals	Test	Dose	Result	Reference
Methanolic (plant organ used is not specified)	Wistar rats	Acute and subchronic toxicity	Acute: 2000 mg/kg Subchronic: 250, 500 and 1000 mg/kg	Acute: does not cause death or symptoms. Subchronic: do not generate significant changes.	Krishna and Sundararajan (2020)
Aqueous three-color bract	Zebra fish	Acute toxicity and teratogenic effect	0.3, 1, 3, 10, 30, 100 and 300 µg/mL	Acute: Pink extract with 85.51 µg/mL of 50% lethal concentration. Teratogenic: 20% edema of the yolk sac with dark pink extract, and purple bract hypopigmentation.	Teh et al. (2019)
Methanol and dichloromethane (DCM) from flowers	MDA-MB-231, MCF-7, CaSKi, DU-145, SW-480 cell lines	Cytotoxicity	500–15.625 µg/mL	IC_{50} (µg/mL) MDA-MB-231 = Methanol: 300.6 DCM: >500 MCF-7= Methanol: 105.7 DCM: >500 CaSKi = Methanol: 88.49 DCM: 180.1 DU-145 = Methanol: 129.9 DCM: 180.9 SW-480 = Methanol: >500 DCM: 304.7	Saleem et al. (2019)
Ethanolic from bracts	Vero cell line and	Cytotoxicity	NA	Vero: 269.10 ± 70.16 µg/mL WRL-68:	Shalini et al. (2018)

Cardona et al. (2017) prepared infusions of leaves and flowers of B. glabra in ethyl alcohol at a concentration of 96%, which were kept for 2 months in the refrigerator. To measure the antibacterial activity, the disc diffusion method (Kirby-Bauer technique) was used against strains of S. aureus, P. aeruginosa and E. coli; each susceptibility test had a control to rule out that 96% ethyl alcohol had antibacterial activity. The results obtained showed that the leaf extracts have a higher inhibition against the strains of S. aureus (15.4 mm) and P.

aeruginosa (16.8 mm); while flower infusions inhibited only 9.4 mm against S. aureus, and 9.8 against P. aeruginosa. An explanation for the fact that leaf extract presented greater inhibition could be due to its difference from the flower in its chemical composition.

The antifungal activity of 15 plants from northeastern Mexico against the fungi Candida albicans, Aspergillus fumigatus, Histoplasma capsulatum and Coccidioides immitis, inducers of pulmonary mycosis, has also been evaluated. The extraction of the aerial part of the plants was carried out with ethanol and water (90:10), subsequently, those extracts which presented antifungal activity were subjected to differential extracts of hexane, ethyl acetate and butanol. B. glabra only showed antifungal activity against C. immitis with a minimum inhibitory concentration (MIC) of 500 µg/mL of the hydroalcoholic extract (Alanís-Garza et al., 2007).

Although there is only one study that reports the minimum inhibitory concentration (MIC) of the B. glabra extract, in Supplementary Table S1 we observe that the different phytochemicals isolated from this plant present antimicrobial activity (MIC, minimum bactericidal concentration MBC, and half maximal inhibitory concentration IC50) against a wide variety of microorganisms (Shi et al., 2016; Wang et al., 2019; Taheri et al., 2020) of clinical importance, such as the pathogens known as ESKAPE, which include Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa and Enterobacter species that are highly infectious and resistant to multiple drugs, which are also classified by the WHO as high priority pathogens (E. faecium and S. aureus) and critical priority (A. baumannii, P. aeruginosa and E. coli) for the search for new drugs (Wijesinghe and Choo, 2022).

Therefore, the great variety of secondary metabolites present in the natural extracts makes it possible to inhibit or retard bacterial growth using different mechanisms of action, but due to he diversity of both compounds and bacteria, these mechanisms are still not well understood. It has been reported that the activity differs according to cell morphology, with coccoid cells being more resistant than rod cells (Nazzaro et al., 2013; Lagha et al., 2019).

Antibiofilm activity

In recent years it has been reported that under natural conditions bacteria commonly live in biofilms, instead of their planktonic form (Rabin et al., 2015; Stiefel et al., 2016). Biofilms are bacterial communities covered by an extracellular matrix composed of extracellular polymeric substances (EPS), such as polysaccharides, proteins, lipids, and extracellular DNA, that adhere to surfaces (Cao et al., 2020; Furner-Pardoe et al.,2020).

The presence of EPS prevents the removal of biofilms from requiring up to 1000 times higher concentrations of antibiotics than planktonically grown bacteria (Stiefel et al., 2016; Furner-Pardoe et al., 2020), which causes around two million illnesses and more than 23,000 deaths per year due to resistant bacteria (Cao et al.,2020).

The high rate of resistance of biofilms to antibiotics is still not very clear, but the search for different and new antimicrobial agents has been chosen, such as the use of plant extracts, antimicrobial nanoparticles, antimicrobial proteins, and peptides (AMP), as well as antimicrobial enzymes (Cao et al., 2020).

Studies show that the use of secondary metabolites has antibiofilm activity since they alter the structure of the biofilm causing bacterial detachment, they also inhibit its adherence and affect the morphology of the biofilm (Roy et al., 2018).

The antibiofilm activity of flavonoids has been reported, which initially allows bacterial aggregation by membrane fusion, but then reduces the absorption of active nutrients, causing their death; in addition, they interact with sortases enzymes of the cytoplasmic membrane of Gram-positive bacteria, catalyzing the assembly of cells that allow infection. On the other hand, the inhibition of the expression and activity of the urease gene by the action of tannic acid reduces the formation of biofilms (Gorzynik-Debicka et al., 2018). A polyphenol extract manages to block the activity of glycosyltransferase (GTF) which affects the formation of the S. mutans biofilm (Cao et al., 2020). The reduction in the expression of virulence genes because of eugenol prevents the adhesion and formation of biofilms (Roy et al., 2018).

The antibiofilm activity of B. glabra extracts has not been explored, despite its potential due to the presence of a variety of metabolites, but a study carried out by Rauf et al. (2019) where zinc oxide nanomaterials (ZnO-NMs) were synthesized from aqueous extract of Bougainvillea sp. flowers, demonstrated their inhibitory effect on the development of S. aureus and E. coli biofilms at a concentration of 100 µg/mL for 48 h. Cao et al. (2020) mention that ZnO-NPs have antimicrobial activity by producing reactive oxygen species (ROS) that cause cell death and alter the stability of the cell membrane; they further hinder the EPS of biofilms and bind and inhibit DNA and enzymes.

Toxicology

The belief that natural treatments are safer is not always true, therefore, it has been decided to evaluate the toxicity of medicinal plants, to guarantee greater safety for the creation of new drugs (Krishna and Sundararajan, 2020). Some studies on toxicology that have been carried out on B. glabra are presented below (Table 4).

To evaluate the cytotoxicity of the ethanolic extract of B. glabra bracts, they were exposed for 72 h with fetal human liver cells (WRL-68) and African green monkey (Vero) kidney cells, resulting in a mean inhibition concentration (IC50) of 269.10 ± 70.16 µg/mL for VERO cells and 135.46 ± 20.43 µg/mL for WRL-68 cells, considered the extract without toxicity since it did not exceed the negative control (Shalini et al., 2018).

To evaluate the acute toxicity and the teratogenic effect of the aqueous extract of three colors of bracts (purple, pink and strong pink) of B. glabra, zebrafish embryos were used. Acute toxicity was evaluated with the following concentrations: 0.3, 1, 3, 10, 30, 100 and 300 µg/mL, the pink bract extract being toxic to embryos with 85.51 µg/mL of 50% lethal concentration. The three extracts caused yolk sac edema as teratogenic results, highlighting a greater growth (20%) with the dark pink bract; in addition to hypopigmentation, which was observed to a greater extent with the purple extract. Despite this, the extract is not considered toxic since the embryos did not undergo major modifications (Teh et al.,2019).

The cytotoxicity of methanolic and dichloromethane extracts from B. glabra flowers was evaluated against different cancer cell lines, such as breast cancer (MDA-MB-231, MCF-7),

cervical cancer (CaSKi), prostate (DU-145) and colon cancer (SW-480), resulting in the methanolic extract with the highest activity against the CasKi line, while the dichloromethane extract presented moderate activity (Saleem et al., 2019). The presence of certain phenolic compounds has been reported to cause apoptosis in cancer cell lines, this may be due to the polarity of the compounds (Yerlikaya et al., 2017).

The acute and subchronic toxicity of methanolic extracts of B. glabra was evaluated in albino Wistar rats, which were subjected for 90 days to a dose of 250, 500 and 1000 mg/kg for the subchronic test. At the end of this period, the animals were sacrificed, and hematological, biochemical, and histopathological parameters were evaluated, which resulted without significant variations compared to the control. For the acute toxicity test, 2000 mg/kg of extract was administered for 14 days, and no mortality or changes in respiratory symptoms, piloerection, tearing, or locomotor symptoms were recorded (Krishna and Sundararajan, 2020).

Toxicological tests offer us a different perspective since it is commonly believed that plant extracts using solvents such as methanol can cause some damage to the organism, but results such as Krishna and Sundararajan (Krishna and Sundararajan, 2020), it reminds us that the appropriate solvent can extract a greater amount of phytochemicals from the plant, which will provide better therapeutic properties.

With previous studies we can consider that the toxicity of B. glabra is null, this may be because the main compound present in the involucre is betalains, which have reported minimal toxicity and side effects (Sadowska and Bartosz, 2021). However, it is necessary to increase the number of in vitro and in vivo studies of this plant to provide greater security. In addition, it is important to add evidence on the toxicological effect of B. glabra in aquatic environments since bioassays using algae and invertebrates are extremely important to know the trophic impact of a substance in an ecosystem (Cangiano et al., 2002).

Conclusion and future perspective

Ethnobotany is a tool that has allowed the search and choice of medicinal plants that are a novel alternative for the treatment of infections. Due to the current increase in microbial

resistance to antibiotics, medicinal plants represent the largest reserve of phytochemical compounds available to counteract this problem; but currently this reserve needs numerous studies to correctly identify the secondary metabolites, as well as their mechanisms of action on the inhibition of bacterial growth.

The phytochemical profile of the involucre of B. glabra contain a variety of compounds, mainly betalains and phenols, providing a new opportunity to study their potential as antimicrobial agents and antibiofilm, but currently, there are no studies that demonstrate the values of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and half maximal inhibitory concentration (IC50) necessary to validate the antibacterial activity with adequate concentrations to provide safety when using it in the health sector.

Current research should not focus only on the antimicrobial activity of the extracts, but on their antibiofilm activity, since this adherence gives them greater resistance to antibiotics and there are no drugs that specifically target this infection mechanism. Therefore, the potential of B. glabra as an antibiofilm should be investigated since it has an action on planktonic bacteria.

At the same time, taking advantage of the use of scanning electron microscopy is an option that would allow us to know how the phytochemistry of B. glabra extracts affects the structure of bacterial cell morphology and biofilms.

The use of B. glabra as a therapeutic agent based on traditional medicine still presents different challenges, first, to know its potential it is necessary to carry out a correct identification of the plant organ that allows to identify the diversity of secondary metabolites present, to enhance its therapeutic properties.

Finally, it is important to highlight the potential of this ornamental plant, not only to beautify landscapes but also its role in mitigating another current problem, which is air pollution in large cities.

We are invited to continue studying all those plants used in traditional medicine.

Author contributions

IO: Conceptualization, Writing–original draft. AG: Conceptualization, Funding acquisition, Project administration, Writing–review and editing. FA: Writing–review and editing. NC: Writing–review and editing. DG: Conceptualization, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2023.1276514/full#supplementary-material

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6. Conclusions

Psidium guajava L. leaves are a highly available agro-industrial residue that is not valued in guava producing countries despite its great phytochemical and bioactive potential. In the present study, three methods of phytochemical extraction were evaluated: Soxhlet, maceration and ultrasound assisted extraction (UAE). Soxhlet is postulated as an advantageous option for the recovery of phenolic compounds, given that it does not require a significant amount of time, no processes are needed to separate the plant material from the solvent, the solvent can be reused and the extract obtained an outstanding yield in phenolic compounds compared to maceration and ultrasound-assisted extraction. In addition, different commercially available systems allow the Soxhlet extraction method to be scaled and even automated for industrial applications.

Thirteen compounds were identified in guava leaf extract, including catechin, quercetin glucuronide, vescalagin, casuarinin, and guajaverin, all of them with reported bioactivities. In addition, the extract exhibited antimicrobial activity, reducing the growth of more than 15 important pathogens such as *S. aureus*, *S. epidermidis*, and *C. acnes*, most of them clinical isolates, representing the bacteria we encounter in everyday life. Highlighting its activity against ten isolates of XDR *A. baumannii*, the microorganism of highest priority by the World Health Organization (WHO) for the discovery of new antibiotics. Furthermore, the purification of polyphenolic compounds was a strategy that allowed us to improve the activity by reducing the dose up to twenty times. Likewise, the combination with gentamicin resulted in a synergistic effect, restoring the effectiveness of the antibiotic. The purification of polyphenols also improved the antioxidant activity of the guava leaf extract, so we can say that polyphenols are the main bioactive compounds in guava leaf and the elimination of other compounds present in the extract such as saponins, sugars and terpenes allow to improve the activity. On the other hand, antibiofilm activity against *S. epidermidis* and *E. faecalis* is also reported, recording inhibition percentages of bacterial adhesion close to 100% in the microplate experiments, which could be corroborated by visualizing the biofilms with the commercial dye Film-tracer. The prevention of adhesion is an attractive strategy for the development of new treatments, since it is a fundamental phase for the formation of biofilms. Finally, guava leaf extract generated changes in the viability and metabolism of different cell lines when exposed to concentrations of crude extract that ranged between 3

and 10 mg/ml, which are lower than the effective concentrations for it to exert antimicrobial activity, so for its future application it is necessary to look for strategies to reduce these effective doses, reduce their potential cytotoxic effects and even improve their long-term stability.

Guava leaf extract is a potential antimicrobial and disinfectant for both biotic and abiotic surfaces, so its application and valorization are an opportunity for all guava producing countries, including Mexico.

6.1. Proposal for Measurement and Monitoring of Results

Table 16. Proposal for the calculation of the application index of Psidium guajava L. leaf extract.

Variable	Dimension	Subdimension 1	Subdimension 2	Indicator	
				Value	Rating scale
Application Index of Guava Leaf Extract (I_{AGLE})	**Wide** From 0.86 to 1 of the I_{AGLE}	Phytochemical extraction	Time	Fast, duration ≤ 4 hours .	8
			Price	All the supplies and materials necessary for obtaining and processing the extract are affordable.	10
			Solvent	No toxic solvents are used.	9
			Complexity	Simple, no specialized equipment or personnel required.	8
		Antimicrobial activity	Spectrum	Broad spectrum, bactericidal activity against Gram positive and Gram negative bacteria.	9
			Effectiveness	Activity against microorganisms extremely resistant to antibiotics.	10
			Dose	MIC values ≤ 5 mg/ml .	8
		Antibiofilm activity	Adhesion inhibition	Inhibition percentages ≥ 80%.	8
		Cytotoxic effects	Metabolic activity	No decrease in metabolic activity.	9
			Viability	No decrease in cell viability.	9
			Morphology	No changes in cell morphology.	9
	Moderate From 0.73 to 0.85 of the I_{AGLE}	Phytochemical extraction	Time	Duration ≥ 4.01 hours ≤ 6 hours.	7
			Price	Requires low to medium investment.	7
			Solvent	Use of toxic solvents but they are reused and/or limited.	8
			Complexity	Use of equipment and reagents that require trained personnel.	8
		Antimicrobial activity	Spectrum	Decreases the growth of Gram positive and Gram negative bacteria.	8
			Effectiveness	Activity against moderately antibiotic-resistant bacteria	8
			Dose	MIC values from 5.01 mg/ml to 15 mg/ml	7
		Antibiofilm activity	Adhesion inhibition	Inhibition percentages ranging from 50 to 79%	7
		Cytotoxic effects	Metabolic activity	Changes in metabolic activity but at concentrations higher than the effective doses.	7
			Viability	Changes in cell viability but at concentrations higher than the effective doses.	7
			Morphology	Changes in cell morphology but at concentrations higher than the effective doses.	7
	Minimum Less than or equal to 0.72 of the I_{AGLE}	Phytochemical extraction	Time	Duration ≥ 6.01 hours.	6
			Price	Requires a significant investment.	5
			Solvent	Use of toxic solvents.	5
			Complexity	Use of equipment and reagents that require highly specialized personnel.	6
		Antimicrobial activity	Spectrum	Narrow spectrum, activity against a selected group of bacteria.	5
			Effectiveness	Activity against ATCC bacteria.	5
			Dose	MIC values ≥ 15.01 mg/ml	5
		Antibiofilm activity	Adhesion inhibition	Inhibition percentages ≤ 49%	5
		Cytotoxic effects	Metabolic activity	Changes in metabolic activity at effective doses.	5
			Viability	Changes in cell viability at effective concentrations.	5
			Morphology	Changes in cell morphology at effective concentrations.	5
Index	$I_{AGLE} = \sum_{n=1}^{n} \left(20\%\left(\frac{01+O2+O3+04}{\text{Max}\,(01+O2+O3+04)}\right) + 30\%\left(\frac{M1+M2+M3}{\text{Max}\,(M1+M2+M3)}\right) + 20\%\left(\frac{B1}{\text{Max}\,(B1)}\right) + 30\%\left(\frac{C1+C2+C3}{\text{Max}\,(C1+C2+C3)}\right) \right)$				

Source: Own elaboration.

6.2. Current Results Situation

Table 17. Application index of guava leaf extract obtained in the present study.

Variable	Dimension	Subdimension 1	Subdimension 2	Indicator	
				Value	Rating scale
Application Index of Guava Leaf Extract (I_{AGLE})	**Moderate From 0.73 to 0.85 of the (I_{AGLE})**	**Phytochemical extraction**	Time	Fast, duration ≤ 4 hours .	**8**
			Price	All the supplies and materials necessary for obtaining and processing the extract are affordable.	**10**
			Solvent	Use of toxic solvents but they are reused and/or limited.	**8**
			Complexity	Use of equipment and reagents that require trained personnel.	**8**
		Antimicrobial activity	Spectrum	Broad spectrum, bactericidal activity against Gram positive and Gram negative bacteria.	**9**
			Effectiveness	Activity against microorganisms extremely resistant to antibiotics.	**10**
			Dose	MIC values ≥ 15.01 mg/ml	**5**
		Antibiofilm activity	Adhesion inhibition	Inhibition percentages ≥ 80%.	**8**
		Cytotoxic effects	Metabolic activity	Changes in metabolic activity at effective doses.	**5**
			Viability	Changes in cell viability at effective concentrations.	**5**
			Morphology	Changes in cell morphology at effective concentrations.	**5**
(I_{AGLE})= 0.82					

Source: Own elaboration.

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