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Antibacterial and hemolytic activity of extracts and compounds obtained from epicarps and seeds of *Garcinia madruno* (Kunth) Hammel

[Actividad antimicrobiana y hemolítica de extractos y compuestos obtenidos del epicarpio y las semillas de *Garcinia madruno* (Kunth) Hammel]

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Abstract: This paper describes the evaluation of the antimicrobial and hemolytic activity of the hexane, dichloromethane, ethyl acetate and methanol extracts from seeds and epicarps of *Garcinia madruno*; as well garcinol, morelloflavone and volkensiflavone isolated from the same species. In the preliminary test of bacterial susceptibility, hexane extracts from seeds and epicarps and the three compounds tested only displayed inhibitory growth effect against Gram-positive bacteria. The minimum inhibitory concentrations of extract and compounds ranging from 86.6 to 1253.4 µg/mL. The hemolytic activity was assessed; however, except for the methanol extract from seeds, none of the samples studied induced hemolysis. Thus, our results suggest that extracts and compounds from *G. madruno* have the potential to be used in the control of pathologies associated to Gram-positive bacteria. This is the first report of the antimicrobial and hemolytic activity of extracts of different polarity obtained from seeds and epicarps of this edible species.

Keywords: *Garcinia madruno*; Antibacterial activity; Hemolytic activity; Biflavonoids; Garcinol.

Resumen: El presente artículo describe la evaluación de la actividad antimicrobiana y hemolítica de los extractos de hexano, diclorometano, acetato de etilo y metanol, obtenidos de la semilla y el epicarpio de *Garcinia madruno*; así como de garcinol, morelloflavona y volkensiflavona; aislados de la misma especie. En el ensayo de susceptibilidad bacteriana, tanto el extracto de hexano obtenido a partir de la semilla y el epicarpio, y los tres compuestos aislados, únicamente mostraron actividad inhibitoria del crecimiento contra bacterias Gram-positivas. La concentración mínima inhibitoria presentó valores entre 86.6 y 1253.4 µg/mL. También se estableció la actividad hemolítica; sin embargo, con excepción del extracto metanólico obtenido a partir de las semillas, ninguna de las muestras evaluadas indujo hemólisis. Por lo tanto, los resultados sugieren que los extractos y compuestos de *G. madruno* tienen el potencial de ser usados en el control de bacterias Gram-positivas asociadas a diversas patologías. Este es el primer reporte de actividad antimicrobiana y hemolítica de extractos de diferente polaridad obtenidos de las semillas y epicarpios de esta especie comestible.

Palabras clave: *Garcinia madruno*; Actividad antibacteriana; Actividad hemolítica; Biflavonoides; Garcinol.

INTRODUCTION

The family Clusiaceae consist of more than 1000 species that are concentrated mainly in the tropics. *Garcinia* is a genus of the Clusiaceae family that includes more than 250 species of shrubs and trees that are widely distributed in Africa, tropical Asia, and some countries in America (Kumar *et al*., 2013). *Garcinia* species are an important source of biologically active secondary metabolites with simple and complex structures, including xanthones, benzophenones, lactones, phenolic acids and flavonoids. Some species of *Garcinia* are well known for their antiprotozoal, antibacterial, anti-inflammatory and anti-immunosuppressive properties; and have been used in the treatment of cancer, inflammatory disorders, ulcers, hypertension, liver damage, among others (Lim, 2012; Carrillo-Hormaza *et al*., 2016; Olmedo *et al*., 2018).

Garcinia madruno (Kunth) Hammel (Clusiaceae) is a tropical native tree from Central and South America (Lim, 2012). In Colombia, this plant called by locals "madroño"is widely distributed and can be found in a range from 0 -1800 m.a.s.l. This species is known by its ornamental value and its wood is employed in construction (Varón & Morales, 2013). The mesocarp of its yellow fruit is edible, and used to prepare jams and drinks (Lim, 2012). In Colombia, farmers use this plant for different environmental services and as a source of edible fruits (Estupiñan-González & Jimenez-Escobar, 2010).

A comprehensive study about the chemical composition of *G. madruno* has been done. Biflavonoids and organic acids were mainly identified; as well polyisoprenylated benzophenones were detected (Carrillo-Hormaza *et al*., 2016). These groups of compounds, together with xanthones, are commonly found in species belonging to the *Garcinia* genus (Hemshekhar *et al*., 2011).

Due to the great number of medicinal uses described for species of the genus *Garcinia*; many of them have been screened in order to determine their pharmacological/therapeutic potential (Hemshekhar *et al*., 2011; Kumar *et al*., 2013**)**. Previous pharmacological studies with *G. madruno* have focused on antioxidant activity of biflavonoids (Osorio *et al*., 2009; Osorio *et al*., 2013**)**. Recently, Olmedo *et al*. (2018), carried out a bioprospecting study of the Panamanian flora in order to find bioactive molecules against tuberculosis, cancer, Alzheimer's, and parasitic diseases. In particular, they found that *G. madruno* extracts were active against three human cancer cell lines, and selectively inhibited the enzyme acetylcholinesterase. Additionally, a standardized extract obtained from this species has been evaluated as an option to treat Alzheimer's disease (Sabogal-Guáqueta *et al*., 2018) and atherogenicity (Tabares-Guevara *et al*., 2014; Tabares-Guevara *et al*., 2017**).**

On the other hand, some biflavonoids such as agathisflavone, amentoflavone, tetrahydroamento-flavone, isoginkgetin among others, isolated from different plants, have shown antimicrobial activity against Gram-positive and Gram-negative bacteria such as *S. aureus*, *E coli* and *E. faecalis* among others (Menezes & Campos, 2021). In addition, the biflavonoid macrophylloflavone, isolated from *Garcinia macrophylla* Mart, showed strong activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. The high antioxidant capacity of macrophylloflavone could explain the antibacterial activity through the generation of reactive oxygen species that can cause disruption of the cell membrane (Górniak et al., 2019).

To the best of our knowledge, there is only one report on antimicrobial properties of *G. madruno*. In this study, methanol:CH_2Cl_2 (1:1) extracts from leaves and stems displayed inhibition of the growth of *S. aureus*; and no showed lethal activity against brine shrimp nauplii (Suffredini *et al*., 2006). Due to the lack of studies of activity of *G. madruno* against microorganisms, the objective of the current work was to examine the antibacterial properties of hexane, dichloromethane, ethyl acetate and methanol extracts obtained from epicarps and seeds of *G. madruno*; as well three isolated compounds from seeds of this species. In addition, hemolytic activity of extracts and pure compounds was also assessed.

METHODS AND MATERIALS

Plant material

Fresh and ripe fruits of *G. madruno* were acquired from local market in Medellín, Colombia. The voucher specimen was identified by a botanist (MEDEL-66811) and deposited at the Herbarium "Gabriel Gutiérrez Villegas" of the Universidad Nacional de Colombia-Sede Medellín.

Extraction and Isolation of metabolites

Epicarps (411.9 g) and seeds (735.9 g) were removed and separated from previously washed ripe fruits of *G. madruno*. Shade dried parts were powdered using a blender. To prepare the extracts, a weighted amount of the powder was exhaustively extracted by percolation with *n*-hexane (H), dichloromethane (D), ethyl acetate (A) and methanol (M). Eight extracts of different polarity were obtained, four from the epicarps (HE, DE, AE and ME) and four from the seeds (HS, DS, AS and MS). Extracts were filtered and evaporated under reduced pressure at 40°C by the rotary evaporator (Buchi R-200) and used for the determination of the antibacterial and hemolytic activities. Isolation, purification, as well as the structural characterization of the secondary metabolites tested, was previously described in detail by Ramirez *et al*. (2019).

Bacterial strains used

Gram-positive bacteria strains employed were *Streptococcus mutans* (ATCC 31989), *Staphylococcus aureus* (ATCC 65380), *Enterococcus faecalis* (ATCC 29212); and Gram-negative strains were *Escherichia coli* (ATCC 25922), and *Salmonella typhimurium* (ATCC 14028).

Bacterial culture media

Gram-positive bacterial strains were cultured and sub-cultured in trypticase soy broth (TSB, Difco Laboratories, Detroit, MI, USA) and Gram-negative strains were grown employing a Luria broth base (LB; Gibco, Cerdanyola del valle, Barcelona, Spain).

Sample preparation

In order to conduct the biological tests; 10 mg/ mL stock solutions of both plant extracts and pure compounds were prepared by dissolving samples in 40% DMSO to a final volume of 1 mL in PBS.

Determination of bacterial sensitivity to plant extracts and compounds by radial diffusion assays

Bacterial sensitivity was carried out following the methodology described by Lehrer *et al*. (1991). To perform radial diffusion assays, both Gram-positive and Gram-negative bacteria were poured on a low nutrient medium consisting of 10 g of low electroendosmosis agarose (Sigma Chem. Co, St. Louis, MO, USA), 0.02% (v/v), Tween-20 (Merck, Darmstadt, Germany) 0.3 g TSB (Difco Laboratories, Detroit, MI, USA) to final volume of 1 L. An overlay of highly nutritive medium was used for assessing bacterial growth in radial diffusion assays and therefore determining the antibacterial activity of control standard antibiotics, plant extracts and pure compounds. The overlay medium was composed of 20 g agar–agar (Difco Laboratories, Detroit, MI, USA), 0.02% (v/v) Tween-20 (Merck, Darmstadt, Germany) and 10 g TSB (Difco Laboratories, Detroit, MI, USA). Phosphate buffered saline (PBS), pH 7.2–7.4, was prepared by mixing 0.2 g KH_2PO_4, 32 g NaCl, 0.8 g KCl, 4.6 g Na_2HPO_4 to a final volume of 1 L. Standard antibiotics were employed as positive antibacterial compounds, these consisted in stock solutions of 50.8 mg/mL ampicillin (Binotal; Bayer1, Germany), 10.25 mg/mL kanamycin sulphate (Gibco, Cerdanyola del valle, Barcelona, Spain), 5.78 mg/mL tetracycline monohydrate (Sigma Chem. Co, St. Louis, MO, USA).

Gram-negative and Gram-positive bacteria were cultured overnight at 37ºC under constant shaking. Subsequently, a sub-culturing of each bacterial strain was obtained by inoculating an aliquot into approximately 20 mL of the corresponding medium. Fresh cultures were then incubated for 4 to 5 additional hours at 37ºC under constant shaking. Then bacteria were harvested by spin-down subculture at 2400 x *g* for 10 min, washed twice with PBS, pH 7.2 at 4ºC, resuspended in fresh PBS and maintained at 4ºC until used. The sample's optical density (OD) was measured at 620 nm and precise amounts of bacteria were measured according to a previously reported ratio of OD620 = 0.2 arbitrary absorbance units (au) = 5 x 10^7 CFU/mL, as needed. A total of 4 x 10^7 CFU were carefully dispersed into 15 mL of low nutrient medium previously heated at 45-50ºC and subsequently poured into Petri dishes. Once solidified, rounded wells were made using a 3-mm diameter sterile cylindrical pouncher. Wells were carefully filled with 2, 4 and 8 µL aliquots of ampicillin, kanamycin sulphate and tetracycline, which corresponded to 0.508, 1.025 and 0.578 mg/mL concentrations, respectively. Systematically, in the same petri dish samples of 2, 4 and 8 µL aliquots of 5.0 mg/mL plant extract and pure compound´s stocks were poured to be analyzed. Sample diffusion was allowed by incubating Petri dishes for 30 min at 37ºC and then plates were coated with 14 to 15 mL of nutrient-rich medium previously heated at 45-50ºC and were

incubated overnight at 37ºC. Antibacterial activity was observed as discrete clear halos caused by the radial diffusion of active compounds present on tested plant extracts and compounds. The diameters of halos were carefully measured and expressed as activity units (AU) according to the relation 0.1 mm = 1 AU.

Determination of Minimum Inhibitory Concentration (MICs)

The experiment was performed according to standardized procedures (Wiegand *et al*., 2008). Briefly, 200 µL of each plant extract and pure compounds from stock solutions at 5.0 mg/mL concentration were initially placed in each well of flat-bottomed, 96-well dishes and 1:2 serial dilutions were made of each for a 5.0 mg/mL and 1.9 x 10^{-3} mg/mL final concentration range. Control antibiotics were prepared from stocks concentrated at 1:10 aqueous dilution at the following concentrations: 1.0 mg/mL (2.862 µM) ampicillin; 1.0 mg/mL (2.064 µM) kanamycin; and 1.0 mg/mL (2.080 µM) tetracycline from which serial dilutions were made, as above. A 1 x10^4 total colony forming unit (CFU) inoculum was put into 100 µL concentration of the bacterial strain being tested. Luria broth (LB) liquid bacterial culture medium was used as assay target and optical density (OD) was read at different time intervals from 0 up to 24 hours at 620 nm wavelength on a Multiskan FC-51119000 – Thermo Scientific, microplate reader (Vantaa, Finland) for monitoring bacterial viability.

Hemolytic activity test

The hemolytic activity of plant extracts and pure compounds was determined in flat-bottomed, 96-well dishes according to Lozano *et al*. (2014). Thus, 100 µL saline solution was initially placed in the wells destined for serial dilutions. 100 µL of the positive control solution was then used for hemolysis: 1% (v/v) Triton X-114 (Sigma-Aldrich Chemical Co., Saint Louis, MO, USA), 1% (v/v) saponin (Sigma-Aldrich Chemical Co., Saint Louis, MO, USA), both in PBS solution. The same amount of DMSO-PBS was used as target in all assays in duplicate. 200 µL of plant extracts and compounds were prepared; and then 1:2 serial dilutions were made for a 5.0 mg/mL initial concentration and 4.94 x 10^{-3} mg/mL final concentration. A 4% (v/v) of red blood cells (RBC) dilution was then prepared in saline solution from RBC obtained from humans' O^+ blood, 100 µL of which was poured into each well. This was incubated for 1h at 37°C and then each dish was spun at 5,000 rpm for 10 minutes and then removed and 100 µL of the supernatant were transferred to another flat-bottomed, 96-well dish and OD was read at 405 nm wavelength on a Multiskan FC-51119000 - Thermo Scientific, microplate reader (Vantaa, Finland). The hemolysis produced in the RBC was expressed as: ((OD sample - OD target/OD triton X-114- OD target)) x 100.

RESULTS

In this work the qualitatively and quantitatively antibacterial activity of extracts from epicarps and seeds obtained with solvents of different polarity; as well three isolated compounds from seeds of *G. madruno* was assessed. Extracts and pure compounds were tested against three Gram-positive bacteria and two Gram-negative bacteria. Results of the preliminary activity, obtained in the agar well diffusion method, are shown in Table No. 1. Hexane extracts from epicarps (HE) and seeds (HS) were the most active. Both extracts showed antibacterial activity against all the Gram-positive strains tested. The most susceptible bacteria was *E. faecalis*. In addition to previously mentioned extracts, which were active against this bacteria, DE and ME extracts exhibited activity against *E. faecalis* and DS against *E. faecalis* and *S mutans*. AE, AS and MS extracts failed to inhibit growth of the bacterial strains used in this study. Finally, the three pure compounds evaluated; morelloflavone, volkensiflavone and garcinol, showed inhibitory effects against all the Gram-positive bacterial strains. None of the extracts and compounds evaluated were effective against Gram-negative bacteria. Several authors have reported the limitations and advantages of this methodology. The low rate of diffusion of non polar metabolites is commonly cited as a pitfall (Cushnie & Lamb, 2005; Scorzoni *et al*., 2007; Schumacher *et al*., 2018; Górniak *et al*., 2019). To obtain reliable results, the agar diffusion test requires an adequate diffusion of the compounds in the medium. To minimize this problem, we used DMSO-PBS to dissolve the samples and improve the diffusion across the agar matrix (Valgas *et al*., 2007; Górniak *et al*., 2019). Aware of this, we used this methodology as a qualitative preliminary assay; having into account its relative importance.

Table No. 1
Susceptibility of Gram-positive bacteria to extracts and isolated compounds from *G. madruno*.
AU: activity units 0.1 mm = 1AU

		Diameter (cm) and (AU) in Gram-positive bacteria		
Antibiotic/ Sample*	**Volume (µL)**	***E. faecalis* 29212**	***S. aureus* 65380**	***S. mutans* 31989**
HS	2	0.7 (70)	1.0 (100)	0.7 (70)
	4	0.6 (60)	0.6 (60)	0.6 (60)
	8	0.6 (60)	0.5 (50)	0.5 (50)
DS	2	0.6 (60)	0	0.6 (60)
	4	0.5 (50)		0.6 (60)
	8	0.4 (40)		0
AS	2	0	0	0
	4			
	8			
MS	2	0	0	0
	4			
	8			
HE	2	0.8 (80)	0.7 (70)	0.6 (60)
	4	0.5 (50)	0.7 (70)	0.6 (60)
	8	0.5 (50)	0.7 (70)	0.5 (50)
DE	2	0.5 (50)	0	0
	4	0.2 (20)		
	8	0.4 (40)		
AE	2	0	0	0
	4			
	8			
ME	2	0.6 (60)	0	0
	4	0.5 (50)		
	8	0		
Volkensiflavone	2	0	0.2 (20)	0.5 (50)
	4	0.3 (30)	0.4 (40)	0.5 (50)
	8	0.4 (40)	0.4 (40)	0.7 (70)
Morelloflavone	2	0	0.3 (30)	0.4 (40)
	4	0.2 (20)	0.3 (30)	0.5 (50)
	8	0.4 (40)	0.5 (50)	0.6 (60)
Garcinol	2	0.8 (80)	0.8 (80)	0.7 (70)
	4	0.7 (70)	0.7 (70)	0.7 (70)
	8	0.6 (60)	0.6 (60)	0.6 (60)
Tetracycline		1.8 (180)	1.1 (110)	0.5 (50)
Kanamycin		0.9 (90)	0.5 (50)	0.6 (60)
Ampicillin		1.5 (150)	0.8 (80)	0.9 (90)

***Sample abbreviations: HS (Hexane seeds), DS (Dichloromethane seeds), AS (Ethyl acetate seeds), MS (methanol seeds), HE (Hexane epicarp), DE (Dichloromethane epicarp), AE (Ethyl acetate epicarp), ME (methanol epicarp). Tetracycline, kanamycin and ampicillin were used as positive antibacterial compounds**

MIC values of samples from *G. madruno* ranging from 188.7 µg/mL to 1253.4 µg/mL against *Staphylococcus aureus*, 162.2 to 329.8 µg/mL against *E. faecalis* and 86.6 µg/mL to 515.7 µg/mL against *S. mutans* (Table No. 2). The strongest activity was observed for garcinol, it was the most active sample against *S. aureus* (188.7 µg/mL), *E. faecalis* (162.2 µg/mL) and *S. mutans* (86.6 µg/mL). Furthermore, biflavonoids morelloflavone and volkensiflavone presented low MIC values against *S. mutans* (each 97.4 µg/mL). In addition to the isolated compounds, the extracts HS and DS exhibited inhibitory effects against *E. faecalis* and *S. mutans* with MIC values ranging from 118.6 µg/mL to 201.9 µg/mL; and DE only to *E. faecalis* (201.9 µg/mL). These results are in agreement with the results obtained in the qualitatively agar diffusion test where these three extracts produced significant growth inhibition zones.

Table No. 2
Minimum inhibitory concentration (MIC) of extracts and isolated compounds from *G. madruno* against Gram-positive bacteria

Sample	Gram-positive bacteria*		
	***S. aureus* 65380**	***E. faecalis* 29212**	***S. mutans* 31989**
	MIC (µg/mL)	**MIC (µg/mL)**	**MIC (µg/mL)**
HS	280.2	182.1	118.6
DS	280.2	201.9	118.6
AS	280.2	329.8	140.5
MS	280.2	222.3	515.7
HE	1253.4	329.8	140.5
DE	280.2	201.9	118.6
AE	280.2	222.3	140.5
ME	298.9	245.5	140.5
Volkensiflavone	298.9	245.5	97.4
Morelloflavone	280.2	245.5	97.4
Garcinol	188.7	162.2	86.6
Tetracycline	**15.6**	**62.5**	**125.0**
Kanamycin	**15.6**	**3.92**	**>500**
Ampicillin	**0.98**	**0.98**	**0.98**

***MICs for the samples against Gram-negative were not determined because samples evaluated in the agar well diffusion method did not show inhibitory growth effect. Tetracycline, kanamycin and ampicillin were used as positive antibacterial compounds**

The hemolytic capacity of extracts and compounds obtained from *G. madruno* are given in Table No. 3. In general, none of the samples tested displayed a significant hemolytic activity. The most potent sample tested was MS with a HC_{50} value of 858.28 µg/mL.

Table No. 3
Hemolytic activities (HC_{50} (µg/mL)) of extracts and isolated compounds from *G. madruno* against O^+ human red blood cells

Sample	HC_{50} (µg/mL)
HS	2470.5
DS	3737.4
AS	> 5000
MS	858.28
HE	3809.0
DE	3393.3
AE	> 5000
ME	> 5000
Volkensiflavone	> 5000
Morelloflavone	> 5000
Garcinol	> 5000
PBS	0.00
Triton-X114 (1%)	96.73
Saponin (1%)	103.33
PBS-DMSO*	0.82

***PBS-DMSO: Phosphate-buffered saline solution-dimethyl sulfoxide**

DISCUSSION

In this work the antibacterial activity of extracts obtained with solvents of different polarity and pure compounds from pericarps and seeds of *G. madruno* was evaluated. The samples were tested using agar disc diffusion and MIC determination against three strains of Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus mutans*) and two strains of Gram-negative bacteria (*Salmonella typhimurium* and *Escherichia coli)*. According to the criteria established by Dzotam *et al*. (2018), and Kuete & Efferth (2010), the antibacterial activity of a plant extract is considered significant when MIC < 100 µg/mL, moderate when 100 ≤ MIC ≤ 625 µg/mL, and low when MIC > 625 µg/mL. For compounds, a high, moderate and low activity was considered when the MIC < 10 µg/mL, 10 ≤ MIC ≤ 100 µg/mL, and MIC > 100 µg/mL, respectively. Based on these references, all the extracts from seeds and epicarps of *G. madruno* showed moderate activity against Gram-positive bacteria. No activity of extracts and compounds evaluated against *E. coli* and *S. typhymurium* was observed. Our results are consistent with the data reported by Suffredini *et al*. (2006), where *G. madruno* extracts from leaves and stems only exhibited inhibitory effects against the Gram-positive bacteria, *S aureus*. Furthermore, in that work no activity against Gram-negative bacteria was described. These differences in bacterial susceptibility are mainly attributed to cell wall composition. Gram-negative microorganisms are considered more resistant to antimicrobial compounds because they possess an external membrane rich in lipopolysaccharides that avoid the pass of substances, including inhibitor agents (Negi *et al*., 2008; Rana et al., 2014).

Similar results with other *Garcinia* species have also been reported. For example, hexane and chloroform extracts obtained from fruit rinds of *G. cowa* and *G. pedunculata* displayed a marked effect against Gram-positive bacteria (Negi *et al*., 2008). Naldoni *et al*. (2009), reported that the extract of hexane from pericarp of *G. brasiliensis* displayed a better antibacterial activity against Gram-positive *S. aureus* and *B. cereus* strains than the ethanol extract from seeds of the same species. Both extracts evaluated did not show an inhibitory effect against *E. coli*. Penduka & Okoh (2011), described the antibacterial activity of *n*-hexane extract from *G. kola* seeds against 42 isolates of the Gram-positive bacteria *Listeria* spp. The antimicrobial potency exhibited by *n*-hexane extracts from pericarp and seeds of *G. madruno*, could be attributed to metabolites soluble in non-polar solvents such as

xanthones and benzophenones; which together with flavonoids are the main constituents of the *Garcinia* genus (Bakana *et al*., 1987; Ho *et al*., 2018; Tang *et al*., 2013). Furthermore, Ramirez *et al*. (2019), demonstrated that the *n*-hexane soluble fraction obtained from epicarps, seeds, and leaves of *G. madruno* presented the highest levels of garcinol. Hence, this compound could be the main responsible for the antimicrobial activity of hexane extract from *G. madruno* against Gram-positive strains.

On the other hand, the two biflavonoids and the polyisoprenylated benzophenone, garcinol, were also active against the Gram-positive strains. This finding is expected, because these metabolites, widely reported in *Garcinia* species, have demonstrated antimicrobial activity (Verdi *et al*., 2004; Kuete *et al*., 2011; Tang *et al*., 2013). Morelloflavone and volkensiflavone purified from *G. madruno* seeds displayed low to moderate antibacterial activity against Gram-positive bacteria. In addition, both biflavonoids showed moderate activity (MIC < 100 µg/mL) against *S. mutans* 31989 (97.4 µg/mL). Biflavonoids are a group of metabolites formed by two monomeric flavonoid units (flavones, flavanones, flavonols, isoflavones or flavanonols) linked, in different positions, by Carbon-Carbon or Carbon-Oxygen-Carbon connections (Jiang *et al*., 2017; Menezes & Campos, 2021) In recent years biflavonoids have gained attention due to their recognition as regulators of physiological functions and the wide array of biological and pharmacological properties that they display. Several activities have been reported, including anti-inflammatory, anti-cancer, antioxidant, anti-clotting, vasorelaxant, antiviral, among others (Gontijo *et al*., 2017; Menezes & Campos, 2021). Moreover, various studies have demonstrated the potential of biflavonoids as antibacterial agents and their role in the response to bacterial infections (Xie *et al*., 2015; Gontijo *et al*., 2017). Although the mechanism of antibacterial action of this type of compounds has not been completely elucidated, a probable explanation can be proposed based on the flavonoid nature of the molecule (Menezes & Campos, 2021). The antibacterial action of flavonoids have been attributed to a multitarget effect and different modes of action have been described: bacterial plasma membrane disruption, inhibition of nucleic acid synthesis, inhibition of cell envelope synthesis and biofilm formation, inhibition of electron transport and ATP; and inhibition of bacterial enzymes by metal complex formation (Farhadi *et al*., 2019; Górniak *et al*., 2019**)**. These mechanisms can also be a plausible explanation of the antibacterial activity of biflavonoids. Our results showed a moderate activity of both flavonoids, volkensiflavone and morelloflavone, against the Gram-positive bacteria *S. mutans*. A probable explanation of this action is the lipophilic nature of the molecules and the characteristics of the cell wall structure (Bagla *et al*., 2014). In addition, a biflavonoid isolated from *Garcinia kola*, named GB1, showed good activity against *S. mutans* through the inhibition of aggregation and glucan synthesis, without affecting protein synthesis (Xu *et al*., 2013).

In terms of structure activity relationship analysis of biflavonoids the information is scarce. Menezes & Campos (2021), have proposed the linkage between the monomer units or substitution, specifically in 8-position, as an important factor for antibacterial activity. The degree of oxidation and lipophilicity have also been considered (Linden *et al*., 2020). For our case, both biflavonoids comply with this attribute. They are composed of monomers of flavone and flavanone connected by a 3-8" C-C bond. Likewise, flavonoid monomers can offer cues about the structural requirements for antibacterial action of biflavonoids. SAR of flavones indicate that an hydroxyl substituent in Ring A is essential for antibacterial activity; and the additional presence in C-5 and C-6 favor the activity (Farhadi *et al*., 2019). On the other hand, SAR of flavanones have proposed that dihydroxylation at C2',C4' or C2',C6' positions of the Ring B; as well as dihydroxylation at C5-C7 of ring A are a major contribution for the antibacterial properties of this subgroup of flavonoids (Cushnie & Lamb, 2005). The biflavonoids tested in this study share some of the abovementioned characteristics. They are substituted with hydroxyls at position C5 and C7 in the Ring A of the flavanone and flavone units; and in the Ring B with an hydroxy at position C4'. In addition, a reduced C-ring has been considered convenient for activity (Linden *et al*., 2020). These features can explain, in part, the antibacterial effects of morelloflavone and volkensiflavone.

The most active compound in both antibacterial assays was garcinol. In the agar disc diffusion had significant activity against all the Gram-positive strains tested. Likewise, in the MIC

determination, garcinol had the lowest values (*S. aureus* 188.7 µg/mL; *E. faecalis* 162.2 µg/mL; *S. mutans* 86.6 µg/mL). This benzophenone, also known as camboginol, has been isolated from several species of *Garcinia* (Yamaguchi *et al*., 2000; Cuesta-Rubio *et al*., 2005) and in recent years has received a lot of attention mainly due to its properties as anti-inflammatory, antioxidant and anti-cancer compound (Liu *et al*., 2015). Previous studies have evaluated the action of this molecule against different microorganisms (Kapadia & Rao, 2011). Specifically, against bacteria, garcinol has demonstrated growth inhibition of Gram-positive *Bacillus cereus*, *B. coagulans* and *B. subtilis*; as well as *S. aureus* and *L. monocytogenes* at concentrations lower than 2.0 µg/mL (Negi & Jayaprakasha, 2004). In addition, Bakana *et al*. (1987), described a moderate activity against Gram-positive bacteria but no effect against the Gram-negative strains tested in the investigation. Moreover, garcinol have exhibited a strong inhibitory growth action against methicillin resistant and methicillin sensitive *S. aureus* strains; with MIC values ranging from 6.25 to 12.5 µg/mL (Iinuma *et al*., 1996; Rukachaisirikul *et al*., 2005). Above-mentioned studies clearly illustrate the great effect of garcinol against Gram-positive bacteria, mainly *S. aureus*; which in turn supports our findings. Although there are many proposals about the mechanism of anti-cancer and anti-inflammatory activity of garcinol (Hemshekhar *et al*., 2011); the antibacterial effects of this compound have not been studied in detail. The specific activity against Gram-positive bacteria can be mainly explained by the disruption of the bacterial cell membrane permeability (Messi *et al*., 2014). Some other modes of action such as interference with nucleic acid function and synthesis; disturb cellular metabolism and blocking intercellular communication; and topoisomerases inhibition have been proposed (Tosa *et al*., 1997; Kumar *et al*., 2013; Tamhid, 2019). Furthermore, some structural features have been described as indispensable for the antibacterial activity. First, the presence of a chelated hydroxyl group at C-1; which exert their action through the binding of cations that are crucial for microbial growth (Bakana *et al*., 1987; Iinuma *et al*., 1996; Fernando *et al*., 2019); second, the existence of phenol and enol groups (Wu *et al*., 2014); and third, the presence of prenyl units which affect the lipophilicity of the molecule (Anholeti *et al*., 2015).

Red blood cells are simple structures mainly formed by a complex membrane composed of a lipid bilayer and proteins. RBCs do not present nucleus, cytoplasmic organelles and cytoskeleton; even so they are responsible for a vital process, oxygen transport (Reinhart *et al*., 2014; Mameri *et al*., 2021). The membrane integrity of RBCs may be a measure of the physiological condition of the cell (Pagano & Faggio, 2015). One of the most common models employed to estimate the toxic effect of xenobiotics and external factors in RBCs is the hemolytic activity assay. In this method, red blood cells are exposed to xenobiotics or external factors and induction of hemoglobin release, damage, alterations of RBCs membrane structure and lysis; are used as indicators of cytotoxicity (De Oliveira *et al*., 2009; Podsiedlik *et al*., 2020). Several studies have reported the use of this *in vitro* model to predict the probable cytotoxic action of natural or synthetic compounds, organic and inorganic molecules, as well as plant, fungi and algal extracts (Pagano & Faggio, 2015; Podsiedlik *et al*., 2020) Furthermore, some advantages of this assay such as easy implementation, sensitivity, inexpensive and fast execution can explain the widespread use of this test in routine evaluations of toxicity (Ribeiro *et al*., 2020). The hemolytic activity was assessed because, independent of the biological property evaluated; the damage of human erythrocytes will prevent the potential use of extracts and compounds obtained from *G. madruno*. Overall, the hemolytic activity of the samples tested did not affect the O^+ human red blood cells. Our results agree with previous works where extracts from different species of *Garcinia* showed a negligible toxic action against normal cells **(**Priya *et al*., 2010; Sangkitikomol, 2012; Paul *et al*., 2017**).** Moreover, extracts of *Garcinia* species have proven to protect and stabilize the erythrocyte membrane; probably due to the high concentration of phenolic metabolites (Luqman *et al*., 2009; Arwa *et al*., 2015; Ghosh *et al*., 2018).

The MS extract was the most active sample; despite the extract displayed the lowest HC_{50} value (858.28 µg/mL); the hemolysis action is still considered weak. This result could be attributed to the presence of saponins, which are soluble in methanol and have already been reported in the genus *Garcinia* **(**Olalekan, 2015; Policegroudra *et al*., 2012; Sripradha *et al*., 2016). Although our results gave us a preliminary idea about non-toxic effects of *G. madruno* samples; it is not possible to have a

definitive conclusion about its safety. In order to determine the toxicity of *G. madruno* more studies should be conducted.

Considering our findings, the tested compounds present low toxicity and can be a good point of departure in the search of new promising antimicrobial agents. Based on these templates (biflavonoid and polyisoprenylated benzophenone skeleton), the elaboration of synthetic analogs with different patterns of substitution or an appropriate hydrophilic-lipophilic balance can lead to safe molecules with enhanced antibacterial action.

Finally, many investigations have reported the antimicrobial effects of several species of the *Garcinia* genus (Kapadia & Rao, 2011). To the best of our knowledge, information regarding antibacterial and hemolytic activity of *G. madruno* is limited. Therefore, this is the first report of the antimicrobial and hemolytic activity of extracts from seeds and epicarps of this edible species. Further studies are needed to investigate the mode of action through which the extracts and compounds from *G. madruno* exert their effects.

CONCLUSION

In this work, the extracts of different polarity and three pure compounds, obtained from the seeds and epicarps of *Garcinia madruno*, were evaluated against Gram-positive and Gram-negative bacteria. The extracts showed antibacterial activity levels between low to moderate. The inhibitory effect was only observed towards Gram-positive bacteria. The extracts of lower polarity (HS and HE) and the nonpolar polyisoprenylated benzophenone; garcinol, were the most active samples. Specifically, garcinol showed the highest activity against *S. aureus*, *E. faecalis* and *S. mutans*. In addition, none of the extracts and compounds evaluated showed significant hemolytic activity against O^+ human red blood cells. Although our results gave us a preliminary idea about non-toxic effects of *G. madruno* samples, it is not possible to have a definitive conclusion about its safety. Consequently, additional toxicity studies should be conducted.

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