ANTIMICROBIAL ACTIVITY OF FRACTIONS AND SUBFRACTIONS OF *ELAEAGIA UTILIS* AGAINST MICROORGANISMS OF IMPORTANCE IN DENTAL CARIES

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ABSTRACT

Dental caries is a multifactorial infectious disease that leads to the destruction of dental hard tissue. The main goal of research into medicinal plants is to seek compounds with antimicrobial activity for subsequent use in prevention strategies and control of infectious diseases. The aim of this study was to evaluate the antimicrobial activity of fractions and subfractions obtained from Elaeagia utilis against Streptococcus mutans, Streptococcus sobrinus and Lactobacillus acidophilus. The plant material was collected in the town of Alban (Cundinamarca, Colombia), which is located at an altitude of 2245 meters above sea level. Two extracts were obtained by cold maceration of E. utilis leaves in (a) petroleum ether extract and (b) ethanol extract. Fractions were obtained from the petroleum ether extract by column vacuum chromatography, and from the ethanol extract by continuous liquid / liquid partitioning. The antimicrobial activity of fractions and subfractions was evaluated by the well diffusion method. At a concentration of 10 mg /well, several fractions from both extracts showed antimicrobial activity against S. mutans, S. sobrinus and L. acidophilus. Among the ethanol extract fractions, the dichloromethane fraction had notably greater antimicrobial activity. It was sub-partitioned, yielding three subfractions with inhibitory activity, of which the most active was MeOH: H_2O (Bp) with minimum inhibitory concentration 0.1 mg /well on the 3 study bacteria. Terpenes, sesquiterpenlactones and simple phenolic compounds were identified in it. In conclusion, this study shows the antimicrobial potential of fractions and subfractions obtained from extracts of E. utilis leaves against bacteria that are important in dental caries.

Keywords*: Plants, medicinal, Dental caries, Streptococcus mutans, Streptococcus sobrinus, Lactobacillus acidophilus.*

ACTIVIDAD ANTIMICROBIANA DE FRACCIONES Y SUBFRACCIONES DE *ELAEAGIA UTILIS* SOBRE MICROORGANISMOS DE IMPORTANCIA EN CARIES DENTAL

RESUMEN

La caries dental es una enfermedad infecciosa multifactorial que conduce a la destrucción del tejido duro dental. El principal objetivo de la investigación en plantas medicinales es la búsqueda de compuestos con actividad antimicrobiana para su posterior uso en estrategias de prevención o control de enfermedades infecciosas. El objetivo de este estudio fue evaluar la actividad antimicrobiana de fracciones y subfracciones obtenidas de la planta Elaeagia utilis contra Streptococcus mutans, Streptococcus sobrinus y Lactobacillus acidophilus. El material vegetal fue colectado en la ciudad de Albán (Cundinamarca-Colombia) situada a una altitud de 2245 metros sobre el nivel del mar. Mediante el método de maceración en frio de hojas de E. utilis se obtuvieron dos extractos, uno en éter de petróleo y otro en etanol. Del extracto etéreo se obtuvieron fracciones mediante cromatografía en columna al vacío y al extracto etanólico se le realizó fraccionamiento líquido/líquido continuo. La evaluación de la actividad antimicrobiana de las fracciones y subfracciones se realizó por el método de difusión en pozo. A una concentración de 10 mg/pozo, múltiples fracciones obtenidas de los dos extractos presentaron actividad antimicrobiana sobre S. mutans, S. sobrinus y L. acidophilus. De las fracciones del extracto etanólico se destaca la fracción diclorometano, por presentar mayor actividad antimicrobiana, razón por lo cual se subfracciona y se obtienen tres subfracciones con actividad inhibitoria. La subfracción más activa fue MeOH:H_2O (Bp) con una concentración mínima inhibitoria de 0.1 mg/pozo sobre las 3 bacterias en estudio. En esta subfracción se determinaron terpenos, sesquiterpenlactonas y compuestos fenólicos simples. En conclusión, en este estudio se presenta el potencial antimicrobiano de fracciones y subfracciones obtenidas de extractos de hojas de E. utilis contra microorganismos de importancia en caries dental.

Palabras claves: *Plantas medicinales, caries dental, S. mutans, S. sobrinus, L. acidophilus.*

INTRODUCTION

Dental caries is one of the most common chronic, multifactorial, transmittable infectious diseases in the world[1]. *Streptococcus mutans* and to a lesser extent, *Streptococcus sobrinus, Streptococcus gordonii,* and species of *Lactobacillus* and *Actinomyces* are the primary microorganisms related to the development and progression of dental caries[1-3]. Recognizing their importance in the initiation and progression of caries leads to designing meas-

ures aimed at eliminating or reducing them in the oral cavity[4].

Plant species have been widely used around the world as a source of traditional medicines for treating diseases.[5] The primary aim of research into medicinal plants is to identify plants with pharmacological activity in order to discover new substances with antimicrobial activity, which through various chemical procedures can be made into medications to control or prevent infectious diseases[6].

Many substances from various plant families have antimicrobial activity useful for oral health.[6-10] Allicine, from *Allium sativum* (Amaryllidaceae)[7], macelignan from *Myristica fragrans* (Myristicaceae)[8], bakuchiol from *Psoralea coryfolia* (Leguminosae)[9], and isopanduratin A from *Kaempferia pandurata* (Zingiberaceae)[10], are examples of molecules of natural origin with antimicrobial activity against *S. mutans, S. sobrinus, S. salivarius* and other microorganisms that are important in the oral cavity.

The family Rubiaceae, one of the 5 plant families with greatest ecological and taxonomical diversity in the world, has a large number of genera and species with special distribution in tropical Andean rainforests[11]. In Colombia, it is represented by 105 native genera including over 960 species, which grow mainly in the Andean, Amazonian and Chocó biogoegraphic regions[11].

Rubiaceae family members which are known to have antimicrobial potential against microorganisms that are important in oral infections are *Uncaria tomentosa* against *S. mutans*[12] and fractions obtained from of *Isertia laevis* leaves against *S. mutans* and *S. sobrinus*[13]. This draws attention to the need for further research to foster the discovery of molecules or substances that could be extracted from plants in this family and may help eliminate or reduce cariogenic microorganisms.

Species of the genus *Elaeagia* (family Rubiaceae) are distributed throughout the Colombian Andes region, at elevations of 100 to 2,600 meters above sea level[11], and are known as "Barniz" and "Mopamopa[11,14]. There is currently no information on evaluation of the antimicrobial activity of *Elaeagia utilis* against microorganisms that are important to dental caries and oral health. The aim of this study was to assess the antimicrobial activity of fractions and subfractions obtained from *Elaeagia utilis* leaves against *S. mutans*, *S. sobrinus* and *L. acidophilus*.

MATERIALS AND METHODS

1. Obtaining and processing plant material

The plant material was collected in the municipality of Albán (Cundinamarca, Colombia), at Padre Luna's Foundation "Granjas Infantiles" (Children's Farms). The specimen was taxonomically determined at the Herbarium of Javeriana University as *Elaeagia utilis* (Goudot) Wedd, of the family Rubiaceae, collection number HPUJ 27413. The leaves were dried at room temperature, then ground and powdered using a chopper. Cold maceration of the dried plant material (960 g) in petroleum ether (petrol) for 72 hours yielded an extract in petrol. The plant material filtered out of the first extraction was placed in cold maceration in ethanol (EtOH), and yielded an ethanol extract.

2. Obtaining fractions

A. Fractions from the petroleum ether extract

One gram of the extract in petrol was passed through a chromatography column under vacuum with a stationary phase composed of silica 60-H (0.063-0.200mm; Merck, Germany), in a proportion of 30:1 for stationary phase:sample. It was eluted with solvents of different polarities: Petrol, Petrol: Dichloromethane (CH_2Cl_2) (1:1), CH_2Cl_2, CH_2Cl_2:ethyl acetate (AcOEt) (1:1), AcOEt, AcOEt:EtOH (1:1) and EtOH.

B. Fractions from the ethanol extract

The ethanol extract was subject to continuous liquid/liquid partitioning (CLLP) and yielded four fractions: Petrol, CH_2Cl_2, AcOEt and Butanol (BuOH). Each fraction was concentrated at 40ºC, under reduced pressure in a rotary evaporator (Buchi B-169, Vacuum-System; Germany) until it was dry. The fraction with greatest antimicrobial activity was sub-partitioned by column vacuum chromatography with stationary phase RP-18 (40-63 µm; Merck, Germany) and eluted with methanol:water (MeOH:H_2O), MeOH and MeOH:CH_2Cl_2.

3. Methods for chemical characterization of active subfractions

Qualitative chemical tests were performed on the subfraction with greatest antimicrobial activity to determine types of secondary metabolites. Then it was partitioned by gas chromatography mass spectrometry (GC-MS) in a chromatograph (Agilent Technology 6850 series II) connected to an electron

impact mass spectrometer (70eV) model Agilent MS 5975B. The device has a fused-silica capillary column with 5% polydimethylsiloxane stationary phase (30 m long, 0.25 mm diameter and 0.25 µm phase thickness), using as carrier gas 99.995%, Aga Fano, S.A, grade 5, with a constant flow of 1mL/min. The sample was dissolved in methanol ($1mg.mL^{-1}$) and 1µL was injected in Split 15:1 mode. The initial temperature was 80°C, for 2 minutes, increasing by 10°C/min up to 280°C sustained for 5 minutes. Data from the 7th edition of the Wiley Mass Spectra library were compared to those obtained from the sample for identification. Spectra with matches better than 90% were considered to provide adequate identification.

4. Evaluation of the antimicrobial activity of fractions and subfractions

A. Study strains

Antimicrobial activity was evaluated on three reference strains: *S. mutans* ATCC 25175, *S. sobrinus* CIO 428 and *L. acidophilus* ATCC 4365, which had been preserved by freezing at -70°C at the Dental Research Center at Javeriana University. In order to reconstitute them and confirm viability, 20 µL from the preservation vials were thawed and cultured in brain heart infusion (BHI) broth for 4 hours at 37º C in an anaerobic atmosphere (H_2:CO_2:N_2; 10:10:80). Bacteria grown in the BHI broth were plated on BHI agar and incubated for 16 hours at 37ºC in an anaerobic atmosphere (H_2:CO_2:N_2; 10:10:80). Pure, viable colonies of *S. mutans* ATCC 25175, *S. sobrinus* CIO 428 and *L. acidophilus* ATCC 4365 were reconfirmed using Gram stain and biochemical tests.

B. Well diffusion method

The antimicrobial activity of the *E. utilis* extracts, fractions and subfractions against bacteria was evaluated using the well diffusion technique described by Dobner et al. [15] A suspension was prepared from each culture of pure bacteria and adjusted by turbidimetry to a 0.5 McFarland standard. Then 100 µl of the suspension were added to 20 mL liquid Mueller Hinton agar, mixed and poured into Petri dishes. Twenty minutes after it had solidified, a sterilized glass Pasteur pipette was used to make wells 0.5 cm in diameter on the agar. Fifty µL of the extract, fraction or subfraction dissolved in dimethyl sulfoxide (DMSO), were placed individually into each well. Fifty µL of Vancomycin at a concentration of 150 µg/mL were used as a positive control and 50 µL DMSO as a negative control. The dishes were immediately incubated at 37º C for 24 hours. Tetrazolium salt (MTT; 2.5 mg/mL aqueous solution) was added to the surface to reveal bacterial viability[16], and the dishes were left to incubate for another six hours. After incubation, the zones of inhibition produced by the fractions and/or subfractions were measured and the minimum inhibitory concentration (MIC; the lowest concentration producing a zone of inhibition of at least 6 mm) was determined. Each test was performed in triplicate and the average reported in mm.

4. Evaluation of the antimicrobial effect of the active subfraction by bioautography

The subfraction which was shown to have antimicrobial activity according to the well diffusion method was also evaluated using the bioautographic method described by Cos et al. [17] and Valgas et al. [18]. It was applied on chromatographic plates 7.5 cm long and 2.5 cm wide with silica gel stationary phase (60µm F254, Merck, Germany). The mobile phase was CH_2Cl_2:MeOH (9:1). The chromatographic plates were sterilized by exposure to UV radiation (wavelength 260nm) for 30 minutes. Suspensions of each of the 3 study bacteria were prepared and adjusted to a 0.5 McFarland standard. One hundred µL of each suspension was added to 10 mL liquid Mueller Hinton agar, mixed and poured onto the chromatography plate in the Petri dish. The chromatography plates were incubated for 24 hours at 37ºC, after which MTT was added and they were returned to incubation at 37ºC for another 6 hours. Rf (distance travelled by solute / distance travelled by solvent) was measured in the zones of inhibition (colourless areas).

RESULTS

Antimicrobial activity of the fractions from the extract in petroleum ether

Table 1 shows the antimicrobial activity of the extract in petrol and the fractions derived from it against the three study bacteria with 10 mg/well. Only two fractions (Petrol:CH_2Cl_2 and CH_2Cl_2) had no antimicrobial activity against the bacteria. The other 4 fractions produced zones of inhibition ranging from 7 to 15 mm.

Table 1: Antimicrobial activity of the Petroleum Ether extract and its subfractions against *S. mutans* ATCC 25175, *S. sobrinus* CIO 428 and *L. acidophilus* ATCC 4365 at a concentration of 10mg/well. Inhibitory activity (zones of inhibition) in mm.

Total extract and fractions		*S. mutans* ATCC 25175	*S. sobrinus* CIO 428	*L. acidophilus* ATCC 4365
Total extract	Petroleum Ether extract	9.5	9	9
Fractions	Petrol:CH_2Cl_2	0	0	0
	CH_2Cl_2	0	0	0
	CH_2Cl_2:AcOEt	11	9	9
	AcOEt	8.5	9	7
	AcOEt:EtOH	15	8.5	8
	EtOH	11	8.5	8.5
Positive control	Vancomycin (150 µg/mL)	19	17	19
Negative control	DMSO	0	0	0

Antimicrobial activity of the fractions obtained by CLLP from the extract in ethanol

Antimicrobial activity was tested for the Petrol, CH_2Cl_2, AcOEt and BuOH fractions obtained by liquid/liquid partitioning of the ethanol extract, plus some precipitates which appeared upon partitioning with AcOEt and BuOH, which were named AcOEt(p) and BuOH(p). Fig. 1 shows the antimicrobial activity of the fractions and precipitates on the 3 study bacteria. The dichloromethane fraction stands out with a larger zone of inhibition.

Antimicrobial activity of the subfractions yielded by the active CH_2Cl_2 fraction from the extract in ethanol

Because the CH_2Cl_2 fraction in the ethanol extract had outstanding antimicrobial activity, it was separated using CVC with stationary phase RP-18, 30:1 (Stationary Phase:Sample), and mobile phase MeOH:H_2O (10:1), MeOH and MeOH:CH_2Cl_2 (9:1). This procedure yielded seven subfractions: MeOH:H_2O (A)(0.5 mg), MeOH:H_2O (B)(1.52 mg), MeOH:H_2O (C)(0.2 mg), MeOH:H_2O (D)(0.08 mg), MeOH:H_2O (E)(0.05 mg), MeOH:H_2O (F)(0.15 mg) and MeOH:CH_2Cl_2 (0.63 mg). Subfraction MeOH:H_2O (B) had a precipitate, which was separated from the supernatant and named MeOH:H_2O (Bp). Fig. 2 shows the antimicrobial activity of the eight subfractions obtained from the dichloromethane fraction. Subfractions MeOH:H_2O A, MeOH:H_2O B and MeOH:H_2O Bp have outstanding inhibitory activity. Subfraction MeOH:H_2O (Bp) at 10 mg/well had the greatest biological activity against the bacterial strains, so we decided to evaluate it at lower concentrations, and found it was active to a concentration of 0.1 mg/well against the three study bacteria (Fig. 3).

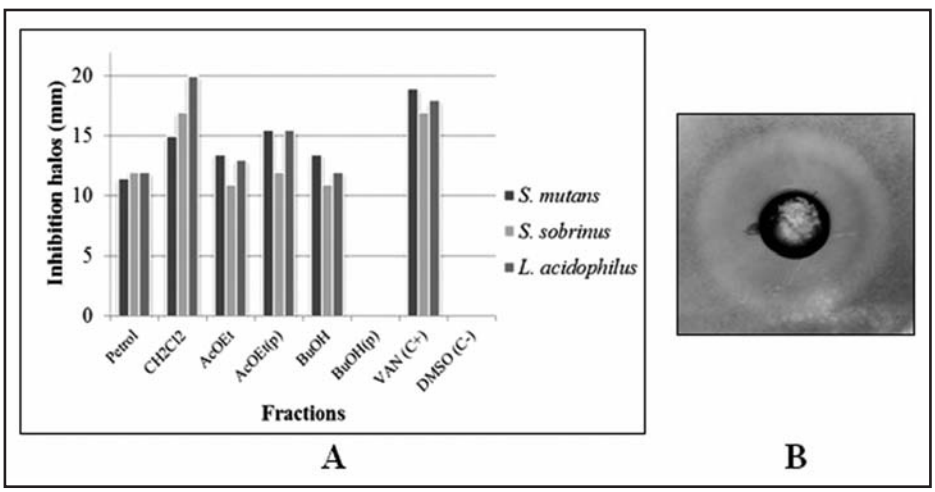

Fig. 1: A: Antimicrobial action of the fractions and precipitates (p) at 10 mg/well obtained by continuous liquid/liquid partitioning (CLLP) of the ethanol extract. B: Zone of inhibition (17 mm) of fraction CH_2Cl_2 against S. sobrinus CIO 428. Vancomycin and dimethyl sulfoxide were used, respectively, as positive control -VAN (C+)- and negative control-DMSO (C-)-.

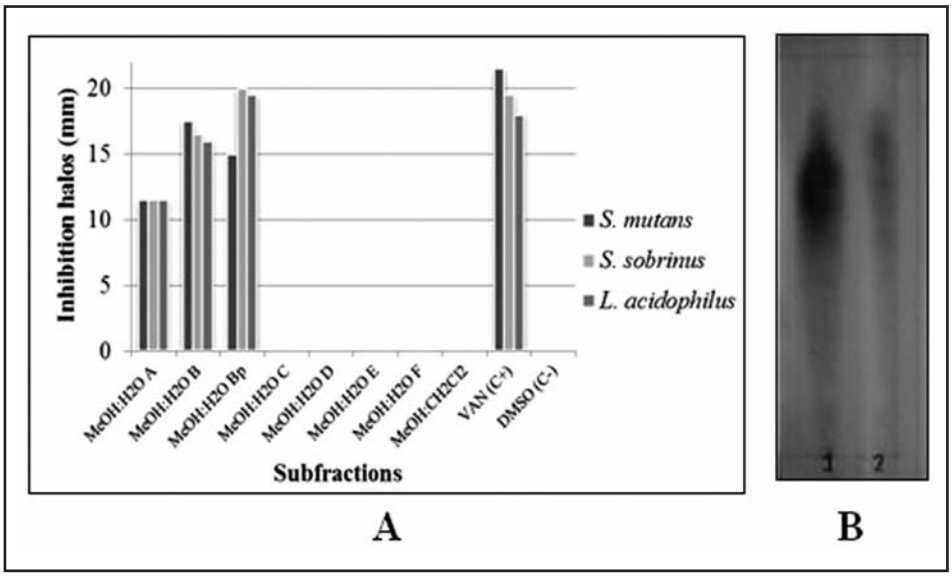

Fig. 2: A: Antimicrobial activity of the subfractions (10 mg/well) obtained from the CH_2Cl_2 fraction of the ethanol extract. B: Thin layer chromatography (stationary phase: RP-18, mobile phase: MeOH:H_2O;10:1); of subfractions MeOH:H_2O B (1) and MeOH:H_2O Bp (2).

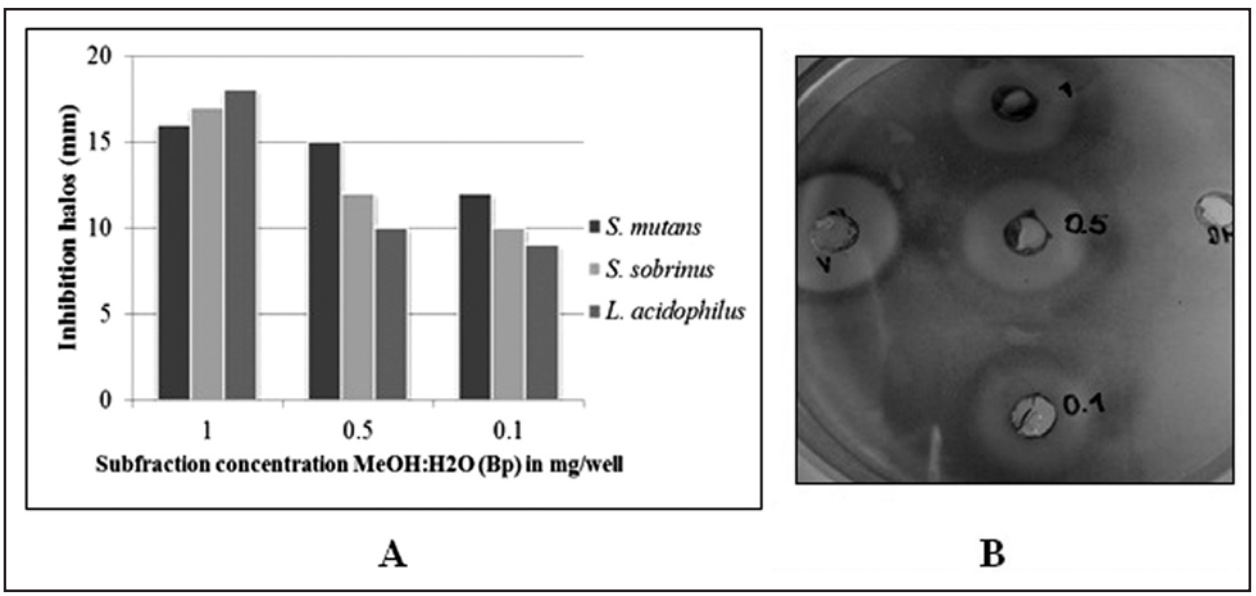

Fig. 3: A: Antimicrobial activity of subfraction MeOH:H_2O (Bp) at three different concentrations. B: Zones of inhibition on S. mutans ATCC 25175 produced by subfraction MeOH:H_2O (Bp) at concentrations of 1mg/well, 0.5mg/well and 0.1mg/well.

Antimicrobial activity of the active subfraction by bioautography

Fig. 4 shows the antimicrobial activity of subfraction MeOH:H_2O (Bp) against the three study bacteria using the bioautographic technique. The antimicrobial activity of the compounds was located at an Rf zone located between 0.44 and 0.49.

Chemical study of the active subfraction

The following qualitative chemical tests were performed: Baljet, Lieberman-Burchard, Salkowski, ammonium molybdate, iron (III) chloride, Dragendroff, foam test, anthrone, ferric hydroxamate. The Baljet, Salkowski, ammonium molibdate, foam and ferric hydroxamate tests were positive, indicating presence of diterpenes, steroids and saponins. The others were negative.

Table 2 shows the compounds identified by GC-MS in the active subfraction MeOH:H_2O (Bp). The most outstanding compounds found in the mixture were simple phenolic compounds, benzene derivatives and hydrocarbons.

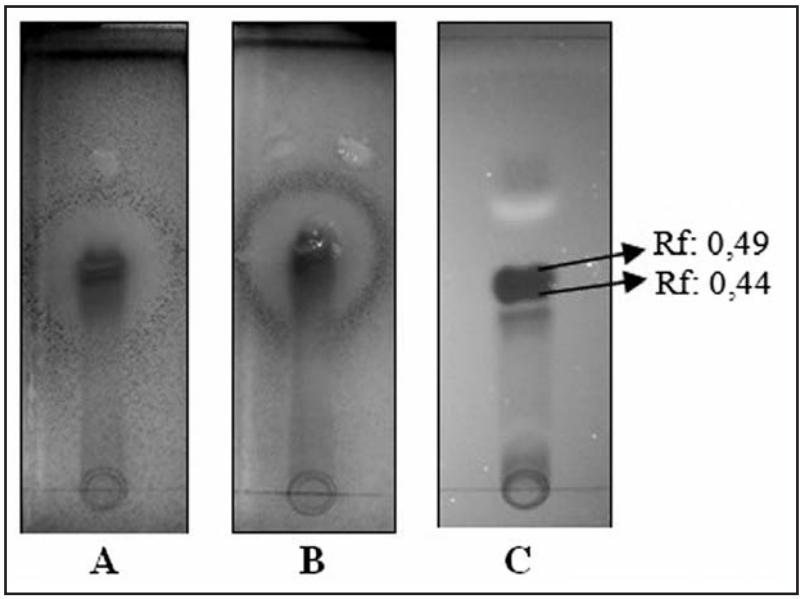

Fig. 4: Antimicrobial activity of subfraction MeOH:H_2O (Bp) by bioautography (Chromatographic plate: stationary phase silica gel and mobile phase CH_2Cl_2:MeOH, 9:1, with MTT reagent) on S. mutans ATCC 25175 (A) and L. acidophilus ATCC 4365 (B). C shows the compounds responsible for the inhibition under UV light (wavelength 254 nm) with Rf values.

Table 2: Compounds identified in subfraction MeOH:H2O (Bp) by GC-MS (Agilent Technology 6850 series II, connected to an electron impact mass spectrometer (70eV) Agilent MS 5975B).

Retention time (seconds)	Name of compound	Area covered (%)	Nature of the Compound
5.885	Benzyl alcohol	1.793	Benzene derivative
7.475	Benzoic acid	1.409	Benzene derivative
8.491	4-vinylphenol	1.186	Simple phenolic compound
8.504	2,3-dihydrobenzofuran	1.186	Aromatic hydrocarbon
9.807	2-methoxy-4-vinylphenol	2.427	Simple phenolic compound
11.775	3-methoxy-4-hydroxybenzaldehyde (vanillin)	0.430	Simple phenolic compound
13.483	2,5-dimethyl-(3-methoxymethyl)-p-benzoquinone	1.116	Benzene derivative
13.732	2,6-dimethoxy-4-(2-propenyl) phenol	0.553	Simple phenolic compound
16.483	1,Z-5,E-7-dodecatrien	2.344	Hydrocarbon
16.662	Loliolide	0.808	Sesquiterpene lactone
16.973	Benzoic acid, 2-hydroxy-, phenyl methyl ester	1.846	Benzene derivative
16.014	4-((1e)-3-hydroxy-1-propenyl)-2- methoxyphenol	9.443	Simple phenolic compoound

DISCUSSION

Colombian flora is widely known, and is considered to be a potential source of products with pharmacological activity.[19] Many substances obtained from plant species have been evaluated against pathogenic microorganisms, and their antimicrobial activity has been proven and/or reconfirmed.[7-10,17,19] Natural compounds with antimicrobial activity are the basis for a line of research that seeks to discover structural and functional components, called active principles, most of which are secondary metabolites.[6,12,13,19-21] Research into these compounds is a strategic route for the development of efficacious affordable drugs which can be used for treating diseases that are important to the public.[6-10] Prior research into plant species of the Rubiaceae family reports antimicrobial activity against different microorganisms.[19,21-23] The ethanol extract from *Cinchona officinalis* had antimicrobial activity against *S. aureus*, *Bacillus cereus* and β-hemolytic *Streptococcus.*[19] Similarly, the ethanol extract from *Uncaria tomentosa* bark inhibited the growth of 6

bacteria, of which the most outstanding are *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*.[21] Another study on *Uncaria tomentosa* aqueous extracts showed antimicrobial activity against *S. aureus* and *Candida albicans*.[22] Studies using *in vitro* models report that *Coffea arabica* aqueous extracts reduce the adherence of *S. mutans* to dental enamel and dentin.[23]

The microbiological evaluation of *E. utilis* leaves showed that both the petroleum ether extract and 4 of the 6 fractions derived from it had antimicrobial activity against the 3 study bacteria. Similarly, 5 of the 6 fractions obtained from the ethanol extract by liquid/liquid extraction had antimicrobial activity against *S. mutans*, *S. sobrinus* and *L. acidophilus* at a concentration of 10 mg/well. Of these, the dichloromethane fraction was outstanding because it produced larger zones of inhibition against all 3 microorganisms, which is why it was sub-partitioned.

Three of the 8 subfractions obtained from the dichloromethane fraction had inhibitory action against the 3 study bacteria. Of these three subfractions, MeOH:H_2O (Bp) was outstanding because it produced the largest zones of inhibition (15-20 mm). The fact that the activity of subfraction MeOH:H_2O (Bp) was greater than that of subfraction MeOH:H_2O (B) may be due to the fact that the former has a precipitation process leading to the separation of additional secondary metabolites which may act as interference with biological activity. Because subfraction MeOH:H_2O (Bp) had less mixture of components and higher biological activity, it was selected for microbiological assays at lower concentrations and determination of MCI, which was found to be 0.1 mg/well.

In the bioautography, subfraction MeOH:H_2O (Bp) showed antimicrobial activity against all 3 microorganisms and when vanillin was used as a reagent, it showed as a uniform yellow patch; nevertheless, exposure to UV light revealed two patches located very close together, with Rf 0.44 and 0.49, suggesting that the compounds in it are highly complex.

The qualitative chemical study performed on subfraction MeOH:H_2O (Bp), primarily determined presence of terpenes, sesquiterpene lactones and saponins. These findings agree with De Rosa et al.[24] and Zhao et al.[25], who report the presence of triterpenic saponins in other Rubiaceae species. Moreover, Kloucek et al.[21] report that triterpenes, are major components in mixtures with antimicrobial activity obtained from species from the family Rubiaceae.

GC-MS analysis of subfraction MeOH:H_2O (Bp) showed presence of various classes of compounds, among which simple phenols and benzene derivatives are outstanding. Cowan[26] reports that simple phenols, phenolic acids and quinones are the main components of plant origin that have antimicrobial activity. Gopalakrishnan et al.[27] report antimicrobial activity of 4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol, a compound which is present in the subfraction of this study. Moreover, Friedman et al.[28] report that the commercial compounds 3-methoxy-4-hydroxybenzaldehyde and benzoic acid, 2-hydroxy-phenyl methyl ester, which are also present in subfraction MeOH:H_2O (Bp), have antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. With regard to the compounds 4-vinylphenol and 2-methoxy-4-vinylphenol found in the mixture of subfraction MeOH:H_2O (Bp), other studies report that they are major components in leaf extracts from the species,[29] while in acetone extract from *Rumex vesicarius* (Polygonaceae) leaves with activity against *E. coli* and *C. albicans*, one of the main components is 2-methoxy-4-vinylphenol.[30]

The results of this study show the potential of fractions and subfractions obtained from *E. utilis* leaf extracts as sources of various compounds with antimicrobial activity against bacteria that are important in dental caries. In this regard, further studies are needed to isolate, characterize and identify substances present in active fractions and subfractions, and to determine their antibacterial activity against a wide range of microorganisms that are important in other oral infections. In the future, these substances may be used in toothpastes, mouth rinses and other products for oral hygiene and health.

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