INTRODUCTION
Dental caries is one of the most common chronic, multifactorial, transmittable infectious diseases in the world. 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. Recognizing their importance in the initiation and progression of caries leads to designing meas-

ACTIVITY ANTIMICROBIAL 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 frío 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 etánol 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. Las fracciones del extracto etánolico se destacaron como las más activas. En conclusion, 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.

Keywords: Plants, medicinal, Dental caries, Streptococcus mutans, Streptococcus sobrinus, Lactobacillus acidophilus.
ures aimed at eliminating or reducing them in the oral cavity.

Plant species have been widely used around the world as a source of traditional medicines for treating diseases. 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.

Many substances from various plant families have antimicrobial activity useful for oral health. Allicine, from *Allium sativum* (Amaryllidaceae), macelignan from *Myristica fragrans* (Myristicaceae), bakuchiol from *Psoralea coryfolia* (Leguminosae), and isopanduratin A from *Kaempferia pandurata* (Zingiberaceae), 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. In Colombia, it is represented by 105 native genera including over 960 species, which grow mainly in the Andean, Amazonian and Chocó biogeographic regions.

Rubiaceae family members which are known to have antimicrobial potential against microorganisms that are important in oral infections are *Uncaria tomentosa* 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. In Colombia, it is represented by 105 native genera including over 960 species, which grow mainly in the Andean, Amazonian and Chocó biogeographic regions.

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 (CH₂Cl₂) (1:1), CH₂Cl₂, CH₂Cl₂: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, CH₃Cl, AcOEt and Butanol (BuOH). Each fraction was concentrated at 40°C, 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:H₂O), MeOH and MeOH:CH₃Cl₂.

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 37°C in an anaerobic atmosphere (H₂:CO₂:N₂; 10:10:80). Bacteria grown in the BHI broth were plated on BHI agar and incubated for 16 hours at 37°C in an anaerobic atmosphere (H₂:CO₂:N₂; 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 37°C for 24 hours. Tetrazolium salt (MTT; 2.5 mg/mL aqueous solution) was added to the surface to reveal bacterial viability16, 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 CH₂Cl₂: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 37°C, after which MTT was added and they were returned to incubation at 37°C 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:CH₂Cl₂ and CH₂Cl₂) 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.

<table>
<thead>
<tr>
<th>Total extract and fractions</th>
<th><em>S. mutans</em> ATCC 25175</th>
<th><em>S. sobrinus</em> CIO 428</th>
<th><em>L. acidophilus</em> ATCC 4365</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total extract</td>
<td>Petroleum Ether extract</td>
<td>9.5</td>
<td>9</td>
</tr>
<tr>
<td>Fractions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Petrol:CH$_2$Cl$_2$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$:AcOEt</td>
<td>11</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>AcOEt</td>
<td>8.5</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>AcOEt:EtOH</td>
<td>15</td>
<td>8.5</td>
<td>8</td>
</tr>
<tr>
<td>EtOH</td>
<td>11</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Positive control</td>
<td>Vancomycin (150 µg/mL)</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMSO</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

Antimicrobial activity was tested for the Petrol, CH$_2$Cl$_2$, 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$_2$Cl$_2$ fraction from the extract in ethanol**

Because the CH$_2$Cl$_2$ 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$_2$O (10:1), MeOH and MeOH:CH$_2$Cl$_2$ (9:1). This procedure yielded seven subfractions: MeOH:H$_2$O (A)(0.5 mg), MeOH:H$_2$O (B)(1.52 mg), MeOH:H$_2$O (C)(0.2 mg), MeOH:H$_2$O (D)(0.08 mg), MeOH:H$_2$O (E)(0.05 mg), MeOH:H$_2$O (F)(0.15 mg) and MeOH:CH$_2$Cl$_2$ (0.63 mg). Subfraction MeOH:H$_2$O (B) had a precipitate, which was separated from the supernatant and named MeOH:H$_2$O (Bp). Fig. 2 shows the antimicrobial activity of the eight subfractions obtained from the dichloromethane fraction. Subfractions MeOH:H$_2$O A, MeOH:H$_2$O B and MeOH:H$_2$O Bp have outstanding inhibitory activity. Subfraction MeOH:H$_2$O (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).
Antimicrobial activity of the active subfraction by bioautography

Fig. 4 shows the antimicrobial activity of subfraction MeOH:H₂O (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, Dragentroff, foam test, anthrone, ferric hydroxamate. The Baljet, Salkowski, ammonium molybdate, 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:H₂O (Bp). The most outstanding compounds found in the mixture were simple phenolic compounds, benzene derivatives and hydrocarbons.
Fig. 4: Antimicrobial activity of subfraction MeOH:H2O (Bp) by bioautography (Chromatographic plate: stationary phase silica gel and mobile phase CH2Cl2: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).

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

DISCUSSION

Colombian flora is widely known, and is considered to be a potential source of products with pharmacological activity. Many substances obtained from plant species have been evaluated against pathogenic microorganisms, and their antimicrobial activity has been proven and/or reconfirmed. Prior research into plant species of the Rubiaceae family reports antimicrobial activity against different microorganisms. The ethanol extract from Cinchona officinalis had antimicrobial activity against S. aureus, Bacillus cereus and β-hemolytic Streptococcus. Similarly, the ethanol extract from Uncaria tomentosa bark inhibited the growth of secondary metabolites. 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.
bacteria, of which the most outstanding are Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Staphylococcus aureus.\textsuperscript{21} Another study on Uncaria tomentosa aqueous extracts showed antimicrobial activity against S. aureus and Candida albicans.\textsuperscript{22} Studies using in vitro models report that Coffea arabica aqueous extracts reduce the adherence of S. mutans to dental enamel and dentin.\textsuperscript{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 sub-partitioning showed that both the petroleum ether extract and 4 of the 6 fractions derived from it had antimicrobial activity. Gopalakrishnan et al.\textsuperscript{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.\textsuperscript{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$_2$O (Bp), have antimicrobial activity against Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli.

The results of this study show the potential of fractions with antimicrobial activity obtained from species from the family Rubiaceae. GC-MS analysis of subfraction MeOH:H$_2$O (Bp) showed presence of various classes of compounds, among which simple phenols and benzene derivatives are outstanding. Cowan\textsuperscript{26} reports that simple phenols, phenolic acids and quinones are the main components of plant origin that have antimicrobial activity. Gopalakrishnan et al.\textsuperscript{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.\textsuperscript{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$_2$O (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$_2$O (Bp), other studies report that they are major components in leaf extracts from the species,\textsuperscript{29} while in acetone extract from Rumex vesicarius (Polygonaceae) leaves with activity against E. utilis and C. albicans, one of the main components is 2-methoxy-4-vinylphenol.\textsuperscript{30}

The qualitative chemical study performed on subfraction MeOH:H$_2$O (Bp), primarily determined presence of terpenes, sesquiterpene lactones and saponins. These findings agree with De Rosa et al.\textsuperscript{24} and Zhao et al.\textsuperscript{25}, who report the presence of triterpenic saponins in other Rubiaceae species. Moreover, Kloucek et al.\textsuperscript{21} report that triterpenes, are major components in mixtures with antimicrobial activity obtained from species from the family Rubiaceae.

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