ANTICARIOGENIC ACTIVITY OF THE ACTIVE FRACTION FROM ISERTIA LAEVIS AGAINST *S. MUTANS* AND *S. SOBRINUS:* COMPARISON OF TWO EXTRACTION METHODS

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ABSTRACT

Dental caries is considered a multi-factorial, infectious, chronic, localized, post-eruptive, transmissible disease that leads to the destruction of dental hard tissue. The recognition of Streptococcus mutans as the major bacterial species involved in dental caries has led to the implementation of prevention and control measures for eliminating or reducing it in oral cavity. The main goal of research on medicinal plants is the search for substances or compounds with antimicrobial activity. The aim of this study was to evaluate the antimicrobial activity of fractions obtained by two methods from Isertia laevis against S. mutans and S. sobrinus. The plant material was collected in Medina (Colombia), at an elevation of 550 meters above sea level. From the ethanol extract of leaves of I. laevis, fractions were obtained by two methods: extraction by column vacuum chromatography (CVC) and extraction by continuous liquid / liquid partitioning (CLLP). The evaluation of the antimicrobial activity of fractions against S. mutans and S. sobrinus was performed by well diffusion and bioautography assays. From the CVC technique, only the methanol and methanol-dichloromethane fractions showed activity against S. mutans and S. sobrinus, with a minimum inhibitory concentration of 2 mg/well. From the CLLP technique, only the dichloromethane fraction showed activity against both microorganisms, with a minimum inhibitory concentration of 1 mg/well. Compounds C1 and C2 were isolated from the three active fractions, and showed a minimum inhibitory concentration of 0.4 mg/well for S. mutans and S. sobrinus, with zones of inhibition measuring 6.5 and 6.2 mm, respectively. In conclusion: 1) the three active fractions of I. laevis showed activity against S. mutans and S. sobrinus, 2) compounds C1 and C2 were present equally in the three active fractions showing activity against the two bacteria, 3) compounds C1 and C2 may be triterpenoid and/or steroidal saponin structures, and 4) the two extraction methods lead equally to obtaining the active fractions.

Key words: dental caries, Streptococcus mutans, Streptococcus sobrinus.

ACTIVIDAD ANTICARIOGÉNICA DE LA FRACCIÓN ACTIVA DE ISERTIA LAEVIS SOBRE *S. MUTANS* Y *S. SOBRINUS:* COMPARACIÓN DE DOS METODOLOGÍAS DE EXTRACCIÓN

RESUMEN

La caries dental es considerada una enfermedad infecciosa multifactorial, crónica, localizada, poseruptiva y transmisible que conlleva a la destrucción del tejido dental duro. El claro reconocimiento de Streptococcus mutans como la principal especie bacteriana implicada en caries dental, ha conducido a la implementación de medidas de prevención y control para la eliminación o disminución de este microorganismo en cavidad oral. El objetivo fundamental de la investigación en plantas medicinales, es la búsqueda de sustancias o compuestos con actividad antimicrobiana para ser utilizadas en el control o prevención de enfermedades infecciosas. En este sentido, en salud bucal muchas sustancias obtenidas de plantas han mostrado actividad antimicrobiana. El objetivo de este estudio fue evaluar la actividad antimicrobiana de fracciones obtenidas de la planta Isertia laevis mediante dos metodologías contra S. mutans y S. sobrinus. El material vegetal se colectó en el municipio de Medina (Cundinamarca-Colombia) situado a una altura de 550 metros sobre el nivel del mar. A partir del extracto etanólico de hojas de I. laevis se obtuvieron fracciones mediante dos metodologías, extracción por cromatografía en columna al vacío (CCV) y extracción por fraccionamiento líquido/líquido continuo (FLLC). La evaluación de la actividad antimicrobiana de las fracciones frente a S. mutans y S. sobrinus se realizó por el método de difusión en pozo y bioautográfico. De las fracciones obtenidas por CCV, solamente las fracciones metanol y metanol-diclorometano presentaron actividad antimicrobiana sobre S. mutans y S. sobrinus, con una concentración mínima inhibitoria de 2 mg/pozo. De las fracciones obtenidas por FLLC solamente la fracción diclorometano presentó actividad antimicrobiana sobre S. mutans y S. sobrinus, con una concentración mínima inhibitoria de 1 mg/pozo. De las 3 fracciones activas se aislaron los compuestos C1 y C2, que presentaron una concentración mínima inhibitoria de 0.4 mg/pozo tanto para S. mutans como para S. sobrinus con halos de inhibición, respectivamente, de 6.5 y 6.2 mm. En conclusión, 1. Las fracciones metanol y diclorometano obtenidas por CCV y la fracción diclorometano obtenida por FLLC de hojas de I. laevis presentaron actividad antimicrobiana sobre S. mutans y S. sobrinus; 2. Los compuestos C1 y C2 presentes por igual en las tres fracciones activas tuvieron acción inhibitoria sobre las dos bacterias en evaluación; 3. Las pruebas químicas cualitativas para los compuestos C1 y C2 indican que posiblemente corresponden a estructuras de saponinas triterpénicas y/o esteroidales; y 4. Las dos metodologías de extracción conducen por igual a la obtención de las fracciones activas.

Palabras claves: caries dental, Streptococcus mutans, Streptococcus sobrinus.

INTRODUCTION

Dental caries is a pathological, infectious, transmissible, localized process that leads to the destruction of dental hard tissue[1]. It is a multi-factorial disease that requires three main factors sustained over time: a susceptible host, cariogenic microflora located in the bacterial plaque and an adequate substrate[1].

Streptococcus mutans (mainly serotype c), and to a lesser degree, *Streptococcus sobrinus* and *Streptococcus gordonii,* and species of *Lactobacillus* and *Actinomyces*[1-3], are, in order of frequency, the main microorganisms associated to the production of dental caries.

The recognition of the participation of the microorganisms involved in dental caries has led to the design of prevention and control measures for eliminating them or reducing their number in the oral cavity[4]. Different strategies have been suggested[5]; but due to the lack of continuity, systematization, regulation and surveillance, they have not been as effective as possible in many countries in the world[6]. As a result, current information on oral health in countries of Latin America and the Caribbean indicates high prevalence of dental caries[7].

The main aim of research into medicinal plants is the search for substances or compounds having antimicrobial activity that could be used to control or prevent infectious diseases[8]. In this regard, the World Health Organization recommends that scientific criteria and adequate methods should be established in order to ensure the quality of the preparations obtained from medicinal plants[9].

In oral health, many substances obtained from plants show antimicrobial activity[8]. Chung et al.[10] report high antimicrobial activity of macelignan, a product obtained from *Myristica fragrans*, on *S. mutans.* Another study by Katsura H. et al.[11] reports bactericidal activity of bakuchiol (obtained from *Psoralea coryfolia*) on *S. mutans, S. sanguis, S. salivarus, S. sobrinus, L. acidophilus, L. casei* and other important microorganisms in the oral cavity. Hwang J.K. et al.[12] report inhibitory capacity of isopanduratin A, obtained from the species *Kaempferia pandurata*, on *S. mutans, S. sobrinus, S. sanguinis* and *S. salivarus* at very low concentrations.

There are many studies of plants, providing a wide range of substances that have antibacterial activity. Plants are still an excellent source for the search and application of products or molecules that help eliminate or reduce cariogenic microorganisms in the oral cavity.

The family *Rubiaceae* includes about 6000 species distributed around the world, mostly in the tropics[13]. Its genus *Isertia* is widely distributed in many regions in Colombia (Andes, Amazon and Chocó) located between 100 and 2000 meters above sea level[13].

The aim of this study was to evaluate the antimicrobial activity of the active fractions of *Isertia laevis*, obtained by means of two methods, against *S. mutans* and *S. sobrinus*.

MATERIALS AND METHODS

1. Obtaining and processing the plant material

The plant material was collected in Medina township (Cundinamarca, Colombia), which is located at 550 meters above sea level. Leaves were picked and air-dried, then powdered in a knife mill, weighed and stored in a clean, dry container. A plant specimen was sent to the Herbarium at the Institute of Natural Science of Colombia National University for taxonomical classification. It was identified as *Isertia laevis* (Triana) B.M. Boom[14], of the family *Rubiaceae*, and filed under number COL: 506495. The dried, powdered *I. laevis* plant material (1.5 Kg) was degreased in a Soxhlet extractor with petroleum ether for 72 hours. Then the extract in petroleum ether was concentrated in a rotary evaporator (Buchi B-169, Vacuum-System; Germany) at low pressure until it was dry. For extraction, the degreased product was soaked in cold ethanol (EtOH) and concentrated at low pressure in a rotary evaporator (Buchi B-169, Vacuum-System; Germany). The extract in EtOH was used as the base for the two extraction methods.

2. Obtaining fractions

Two different methods were used to obtain fractions from the *I. laevis* leaf extract in EtOH (28 grams): **A)** extraction by column vacuum chromatography (CVC) and **B)** extraction by continuous liquid-liquid partitioning (CLLP).

A. Extraction by CVC

Five grams of the EtOH extract were separated using CVC with stationary phase RP-18 (40-63 um, Merck, Germany), with a 1:10 stationary phase: sample ratio, and eluted with 120 ml of the mixtures methanol (MeOH): H_2O (10:2), MeOH, MeOH: dichloromethane (CH_2Cl_2) (1:1) and CH_2Cl_2. From each phase, 1.35 g, 1.23 g, 1.10 g and 1.32 g were obtained, respectively.

B. Extraction by CLLP

Solvents were used in ascending order of polarity. Ten grams of EtOH extract were subject to continuous liquid-liquid partitioning with 1000 ml of petroleum ether, 2000 ml CH_2Cl_2, 1000 ml ethyl acetate and 500 ml butanol. From each phase, 1.57 g, 4.46 g, 2.71 g and 1.26 g were obtained, respectively.

3. Evaluation of the antimicrobial activity of fractions

A. Strains used

Two reference strains were used to evaluate antimicrobial activity: *S. mutans* ATCC 25175 and *S. sobrinus* (University of Granada, Spain), which were preserved by freezing at -70°C at the Center for Dental Research at Javeriana University. In order to reconstitute them and confirm viability, small thawed aliquots from the preservation vials were cultured in brain heart infusion (BHI) broth for 4 hours at 37ºC 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 *S. mutans* and *S. sobrinus* colonies were reconfirmed using biochemical tests.

B. Well diffusion method

The antimicrobial activity of fractions obtained from *I. laevis* against *S. mutans* and *S. sobrinus* was evaluated according to the technique described by Dobner et al.[15]. From a pure culture of each strain to be evaluated, a suspension was prepared in BHI broth adjusted by turbidimetry to a 0.5 McFarland standard. Then 100 ml of the suspension of bacteria was added to 20 ml melted (non-solidified) trypticase soy agar (TSA), mixed and poured into the Petri dish. About 20 minutes after the agar had set, 4 or 5 wells 0.5 cm in diameter were made using a sterile pipette in each Petri dish containing bacteria. Into the wells were placed separately 50 ml of the fractions prepared at different concentrations in dimethyl sulfoxide (DMSO), 50 ul azithromycin at a concentration of 150 ug/ml (positive control) and 50 ul DMSO (negative control). Finally, the assays were incubated under anaerobiosis (H_2:CO_2:N_2; 10:10:80) at 37°C for 24-30 hours. After incubation, the zones of inhibition produced by the fractions were measured and the minimum inhibitory concentration was determined. The assays were performed in triplicate, and the average reported in mm.

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

The fractions that were positive for antimicrobial activity were set up for bioautography[16-20]. The method comprises two parts: A and B. In part A, the active fraction is applied on chromatographic silica gel plates measuring 7.5 cm x 2.5 cm (60 um F254, Merck, Germany) and run with CH_2Cl_2-MeOH (9.5:0.5). The mounted chromatographic plates are then exposed to UV radiation (260 nm) for 30 minutes to sterilize them for subsequent use in antimicrobial evaluation. For part B of the procedure, suspensions of S. *mutans* and S. *sobrinus* are prepared in BHI broth and adjusted by turbidimetry to a 0.5 Mc Farland standard. Of each suspension, 100 ml are added to 10 ml melted TSA agar, mixed and poured into the Petri dish containing the chromatographic plate prepared in part A of the procedure; the TSA agar should cover the entire plate. The Petri dishes containing the chromatographic plate are incubated under anaerobiosis (H_2:CO_2:N_2; 10:10:80) at 37°C for 24 - 30 hours. After incubation, the bioautographic plates are developed by spraying the surface with tetrazolium salt (MTT) prepared with aqueous solution at a final concentration of 2.5 mg/ml. To observe the antimicrobial effect, the plates are incubated at 37°C for 12 - 24 hours, after which the bioautographic plates turn bright violet colored, except for the areas where the active metabolites have inhibited bacterial growth. The inhibition zones are ivory-colored, and their Rf (rate of flow) is determined.

RESULTS

Antimicrobial activity of the fractions obtained by CVC

Of the fractions obtained by CVC with RP- 18, only the MeOH and MeOH-CH_2Cl_2 fractions had antimicrobial activity on *S. mutans* and *S. sobrinus*. A minimum inhibitory concentration of 2 mg per well was found (Fig. 1).

Antimicrobial activity of the fractions obtained by CLLP

Of the fractions obtained by CLLP, only the CH_2Cl_2 fraction had antimicrobial activity on *S. mutans* and *S. sobrinus*, with a minimum inhibitory concentration of 1 mg per well (Fig. 2). Interfering substances (chlorophyll) were eliminated from this active fraction in a Sephadex column eluted with mobile phase MeOH:CH_2Cl_2 (9:1) and 4 sub-fractions were

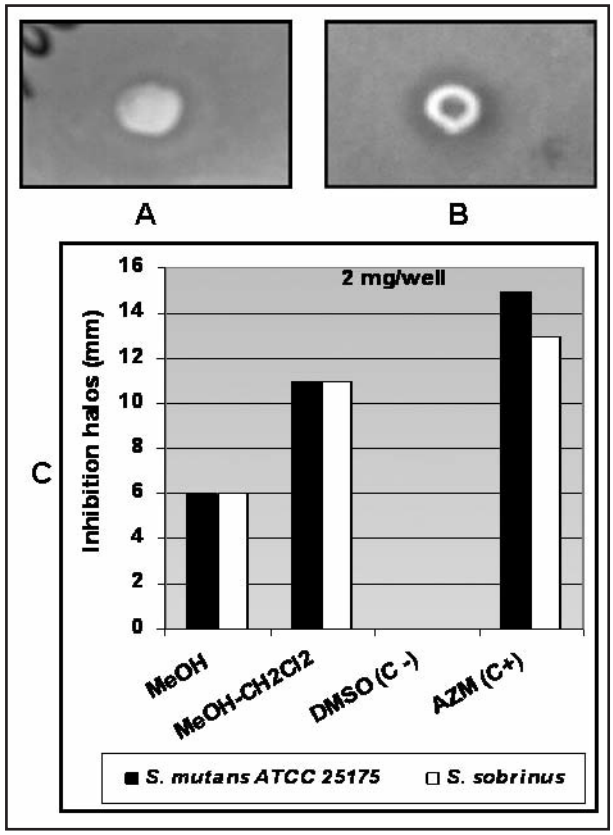

Fig. 1: Antimicrobial activity of the MeOH (A) and MeOH-CH_2Cl_2 (B) fractions obtained from leaves of I. laevis on S. mutans ATCC 25175 at a concentration of 2 mg/well. Inhibition halos were 6 mm for MeOH and 11 mm for MeOH-CH_2Cl_2 on S. mutans and S. sobrinus (C).

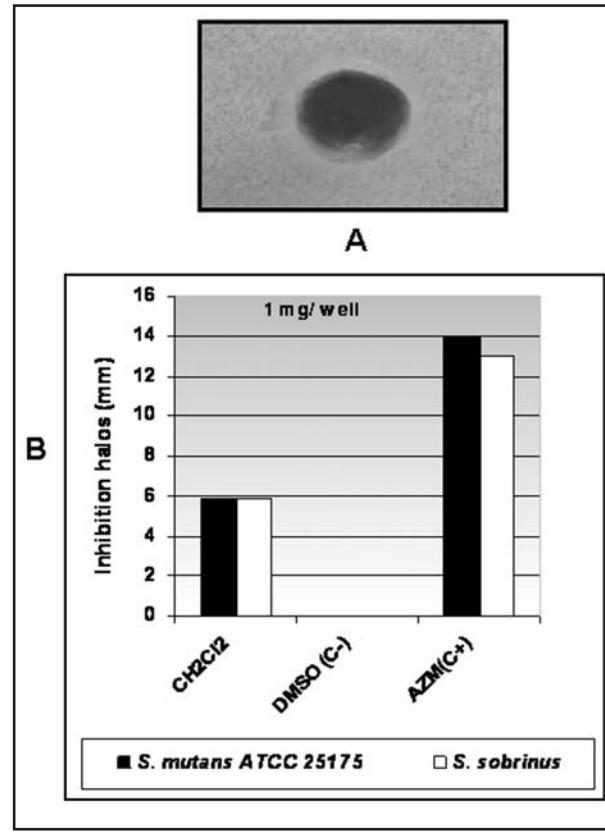

Fig. 2: Inhibitory action on S. mutans ATCC 25175 produced by the CH_2Cl_2 fraction of I. laevis at 1 mg per well, developed with MTT (A). Antimicrobial action of the CH_2Cl_2 fraction obtained from leaves of I. laevis on S. mutans ATCC 25175 and S. sobrinus, at a final concentration of 1 mg per well (B).

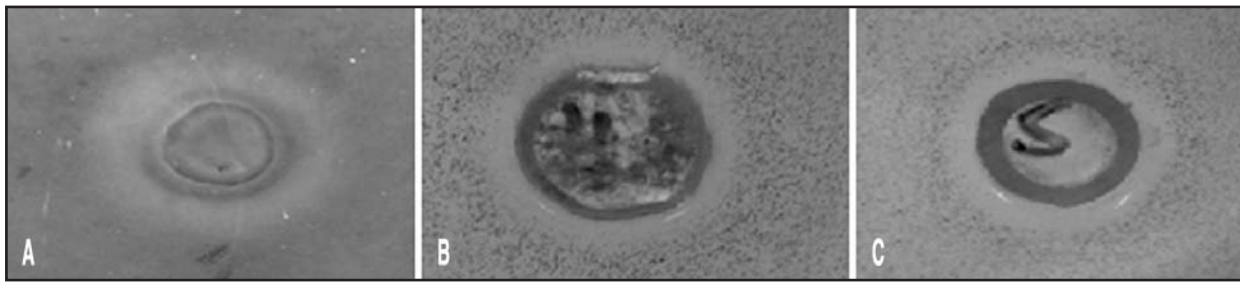

Fig. 3: Inhibition halos in mm produced by the CH_2Cl_2 sub-fractions on S. sobrinus. A: Sub-fraction 2 (13 mm), B: Sub-fraction 3 (7 mm), C: sub-fraction 4 (8 mm).

obtained that were monitored by thin layer chromatography (stationary phase RP–18 and mobile phase MeOH:CH_2Cl_2 9:1) and developed with perchloric acid. The antimicrobial activity of each subfraction on *S. mutans ATCC 25175* and *S. sobrinus* was evaluated. It was found that sub-fractions 2, 3 (with chlorophyll) and 4 produced zones of inhibition, as shown in Fig. 3, at a minimum inhibitory concentration of 0.6 mg per well.

Antimicrobial effect of active fractions by bioautography

Bioautography was performed on the active fractions from method 1 (MeOH and MeOH:CH_2Cl_2 fractions) and the active fraction from method 2 (CH_2Cl_2 fraction). All three fractions had antimicrobial activity on the two study bacteria. Figure 4 shows the bioautography results obtained for the MeOH and CH_2Cl_2 fractions.

Chemical study of the fractions with antimicrobial activity

To determine the Rf values, the 3 active fractions obtained by the two methods were run on thin-layer chromatography (stationary phase RP-18 and mobile phase MeOH:CH_2Cl_2 (9.5:0.5)) and developed with perchloric acid. All 3 fractions were found to contain two compounds with the same Rf values: 0.23 and 0.42. For further studies the components of the active fractions were separated using repetitive column chromatography (stationary phase RP-18 and mobile phase MeOH-CH_2Cl_2 9:1). For the qualitative chemical study and measurement of antimicrobial activity, the concentrated compound with Rf 0.23 was named C1, the concentrated compound with Rf 0.42 was named C2 and the mixture of both compounds was named C3. Individually, compounds C1 and C2 had a minimum inhibitory concentration of 0.4 mg /well, for both *S. mutans* and *S. sobrinus*, with zones of inhibition of 6.5 y 6.2 mm, respectively. The mixture C3 had a minimum inhibitory concentration of 0.4 mg/well for both *S. mutans* and *S. sobrinus*, with a zone of inhibition of 8.5 mm (Fig. 5).

The following qualitative chemical assays were performed for the chemical study: Lieberman-Bürchard, ammonium molybdate, Börntrager, Dragendorff, ferric chloride and foam index. The Lieberman-Bürchard, ammonium molybdate and foam index assays were positive, indicating presence of terpenes, steroids and saponins. The positive qualitative assays were the same for both compounds (C1 and C2).

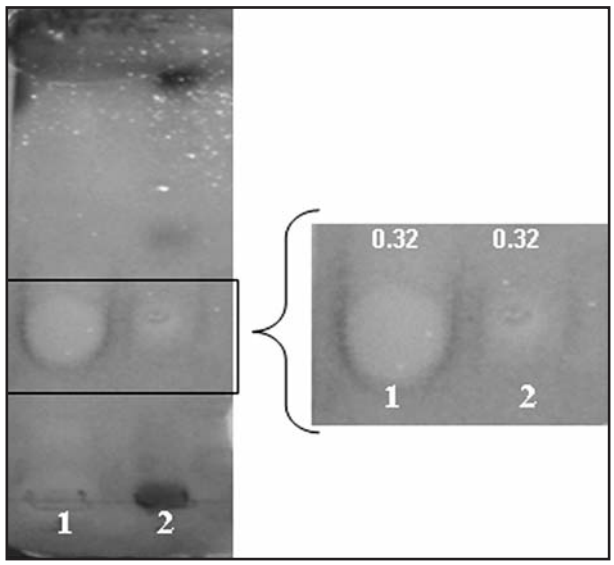

Fig. 4: Bioautography on silica gel of active fractions MeOH (1) and CH2Cl2 (2) on S. sobrinus. They both show zones of inhibition of 6 mm. Rf (rate of flow) values are indicated on the plates developed with MTT salt.

DISCUSSION

Thanks to its excellent geographical location, Colombia enjoys a wide range of biological resources, including flora. The flora is well-known and believed to be a potential source of products with pharmacological activity, and has been the object of much research[21]. Most plants produce secondary metabolites as part of their development, growth and response to the attack of pathogens or conditions of stress[8]. The ability of these compounds to provide protection for the plants has now been clearly defined, and therefore it is important to evaluate their action against infection-producing pathogens in humans[22].

Many substances produced by plants have been evaluated against pathogenic microorganisms and have been shown and/or reconfirmed as having great antimicrobial activity. Thus, for example, extracts obtained from species belonging to the families *Rubiaceae, Rhamnaceae* and *Teloschistaceae* have been shown to possess high inhibitory activity against fungi. Other species, such as *Borrichia frutences* and *Sarcocephalus coadunatus,*

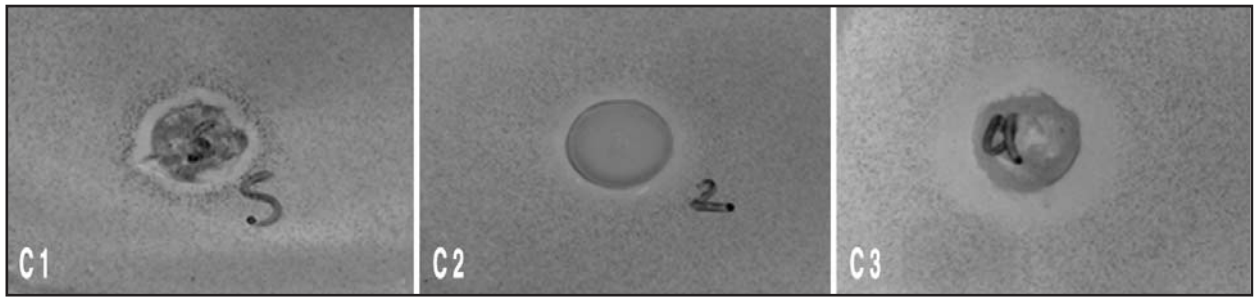

Fig. 5: Antimicrobial activity of compounds C1 and C2 and mixture C3 at a concentration of 0.4 mg/well on S. mutans. Note the larger inhibition halo in C3 (C1+C2) (8.5 mm) due to sinergic interaction of the compounds.

among hundreds of others, have been evaluated and found to have inhibitory activity against the growth of several pathogenic bacteria[23,24].

Species in the family *Rubiaceae* have substances in common which have been shown to possess antimicrobial activity against different pathogenic microorganisms[22]; this is an important reason for continuing with the search for substances that are active against microorganisms which are important in oral infectious pathologies. The aim of this study was to evaluate the antimicrobial activity of the active fraction obtained using two methods from the plant *Isertia laevis*, (family *Rubiaceae*) against *S. mutans* and *S. sobrinus*.

From an ecological standpoint, dental decay is considered to be the consequence of an imbalance in the oral system leading to the predominance of a certain type of flora, previously considered normal in the oral cavity, and then converted into pathogenic. The microbial imbalance, the existence of different external factors and the patient's socio-economic level all influence the development of this infectious disease. Among the main microorganisms associated to dental caries are, in order of importance, *S. mutans* and *S. sobrinus*[25]. Prevention and control strategies against them should be aimed at inhibiting their adhesion to the tooth surface and reducing the production of insoluble glucans that depend on microbial cell metabolism [1]. There is thus a need to search for efficient alternatives for eliminating or reducing the number of these microorganisms in the oral cavity, when appropriate.

Compounds or secondary metabolites against *S. mutans* have been identified in several plant families, including, among others, *Fabaceae, Moraceae*, *Clusiaceae*, *Theaceae*, *Rubiaceae*, *Vitaceae*, *Asteraceae* and *Zingiberaceae*[11,26-32].

Two of the fractions, MeOH and MeOH:CH_2Cl_2 (1:1), obtained by method 1 (CCV), and one of the fractions, CH_2:Cl_2, obtained by method 2 (CLLP) were active against *S. mutans* and *S. sobrinus.* The 3 active fractions were evaluated at concentrations of 0.5 to 20 mg per well: the fractions from method 1 had a minimum inhibitory concentration of 2 mg per well on the two study bacteria, while the fraction from method 2 had a minimum inhibitory concentration of 1 mg per well, also on both study bacteria. This difference may be explained by the fact that the whole of the only fraction (CH_2:Cl_2) obtained by method 2 contains all the substances present in both active fractions from method 1.

In contrast to the fractions obtained by method 1, the CH_2:Cl_2 fraction obtained by method 2 had high chlorophyll content, which therefore had to be removed before evaluation. After removing the chlorophyll, 3 sub-fractions were identified which had a minimum inhibitory concentration of 0.6 mg (Fig. 3). Of the 3 sub-fractions, sub-fraction 2 showed the best antimicrobial activity (zone of inhibition: 13 mm).

The aim of using bioautography in this study was to show the antimicrobial activity of the active fractions in a different way, and it did in fact show the antimicrobial effect of all 3 fractions on the two study bacteria. It also enabled active fractions to be separated and the similarity in their chromatographic runs to be seen (Fig. 4).

The results (inhibitory activity) of both the bioautography and the well diffusion method were developed with tetrazolium salt or MTT. In this method, viable bacteria metabolically reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and convert it into formazan, producing a purple-blue color in the zone of non-inhibition (bacterial growth) but not in the zone of inhibition. The tetrazolium salt allows the zones of inhibition produced by the antimicrobial activity of the fractions to be seen more clearly[33].

Thin-layer chromatography of the active fractions showed that each fraction contained two compounds, which were named C1 y C2, with Rf values 0.23 and 0.42 respectively. These findings confirm that the 3 fractions contain the same substances. Separate microbiological evaluation of compounds C1 and C2 showed that they are capable of inhibiting the study bacteria, and that this activity increases when the two compounds are mixed.

The qualitative chemical tests for compounds C1 and C2 indicate that they might be triterpenoid and/or steroidal saponin structures. The presence of these substances as responsible for the antimicrobial action of the fraction is in agreement with Murphy M.[34], who reports the inhibitory action of terpenes and steroids on bacteria, and that they act specifically by disrupting bacterial cell wall formation. Another study reports the inhibitory action of saponins on both Gram-positive and Gram-negative bacteria[35].

This study showed the reproducibility of the active fractions obtained by the two extraction methods. CLLP method allowed a larger amount of the active fraction to be obtained at low cost (data not shown). It is therefore an economical alternative for extract-

ing the active principles, which is easy to implement industrially. The fraction may be extracted at a higher cost by CVC, although in this case it is obtained directly, without pigments, making it easier to purify the components in subsequent studies for the identification and standardization of the plant medicine.

The findings of this study using *I. laevis* leaves, added to the fact many plants the family *Rubiaceae* grow in Colombia, suggest the need for further studies and characterization of other substances with antimicrobial activity on a wide range of genera and species involved in oral infections.

To conclude: 1. the MeOH and MeOH:CH_2Cl_2 fractions obtained by Method 1 (CVC), and the CH_2:Cl_2 fraction obtained by Method 2 (CLLP) from *Isertia laevis* leaves showed antimicrobial activity on *S. mutans* and *S. sobrinus*; 2. the compounds C1 and C2, which are both present in all three active fractions, had inhibitory action on the two study bacteria; 3. the qualitative chemical tests for compounds C1 and C2 indicate that they might be triterpenoid and/or steroidal saponin structures; and 4. the two extraction methods were effective for obtaining the active fractions.

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