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Antimicrobial and Antibiofilm Effect of Hydrogel with *Origanum vulgare* on Culture of *Streptococcus mutans* and *Streptococcus sobrinus*

Efecto Antimicrobiano y Antibiofilm de Hidrogel a Base de *Origanum vulgare* sobre Cultivo de *Streptococcus mutans* y *Streptococcus sobrinus*

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ABSTRACT: The oral cavity is an ecosystem that provides ideal conditions for the growth of bacteria, the Streptococcus genus is important for the formation of biofilms that lead to the development of dental caries, which affects the population worldwide. The world health organization encourages the use of plants thanks to its various therapeutic actions. *Origanum vulgare* L. (oregano), is an aromatic plant with medicinal and culinary properties. The objective of this study was to investigate the *in vitro* antimicrobial and antibiofilm activity of the ethanolic extract of oregano, against the growth of *Streptococcus mutans* and *Streptococcus sobrinus* ATCC. Leaves of the plant were obtained and the ethanolic extract was made by maceration. Antimicrobial activity was evaluated using the Kirby-Bauer method and compared with 2% chlorhexidine, subsequently the extract was incorporated into a hydrogel and its effect on biofilm formation was assessed by fluorescence microscopy and the main compounds were identified. present in the extratco. The study revealed that the extract presented antimicrobial effect against both strains and at 2% it showed high antimicrobial action compared to chlorhexidine at the same concentration, with average inhibition halos of 26.3 mm and 19 mm for each microorganism analyzed, ($p < 0.05$). Likewise, the hydrogel prepared with 2% extract significantly eliminated the preformed Streptococcus biofilm, at 24 hours of exposure, due to the presence of a variety of chemical groups, such as sterols, triterpenes, flavonoids, flavanones, flavanonols, lactones. sesquiterpenic, tannins and coumarins. The oregano extract presented high antimicrobial action for both species, with a greater effect towards *Streptococcus mutans* and an interesting antibiofilm action; These results show the importance of exploring treatment alternatives of plant origin, to be considered as interesting complementary aids in dental therapy.

KEY WORDS: biofilms, dental plaque, *Origanum vulgare*, phytotherapy, *Streptococcus mutans*, *Streptococcus sobrinus*.

INTRODUCTION

Dental plaques are microbial biofilms composed of approximately 100 different types of microorganisms, mainly bacteria, that are embedded in a matrix of extracellular polymeric substances that adhere to tooth surfaces and can cause carious lesions (Larsen & Fiehn, 2017; Yu *et al*., 2017). *Streptococcus mutans* and *Streptococcus sobrinus* are the main oral biofilm-forming microorganisms responsible for the development of dental caries (Fragkou *et al*., 2016). Dental caries is a prevalent chronic oral disease that

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occurs due to the demineralization of the hard tissues of the tooth by the organic acids formed by oral bacteria present in the biofilm. Worldwide, approximately 90 % of children have dental caries (Marcenes *et al*., 2013; Marinho *et al*., 2003), and in Mexico, it is estimated that 73.3 % of children between 2 and 5 years have this disease (Lomelí Buyoli & Rodríguez González, 2017).

Over the years, it has been shown that plants can produce a large number of bioactive compounds, justifying their use in traditional medicine to controlvarious pathogens (Altemimi *et al*., 2017). *Origanum vulgare* is an aromatic plant belonging to the Lamiaceae family used as a spice and in traditional medicine because of its antimicrobial, antispasmodic, anti-inflammatory, analgesic and antipyretic properties (Al-Tameme *et al*., 2015). Its biological activity has been attributed to compounds such as thymol, carvacrol, p-cymene, y-terpinene and linalool (Sarikurkcu *et al*., 2015).

Among the different strategies for incorporating therapeutic agents that can be administered in a localized area are hydrogels, defined as fluid, semisolid or solid compounds formed by polymer chains with hydrophilic structures capable of retaining large amounts of water in their three-dimensional networks (Ahmed, 2015) that exhibit viscosity, that is, resistance of the gel to be deformed (Choe *et al*., 2018). They are used in various biomedical areas, including dentistry, because by integrating biologically active agents in their structure, they allow controlled release and provide protection (Buwalda *et al*., 2017), in addition to being able to eliminate pathogens from biofilms associated with major oral cavity diseases (Marsh & Zaura, 2017). Chlorhexidine is an antimicrobial agent used in the mouth to eliminate pathogens; its adverse effects have been reported: toxicity, bacterial resistance, and discoloration of teeth, restorations and the oral mucosa (López *et al*., 2009; Wand *et al*., 2016; Cieplik *et al*., 2019). Therefore, the aim of the present study was to evaluate the antimicrobial and antibiotic effects using *Origanum vulgare* extract incorporated in a hydrogel against *Streptococcus mutans* and *Streptococcus sobrinus* ATCC, oral pathogens that form biofilms and cause dental caries.

MATERIAL AND METHOD

Obtaining the extract. Ethanolic extract was obtained by cold maceration. The dried leaves of *Origanum vulgare* available in a market in the city of Monterrey, N.L., Mexico, were acquired and ground, and 76.98 g of plant was placed into 400 mL of ethanol in an amber flask for 9 days at room temperature. The mixture was filtered through Whatman No. 2 paper, and the solvent was removed by evaporation under reduced pressure for 2 hours at 40 °C using a Büchi® Rotavapor R-205. The resulting extract was. Placed in an amber vial at 4 °C until use (Habibi *et al*., 2015; Coccimiglio, *et al*., 2016).

Antimicrobial testing of the extract. The antimicrobial effect of the *Origanum vulgare* extract against *Streptococcus mutans* (700611) and *Streptococcus sobrinus* (27607) ATCC was analysed. First, the microorganisms were reactivated and reseeded in trypticasein soy broth; two to three colonies were inoculated, and the inoculum was adjusted to a 0.5 McFarland standard equivalent to 1x106 CFU/mL. Subsequently, antimicrobial analysis was performed by the disc diffusion method (Kirby-Bauer method) six times, and 100 mL of the adjusted inoculum was placed on Müller Hinton agar culture medium. Five serial concentrations of the extract were evaluated [from 2 % to .02 % w/v] and incubated at 37 °C for 24 hours. The inhibition halos were measured with a calliper, and the results were compared with a positive chlorhexidine 2 % w/v control and negative ethanol control (Bauer *et al*., 1966; Roozegar *et al*., 2016). The percentage of the relative inhibitory effect of the extract was calculated, interpreted as high inhibitory activity (> 70 %), intermediate inhibitory activity (between 50 and 70 %) and low inhibitory activity (< 50 %) using the following formula (Ramírez & Díaz, 2007; Cruz-Carrillo *et al*., 2010).

$$\% \text{ inhibition} = \frac{\bar{x} \text{ halo diameter of extract}}{\bar{x} \text{ halo diameter of positive control}} \text{ X } 100$$

Hydrogel preparation. A hydrogel with 2 % *Origanum vulgare* extract was prepared. One hundred millilitres of sterile distilled water was placed in a beaker, and the acrylic acid polymer Carbopol ® 940 NF at 1 %, w/v, was incorporated; subsequently, 20 mL of glycerine was added and then mixed with a mechanical stirrer at 200 rpm for 20 min at 25 °C. The extract was incorporated until it was integrated into the gel. Lastly, triethanolamine was added, incorporating three drops, to neutralize and reach the desired viscosity and as a pH balancer (Zhang *et al*., 2016; Singh *et al*., 2016). The viscosity of the hydrogel was evaluated by emulsion flow curves using a rotational test with a Reolab QC rheometer as a function of the shear rate (0.02 to 100 s-1), using cylindrical concentric geometry CC-27 at a temperature of 27 °C (Bociaga *et al*., 2019).

Antimicrobial tests of the hydrogel extract. The antibiotic effect of the bioadhesive gel on a mixed culture of *Streptococcus mutans* and *Streptococcus sobrinus* ATCC was determined in 96-well polystyrene flat bottom plates. Bacterial inocula were cultured in trypticase soy broth in the presence of 2 % extract. As a negative control, inoculum without bacteria and an ethanolic medium were used. The plates were incubated at 37 °C for 24 hours; the supernatant was removed, and the plates were air dried for 30 min; the wells were stained for 15 min with 0.1 % aqueous crystal violet solution, and then 250 mL of ethanol was added to each well. After 15 min of incubation, the optical density was measured using a microplate reader at a wavelength of 560 nm. The biofilm formation index (BFI) was calculated (Teh *et al*., 2010; Han *et al*., 2016) based on the optical density of the adhered crystal violet-stained microorganisms (AB), the negative control culture medium (CW) and the optical density of the growth control (G) using the following formula:

$$BFI = (AB - CW)/G$$

Additionally, all treatments were labelled with DAPI to determine their antibiotic activity, and images were taken using an inverted microscope at a wavelength of 358 nm.

Basic phytochemical analysis. Subsequently, phytochemical screening of the extract by conventional chemical tests was performed to determine the main chemical groups present in the extract and to characterize the extract using the following tests: Liebermann Burchard (sterols and triterpenes), Shinoda (flavonoids, flavanones and flavanonols), Baljet (sesquiterpene lactones), sulfuric acid (quinones), ferric chloride (tannins), Molisch (carbohydrates), sodium hydroxide (coumarins) and Dragendorff (alkaloids) (Pérez Hernández *et al*., 2015; Elizondo-Luévano *et al*., 2018). Lastly, GC-MS chromatography ((6890/5973N), Agilent Technologies, Santa Clara, CA, USA) was performed on a sample of the extract obtained. Gas chromatography (GC) was conducted in a HP-5 MS (30 m x 0.25 mm to 0.25 m) capillary column. The GC conditions were as follows: injection temperature, 250 °C; and oven temperature controlled at 70 °C for 1 min with a heating rate of 10 °C/min, at 200 °C for 2 min, with a heating rate of 10 °C/min, and at 300 °C for 5 min. The following parameters were used for the EM 5973N analysis: ion source, EI; electronic energy, 70 e;, quadrupole temperature, 150 °C; interface temperature, 230 °C; and m/z, 30-400 amu (Torres-Alvarez *et al*., 2017; Rostro-Alanis *et al*., 2019).

All experiments were performed in triplicate at least three times. Statistical analyses were performed using SPSS version 25.0 (SPSS Inc., Chicago, Illinois, USA). Normality was determined with the Kolmogorov-Smirnov test with a level of significance of $p < 0.05$). Subsequently, a comparison was made between the medians of the extract concentrations and the inhibition zone with the Mann-Whitney test, with a level of significance of $p < 0.05$).

RESULTS

The *Origanum vulgare* extract showed an antimicrobial effect against both strains at various concentrations. For *Streptococcus mutans*, the mean inhibition halo was 26.3 mm with 2 % extract and 16 mm with 0.25 % extract (Fig. 1). Against *Streptococcus sobrinus*, mean inhibition halos of 19 mm and 14 mm were obtained with 2 % extract and 0.12 % extract, respectively (Fig. 2). For both bacteria, the extract presented a high inhibition compared to that with the positive control, i.e., inhibition halos of 15 mm. The data did not present a normal distribution based on the Kolmogorov-Smirnov normality test, either for *S. mutans* ($Z = 0.288$, $p = 0.000$) or for S. sobrinus ($Z = 0.237$, $p = 0.003$), because the p was less than 0.05. With these results, a nonparametric Kruskal-Wallis analysis was performed, with a level of significance of $p \leq 0.05$). There was only a significant difference in extract

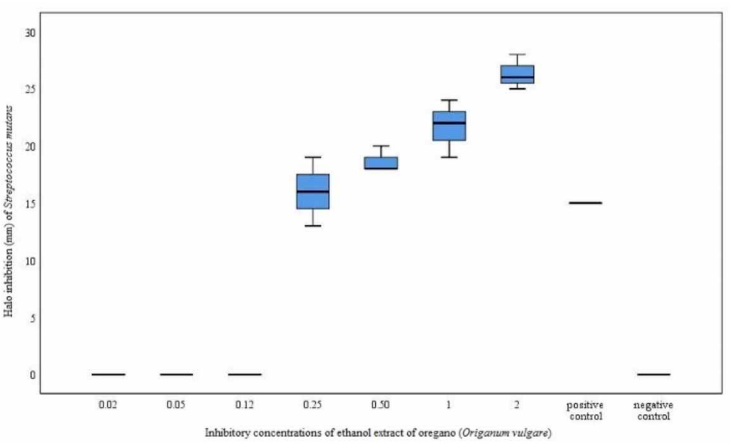

Fig. 1. Inhibition of the extract of *Origanum vulgare* against *Streptococcus mutans*.

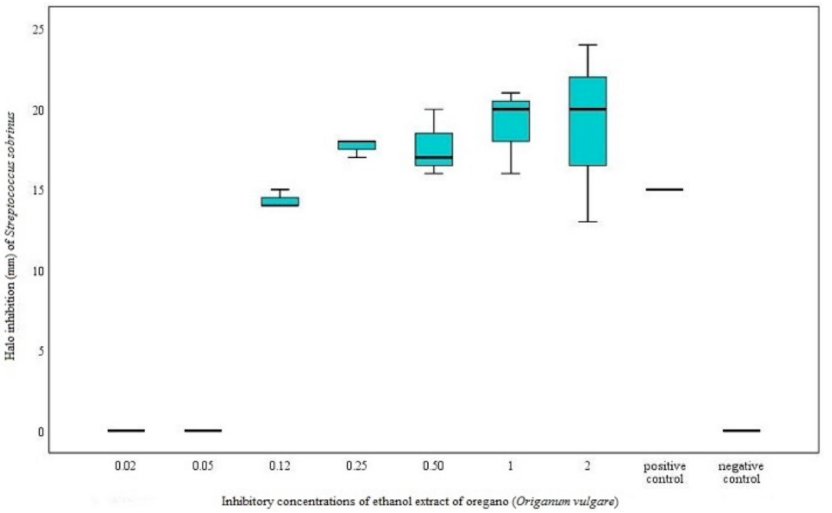

Fig. 2. Inhibition of the extract of *Origanum vulgare* against *Streptococcus sobrinus*.

inhibitory concentrations against *S. mutans* (H = 19.215, df = 6, p = 0.004), not S. sobrinus (H = 15.337; df = 6, p = 0.18). For this, a Mann-Whitney test was performed to compare the medians of the *S. mutans* treatments. Likewise, a comparison of the medians of the positive control and negative control was performed with the *S. mutans* and S. sobrinus treatments using the Mann-Whitney test (Table I).

Table I. Inhibitory concentrations of the extract of *Origanum vulgare* on *Streptococcus mutans* and *Streptococcus sobrinus*.

Extract concentration (%)	Halo inhibition (mm)		% relative inhibition		Significance (*P* > 0.05)	
	S. mutans	*S. sobrinus*	*S. mutans*	*S. sobrinus*	*S. mutans*	*S. sobrinus*
0.02	0	0	0	0	a	a*
0.05	0	0	0	0	a	a*
0.12	0	14	0	95	a*	a*_
0.25	16	17	106	117	b*_	a*_
0.5	18	17	124	117	b*_	a*_
1	21	19	144	126	b*_	a*_
2	26	19	175	126	c*_	a*_
C-	0	0	-	-	_	_
C+	15	15	-	-	*	*

The data did not show normal distribution (P ≤ 0.05, Kolmogórov-Smirnov). Negative control (C-), positive control (C +).
a, b, c. Average range (median) with similar letters were not significantly different. Comparison between the median concentrations of the extract and the inhibition halo (P ≤ 0.05, Mann-Whitney test).
Comparison between the positive control group * (P ≤ 0.05, Mann Whitney) and the treatment groups.
Comparison between the negative control group? (P ≤ 0.05, Mann Whitney) and the treatment groups.

The hydrogel with 2 % *Origanum vulgare* extract presented a flow curve, and when plotting the shear stress (z, Pa) vs. strain rate (° ˙, s-1), as the shear rates increased, the shear stress also increased (Fig. 3a); with a viscosity that decreased as the rate with which the fluid in motion increased, the initial viscosity at a rate of 0.1 was 240 Pa•s and ended at 0.908 Pa•s at a strain rate of 100 s-1. Compared to the viscosity of water, i.e., 0.001 Pa•s, the hydrogel had higher viscosity (Fig. 3b); that is, a non-Newtonian pseudoplastic behaviour was obtained.

Regarding the antibiotic effect of the 2 % *Origanum vulgare* hydrogel on a mixed culture of *S. mutans* and S. sobrinus, the biofilm was reduced by 91 % at 24 hours, and for the positive control

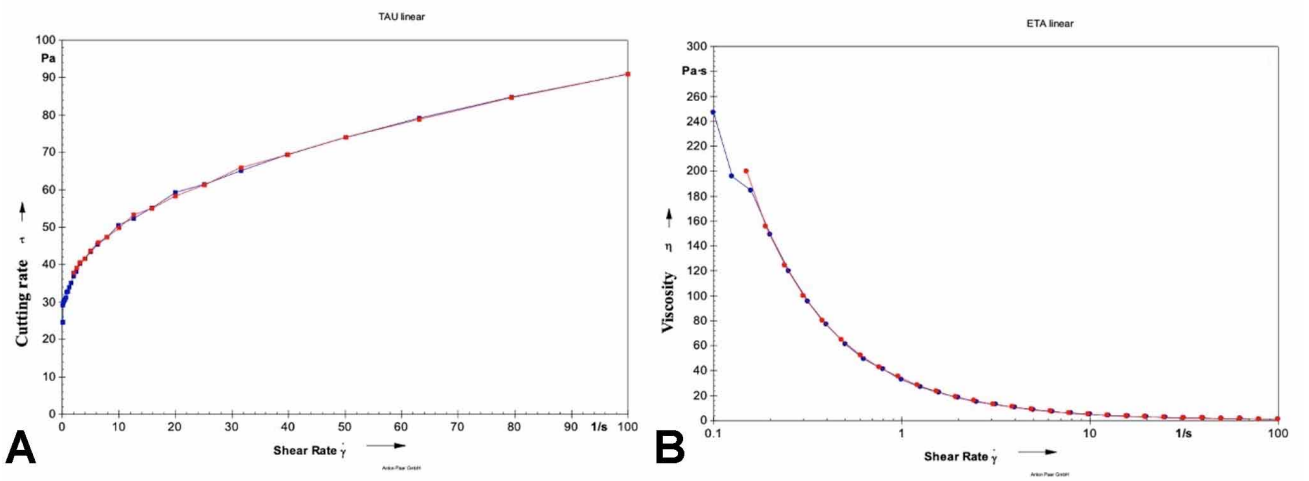

Fig. 3. Hydrogel characteristics. a) Hydrogel flow curve, b) Hydrogel viscosity curve.

(chlorhexidine 2 %), the biofilm was reduced by 56 %, results that were corroborated with fluorescence images (Fig. 4). Therefore, the Wilcoxon signed-ranks test was performed for the subsequent treatment comparisons; the differences were all significant ($P \leq 0.05$) (Tables II and III).

Table II. Descriptive statistics of antibiofilm activity.

	Gel.chx.2 - Gel.tx.25	Gel.tx.25 - C. growth	Gel.chx.2 - C.growth	Gel.tx.25 - Gelc.negative	Gel.chx.2 - Gelc.negative
Z	-3.059	-2.803	-2.191	-3.059	-2.903
P	0.002	0.005	0.028	0.002	0.004

Table III. Partial phytochemical characterization of *Origanum vulgare.*

Chemical test	Compound to identify	Result
Liebermann Burchard	Sterols Triterpenes	+
Shinoda	Flavonoids Flavanones Flavanonols	+
Baljet	Sesquiterpenlactones	+
Sulfuric acid	Quinones	-
Ferric chloride	Tannins	+
Molisch	Carbohydrates	-
Sodium hydroxide 10 %	Coumarins	+
Dragendorff	Alkaloids	-

The *Origanum vulgare* extract was positive for sterols, triterpenes, flavonoids, flavanones, flavanonols, sesquiterpene lactones, tannins and coumarins (Table IV). The principally identified components were carvacrol, phenol, p-cymene, thymol, 2-methyl-5-isopropylphenol (RT 10.89 - 31.39 %a), 3-benzenetriol, 3-trihydroxybenzene, 3-dihydroxyphenol (RT 11.93 - 28.28 %a), n-hexadecanoic acid (RT 19.31 - 8.63 %a), 2-furaldehyde, 2-furancarboxaldehyde (RT 9.82 - 6.23 %a), linoleic acid, methyl linolenate (RT 21.59 - 6 %a), and D-glucopyranose, levoglucosan (RT 13.63 - 5.84 %a), to which the antimicrobial and antibiotic activities of the *Origanum vulgare* extract were attributed (Fig. 5).

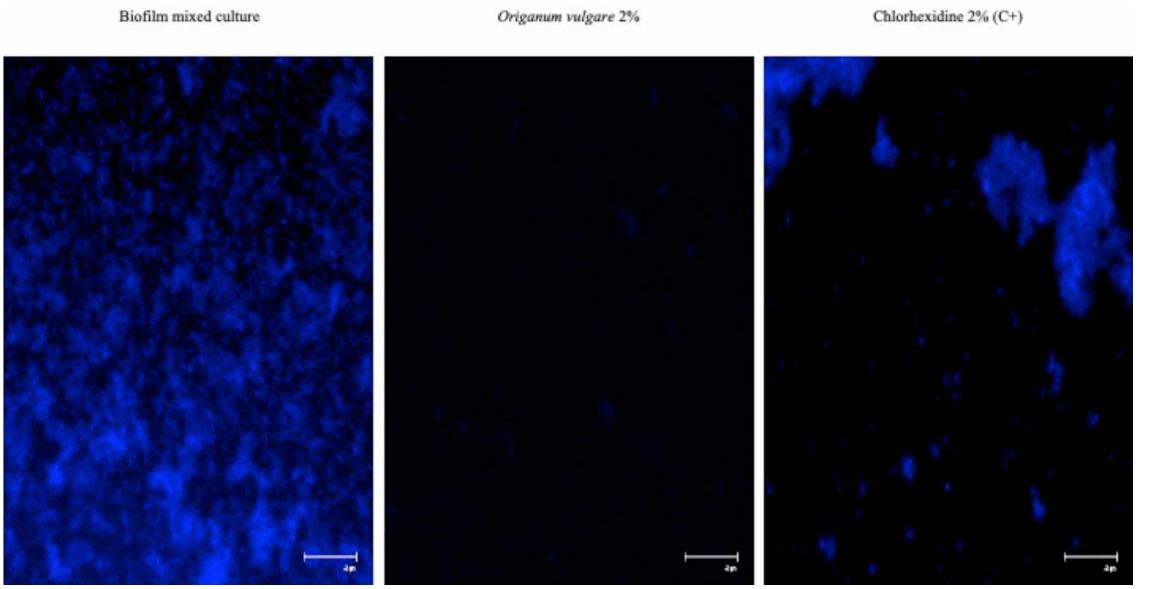

Fig. 4. Images of mixed culture plates of S. mutans and S. sobrinus stained under fluorescence microscopy. Positive control (C +).

Table IV. Compounds obtained in the chromatogram obtained from the GC / MS analysis of the *Origanum vulgare* extract.

Compounds	RT	%[a]
Carvacrol, phenol, p-cymene, thymol, 2-methyl-5-isopropylphenol	10.89	31.39
3-benzenetriol, 3-trihydroxybenzene, 3-dihydroxyphenol	11.93	28.28
n-Hexadecanoic acid	19.31	8.63
2-furaldehyde, 2-furancarboxaldehyde	9.82	6.23
linoleinic acid, methyl linolenate	21.59	6
D-glucopyranose, levoglucosan	13.63	5.84

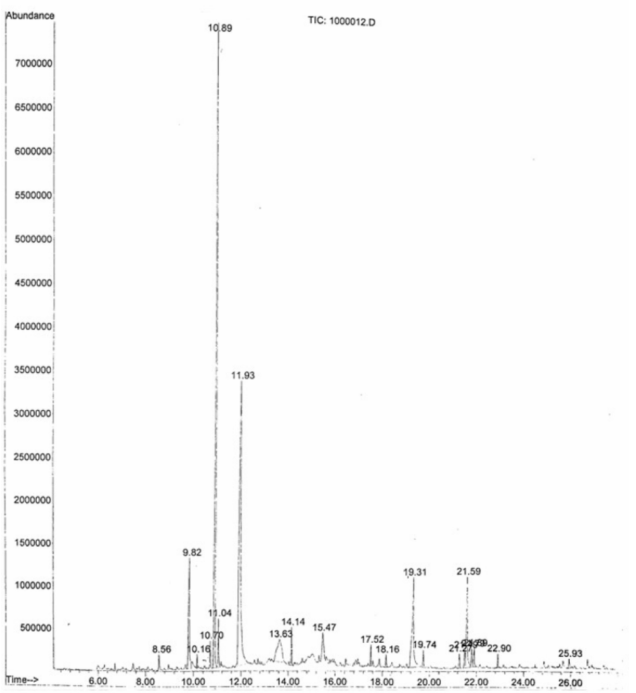

Fig. 5. Chromatogram obtained from the GC / MS analysis of the *Origanum vulgare* extract.

DISCUSSION

The present work highlights *Origanum vulgare* ethanolic extract as a good antibacterial agent. The reported results show good growth inhibition against *Streptococcus sobrinus*, presenting an even greater effect against *Streptococcus mutans*, important pathogens in the formation of oral biofilms and dental caries. These results are consistent with various studies that support its antibacterial activity. Its activity has been reported against Gram+ bacteria, such as *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis*, and *Streptococcus mutans*, and Gram- bacteria, such as *Escherichia coli*, and *Pseudomonas aeruginosa.* The concentrations at which the extract has been shown to be active range from 2.36 mg/mL for *S. aureus* and *B. subtilis*, to 9.44 mg/mL for *E. faecalis*, 200 mg/mL for *S. mutans*, 4.72 mg/mL for *E. coli*. and 9.44 mg/mL for *P. aeruginosa*, showing inhibition of 20.5, 26.9, 13.6, 9, 12.9 and 9.3 mm, respectively (Fikry *et al*., 2019). In this study, inhibitory action was observed

at 1.2 mg/mL for S. sobrinus and 2.5 mg/mL for *S. mutans*, concentrations similar to those reported.

The effectiveness of chlorhexidine present in mouthwash or 2 % w/v bioadhesive gel as the main antiseptic agent used to eliminate oral bacteria (de Queiroz *et al*., 2016; Brambilla *et al*., 2017) has been described. Likewise, its adverse effects, toxicity and the emergence of antimicrobial resistance have been reported (Karpin´ski & Szkaradkiewicz, 2015; Saleem *et al*., 2016; Cieplik *et al*.); therefore, it is important to search for alternatives based on herbal products that control oral biofilms. In this study, *Origanum vulgare* extract showed inhibitory activity superior to that of chlorhexidine at the same concentration. This is relevant because it is a crude extract that has been studied due to the presence of a significant variety of compounds present. Khan *et al*. (2017), evaluated the inhibition of *S. mutans* biofilm formation by thymol and carvacrol and by chlorhexidine and reported that the biofilm was significantly reduced in the presence of both thymol and carvacrol, with inhibition similar to that of chlorhexidine.

The phytochemical analysis performed on the extract revealed the presence of major compounds such as thymol and carvacrol. When the extract was incorporated at 2 % w/v in hydrogel, the formation of biofilm after 24 hours of exposure was considerably reduced, presenting a greater inhibitory effect with better performance than the free extract at the same concentration, i.e., extract that had not been incorporated into hydrogel. An advantage of hydrogels is the slow release of the products incorporated in their structure, in addition to protecting active agents, biocompatibility with tissues and controllable degradability (Li & Mooney, 2016). The hydrogel formulation tested in this study had a higher inhibitory effect than did the free extract at the same concentration evaluated. There have been several recent studies in which an active agent has been incorporated due to the advantages of this system; for example, Agarwal *et al*. (2019) developed a hydrogel formulation loaded with Theobroma cacao extract to increase the antioxidant and antimicrobial effects against different bacteria and found that the extract incorporated in hydrogel increased its bioavailability, maintaining an effective prolonged release up to 12 hours. These results agree with what was found in this study because when the *Origanum vulgare* extract was incorporated at 2 % w/v in hydrogel, biofilm formation after 24 hours of exposure was considerably reduced. Raei *et al*. (2017), and Jafri *et al*. (2019), also demonstrated that thymol and carvacrol inhibit the formation of biofilm of Gram-negative bacilli, which produce carbapenemase enzymes that are important in bacterial resistance.

It has been reported that in the ethanolic extract of oregano leaf, the main compounds identified were ferulic acid, rosmarinic acid, quercetin, luteolin, apigenin, carvacrol and thymol (Chuang *et al*., 2018; Moghrovyan *et al*., 2019). In the analysis of the essential oil of oregano, a-pinene, b-myrcene, a-terpinene, limonene, g-terpinene, thymol and carvacrol were present (Rostro-Alanis *et al*.). In this study, the presence of previously reported compounds was also identified in the ethanolic extract of *Origanum vulgare* by GC/MS, highlighting phenols such as thymol and carvacrol; therefore, it could be suggested that these agents are responsible for its biological activity and that their application through a polymeric formulation in hydrogel facilitates the control of dental caries.

CONCLUSION

Hydrogels improve the safety and topical application of active ingredients, providing greater action in the therapeutic area, and according to the study performed, the ethanolic extract of *Origanum vulgare* incorporated in hydrogel at a concentration of 2 % showed important antimicrobial effects against *Streptococcus mutans* and *Streptococcus sobrinus* and showed that, at a concentration of 2 %, it reduced the biofilm of these microorganisms by 91 %, obtaining a better result than the positive control gel (2 % chlorhexidine), which reduced the same biofilm by 56 %. This result is associated with the presence of a wide range of active substances in the plant material; therefore, studies with natural products are of interest because they contain compounds capable of providing favourable properties for the medical and odontological fields, reducing the risk of producing adverse effects and being a complementary alternative in oral therapy.

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RESUMEN: La cavidad oral es un ecosistema que proporciona condiciones ideales para el crecimiento de bacterias, el género Streptococcus es importante para la formación de biopelículas que conducen al desarrollo de caries dental, que afecta a la población a nivel mundial. La organización mundial de la salud, fomenta el uso de plantas gracias a sus diversas acciones terapéuticas. *Origanum vulgare* L. (orégano), es una planta aromática con propiedades medicinales y culinarias. El objetivo de este estudio fue investigar la actividad antimicrobiana y antibiofilm *in vitro* del extracto etanólico de oregano, contra el crecimiento de *Streptococcus mutans* y *Streptococcus sobrinus* ATCC. Se obtuvierón hojas de la planta y se realizó el extracto etanólico mediante maceración. La actividad antimicrobiana se evaluó mediante el método de Kirby-Bauer y se comparó con la clorhexidina al 2 %, posteriormente se incorporó el extracto en un hydrogel y se valoró su efecto sobre la formación del biofilm mediante microscopía de fluorescencia y se identificó los principales compuestos presentes en el extratco. El estudio reveló que el extracto presentó efecto antimicrobiano contra ambas cepas y al 2 % mostró alta acción antimicrobiana en comparación con la clorhexidina a la misma concentración, con halos de inhibición promedio de 26.3 mm y de 19 mm para cada microorganismo analizado, ($p < 0.05$). Así mismo, el hidrogel preparado con extracto al 2 %, eliminó significativamente la biopelícula preformada de Streptococcus, a las 24 horas de exposición, debido a la presencia de una variedad de grupos químicos, como esteroles, triterpenos, flavonoides, flavanonas, flavanonoles, lactonas sesquiterpénicas, taninos y cumarinas. El extracto de orégano presentó alta acción antimicrobiana para ambas especies, con mayor efecto hacia el *Streptococcus mutans* y una acción antibiofilm interesante; estos resultados muestran la importancia de explorar en alternativas de tratamiento de origen vegetal, para considerarse como auxiliares complementarios interesantes en la terapia dental.

PALABRAS CLAVE: biofilms, placa dental, origanum, fitoterapia, *Streptococcus mutans*, *Streptococcus sobrinus*.

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