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Inhibitory effect of the essential oil of *Schinus molle* L. against pathogens causing periodontal disease

Carlos Cadena-Viteri[1], Miriam Lima-Illescas[1,2,3], Edisson-Mauricio Pacheco-Quito[1,2,3], Mariela Cumandá Balseca-Ibarra[4], Fernanda Sacoto-Figueroa[2], Katherine Cuenca-León [1,2,3,5*]

[1]Specialty in Orthodontics, Catholic University of Cuenca, Azogues 030102, Ecuador; carlos.cadena.47@est.ucacue.edu.ec (C.C.-V)

[2]Academic Unit of Health and Wellness, Faculty of Dentistry, Catholic University of Cuenca, Cuenca 010105, Ecuador; mlimai@ucacue.edu.ec (M.-V.L); epachecoq@ucacue.edu.ec (E..P.-Q.); kacuen-ca22@gmail.com (M.B.-I.); fsacotof@ucacue.edu.ec (F.S.-F.); kcuencal@ucacue.edu.ec (K.C.-L.)

[3]Research Group: Innovation and Pharmaceutical Development in Dentistry Research Group, Faculty of Dentistry, Head of Research and Innovation, Catholic University of Cuenca, Cuenca 010105, Ecuador

[4]Central University of Ecuador. Quito 170521, Ecuador.

[5]Pharmaceutical Sciences Department, University of Salamanca, 37007 Salamanca, Spain

*Correspondence: kcuencal@ucacue.edu.ec

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Summary

Introduction: There are several oral diseases caused by various microorganisms. In this work, we discuss periodontal pathogens, which cause chronic degenerative damage in the supporting tissues of teeth. This is why several treatments have been developed for their eradication, including phytocomponents and essential oils as an option in antimicrobial therapy. **Objective:** The aim of this study was to determine the inhibitory effect of the essential oil of the plant species *Schinus molle* L. native to Ecuador on strain of *Porphyromonas gingivalis* at different concentrations. **Methodology:** This was a laboratory and longitudinal study in which the *Porphyromonas gingivalis* ATCC 33277 strain was cultured in 20 Petri dishes, working with several exposure subgroups, including Group 1 - 50% essential oil of *Schinus molle* leaves; Group 2 - 100% essential oil of *Schinus molle* L.; Group 3 - 0.12% chlorhexidine (positive control); Group 4 - saline solution (negative control)

with different incubation periods of 24 and 72 hours. **Results:** The *Porphyromonas gingivalis* ATCC 33277 sample exposed to 100% plant species *Schinus molle* L. for 24 hours had an inhibition zone of 15 mm, demonstrating high sensitivity, and exposure for 72 hours produced a zone of 14 mm, also suggesting sensitivity. Exposure to *S. molle* L. at 50% for 24 hours produced a zone of inhibition of 9.65 mm, showing sensitivity; however, it is worthwhile to continue developing and evaluating this area of study. **Conclusions:** This study demonstrates that phytotherapy using the essential oil of the plant species *Schinus molle* L. represents a therapeutic option in cases of infections caused by *Porphyromonas gingivalis*.

Keywords: Oils, periodontal diseases, anti-bacterial agents

Resumen

Efecto inhibitorio del aceite esencial de *Schinus molle* L. contra patógenos causantes de la enfermedad periodontal

Introducción: Existen varias enfermedades orales causadas por diversos microorganismos. En este trabajo, discutimos los patógenos periodontales, que causan daños crónicos degenerativos en los tejidos de soporte de los dientes. Por ello, se han desarrollado diversos tratamientos para su erradicación, entre los que destacan los fitocomponentes y aceites esenciales como opción en la terapia antimicrobiana. **Objetivo:** El objetivo de este estudio fue determinar el efecto inhibitorio del aceite esencial de la especie vegetal *Schinus molle* L. nativa del Ecuador sobre una cepa de *Porphyromonas gingivalis* a diferentes concentraciones. **Metodología:** Se trató de un estudio de laboratorio y longitudinal en el que se cultivó la cepa *Porphyromonas gingivalis* ATCC 33277 en 20 placas Petri, se trabajó con varios subgrupos de exposición, tales como: Grupo 1 - 50% aceite esencial de hojas de *Schinus molle*; Grupo 2 - 100% aceite esencial de *Schinus molle* L.; Grupo 3 - clorhexidina al 0,12% (control positivo); Grupo 4 - solución salina (control negativo) a diferentes periodos de incubación de 24 a 72 horas. **Resultados:** La muestra de *Porphyromonas gingivalis* ATCC 33277 expuesta al 100% de la especie vegetal *Schinus molle* L. durante 24 horas presentó un halo de inhibición de 15 mm, lo que demuestra una alta sensibilidad, y la exposición durante 72 horas produjo un halo de 14 mm, lo que también sugiere sensibilidad. La exposición a *S. molle* L. al 50% durante 24 horas produjo un halo de inhibición de 9,65 mm, demostrando sensibilidad; sin embargo, vale la

pena continuar desarrollando y evaluando esta área de estudio. **Conclusiones:** Este estudio demuestra que la fitoterapia utilizando el aceite esencial de la especie vegetal *Schinus molle* L. representa una opción terapéutica, en casos de infecciones ocasionadas por *Porphyromonas gingivalis.*

Palabras clave: Aceites, enfermedades periodontales, agentes antibacterianos.

Resumo

Efeito inibitório do óleo essencial de *Schinus molle* L. contra patógenos causadores de doença periodontal

Introdução: Existem diversas doenças bucais causadas por diversos microrganismos. Neste trabalho, discutimos os patógenos periodontais, que causam danos degenerativos crônicos aos tecidos de suporte dos dentes. Por este motivo, vários tratamentos têm sido desenvolvidos para a sua erradicação, entre os quais se destacam os fitocomponentes e os óleos essenciais como opção na terapia antimicrobiana. **Objetivo:** O objetivo deste estudo foi determinar o efeito inibitório do óleo essencial da espécie vegetal *Schinus molle* L. nativa do Equador sobre uma cepa de Porphyromonas gingivalis em diferentes concentrações. M**etodologia:** Trata-se de um estudo laboratorial e longitudinal em que a cepa *Porphyromonas gingivalis* ATCC 33277 foi cultivada em 20 placas de Petri, trabalhando com diversos subgrupos de exposição, tais como: Grupo 1 - 50% de óleo essencial de folhas de *Schinus molle* L.; Grupo 2 - óleo essencial 100% de Schinus molle L.; Grupo 3 - clorexidina 0,12% (controle positivo); Grupo 4 - solução salina (controle negativo) em diferentes períodos de incubação de 24 a 72 horas. **Resultados:** A amostra de *Porphyromonas gingivalis* ATCC 33277 exposta a 100% da espécie vegetal *Schinus molle* L. por 24 horas apresentou zona de inibição de 15 mm, o que demonstra alta sensibilidade, e a exposição por 72 horas produziu zona de inibição de 14 mm, o que também sugere sensibilidade. A exposição a *S. molle* L. a 50% durante 24 horas produziu uma zona de inibição de 9,65 mm, demonstrando sensibilidade; no entanto, vale a pena continuar a desenvolver e avaliar esta área de estudo. **Conclusões:** Este estudo demonstra que a fitoterapia utilizando o óleo essencial da espécie vegetal *Schinus molle* L. representa uma opção terapêutica em casos de infecções causadas por *Porphyromonas gingivalis.*

Palavras-chave: Óleos, doenças periodontais, agentes antibacterianos.

Introduction

The mouth constitutes a reservoir for many microorganisms, including bacteria. These bacteria, along with mucus and other components, constantly form a sticky, color-less "plaque" that adheres to the teeth [1, 2]. Brushing and flossing help to remove this plaque, but in situations wherein plaque is not removed, it hardens and forms deposits called "calculus" or "tartar", which brushing alone cannot remove; only professional cleaning by a dentist or dental hygienist can re-move it [3-6].

Periodontitis is a chronic inflammatory condition in which the progressive destruction of the tissues that support the teeth prevails; the etiological factor of this is the bacterial interaction of cell groups and the susceptibility of individuals to suffer it [7, 8]. Among the bacteria that generate this disease, the most predominant is *Porphyromonas gingivalis*, which has been found in patients with compromised periodontium, and curiously also in healthy patients. This microorganism can evade the host's immune system because it features a conglomerate of virulence factors, which allow it to become an aggressive pathobiont [9, 10].

Global records indicate that the incidence rate of periodontal disease in the adult population is approximately 1% in all ethnic groups, while its prevalence in the female sex is two to three times that in the male sex [4, 11-13]. The numbers are increasing, and preventive measures are sometimes not enough to reduce these numbers. For this reason, other alternatives are sought to help reduce the percentage of these oral diseases [14]. In recent years, phytotherapy has become a possible solution to this complex of microbial resistance, and it has begun to feature more prominently among new therapeutic options, raising hope with respect to combating bacterial resistance and the secondary effects that arise in most cases [15-19].

Essential oils have demonstrated their antioxidant capacity to act in metabolic response to endogenous production of free radicals and other oxidative species [20], showing *in vitro* studies, antimicrobial properties against various pathogens. *Schinus molle* L., commonly known as pink pepper or American pepper, is a tree of the Anacardiaceae family native to the subtropical regions of South America. It was introduced and naturalized in southern Europe, including Portugal, as an ornamental plant. In the health field, *Schinus molle* has been used for its antibacterial, antiviral, topical antiseptic, antifungal, antioxidant, anti-inflammatory, antitumor, antispasmodic, analgesic, stimulant and antidepressant properties, so its activity on bacteria such as *Porphyromonas gingivalis* is the focus of this study [21, 22].

Thus, the use of adjuvants with natural components in the treatment of periodontal disease suggests advantages such as a decrease in side effects; as it is a different treatment, resistance to usual treatments would be eliminated, and in terms of costs it would be very beneficial [20]. Now, the formulation of pharmaceutical substances based on plant species should take a more central role and should move beyond the level of theory. For this reason, we suggest that these types of study be developed, since there is a very broad range of plant species whose applications are not yet known, and it is necessary to develop this field so as to extend our knowledge [21-24].

In this context, chlorhexidine gluconate at a concentration of 0.12% is currently still used to combat diseases such as periodontitis. This antiseptic has been traditionally used since the 1970s in periodontal odontology. It continues to be the mouthwash of first choice for cases of periodontal diseases [25]. Consequently, and due to the need to explore new forms of treatment that are less invasive and have low toxicity, this research has been proposed to evaluate the inhibitory effects of one of our country's own plants, with anti-septic antecedents, on the microorganism that predominantly generates periodontitis [15].

Methodology

This was a longitudinal laboratory study that tested the inhibitory efficacy of *Schinus molle* oil at different concentrations and times on *Porphyromonas gingivalis* strain [26]. The inhibition halos produced by *Schinus molle* oil at 50% and 100% at 24 and 72 hours were compared with those of the control (chlorhexidine 0.12%) and blank (physio-logical serum 0.9%) when applied on strain of *Porphyromonas gingivalis* (ATCC 33277).

Obtaining the essential oil

After the selection of the sample, 1.5 kg of fresh plant was subjected to dehydration to a final weight of 140 g, which was crushed. This was then subjected to hydrodistillation with the reflux of condensation vapors using a Dean Stark grid for greater efficiency in the collection of the oil. It was expected that from 140 g of *Schinus molle*, between 4 and 5 mL of oil could be obtained. This was stored and refrigerated in amber containers to avoid de-composition by exposure to atmosphere. A concentration of 100% was obtained by separating residual water via distillation. To obtain a concentration of 50%, dilution with dis-tilled water was performed. To obtain a 50% concentration, dilution was carried out with distilled water; vortex was used to homogenize the dilution. The discs were soaked with 25 mL of this compound. Thus, the discs that represented 100% concentration were soaked with undiluted essential oil, and those that represented 50% concentration were soaked with oil diluted in a 1:1 ratio (oil-distilled water) [27, 28] (Figure 1. A).

Strain activation

To develop the microbiological part, a periodontal pathogenic bacterium, *Porphyromonas gingivalis* (strain ATCC 33277), was acquired and activated in a class 2 laminar flow chamber type BII. The turbidity of the bacterial inoculum was standardized until a dilution of 1.5×10^8 CFU/mL was obtained, equivalent to 0.5 on the McFarland scale. The bacteria were seeded on Agar blood culture medium and incubated in an anaerobic jar for 48 hours at 37 °C.

Inoculation of *Porphyromonas gingivalis* strain ATCC® 3327 TM

Using a sterile loop or swab, a small sample of the previously reactivated *Porphyromonas gingivalis* strain was extracted and immersed in a test tube with saline solution. The test tube was shaken until a turbidity of 1×10^8 CFU/m was reached, equivalent to 0.5 on the McFarland scale. This process was carried out visually [28] (Figure 1.B).

Placement of white disks soaked in the extracts of the plant species under study.

We inoculated culture media in Mueller Hinton agar with two drops of blood and with the *Porphyromonas gingivalis* ATCC® 33277TM strain. In total, 20 white disks for each sub-stance studied were placed in labeled Petri dishes; into these disks we embedded *Schinus molle* L. extract in concentrations of 50% and 100%, using a micropipette (Figure 1.C).

Placement of the culture media with the embedded discs in a Gaspak jar

Four antibiogram disks were placed in each Petri dish and soaked in 100% essential oil of *Schinus molle,* as well as 50% and 0.9% saline solutions acting as the negative control, and 0.12% chlorhexidine as the positive control. Each disk had a minimum separation of 15 mm. After the inoculation of the disks, the 20 Petri dishes were incubated at 37 °C for 24 and 72 hours (Figure 1.D).

Measurement of inhibition halos

With the help of a millimeter measuring device, data on the inhibition zones generated by the essential oil of *Schinus molle* at different concentrations were obtained (Figure 1.E).

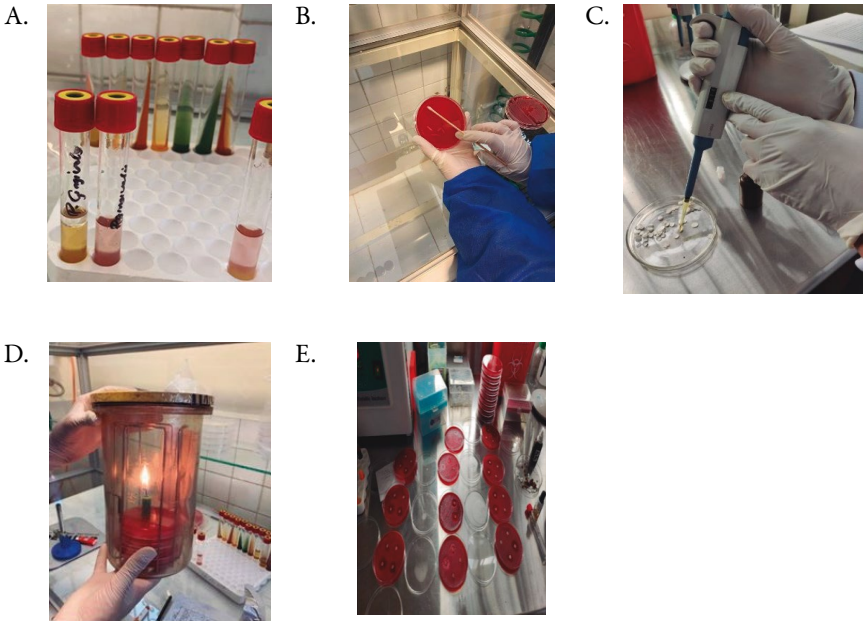

Figure 1. *In-vitro* study process of *Schinus molle* essential oil. A: Extraction of the essential oil. B: Inoculation of the *Porphyromonas gingivalis* ATCC® 3327 TM strain. C: White discs soaked in the study extracts. D: Placement of culture media with the discs soaked in the study substances in a Gaspak jar. E: Measurement of inhibition halos.

Results

The results of this research are based on the selection, preparation and extraction of essential oils from the plant species *A. Schinus molle*, evaluated on ATCC33277 strain of *Porphyromonas gingivalis*. Physiological serum and chlorhexidine were used as the controls. The following results were obtained according to the diameter of the inhibition halo, taking into account the exposure time (Table 1).

Descriptive statistical data for the inhibitory halo (mm) at 24 hours can be observed for the different substances evaluated, such as the essential oils of *Schinus molle* at 50% and 100%, and the physiological serum and chlorhexidine as negative and positive control groups, respectively. The average value of the inhibitory halo of chlorhexidine was the highest (20.80±1.64), and the lowest value was yielded by the control group (6.00±0.00).

Table 1. Concentration of the essential oil and exposure time on *Porphyromonas Gingivalis* strain

Time of exposure	Action composite	N	Mean mm	Standard deviation	Mediana	Minimum	Maximum	*p* value
24 hours	*E. Schinus* 50%	20	9.65	0.74	8	8	11	0.00
	E. Schinus 100%	20	15	0.85	14	13	17	
	Physiological saline solution (Negative Control)	20	6	0	6	6	6	
	Chlorhexidine (Positive Control)	20	20.8	1.64	18	19	25	
	Total	80	12.86	5.7	12	6	25	
72 hours	*E. Schinus* 50%	20	7.75	0.55	8	7	9	0.00
	E. Schinus 100%	20	14	0.64	14	13	15	
	Physiological saline solution (Negative Control)	20	6	0	6	6	6	
	Chlorhexidine (Positive Control)	20	17.5	0.82	18	16	19	
	Total	80	11.31	4.71	12	6	19	

The average inhibition halos of the different substances at 72 hours were the greatest for chlorhexidine (x=17.50±0.82), followed by the essential oil of *Schinus molle* at 100% (x=14.00±0.64) (Table 1). It was observed at 24 hours that the essential oil of the plant species at a concentration of 50% presented a halo of 9.65 mm; at a concentration of 100%, it showed an inhibitory halo of 15 mm, and with chlorhexidine, a halo of 20.80 mm was seen, surpassing all the previous ones. The Shapiro Wilk test determined a value of $p<0.05$; therefore, the non-parametric Kruskall Wallis test was used (Table 1). Significant differences were observed in all groups ($p=0.00$), thus the Dunn test was utilized to establish pairwise comparisons.

The average inhibition halos at 24 hours after subjecting the different substances to the PG strains were analyzed. For the negative control group, it was 6.00 mm, followed by 7.75 mm for *E. Schinus* 50%, 14.00 mm for *E. Schinus* 100%, and 17.50 mm for the

positive control group. Significant differences were found, prompting a further analysis using the Dunn test (Table 2).

It is important to point out that, according to the results presented, there were significant differences, and the effectiveness of *E. Schinus molle* was thus demonstrated. However, it is necessary to point out that according to the inhibition diameters, *E. Schinus* at 100% showed a greater inhibition diameter compared to *Schinus* at 50%.

On the other hand, chlorhexidine was more effective than the plant species *E. Schinus*, presenting a greater inhibitory diameter (Table 3).

Table 2. Dunn's test in the pairwise comparison of the inhibitory effects of essential oilsof *Schinus molle* L. at 50% and 100%, as well as physiological saline and chlorhexidine,at 72 hours on strain of *Porphyromonas gingivalis*.

		Contrast statistic	**Standard error**	**Deviation in contrast statistic**	**p**	**Adjusted p**
E. Schinus 50%	*E. Schinus* 100%	-20.00	7.25	-2.75	0.00	0.03
Control (-)	*E. Schinus* 50%	20.00	7.25	2.75	0.00	0.03
Control (-)	*E. Schinus* 100%	-40.00	7.25	5.51	0.00	0.00
E. Schinus 50%	Control (+)	-40.00	7.25	-5.51	0.00	0.00
E. Schinus 100%	Control (+)	-20.00	7.25	-2.75	0.00	0.03
E. Schinus 100%	Control (+)	-60.00	7.25	-8.27	0.00	0.00

Table 3. Comparison of the inhibitory effects of the essential oils of *Schinus molle* L. at 50% and 100%, as well as physiological saline and chlorhexidine, at 72 hours on strain of *Porphyromonas gingivalis*.

		Contrast statistic	Standard error	Deviation in contrast statistic	p	Adjusted p
Control (-)	*E. Schinus* 50%	20.00	7.25	2.75	0.00	0.03
Control (-)	*E. Schinus* 100%	40.00	7.25	5.51	0.00	0.00
Control (-)	Control (+)	-60.00	7.25	-8.27	0.00	0.00
E. Schinus 50%	*E. Schinus* 100%	-20.00	7.25	-2.75	0.00	0.03
E. Schinus 50%	Control (+)	-40.00	7.25	-5.51	0.00	0.0
E. Schinus 100%	Control (+)	-20.00	7.25	-2.75	0.00	0.03

Discussion

Phytotherapy has shown advancements in the field of health by taking advantage of the effects of active components on pathogenic bacteria. There is a diverse arsenal of essential oils obtained from plant species that have been used as adjuvants in oral problems and/or afflictions, with the bacteria that cause periodontal disease being of interest in this work. In this context, the essential oil of *Schinus molle* has been studied and applied due to its diverse properties, such as antibacterial, analgesic, diuretic, healing, and anti-inflammatory effects [29].

Several studies have evaluated the effectiveness of *Schinus molle* as an antibacterial agent in dentistry. An *in vitro* study conducted by Da Silva *et al.* in 2016, found that *Schinus molle* extracts significantly inhibited the growth of Streptococcus mutans, a bacteria commonly associated with dental caries. Furthermore, research by Turchetti *et al.* (2020) suggest that the active compounds of *Schinus molle* may interfere *in vitro* with the formation of bacterial biofilms, which could be relevant for the control of dental plaque and the prevention of periodontal diseases [30, 31].

Tahtamouni in 2018 [29] showed the antibacterial activity of the ethanolic, methanolic and essential oil extracts of *Schinus molle* leaves, compared to a positive control, which in their case was tetracycline. The ethanolic extract had a strong antibacterial effect against *Bacillus subtilis* and *Micrococcus luteus*, as did the positive control, but no significant differences were noted. On the contrary, in the present investigation, significant differences were found between the inhibitory effects of *Schinus molle* essential oil and 0.12% chlorhexidine as a positive control against *Porphyromonas gingivalis*, wherein the highest inhibitory effect was presented at the highest concentration of the oil (100%) in the first 24 hours.

Alfaro showed that the essential oil of *Schinus molle* at 60% presented a bactericidal effect due to its bioactive contents that are capable of inhibiting the bacterial development of *Staphylococcus aureus*; however, the use of penicillin as a positive control acted as a potent antibacterial agent. The data reported by Alfaro are similar to those obtained in this research, since applying the essential oil of *Schinus molle* at 50% and 100% had an inhibitory effect against *Porphyromonas gingivalis*, but there was no significant effect when com-pared with chlorhexidine 0.12% (positive control) [32].

On the other hand, Loyola and collaborators showed that extracts of *Schinus molle* at concentrations of 50% and 75%, applied for 24 h and 48 h, had inhibitory effects on *Streptococcus mutans*, due to the antibacterial and antifungal properties. This was also evidenced here, as the positive control manifested a halo with a diameter surpassing

that of the vegetable component. In this research, the *Schinus molle* essential oil applied at a concentration of 50% for 24 hours showed an increased inhibition halo according to the Duraffourd scale, and at 72 hours, its effect shifted from low to null, proving once again that applying 0.12% chlorhexidine leads to better bacterial inhibition [33].

Turchetti and Garzoli presented the results of an *in vitro* study carried out with extracts of *Schinus molle* leaves at various concentrations, classified into male and female leaves. They derived better inhibitory results with the extracts obtained from the female leaves against *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans* and *Bacillus subtilis*. In the present work, as mentioned, we used two concentrations (50-100%) and obtained an inhibitory effect equal to that obtained by Turchetti; however, it is necessary to emphasize that a comparison was made with chlorhexidine 0.12% as a positive control, with the latter showing a better inhibitory effect [31].

Despite the encouraging evidence, it is important to recognize the limitations of existing studies, which include the lack of standardization in extraction and evaluation methods, as well as the need for controlled clinical trials to validate their efficacy and safety in dental patients. In addition, a greater understanding of the mechanisms of action and possible interactions with other dental treatments is required.

In conclusion, *Schinus molle* emerges as a promising candidate for the development of antibacterial therapies in dentistry. However, more research is needed to establish its clinical efficacy and determine its exact role in the dental therapeutic armamentarium [15].

Conclusions

The use of the essential oil of *Schinus molle* against *Porphyromonas gingivalis* at a concentration of 50% for 24 hours and 72 hours showed sensitive and null inhibitory effects, respectively. The application of the essential oil at 100% for 24 hours showed high sensitivity, and it was sensitive for 72 hours. However, the chlorhexidine always showed greater sensitivity against the exposed bacteria according to the size of the inhibition halo. It is necessary to continue these studies, since this is a very wide field and can thus con-tribute new therapeutic options that will reduce the adverse effects associated with conventional pharmacological therapy.

In our country (Ecuador), there are innumerable plant species with curative properties that have not yet been studied. This research work represents a clear proposal to continue working in this area, carrying out more research on phytotherapy in relation to pathologies for which traditional treatment is no longer an option.

In the dental field the efficacy of chlorhexidine is clear, but there may also be other disinfection options, as proposed in this research, speaking specifically of the bacterium *Porphyromonas gingivalis*, but it is necessary to continue studying the phytotherapeutic area and in this way obtain new information, new alternatives.

Conflicts of interest

Authors declare that they have no conflicts of interest

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