

RESEARCH ARTICLE

Chemical composition and antimicrobial activity of essential oil from *Minthostachys mollis* against oral pathogens

Composición química y actividad antimicrobiana del aceite esencial de *Minthostachys mollis* frente a patógenos orales

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ABSTRACT

Introduction: *Minthostachys mollis* is medicinal plant frequently used because of its anti-inflammatory and antibacterial properties.

Objective: Identify the chemical composition of the essential oil from *Minthostachys mollis* and evaluate its antimicrobial properties against oral pathogens.

Methods: The essential oil was extracted from the fresh leaves and branches of the plant by steam distillation method, and Gas Chromatography-Mass Spectrometry were performed to identify the principal constituents of the essential oils. Repetitions for the antimicrobial assay were calculated with the Epi info TM program. The antimicrobial activity was tested against *Enterococcus faecalis* ATCC 29212, *Porphyromonas gingivalis* ATCC 33277 and *Candida albicans* ATCC 10231 by disc diffusion assay and microdilution method, the minimum inhibitory concentration, minimum bacterial concentration and minimum fungal concentration were recorded.

Results: The main compounds found were menthone (32.9%) and eucalyptol (28.1%). The essential oil of *M. mollis* demonstrated inhibition zones against *E. Faecalis*, *P. gingivalis* and *C. albicans* of 15.13 ± 0.39 , 17.96 ± 0.21 , and 20.82 ± 0.63 mm, respectively. There were significant differences on the inhibition zones between the three strains tested, being *C. albicans* the most sensible microorganism ($p < 0.05$). The minimum inhibitory concentration ranged from 1.05 to 2.11 $\mu\text{g/mL}$, the minimum bacterial concentration ranged from 2.11 to 4.23 $\mu\text{g/mL}$, and the minimum fungal concentration was 1.05 $\mu\text{g/mL}$.

Conclusions: The essential oil of *Minthostachys mollis* showed as principal compounds menthone and eucalyptol and it has an important antimicrobial activity against *E. faecalis*, *P. gingivalis* and *C. albicans*.

Keywords: *Minthostachys mollis*; plant oils; chemical composition; antibacterial agents.

RESUMEN

Introducción: *Minthostachys mollis* es una planta medicinal de uso frecuente por sus propiedades antiinflamatorias y antibacterianas.

Objetivo: Identificar la composición química del aceite esencial de *Minthostachys mollis* y evaluar sus propiedades antimicrobianas frente a patógenos orales.

Métodos: El aceite esencial se extrajo de las hojas y ramas frescas de la planta mediante el método de destilación al vapor; se realizó una cromatografía de gases-espectrometría de masas para identificar los principales constituyentes del aceite esencial. Las repeticiones del ensayo antimicrobiano se calcularon con el programa Epi infoTM. La actividad antimicrobiana se evaluó frente a *Enterococcus faecalis* ATCC 29212, *Porphyromonas gingivalis* ATCC 33277 y *Candida albicans* ATCC 10231 mediante ensayo de difusión en disco y método de microdilución. Se registró la concentración mínima inhibidora, la concentración mínima bactericida y la concentración mínima fungicida.

Resultados: Los principales compuestos encontrados fueron mentona (32,9 %) y eucaliptol (28,1 %). El aceite esencial de *M. mollis* generó zonas de inhibición frente a *E. Faecalis*, *P. gingivalis* y *C. albicans* de $15,13 \pm 0,39$, $17,96 \pm 0,21$ y $20,82 \pm 0,63$ mm, respectivamente. Existió diferencias significativas en las zonas de inhibición entre las tres cepas, siendo *C. albicans* el microorganismo más sensible ($p < 0,05$). La concentración mínima inhibitoria varió de 1,05 a 2,11 $\mu\text{g/mL}$, la concentración mínima bacteriana varió de 2,11 a 4,23 $\mu\text{g/mL}$ y la concentración mínima de hongos fue 1,05 $\mu\text{g/mL}$.

Conclusiones: El aceite esencial de *Minthostachys mollis* mostró como compuestos principales mentona y eucaliptol y posee una importante actividad antimicrobiana contra *E. faecalis*, *P. gingivalis* y *C. albicans*.

Palabras clave: *Minthostachys mollis*; aceites vegetales; composición química; agentes antibacterianos.

INTRODUCTION

The high Andean region of South America has a great variety of medicinal plants, commonly used as part of the popular culture and traditional medicine.⁽¹⁾ Particularly, Peru has a great diversity of flora, as a result of the diverse ecosystems dependent on the country's geography. One of these plants, *Minthostachys mollis* (*M. mollis*) known as “muña”.⁽²⁾ This plant belongs to the Lamiaceae family consisting of more than 300 species distributed in 200 genera including mint, peppermint, thyme and rosemary.^(3,4)

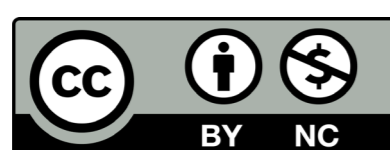
M. mollis is a perennial shrub and grows wild in the valleys of the central and southern highlands of

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Peru. *M. mollis* is frequently used because of its anti-inflammatory, antibacterial, antiemetic, antispasmodic, antitussive and anti-mountain sickness properties. These properties are mainly attributed to the chemical components of their essential oils (EOs).⁽⁴⁾

Several studies have been developed in the Andean region of south America to identified and quantified the chemical components of this plant, considering some variation on species names, due probably to different voucher specimens. The principal compounds reported are neomenthol, menthol, menthone, eucalyptol, piperitone, pulegone, trans- β -karyophyllene, carvacrol, thymol, 1-tetradecene 2S-trans-menthone, γ -terpinene and nerolidol.^(4,5,6) However, there is no evidence of studies that have previously evaluated the chemical composition of *M. mollis* from the southern region of Peru.

On the other hand, antibacterial and antifungal properties of Essential oil (EO) of *M. mollis* have been previously evaluated.^(5,6) The results of these investigations show promising possibilities which would allow the pharmacological and commercial use of products derived from the compounds of this plant.

More than 700 bacterial species have been identified in the oral cavity; the imbalance in the homeostasis conditions due to intrinsic or extrinsic factors is associated with the appearance of various bacterial diseases. Periodontitis, persistent intra root canal infection and oral candidiasis are common conditions observed in clinical management of dental patients.⁽⁷⁾

Enterococcus faecalis is considered the most resistant microorganism associated with persistent periradicular lesions in root canals treatment failures, and it is found in 80-90% in these cases. *E. faecalis* has the ability to adhere to the dentin and invade the dentin tubules, being able to form a biofilm resistant to irrigation solutions, as well as to intracanal drugs.⁽⁸⁾

Periodontitis is an inflammatory disease induced by the presence of bacterial agents, mainly by *Porphyromonas gingivalis*, which is associated with the progression of the disease; *P. gingivalis* is an opportunistic pathogen and can be isolated from the supragingival biofilm and mainly within the gingival sulcus.⁽⁹⁾

Oral candidiasis is a common fungal infection caused by the overgrowth of *Candida albicans* and is considered as an opportunist condition associated with different local or systemic factors such as reduction in salivary flow, intake of some drugs, smoking, diabetes, reduced immunity, and others.⁽¹⁰⁾

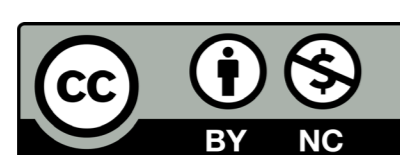
The use of products derived from natural plants has been recommended for the development of new effective and safe products as strategies for the control of various infections. Since the chemical composition of essential oils depends on several factors such as the geographical origin, this study aims to characterize the essential oil obtained from fresh leaves and branches of *Minthostachys Mollis* (Benth.) Griseb. collected from the southern Andes of Peru, report its composition for the first time in the literature and test its antimicrobial activity against *Enterococcus faecalis*, *Porphyromonas gingivalis* and *Candida albicans*, three important oral pathogens.

METHODS

An in vitro experimental study was designed. This study was approved under Rectoral resolution No. 959-2019-UPT-R. The calculation of the repetitions for the antimicrobial assay was set with the Epi info™ program comparing the means of a previous studies.

Plant collection and essential oil extraction

Specimens of *M. mollis* were collected during September 2019 in the province of Tarata, located in the region of Tacna, Peru at coordinates 17°28'27"S 70°01'53"W. This province has an altitude of 3083 m.a.s.l. A total amount of 5 kg was collected, and a voucher specimen were deposited at the herbarium of the Faculty of Agricultural Science of the Jorge Basadre Grohmann National University for taxonomy identification under collection number 3103. The sample was identified as *Minthostachys mollis* (Benth.) Griseb. The EO extraction was performed by triplicate from the fresh leaves and branches of the plant using steam distillation method for 2 hours. A slightly yellow oil was obtained (19 mL). The EO was transferred to an amber glass vial, sealed and kept refrigerated at 2 °C until analysis. Then, the refractive index was determined with an Abbe refractometer (NMX-F074-S-1981), and the density was measured following the directions of the OIML G - 14.



Essential oil analysis

Gas Chromatography-Mass Spectrometry was carried out on the above used instrument, coupled with a QP2010 Ultra Shimadzu mass-spectrometer equipped with a capillary column DB-5 MS (30 m x 0.25 mm, 0.25 µm film thickness). The operative conditions were programmed as follow: the initial column temperature was set at 40 °C and kept for 2 min; heated gradually to 140 °C with a rate of 3 °C/min; the heat was increased to 250 °C at a 10 °C/min rate, kept for 20 min; and finally heated to 300 °C at a 50 °C/min rate, and was kept for 1 min. Helium was employed as carrier gas with an injection volume of 1.0 µL at a constant flow of 1 mL/min with an injection volume set at a ratio of 1:75. The temperatures of transfer line and ion source were set at 250 and 230 °C, respectively. Electron ionization voltage was adjusted at 70 eV, and mass spectra were obtained at a range of 50-350 m/z. The operation conditions were: the EO was diluted to 5% in dichloromethane and injected at 250 °C, with an injection volume of 1 µL at a ratio of 1:75. The compounds were identified by their retention rates of the homologous n-alkane series compared with standards from the literature.⁽¹¹⁾ Mass spectra, reflected by the number of peaks in the chromatogram, was compared with the NIST 08 Library. The acquired percentage values were the mean of three injections of the sample.

Antibacterial and antifungal activity

Antibacterial activity was tested by the disk diffusion method against two bacterial strains; a Gram-positive Bacteria (*Enterococcus faecalis* ATCC 29212), a Gram-negative Bacteria (*Porphyromonas gingivalis* ATCC 33277), and against a yeast strain (*Candida albicans* ATCC 10231). *E. Faecalis* was grown on brain heart infusion agar (BHA) at 37 °C for 18 h, five colonies of the bacteria growth were transferred to a test tube with brain heart infusion broth (BHI) and incubated overnight at the same conditions. The suspension was adjusted to the 0.5 McFarland standard comparing the turbidity with the DensiChek plus standard kit. The suspension was spread with a sterile cotton swap onto Petri dishes containing BHA. Then, 10 µL of the essential oil was charged to a sterile filter paper disk (diameter of 6 mm) and placed onto the agar plates. In the case of *P. gingivalis* tryptic soy agar and broth (TSA, TSB) were used as growth mediums and the plates were incubated at 37 °C in anaerobic conditions using a GasPak EZ Anaerobe Container System. For testing the antifungal activity against *C. albicans* the same method was used, considering yeast mold agar and broth (YMA, YMB) as growth mediums, the incubation conditions were 25 °C for 24 h. Distilled water was used as negative control and chlorhexidine at 0.12% and nystatin were used as positive control for bacteria and fungus, respectively. To evaluate the antimicrobial activity, the growth inhibition zones were measured with a digital caliper (Ubermann©). The test was carried out in triplicate and the mean of the diameter of the inhibition zones was recorded.

The minimum inhibitory concentration was evaluated by the microdilution method. Briefly, 230 µL of the essential oil was diluted in 5 mL of BHI, TSB and YMB for testing the three different microbial strains. 50 µL of the solution were added to the microplate wells and 50 µL of the different inoculum solutions with a concentration of 1x10⁸ UFC/mL were added to the first column and a serial dilution of 1:2 was performed with concentrations from 4.23 to 0.004 µg/mL. In the final column, wells from A to D were filled with 50 µL of medium and 50 µL of the inoculum, and represent the negative control; from wells E to H, 100 µL of medium was added, this was used as the medium control. The microplates were incubated at the same conditions of the disc diffusion assay. Three microplates were used for each microbial strains and MIC was considered as the lower concentration without visible microbial growth. For the determination of the minimum bactericidal and fungicidal concentration (MBC or MFC), 50 µL of the suspension contained in the wells that showed no visual growth were transferred to petri dishes containing the respective growth medium and incubated at the same conditions described above. The MBC or MFC was considered as the lowest concentration of the solutions capable of completely inhibit the microbial growth, with no colony formation.

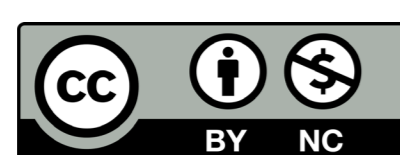
Statistical analysis

All the statistical analysis was performed using SPSS V.23.0 for Mac OS (IBM Corp., Armonk, NY, USA). Student T test was selected to compare the inhibition zone diameter from the groups and the controls. To compare the inhibition growth between the three microbial strains ANOVA test was performed, followed by the Tukey post hoc test. The level of significance was set at p <0.05.

RESULTS

Chemical composition

The extraction yield of the EO of *M. mollis* was 0.38 ± 0.1% (v/w), with a refractive index of 1.47 ± 0.15, and a



relative density of 1.03 ± 0.02 (g/mL). Quantitative data of the compounds isolated from the EO are presented in Table 1. Ten components were identified in the essential oil of *M. mollis*. The most representative compounds identified in the analyzed sample were menthone (32.9%), eucalyptol (28.1%), Trans-menthone (11.9%), and o-Cymene (9.6%).

Table 1 - Chemical composition of EO of *M. mollis* (Benth.) Griseb.

Peak	Compound	Area %	LRI _{exp}	LRI _{lit}
1	.alpha.-Pinene	3.2	939	939
2	.beta.-Pinene	3.5	979	980
3	o-Cymene	9.6	1009	1011
4	Eucalyptol	28.1	1030	1033
5	Linalool	1.4	1104	1103
6	Octen-1-ol, acetate	5.8	1125	1124
7	Isomenthone	2.2	1140	1141
8	Menthone	32.9	1153	1154
9	Trans-menthone	11.9	1161	1163
10	.gamma.-Elemene	1.5	1431	1430

Relative concentrations (Area %), experimental linear retention index (LRI_{exp}) and literature values (LRI_{lit}) are presented.⁽¹¹⁾

Antimicrobial activity

The antimicrobial activity of the essential oil of *M. mollis* was tested against three microbial strains, the results of the disc diffusion assay are presented in Table 2. The essential oil demonstrated antimicrobial activity against the three strains with inhibition zones ranged from 15.13 to 20.82 mm. There were no differences when compared the inhibition zones of the EO of *M. mollis* from *P. gingivalis* and *C. albicans* with the positive controls ($p > 0.05$); chlorhexidine at 0.12% was more effective to inhibit the growth of *E. faecalis* than the EO of *M. mollis* ($p < 0.05$).

Table 2 - Disc diffusion assay for antimicrobial activity of essential oil of *M. mollis*. Inhibition zone (mm)

Microorganism	Essential oil (mm)	Control (mm)		p Value
		Chlorhexidine	Nystatin	
<i>E. faecalis</i> ATCC 29212	15.13 \pm 0.39	18.54 \pm 0.77	-	0.002
<i>P. gingivalis</i> ATCC 33277	17.96 \pm 0.21	18,31 \pm 0.66	-	0.433
<i>C. albicans</i> ATCC 10231	20.82 \pm 0.63	-	20.21 \pm 0.23	0.192

Student T test was performed for comparison.

Comparison between the three strains demonstrated that the inhibition zones generated by the EO of *M. mollis* were significant greater for *C. albicans* than the two bacterial strains ($p < 0.05$). Additionally, the inhibition of growth between *E. faecalis* and *P. gingivalis* was statistically significant ($p < 0.05$) (Figure 1).

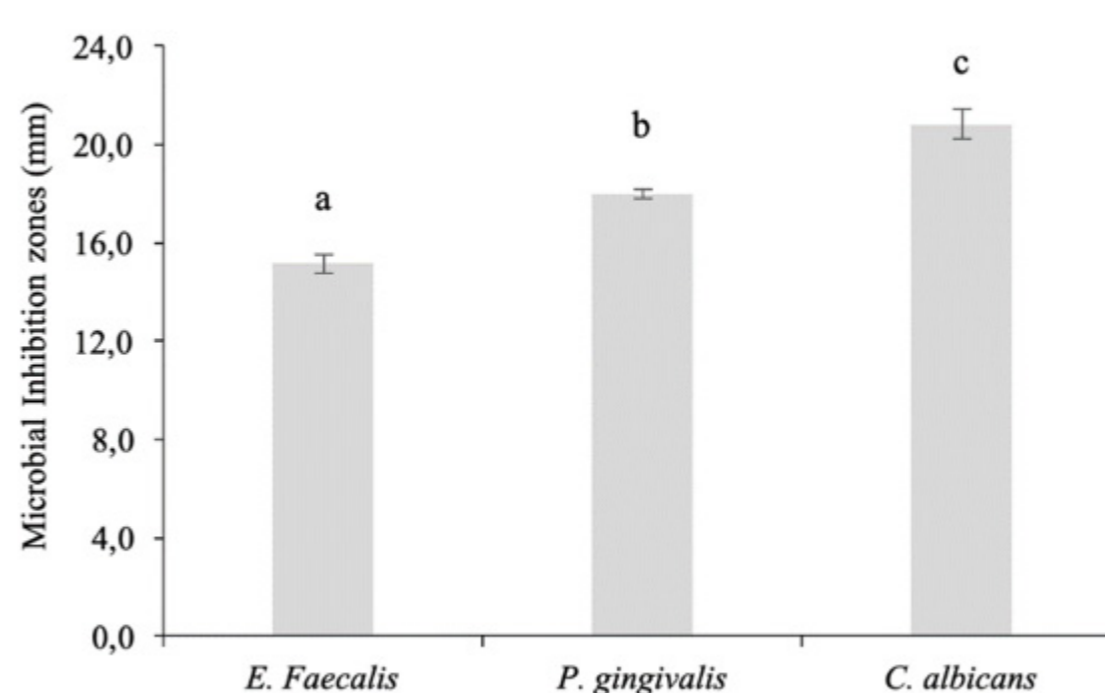


Figure 1 - Comparison of growth inhibition zones (mm) between the three microbial strains. Different letters indicate significant differences. $p < 0.05$, ANOVA test followed by Tukey post hoc test.

The MIC value for *C. albicans* was 1.05 μ g/mL, a higher concentration was necessary to inhibit *E. faecalis* and *P. gingivalis* (2.11 μ g/mL); meanwhile the MBC for *E. faecalis* and *P. gingivalis* were 4.23 μ g/mL and 2.11 μ g/mL respectively, and the MFC was 1.05 μ g/mL for *C. albicans* (Table 3).

Table 3 - Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) of essential oil of *M. mollis*.

Microorganism	MIC (µg/mL)	MBC (µg/mL)	MFC (µg/mL)
<i>E. faecalis</i> ATCC 29212	2.11	4.23	-
<i>P. gingivalis</i> ATCC 33277	2.11	2.11	-
<i>C. albicans</i> ATCC 10231	1.05	-	1.05

DISCUSSION

The results of this study showed that the principal constituents of the EO of *M. mollis* were menthone (32.9%) and eucalyptol (28.1%). It is a different pattern of compounds from other studies, whom analyzed different samples of EO obtained from *M. mollis* collected from Peru and South America. Thereby, Cano et al. reported pulegone (36.6%) and menthone (24.2%) as the main compounds of a sample of EO of *M. mollis* Kunth. from the central Andes of Peru.⁽¹²⁾ Fuertes and Munguía identified the components of EO of *M. mollis* (Kunth) Griseb from three regions of Peru; the principal components of the sample from Tarma were 1-tetradecene (23.1%), 2S-trans-menthone (23%) and pulegone (13.2%); the EO from Huaraz was constituted by 2S-trans-mentona (41.4%), pulegone (16.0%), γ -terpinene (7.5%); and 2S-trans-mentone (34.5%), pulegone (28.6%), and nerolidol (5.0%) were the principal components of the sample from Huancavelica.⁽¹³⁾ In a sample from Cajamarca, the main compounds were menthone (13.2%), pulegone (12.4%) and cis-dihydrocarvone (9.8%).⁽⁴⁾ It is important to notice that pulegone was a common compound in these studies and this differ from our report, where pulegone was not quantified.

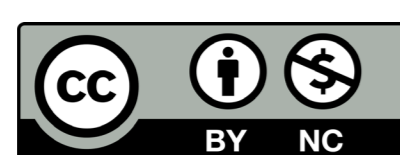
The composition of the EO of *M. mollis* from Ecuador show as principal compounds menthone (16.4%), carvacryl acetate (10.0%) and pulegone (9.9%).⁽¹³⁾ Elechosa et al. identified and quantified samples of *M. mollis* (Kunth.) Griseb. from three regions of central Argentina, the principal components were menthone (23.8-52.6%), pulegone (38.6-49.2%), and linalool (68.5-71.7%).⁽¹⁴⁾ Samples of *M. mollis* Grisebach obtained from two regions with different altitudes from Venezuela showed as the major component pulegone (79.32% at 3600 m, 75.20% at 1600 m), other components as limonene, linalool and piperitone were higher at 3600 m. Other report from Venezuela confirm the present of pulegone as the main component of EO of *M. mollis* (Kunth) Griseb. (55.2%) followed by trans-menthone (31.5%).⁽⁵⁾ These differences could be related to climatic conditions harvest time, place of origin, storage conditions and time and distillation methods, and could explain why are dissimilar from our study.

The EO of *M. mollis* demonstrated an important antimicrobial activity against different strains, and no differences were observed between the growth inhibition zones of *P. gingivalis* and *C. albicans* when compared with chlorhexidine at 0.12% ($p = 0.433$) and nystatin ($p = 0.192$), respectively. However, chlorhexidine at 0.12% was more effective than the EO of *M. mollis* to inhibit the growth of *E. faecalis* ($p = 0.002$). Mora et al. found that the EO of *M. mollis* is particularly effective to inhibit the growth of *Bacillus subtilis* and *Salmonella typhi* (4 µg/mL);⁽⁵⁾ the EO of *M. mollis* (Kunth) at different concentrations also has been tested against fungus, and completely inhibit the growth of *T. tinsurasn*, *T. mentagrophytes* and *M. canis* by the tube dilution method, and against *C. albicans* using the agar diffusion method, the EO of *M. mollis* produced an inhibition zone of 30 mm and 35 mm when were used at 100% and diluted at 50%.⁽¹²⁾ The MIC of the EO of *M. mollis* for *E. faecalis* and *P. gingivalis* was 2.11 µg/mL, and 1.05 µg/mL for *C. albicans*. There were differences between the MBC for the two bacterial strains, being higher for *E. faecalis* than for *P. gingivalis*; the EO of *M. mollis* had a bactericidal activity on *C. albicans* at a concentration of 1.05 µg/mL.

In our knowledge, the present study demonstrated an important antibacterial activity of the EO of *M. mollis* for the first time in the literature against *E. faecalis* and *P. gingivalis*, two important bacteria related with endodontic infection and periodontal disease.

The variety of the results from the mentioned studies might be explained by the interaction of the properties of the different constituents of the essential oils tested. It is worth mentioning that the variation of the EO components, its quantities and properties are related to several factors. In our study the major constituents of the EO of *M. mollis* were menthone (32.9%), eucalyptol (28.1%). Other compounds such as trans-menthone, o-Cymene were present; thus, a synergistic effect should be considered as an important factor in the reported antimicrobial activity.

Menthone was the principal compound found in the sample tested (32.9%). Menthone is a monoterpene analog of menthol, and both share similar structural characteristics.⁽¹⁶⁾ On the other hand, eucalyptol (1,8-cineole) was the second most common compound found in this study (28.1%). Eucalyptol is a monoterpene and it is the



principal component of several aromatic plants and their essential oils, including the Myrtaceae family, particularly in the genus *Eucalyptus* (*E. Globulus* Lab.).⁽¹⁷⁾ Eucalyptol has been used as an antibacterial, anti-inflammatory, and as an expectorant and principally as a relief of the respiratory system.⁽¹⁸⁾

The findings of this study have to be seen in light of some limitations. First, the antibacterial effect was based on an in vitro model and not accurately represent in vivo conditions. Thereby, these results may provide only limited information about the antibacterial effect of the EO of *M. mollis*. On the other hand, the antibacterial model tested the inhibition of growth zones of bacteria and the minimal inhibitory and bactericidal concentrations. Consequently, it would be necessary to test in future studies the behavior of the essential oil on biofilm models.

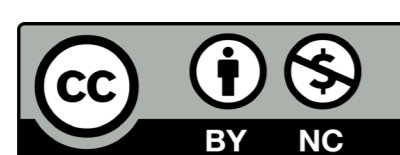
The antimicrobial properties of monoterpenes are associated with increased permeability, protein disturbances and alteration of the cellular membrane transport processes. These effects are different depending on the type of microorganism. It has been suggested that Gram-positive microorganisms are more sensitive to essential oils, because Gram-negative microorganisms present a large amount of lipoproteins and lipopolysaccharides that form a hydrophobic barrier that would make it difficult for the constituents of essential oils to pass into the interior of the cell.^(19,20)

In our results the antibacterial effect against *E. faecalis*, a Gram-positive strain, was lower than the reported for *P. gingivalis*, a Gram-negative strain. *C. albicans* is a Gram-positive yeast and was shown to be more sensitive than the two bacteria strains tested. This could be explained by the interaction between the compounds identified in the essential oil. Being necessary to carry out fractionation assays to isolate the compounds and to be able to establish their antibacterial potential individually.

The essential oil of *Minthostachys mollis* (Benth.) Griseb. collected from the southern Andean region of Peru showed as principal compounds menthone and eucalyptol and has an important antimicrobial activity against *E. faecalis*, *P. gingivalis* and *C. albicans*, its components have demonstrated a high potential use in medical and pharmaceutical industries, since they can be naturally obtained from *M. mollis*, further studies are needed to evaluate its cytotoxicity and others properties prior to recommend its use in medical and dental fields.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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