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Chemical composition and antibacterial activity of *Laureliopsis philippiana* (Looser) essential oil

[Composición química y actividad antibacteriana del aceite esencial de *Laureliopsis philippiana* (Looser)]

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Abstract: *Laureliopsis philippiana* (Looser) is native evergreen specie from Chile and Argentina used in traditional medicine. In this study, chemical composition as well as its *in vitro* antibacterial activity against *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus epidermidis* and *Staphylococcus aureus* of essential oil from leaves of this species was determinated. Chemical analysis by GC-MS resulted in the identification of 19 compounds representing 98.8% of essential oil composition. Oxygenated monoterpenes; linalool (32.3%) and eucalyptol (37.4%) were the main constituents. To evaluate the antibacterial activity disc diffusion method and broth dilution method were used. The essential oil exhibited inhibitory activity against Gram (-) and Gram (+) bacteria, whereas similar activity to essential oil was showed for linalool against *E. aerogenes* and *S. epidermidis* whereas linalool alone, achieves an inhibitory effect against *E. aerogenes* and *S. epidermidis* comparable to the essential oil.

Keywords: *Laureliopsis philippiana*, essential oil, antibacterial activity, linalool.

Resumen: *Laureliopsis philippiana* (Looser) es una especie siempre verde nativa de Chile y Argentina usado en medicina tradicional. En este estudio se determinó tanto la composición química del aceite esencial obtenido a partir de hojas de esta especie, así como su actividad antibacterial *in vitro* contra *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus epidermidis* and *Staphylococcus aureus*. El análisis químico por GC-MS permitió la identificación de 19 compuestos, representando el 98,8% de la composición del aceite. Monoterpenos oxigenados, eucaliptol y linalol fueron los mayores constituyentes del aceite con un 37,4% y 32,3% respectivamente. Para evaluar la actividad antibacteriana se utilizaron los métodos de difusión en agar y dilución en caldo. El aceite esencial muestra actividad inhibitoria contra las bacterias Gram (-) y Gram (+) evaluadas, mientras que linalol por si solo logra un efecto inhibitorio comparable con el aceite esencial contra *E. aerogenes* y *S. epidermidis* mientras que el linalol por si solo, logra un efecto inhibitorio contra *E. aerogenes* y *S. epidermidis* comparable al del aceite esencial.

Palabras Clave: *Laureliopsis philippiana*, aceite esencial, actividad antibacteriana, linalol.

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INTRODUCTION

Natural compounds, such extracts from plant are important sources of active substances with therapeutic potential for the majority of the world's population (Tripathi and Nath, 2003), and its use have been growing in the last years because the extracts from natural sources normally contain molecules such as phenolic compounds with antibacterial and antioxidant properties (Fagundes *et al*., 2010; Piccirillo *et al*., 2013). The essential oils are considered among the most important antimicrobial agents present in plants (Longaray *et al*., 2007), and their constituents are important source of secondary metabolites with potential in medical procedures and applications in the food, cosmetics, aromatherapy and folk medicine (Sonboli *et al*., 2005). Volatile oils are a complex mixture of monoterpenes, sesquiterpenes, and their derivatives (for example, alcohols, aldehydes, esters, ethers, phenols) (Longaray *et al*., 2007). In Chile, the native peoples had developed a rich tradition of healing diseases through the use of plants (Niemeyer, 1995). Extracts of leaves of *Laureliopsis philippiana* have traditionally been used for the treatment of colds, nervous system disorders and headaches (Morales and Ladio, 2009; Staerk *et al*., 2009). To date the information about the chemical constitution of *L. philippiana*, comprise alkaloid compounds, such as asimilobine, anonaine, noreorydine, nornantenine, (+)-reticuline, 4-hydroxyanonaine (Urzúa and Cassels, 1982), laurotetanine (Urzúa *et al*., 1978), and laureliopsine A (Staerk *et al*., 2009), and terpenes, such as 1,8-cineole and 3-carene, and 1,2-dimethoxy-4-(2-propenyl)-phenol (Bittner *et al*., 2009). In fact, the fungistatic activity showed for the essential oil obtained from leaves of *L. philippiana* on *Rhizoctonia solani* Kühn (Donk), *Pythium irregulare* Buisman, *Ceratocystis pilifera* (Fr.) C. Moreau, *Phragmidium violaceum* (Schultz) Winter and *Fusarium oxysporum* Schltdl, has been attributed to these compounds (Bittner *et al*., 2009).

The main objectives of this study were: i) to evaluate the antimicrobial activity of essential oil of *L. philippiana* by disc diffusion method against some pathogen bacteria, ii) to determine the chemical composition of this essential oil by GC-MS and iii) to evaluate the antibacterial effect of their major constituents.

MATERIAL AND METHODS

Plant material

The aerial parts of *L. philippiana* were collected during March 2008 from Rucamanque experimental field of the Universidad de La Frontera (38°39' S; 72°35'W). A voucher specimen was deposited at the herbarium of the Universidad de Concepcion (Voucher N° CONC 173858). The basin and slopes of Rucamanque Valley are covered by native forest, which is evergreen in the lower areas and partially deciduous at higher elevations. The climate of the area is humid and template with a Mediterranean influence. The average annual rainfall is 1,400 mm and the median annual temperature is 12° C. Rainfall is abundant in winter, and summers often have one to two dry months (Ramirez *et al*., 1989).

Microbial strains

The essential oil of *L. philippiana* was tested against bacteria strains, selected as representatives of the classes Gram (+) and Gram (-). The microorganisms used were *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922) and *Enterobacter aerogenes* (ATCC 13048).

Essential oil extraction and analysis

100 g of fresh leaves of *L. philippiana* were distilled for 2 hours using a Clevenger-type apparatus, according to the methodology described by Sonboli *et al*. (2005). The resulting essential oil was dried over anhydrous Na_2SO_4 and stored in sealed ampoules at -20° C in order to use for chemical analysis. The oil was analyzed by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (70 eV) using a ThermoFinnigan chromatograph (Milan, Italy) equipped with a Ultra-1 capillary column (25 m x 0.20 mm x 0.25 µm; SGE, Australia) using helium as carrier gas. The GC oven was programmed to start at 40° C and increased 5° C min^{-1} up to 260° C and held for 5 min. The injector and transfer line temperatures were 250° C. The identification of the compounds was performed by comparison of their Kovats indices and mass spectra with those of commercial standards and library database spectra using the NIST mass spectral search program (ver. 2.0), and NIST webbook (**http://webbook.nist.gov/chemistry**) cited by Babushok *et al.,* (2007). Calibration curves based on peak area ratio were constructed using commercial

standards and docosane as internal standard for quantification of compounds with area percentage higher than 5%, identified in the samples.

Antibacterial activity

Disc diffusion method was employed for the determination of antimicrobial activities of the essential oil (National Committee for Clinical Laboratory Standard, 2001). Briefly, a suspension of the tested microorganism (0.1mL of 10^8 cells per mL) was spread on the solid media plates. Filter paper disks (5 mm in diameter) were impregnated with 2.5; 5.0 and 10 µL of the oil, equivalent to 2.2; 4.5 and 9 mg/disk, respectively, and 1 µL of each linalool and eucalyptol standards and blend of two compounds, for the evaluation of major constituents and placed on the inoculated plates. These plates were held at 4º C for 2 hours and incubated at 37° C for 24 hours. The diameters of the inhibition zones were measured in millimetres. Vancomycin (30 µg/disk), cefalotine (30 µg/disk) and ciprofloxacin (5.0 µg/disk) were individually used as positive controls for bacteria. Values of inhibition zone (mm) represent the average of twelve determinations, and excluding disk diameter of 5 mm.

Inhibition data were checked for normal distribution and variance homogeneity, and analyzed by ANOVA followed by HSD-Tukey test for mean separation ($p < 0.05$) using StatsDirect 2.7.8 (StatsDirect Ltd, UK).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The MIC values against bacterial strains were performed using the broth dilution method (TS broth) (Ericcson and Sherris, 1971). Serial dilutions were made in ethanol for a concentration range between 1 to 100 µg/mL of the essential oil and added to TS broth medium. The bacterial suspensions were aerobically incubated for 24 h at 37° C. The MBC determination was carried out by transferring to the fresh TS broth aliquots of bacterial suspensions from the test tubes containing oil concentrations equal or higher than the MIC and then incubated for 24 h at 37° C. Each oil concentration was tested in triplicate and the experiment was performed three times.

RESULTS AND DISCUSSION

Essential oil analysis

The hydro-distillation of fresh leaves of *L. philippiana* yielded 0.43% w/w, based on the fresh weight of plant with density similar to water. Qualitative analytical results are shown in Table 1. Nineteen components were identified representing 98.8% of the total essential oil. The oil was characterized by a high percentage of oxygenated monoterpenes (73.8%), being linalool (392.34 µg/µL) and eucalyptol (142.06 µg/µL) the main components. Monoterpene hydrocarbons represented twelve of the nineteen compounds, corresponding to 24.1% of the total oil, being β-pinene (21.69 ug/uL) the main component. This essential oil composition is similar to reported by Urzúa *et al*. (2010), for other Monimiaceae specie, but different to the reported by Bittner *et al*., (2009) for *L. philippiana*. The most important differences are the low content of 3-carene and the higher content of eucalyptol, and the presence of linalool. According to Jerkovic *et al*., (2001) and Cimanga *et al*., (2002), the composition of the essential oil can vary depending to geographic region, the age of the plant, method of drying and method of extraction of the oil. In addition the study performed by Donahue *et al.*, (1995), in native populations of *Pinus Greggii* in Mexico, indicated that proportions of α-pinene, myrcene, limonene and longifolene were different in the northern respect to the southern populations. In our study, the *L. philippiana* leave were collected from trees located in a rainforest in Región de La Araucanía (38°39' S; 72°35'W), and the vegetal material used by Bittner *et al*., (2009) was collected ca. 150 km to the north, in Región del Bio-Bío (37º31' S; 71º51' W).

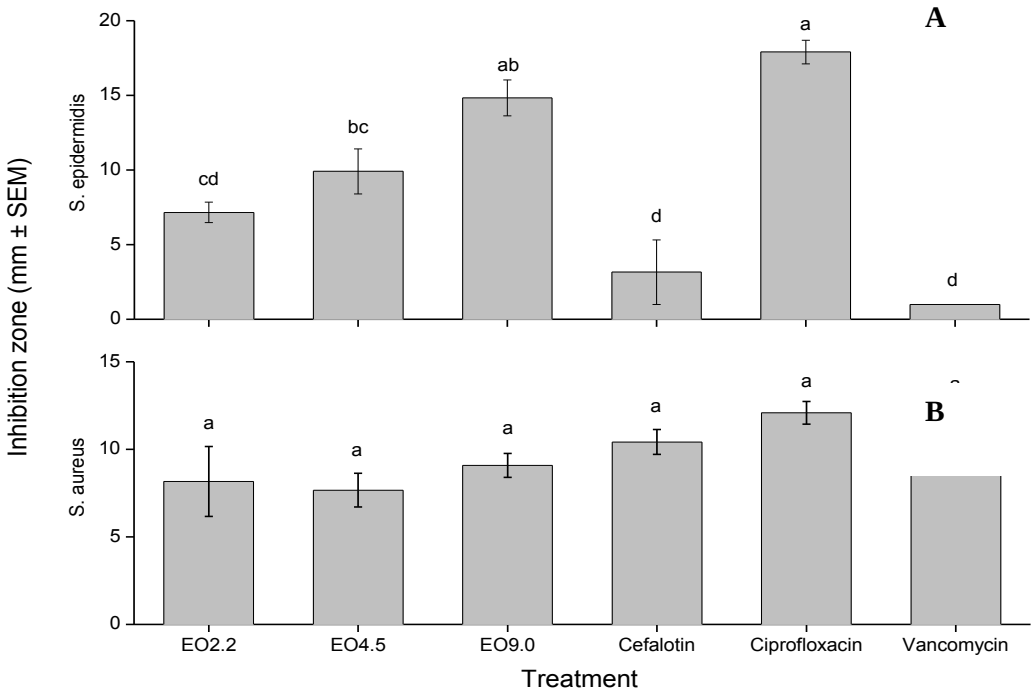
Table 1.
Chemical composition (%) of *L. philippiana* essential oil.

Compounds	% [a]	KI Exp. [b]	KI Lib. [c]	Identification method[d]
Monoterpene Hydrocarbons				
α-Pinene	4.1 ± 0.6	931	934	1
Camphene	0.1 ± 0.0	942	946	2
Sabinene	4.1 ± 0.6	967	969	2
β-Pinene	6.4± 0.6	970	973	1
β-Myrcene	1.2 ± 0.1	984	984	1
Phellandrene	1.9 ± 0.5	995	1000	1

3-Carene	0.1 ± 0.1	1002	1010	1
Cymene	2.1 ± 0.4	1011	1026	1
Limonene	3.6 ± 0.4	1021	1024	1
Z-β-Ocimene	0.1 ±0.1	1041	1041	2
γ-Terpinene	0.2±0.1	1051	1058	2
α-Terpinolene	0.2±0.1	1079	1084	2
Oxigenated Monoterpenes				
Eucalyptol	37.4 ± 3.0	1019	1022	1
Linalool	32.3 ± 3.8	1082	1086	1
Terpinen-4-ol	0.6 ± 0.2	1161	1176	1
α-Terpineol	3.5 ± 0.8	1171	1172	1
Esters				
2-Methylpropyl isobutyrate	0.1 ± 0.1	901	899	2
2-Methylpropyl 2-methylbutanoate	0.2 ± 0.2	991	-	2
Hydrocarbons				
Pentadecane	0.6 ± 0.0	1500	1500	1

[a] Mean ± S.E.M.; n = 10.
[b] Kovats Indices Experimental.
[c] Kovats Indices Library.
[d] The reliability of the identification is indicated by the following letters: 1) mass spectrum (MS), Kovats indices and matching with standard; 2) mass spectrum and Kovats indices agree with corresponding data in the literature, but the data were not confirmed by comparison with the retention time or MS data for an appropriate identical standard compound.

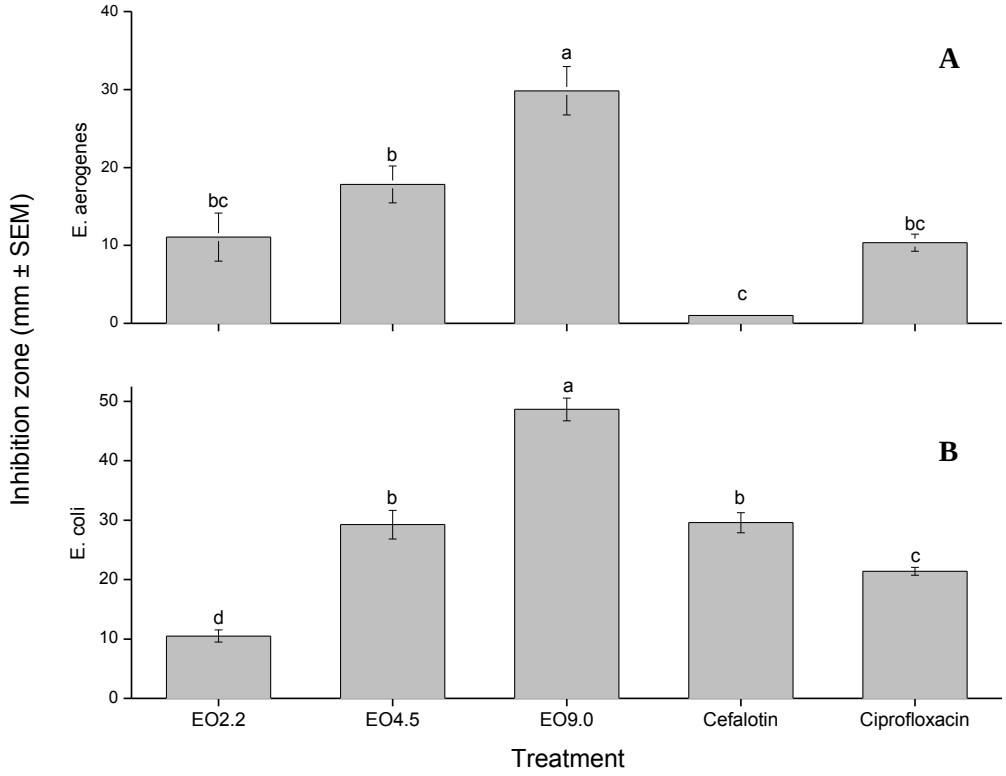
Figure 1
Antibacterial activity of essential oil from leaves of *L. philippiana*.
A: Against *S. epidermidis*; B: Against *S. aureus*. Different letters indicate significant differences according to Tukey HSD test (P ≤ 0.05). EO 2.2 = Essential oil 2.2 mg/disk; EO 4.5 = Essential oil 4.5 mg/disk; EO 9.0 = Essential oil 9.0 mg/disk.



Antibacterial activity

The essential oil exhibited better activity against Gram (-) than Gram (+) tested strains. There was a positive dose-dependent effect of the inhibition activity of *S. epidermidis*. The essential oil at 4.5 mg/disk was significantly more active than cefalotine (30 µg/disk) and vancomycin (30 µg/disk), and the activity was comparable to ciprofloxacin (5 µg/disk) at 2.2 mg/disk of essential oil (Figure 1A). Meanwhile, there were not significant differences among the different concentrations of essential oils and the antibiotics against the *S. aureus* (Figure 1B). The Figures 2A, and 2B show a remarkable effect in the zone of inhibition of *E. aerogenes* and *E. coli* to the increasing in the concentration of essential oil. It can be seen that the highest concentration of essential oil posses a stronger antibacterial potential than the evaluated antibiotics, against Gram (-) bacteria *E. coli* and *E. aerogenes*. Although some authors reported that Gram (+) bacteria are more sensitive to inhibition by plant essential oils than the Gram (-) bacteria (Nakatani, 1994; Smith-Palmer *et al.,* 1998), Deans and Ritchie (1987), indicate that the susceptibility of bacteria to plant volatile oils and the Gram reaction appears do not have relationship.

Figure 2
Antibacterial activity of essential oil from leaves of *L. philippiana*
A: Against *E. aerogenes*; B: Against *E. coli*. Different letters indicate significant differences according to Tukey HSD test (P ≤ 0.05). EO 2.2 = Essential oil 2.2 mg/disk; EO 4.5 = Essential oil 4.5 mg/disk; EO 9.0 = Essential oil 9.0 mg/disk.



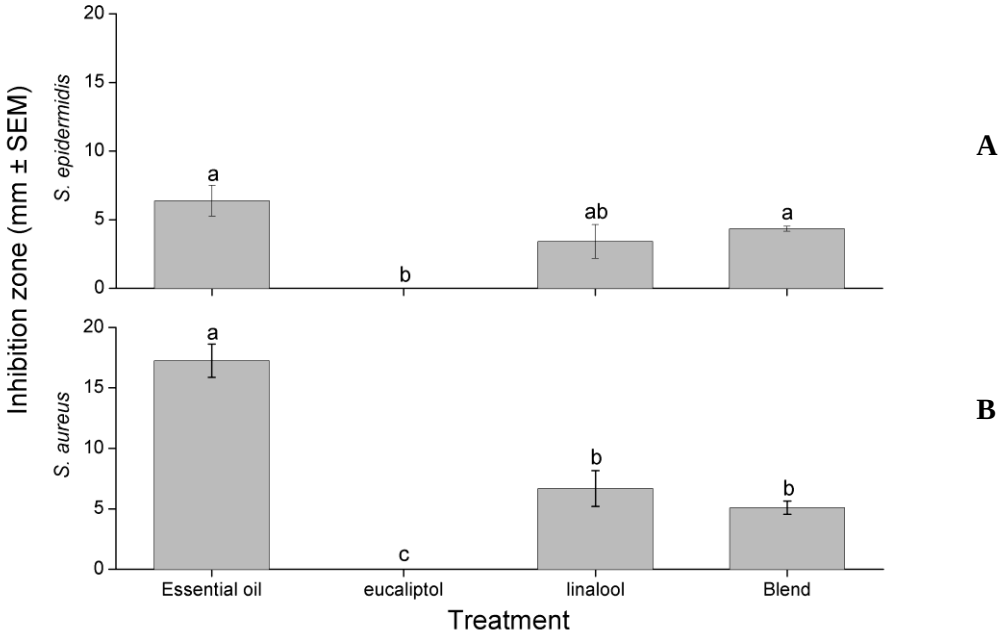
The minimal inhibitory concentrations of essential oil are ranged from 22 µg/mL against *S. aureus* to 48 µg/mL on *E. aerogenes*, whereas the minimal bactericidal concentrations were 32 µg/mL on *E. coli* and *S. aureus* to 51 µg/mL against *E. aerogenes* (Table 2), while the values of MICs and MBCs for the essential oil from *L. philippiana* studied here are much higher if compared with ciprofloxacin, Júnior *et al*., 2012 indicate that natural products derived from plant species showing MIC values equal to or less than 500

µg/mL are considered strong inhibitors. In this study, the composition of essential oil rich in the oxygenated monoterpenes linalool and eucalyptol; could explain its antibacterial activity. In effect, the evaluation of antibacterial activity of two major compounds present in the essential oil and its blend showed that linalool has the same inhibitory effect of essential oil against *E. aerogenes* whereas the blend (linalool + eucalyptol) has the same inhibitory effect of essential oil against *S. epidermidis* (Figure 3 and 4).

Table 2
Minimum inhibitory and bactericidal concentrations (MICs and MBCs) of *Laureliopsis philippiana* essential oil (µg/mL).

Strain	MIC	MBC
	Essential oil	Essential oil
E. aerogenes	48	51
E.coli	31	32
S. aureus	22	32
S. epidermidis	33	40

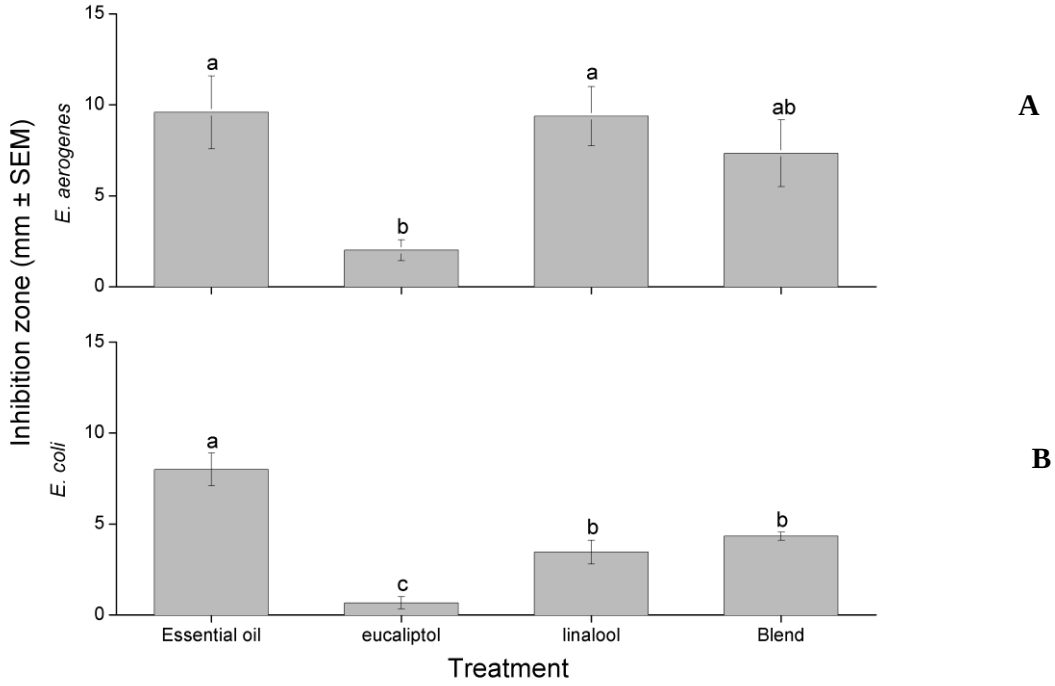
Figure 3
Comparison of antibacterial activity between essential oil from leaves of *L. philippiana* and principal components linalool and eucalyptol.
A: Against *S. epidermidis*; B: Against *S. aureus*. Different letters indicate significant differences according to Tukey HSD test (P ≤ 0.05).



Several authors reported a significant efficiency of essential oils on Gram-positive bacteria, attributing it, to the simple structure of the cell wall (Nikaido, 2003; Bagamboula *et al*., 2004), whereas all Gram (-) bacteria characteristically are surrounded by an outer membrane; where the most important function is to serve as a selective permeation barrier (Nikaido, 2003). The outer membrane provides to the bacterium with a hydrophilic surface, due to the presence of lipopolysaccharide. Small hydrophilic compounds,

such as linalool and eucalyptol, could be able to pass the outer membrane through abundant porin proteins that providing hydrophilic transmembrane channels, whereas the outer membrane serves as a penetration barrier toward macromolecules and to hydrophobic compounds (Helander *et al*., 1998). Moreover, it has been suggested that linalool has the potential to act as either protein denaturing agent or as a solvent dehydrating agent (Stefan *et al*., 2013).

Figure 4
Comparison of antibacterial activity between essential oil from leaves of *L. philippiana* and principal components linalool and eucalyptol.
A: Against *E. aerogenes*; B: Against *E. coli*. Different letters indicate significant differences according to Tukey HSD test (P ≤ 0.05).



Similar antibacterial activity of essential oils obtained of medicinal plants rich in eucalyptol and linalool has been described. Results obtained by Delaquis *et al*., 2002 for essential oils from dill, coriander, cilantro and eucalyptus against Gram (+) and Gram (-) bacteria, showed that linalool present in cilantro oil was responsible for the activity against gram (-) bacteria. In the other hand Cimanga *et al*., 2002, indicated that the antibacterial activity of essential oils from leaves of *Eucalyptus camaldulensis* in those with low percentage of eucalyptol (< 10%) were similar to those with high amount of eucalyptol (> 30%), suggesting that minors components such as nerol, eugenol and linalool could be responsible of the antibacterial activity exhibited. Moreover Panahi *et al*., 2011, evaluated the inhibitory effect of the essential oils from *E. polycarpa*, *E. largiflorence*, *E. malliodora* and *E. camaldulensis* on *S. aureus*. The major antimicrobial activity was showed by the essential oil from *E. largiflorence*, which contained the higher percentage of eucalyptol (70.32%), while that the minor anti *staphylococcus* activity was obtained with essential oil from *E. polycarpa* containing the lower percentage of eucalyptol (50.12%). In addition Randrianarivelo *et al*., 2009, studied the profile components and activity of the essential oils obtained from *Cinnamosma fragrans*, from two regions of Madagascar. Fifty seven components were identified, but the major components were linalool (72.5 ± 23.3%) in the Tsaramandroso cultivar, and eucalyptol (47.3 ± 10.2%) in Mariarano cultivar. From 10 microbial strains assayed, *Bacillus subtilis* and *S. aureus* were the most sensitive to both oils; however, minimum inhibitory concentration values were lower for essential oil than pure eucalyptol, suggesting the occurrence of synergism between minor compounds and major compounds present in the essential oils.

CONCLUSION

In conclusion, the composition and activity of the essential oil of *L. phillipiana* in the current study showed clear differences respect to those reported before. It exhibited a remarkable antibacterial activity, which was higher than those showed by the standards antibiotics against Gram (-) pathogens and similar to the standards antibiotics used to Gram (+), and was also possible to demonstrate that the antimicrobial activity of essential oil against *E. aerogenes* and *S. epidermidis* is due to its major constituent. Despite of these promising results further studies are required in order to establish geographic variations in the chemical composition with other habitats for *L. philippiana*, and determinate the antibacterial contribution of each constituent of the essential oil and establish the synergic and/or antagonistic effect of its components.

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