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Antibiofilm and Antifungal Activities of *Laurelia sempervirens* (Chilean laurel) Essential Oil

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Abstract

Background: *Laurelia sempervirens*, better known as Chilean laurel, is endemic to Chile. For many years, the leaves and branches of this plant have been popularly used as medicinal agents. However, its antifungal properties and antibiofilm activity against bacterial strains have not been studied.
Objectives: To determine antibiofilm and antimicrobial properties of Chilean laurel essential oil on human pathogenic strains.
Methods: The antifungal and antibacterial activities of Chilean laurel essential oil were evaluated through the agar diffusion method, and its minimum inhibitory concentration was determined using the standard microdilution method. Antibiofilm activity was examined based on the formation and disruption of bacterial biofilms and evidenced by the crystal violet technique.
Results: The results showed important antimicrobial activity against human pathogenic yeast strains, and the minimum inhibitory concentration of Chilean laurel essential oil was 64 μg mL^{-1} against *Candida albicans*. The essential oil also showed an important inhibitory effect against the formation of biofilms produced by *Staphylococcus aureus*, since it inhibited the formation of biofilms by over 50% at the concentration of 64 μg mL^{-1}. With increasing the essential oil's concentration to 128 μg mL^{-1}, its antibiofilm activity increased by 60%.
Conclusions: These results approve the domestic use of Chilean laurel essential oil as an antimicrobial agent and provide knowledge about the antibiofilm and antifungal properties of *L. sempervirens*.

Keywords: *Laurelia sempervirens*, Laurel chileno, Antibiofilm, Antifungal Activity, Essential Oil

1. Background

In recent years, there has been a dangerous spike in new antimicrobial resistance mechanisms worldwide, greatly threatening the ability to treat diseases of infectious origin. According to the World Health Organization (WHO), there are few replacement products in the research and development phase, and the world is moving towards a post-antibiotic era in which common infections could be fatal again (1). Thus, an important challenge for the scientific community is finding new antimicrobial compounds that can be used in cases of resistance (2).

Essential oils (EOs) are natural substances produced by aromatic plants and consist of mixtures of volatile compounds (3). The potent antibacterial activity of EOs of various origins has been demonstrated for several years. Furthermore, it has been suggested that bacteria sensitive to oils are inactivated by EOs without developing resistance (4, 5). Essential oils have been shown to have low toxicity in mammals and are rapidly degraded in the environment (6, 7).

Laurelia sempervirens, popularly known as Chilean laurel, belongs to the Atherospermataceae family and is endemic to Chile. This plant grows in the southern part of the country between 35° and 42° south latitude. In Chile, this plant is used in traditional medicine as an anti-inflammatory agent to as an expectorant and treatment for venereal diseases (8), and leaves of *L. sempervirens* are used by Amerindians for treating headache and as a diuretic (9). Although the chemical composition of Chilean laurel EO has been studied, only a few studies have examined its antifungal activity mainly against filamentous fungi, such as *Penicillium* spp. and *Fusarium oxysporum* (10, 11). To our knowledge, there is a scarcity of information on the effect of Chilean laurel EO on the generation and/or

degradation of bacterial biofilms and the activity of this oil against yeast species that are pathogenic for humans.

2. Objectives

To determine antibiofilm and antimicrobial effects of Chilean laurel essential oil on human pathogenic strains.

3. Methods

3.1. Plant Material and Extraction of Chilean laurel EO

Fresh plant of *L. sempervirens* was collected in Ñuble Region (Chile) (36°51'S, 71°40'W). The plant was analysed by Dra. Olga Contreras Perez of Biology Institute of Talca University. Then, the plant was washed with distilled water and submitted to distillation (3 h; using Clevenger-type apparatus). The EO was obtained (0.73% $\pm$ 0.2), dried over anhydrous sodium sulphate, filtered and stored at 4 °C.

3.2. Chemical Composition of Chilean laurel EO

Chemical analysis of *L. sempervirens* EO was performed by gas chromatography coupled to mass spectrometry detector (GC-MS), using a GC Trace 1300 Thermo Fisher Scientific S.p.A equipment made in Italy. Separation was achieved using a column Rtx-5MS w/integra-guard (Cross bond 5% diphenyl - 95% dimethyl polysiloxane), 30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm d.f. The injection conditions were as follows: temperature: 250 °C, septum purge: 3 mL min^{-1} and split valve: 33.3. The column temperature gradient was 40 °C - 280 °C with the ramp rate of 10 °C min^{-1}. The conditions of MS were: temperature of the transfer line: 280 °C, electronic impact ionization energy: 70 eV, mass range: 40 - 500 atomic mass units, scan rate: 20 scan s^{-1} and ion source temperature: 260 °C. The identification of compounds was achieved by comparing mass spectra with the National Institute of Standards and Technology Library (NIST NBS54K). And alkane standard solution C8-C20 was used as an analytical standard for measurement of retention indices (RIs) those that were calculated according to the RI Van den Dool and Kratz equation (12).

3.3. Antifungal and Antibacterial Activity of Chilean laurel EO and Determination of Minimum Inhibitory Concentration (MIC)

3.3.1. Yeast and Bacterial Strains

Candida albicans ATCC 14053, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, *C. utilis* ATCC 9950, *C. krusei* ATCC 6258, *C. guillermondii* ATCC 7350, *C. lusitaneae* ATCC 34449, *Geotrichum* sp. ATCC 96884, *Bacillus cereus* ATCC 14579, *Staphylococcus saprophyticus* ATCC 15305, *Staphylococcus epidermidis* ATCC 14990 and, *Staphylococcus aureus* ATCC 25923), *Acinetobacter baumannii* ATCC 19606, *Proteus vulgaris* ATCC 8427, *Enterobacter aerogenes* ATCC 13048, *Salmonella typhi* ATCC 35664, *Klebsiella oxytoca* ATCC 700324, *Shigella sonnei* ATCC 25931, *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047, *Providencia alcalifaciens* ATCC 51902, *Proteus mirabilis* ATCC 35659, *Enterococcus faecalis* ATCC 29212, *Citrobacter freundii* ATCC 8090, *Shigella flexneri* ATCC 12022 and *Pseudomonas aeruginosa* ATCC 27853.

3.3.2. Antimicrobial Assay by the Agar Diffusion Method

Evaluation of antimicrobial activity of *L. sempervirens* EO was carried by the agar diffusion method. Bacterial suspension ($\approx 10^6$ colony forming unit [CFU] per mL) was streaked over Müller-Hinton (MH) agar (Merck KGaA, Darmstadt, Germany) plates, and then discs (Whatman® Antibiotic Assay Discs, 6 mm) impregnated with 5 μL of EO were placed on the inoculated plates and subsequently incubated at 37 °C for 24 h. Similarly, yeast suspension ($\approx 10^6$ CFU mL^{-1}) was streaked over potato dextrose agar (PDA) (Merck KGaA, Darmstadt, Germany), and discs impregnated with EO were placed on the inoculated plates and were incubated for 48 h at 27 °C. For antibacterial assay, amoxicillin/sulbactam 10/10 μg (Valtek, code: 400 - 117) and discs with sterile distilled water were used as positive and negative controls respectively, and for antifungal assay, fluconazole (30 μg) (Neo-Sensitabs, Rosco diagnostica, Denmark) was used as a positive control, whereas discs without sample were used as a negative control. All the analyses were carried out in triplicate, and after the corresponding incubation time, each plate was analysed for the presence or absence of inhibition zones.

3.3.3. Minimum Inhibitory Concentration

The MIC of Chilean laurel EO was determined using the standard microdilution method (CLSI M100-S25) (13) and only against microorganisms that were susceptible in screening antimicrobial assay. Minimum inhibitory concentration was determined in Mueller Hinton (MH) broth using EO concentrations ranging from 4 μg mL^{-1} to 512 μg mL^{-1}. The cell concentration was standardized to $\approx 1 \times 10^8$ CFU mL^{-1} of bacteria and yeasts using the McFarland standard (OD of 0.1 at 625 nm). Mueller Hinton broth medium with microorganism was tested as a positive control and MH broth without EO and without microorganism was tested as a negative control. Finally, the plates were incubated for 24 h at 37 °C for bacteria and for 24 h at 27 °C for

yeasts. The MIC was determined based on the visual turbidity of the tubes and was examined in triplicate for each bacterium.

3.4. Determination of Antibiofilm Activity of Chilean laurel EO

To determine the inhibitory effect of Chilean laurel EO on the formation of bacterial biofilms, two methods already reported in the literature were used with a few modifications (3, 14, 15). Only two susceptible biofilm-forming bacteria were analysed.

3.4.1. Inhibition of Biofilm Formation

S. aureus and *E. coli* were grown in MH broth at 37 °C overnight. The cultures were centrifuged and rinsed with phosphate-buffered saline (PBS, pH = 7.4), and the bacteria were resuspended in MH broth ($\approx 1 \times 10^6$ CFU mL^{-1}, determined by optical density, OD). Then, 140 μL of the bacterial suspension was placed in wells of polystyrene plates, and EO was added to obtain a final concentration equivalent to 1 MIC and 2 MIC. Sterile water was used as the negative control; sterile broth was used to confirm the sterility of the medium. After incubation for 24 h at 37 °C, the broths were removed, the wells were washed with saline solution (0.89% NaCl) and the biofilm was quantified by the crystal violet staining method (3). Briefly, methanol (200 μL) was added to the wells, and after 15 min it was removed and the wells dried at room temperature. Then, an equal amount of crystal violet dye (0.5%) was added for 15 min, and after this time the stain was removed and the wells were washed and dried. Finally, 200 μL of 95% ethanol was added and absorbance (λ = 450 nm) was read using an AnthosBiochrom 2010 microplate reader.

The percentage of inhibition was obtained using the following equation:

% inhibition = 100 - [(OD450 nm experimental sample with EO/OD450 nm control sample without EO) × 100]

3.4.2. Effect of EO on Disruption of Biofilms

For this assay, 100 μL of bacterial suspension ($\approx 10^6$ CFU mL^{-1}) was contacted with 100 μL MH broth enriched with 1% glucose, and then the plate was incubated at 37 °C for 48 h. Afterwards, the wells were washed with sterile saline solution to remove non-adherent cells, and *L. sempervirens* EO at MIC value was added and incubated at room temperature for 3 h. The wells were washed with saline solution, and the biofilm was quantified by the crystal violet staining method, as described previously (see point 3.4.1).

3.5. Statistical Analysis

Analysis of variance test was used to determine significant differences in the assays. A P-value of less than 0.05 was considered statistically significant.

4. Results

4.1. Chemical Composition

The chemical composition of Chilean laurel EO (see Appendix 1 in Supplementary File) was analysed by GC-MS, and 20 different compounds were identified that represented 98.71% of the total mixture. Safrole and Methyleugenol were the majority compounds with the relative abundance of 47.09% and 44.58%, respectively (Figure 1).

4.2. Antimicrobial Activity

Antagonistic effect of Chilean laurel EO demonstrated extensive activity against the human pathogens studied, and antifungal activity is showed in Table 1 and Figure 2. Antibacterial activity is presented in support information (Appendix 2 in Supplementary File).

Table 1. Antifungal Activity Expressed as Diameter of Inhibition of Chilean laurel EO Against Yeast Strains

Yeast	Inhibition Zone, mm ± RSD[a]
***Candida albicans* ATCC 14053**	10.4 ± 0.7
***Candida parapsilosis* ATCC 22019**	9.8 ± 0.3
***Candida tropicalis* ATCC 750**	12.1 ± 0.6
***Candida utilis* ATCC 9950**	7.9 ± 0.7
***Candida guillermondii* ATCC 7350**	10.0 ± 0.5
***Candida lusitaniae* ATCC 34449**	11.2 ± 0.4
***Candida krusei* ATCC 6258**	9.3 ± 0.8
***Geotrichum* sp. ATCC 96884**	12.8 ± 0.3

Abbreviation: NA, not active.
[a]Values are expressed as means ± SD (n = 3) and include disc diameter (6 mm).

4.3. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration was determined for the microorganisms that were found most susceptible in the screening test, and the results are shown in Table 2.

4.4. Antibiofilm Activity

The antibiofilm activity was tested against *S. aureus* and *E. coli*, and the results are presented in Table 3.

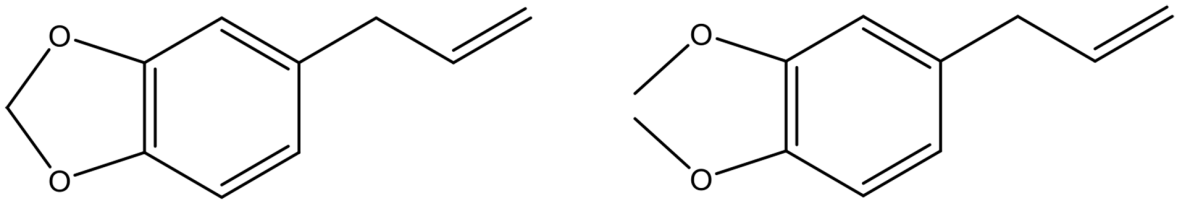

Figure 1. Chemical structure of safrole and methyl eugenol

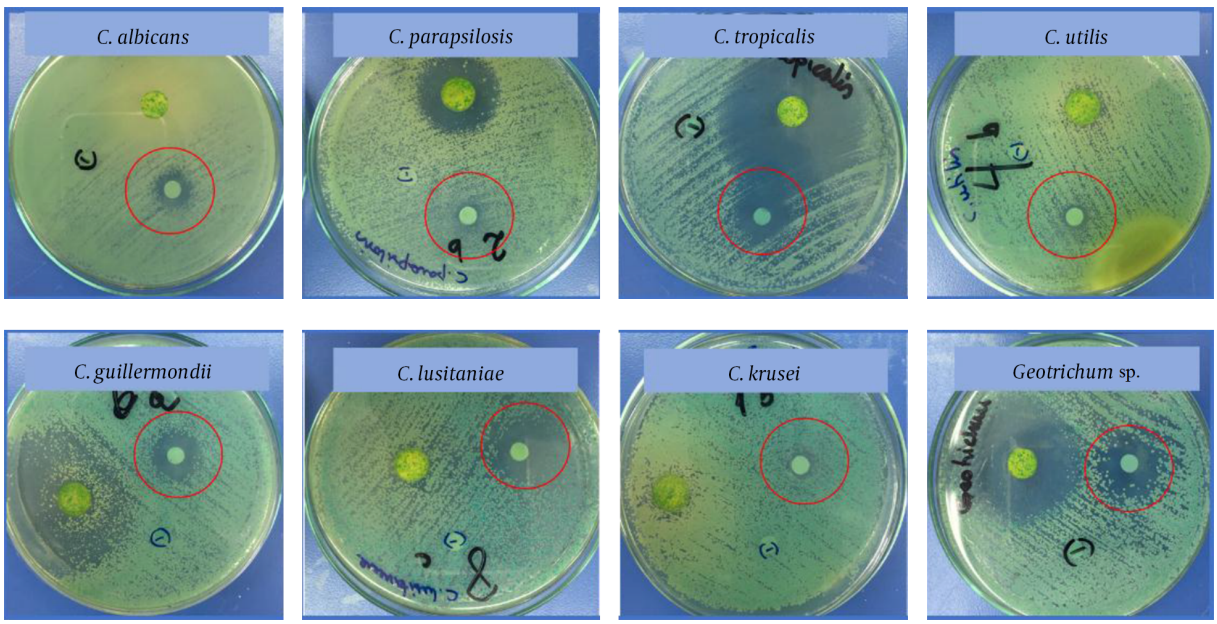

Figure 2. Antifungal activity (in red circle) of Chilean laurel EO on yeast strains

5. Discussion

It has been known that essential oils can contain between 20 to 60 components in different concentrations and two or three that are found in high concentrations (20% - 70%) (7). In this study, the most abundant compounds of Chilean laurel EO were the aromatic compounds safrole and methyl eugenol. Tricyclic sesquiterpenoid, spathulenol and other components identified were present in amounts less than 1.1%. These findings are consistent with other reports published on the composition of *L. sempervirens* EO (8, 16).

Antibacterial activity of Chilean laurel EO against *S. aureus*, *Bacillus subtillis*, *A. baumanni*, *P. aeruginosa*, *Salmonella* spp, *S. marcescens* and *E. coli* has been demonstrated (9, 10). However, this study revealed its activity against important strains of human pathogens such as, *P. vulgaris*, *E. aerogenes*, *S. typhi*, *K. oxytoca*, *S. sonnei*, *E. cloacae*, *P. alcalifaciens*, *S. flexneri*, *P. mirabilis*, *B. cereus*, *S. epidermidis*, *S. saprophyticus*, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. utilis*, *C. guillermondii* and *C. krusei*. The antimicrobial activity of Chilean laurel EO could be largely due to the presence of safrole. It has been documented that this molecule inhibits the production of intracellular enzymes, such as amylases and proteases, causing wall deterioration and a high degree of cell lysis (11). Also, the presence of hydrophobic compounds, such as eugenol, α-terpineol and γ-terpinene, has been shown to cause bacterial cell membrane disruption (17). Some authors have proposed that EO components

Table 2. Minimum Inhibitory Concentration of Chilean Laurel EO Against Selected Bacterial Strains and *C. albicans*

Microorganisms	MIC, μgmL^{-1}
Acinetobacter baumannii **ATCC 19606**	128
Escherichia coli **ATCC 25922**	128
Shigella flexneri **ATCC 12022**	64
Bacillus cereus **ATCC 14579**	32
Staphylococcus aureus **ATCC 25923**	64
Staphylococcus epidermidis **ATCC 14990**	64
Staphylococcus saprophyticus **ATCC 15305**	64
Candida albicans **ATCC 14053**[a]	64

[a] MIC fluconazole to *C. albicans* $\geq$ 64 μg mL^{-1}.

with the lowest proportion play a critical role in antimicrobial activity, possibly due to a synergistic effect between them (10). Due to the variability of compounds present, some possible mechanisms have been proposed that are associated with alteration of the outer membrane of Gram-negative bacteria with the release of lipopolysaccharides, interaction with membrane proteins (ATPases and others), destabilization of the proton motive force with ion leakage, coagulation of cell content and inhibition of enzyme synthesis (18).

Chilean laurel EO also had activity against *Candida albicans*, which is a human pathogen responsible for 90% of vulvovaginal infections caused by fungi. This information is relevant, since it has been reported that the management of fungal infections is currently complicated mainly due to the limited number of antifungal drugs, toxicity, high resistance to antifungal drugs and high costs (19). The mechanisms of antifungal activity of essential oils that have been described so far are quite similar to those described for their antibacterial activity, and they have to do with irreversible damage to the cell membrane (18).

Finally, Chilean laurel EO showed antagonistic activity against the formation of biofilms. Bacterial biofilms are communities of bacteria that adhere to surfaces through the extracellular production of polymeric substances, mainly polysaccharides and proteins (20). In this study, the EO activity decreased when the biofilms were already formed. It must be considered that biofilm formation implies two phases, an initial reversible binding phase followed by an irreversible binding phase (21). Therefore, the decrease in the activity of Chilean laurel EO for the disruption of biofilms may be due to the fact that the biofilms were already in an irreversible binding phase, and an even higher concentration should be considered to exert a considerable effect.

5.1. Conclusions

In sum, although moderate activity has been seen in terms of *S. aureus* biofilm disruption and low *E. coli* antibiofilm activity, we must consider, as mentioned above, that the test carried out in this work (crystal violet staining method) serves only as an indicator of bound biomass in a biofilm and does not reveal the metabolic status of cells. Therefore, we must continue with a metabolic analysis through which we can identify if the cells remain viable or not at the concentration the EO was used.

Taken together, it can be stated that Chilean laurel EO is a promising alternative candidate to the conventional antibacterial and antifungal agents, and it can be used as a possible antibiofilm agent. Thus, it can have various applications in the food and cosmetics industries among others. In view of these promising properties, in vivo studies and clinical trials are required to justify the use of this EO in humans.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: Study concept and design: VCS, OL, and CP. Analysis and interpretation of data: AB, ZLC, CM, and PA. Drafting of the manuscript: VCS, OL, and CP. Critical revision of the manuscript for important intellectual content: VCS. Statistical analysis: AB.

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Table 3. Antibiofilm Activity of *L. sempervirens* EO

Bacteria	Inhibition of Biofilm Formation, % ± RSD		Effect on Disruption of Biofilms, % ± RSD	
	1 MIC	2 MIC	1 MIC	2 MIC
S. aureus	56 ± 5	67 ± 6	29 ± 2	32 ± 4
E. coli	35 ± 6	45 ± 3	22 ± 2	25 ± 2

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