Antimicrobial activity of ethyl acetate extract and essential oil from bark of *Laurelia sempervirens* against multiresistant bacteria

[Actividad antimicrobiana del extracto acetato de etilo y aceite esencial de corteza de *Laurelia sempervirens* contra bacterias multirresistentes]

Iván MONTENEGRO1, Alejandro MADRID VILLEGAS1, Luis ZAROR2, Rolando MARTÍNEZ3*, Enrique WERNER1, Hector CARRASCO-ALTAMIRANO1, Mauricio CUELLAR FRITIS5 & Hernán PALMA-FLEMMING6.

1Departamento de Química, Universidad Técnica Federico Santa María, Av. España 1680, Valparaíso, Chile
2Departamento de Microbiología Clínica, Facultad de Medicina, Universidad Austral de Chile.
3Departamento de Química, Facultad de Ecología y Recursos Naturales, Universidad Andrés Bello, Viña del Mar, Chile
4Departamento De Ciencias Básicas, Universidad del Bio-Bio, Chillán, Chile
5Facultad de Farmacia, Universidad de Valparaíso, Valparaíso, Chile.
6Instituto de Química, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile

Contactos | Contacts: Iván MONTENEGRO - E-mail address: ivan.montenegro@postgrado.usm.cl

Abstract
Context: *Laurelia sempervirens* (R. et P.) Tul., is an evergreen tree that grows in southern Chile, its leaves and bark are used in folk medicine as an infusion. **Objective:** The antimicrobial activities of the essential oil and ethyl acetate extract obtained from the bark of *Laurelia sempervirens* were investigated. **Materials and methods:** Ethyl acetate extract and essential oil were analyzed by GC- mass and the antimicrobial activity was investigated against gram positive and gram negative bacteria. **Results:** The extract and essential oil showed a strong antimicrobial activity against bacteria such as *Acinetobacter baumannii*, a relevant world nosocomial pathogen **Discussion and conclusion:** These findings demonstrate that the ethyl acetate extract and essential oil of *L. sempervirens* bark have excellent antimicrobial activities and thus have great potential as a source for natural health products.

Keywords: *Laurelia sempervirens*, inhibitory activity, bacteria.

Resumen
Contexto: *Laurelia sempervirens* (R. et P.) Tul., es un árbol de hoja perenne que crece en el sur de Chile, sus hojas y corteza se utilizan en medicina popular como infusión. **Objetivo:** Investigar la actividad antimicrobiana del aceite esencial y el extracto de acetato de etilo obtenido de la corteza de *Laurelia sempervirens*. **Materiales y métodos:** El extracto de acetato de etilo y el aceite esencial se analizaron por GC-masa y la actividad antimicrobiana se analizó contra bacterias gram positivas y gram negativas. **Resultados:** El extracto y aceite esencial evidenciaron una fuerte actividad antimicrobiana frente a la bacteria, *Acinetobacter baumannii* patógeno que causa infecciones nosocomiales de relevancia mundial. **Discusión y conclusiones:** Estos hallazgos demuestran que el extracto de acetato de etilo y aceite esencial de corteza de *L. sempervirens* tienen excelentes actividades antimicrobianas y por lo tanto tienen un gran potencial como fuente de productos naturales para la salud.

Palabras Clave: *Laurelia sempervirens*, actividad inhibitoria, bacterias.
INTRODUCTION
Considering the knowledge of Chilean indigenous cultures on the use of medicinal plants, we conducted the research on those that present obvious biological activities, being Laurelia sempervirens one of the most used. Laurelia sempervirens, also called Chilean Laurel, is a species of evergreen tree endemic to Chile. Leaves of Laurelia sempervirens were used by the Mapuche amerindians for treating headache and as a diuretic. Intravenous administration of a hydroalcoholic L. sempervirens extract to rats, elicited a hypotensive response. Bioassay-guided isolation of the active Laurel metabolites led to the alkaloid laurotetanine as the main hypotensive principle of L. sempervirens leaves (Schmeda-Hirschmann et al., 1994). Bittner et al., 2009 demonstrate the fungistatic activity of essential oil of Laurelia sempervirens leaves. Cassels and Urzá in 1985 reported the isolation alkaloids (obaberine, thalrugosine, and oxyacanthine) of the stem bark. Liriödenine and oxonantenina present in bark extracts are powerful broad spectrum antimicrobial agents (Hufford et al., 1980). In recent studies by Mølgaard et al., 2011 demonstrate the antimicrobial activities of aqueous and ethanolic extracts of leaves, these results support the previous comprehensive reports regarding the antimicrobial and/or cytotoxic effects of Laurelia sempervirens.

Based on these data, we carried out the study of compounds through joined gas chromatography present in the phase of ethyl acetate and in the essential oil of L. sempervirens bark. In addition, we perform tests on inhibitory activity in bacterial development considered a problem for its frequency and ability to present resistance to commonly used antibiotics like Acinetobacter baumannii, Pseudomonas aeruginosa and Staphylococcus aureus (Storti et al., 2005; Akers et al., 2010; Perazzi et al., 2010).

EXPERIMENTAL SECTION
Plant Material
The samples of L. sempervirens were collected in September 2003, from trees growing in plantations located at the Arboretum of the Universidad Austral de Chile, Valdivia, Chile. Number of herbal 17472.

Microorganisms
Staphylococcus aureus ATCC29583, Pseudomonas aeruginosa ATCC 27853, Bacillus subtilis ATCC6633, Salmonella sp108,109; Serratia marcescens 1332-2; Pseudomonas aeruginosa1020-1,1020-2,1048-2; Acinetobacter baumannii 999, Staphylococcus aureus 2312 (not ATCC bacteria was isolated from clinical samples at Regional Hospital, Valdivia, Chile) cultures were obtained from the culture collections of Clinical Microbiology Department of Universidad Austral de Chile.

Essential oil extraction
Fresh bark of Laurelia sempervirens (4 kg) was steam-distilled for 5 h using a Clevenger type apparatus. Essential oil yielded 1.0%. The essential oil obtained was stored at 4º C and used in biological assays without further purification.

Preparation of ethyl acetate extract
To extract bioactive compounds, 2.7 kg of Laurelia sempervirens bark with 20 L of ethyl acetate were processed twice. The resulting solution was brought to dryness in a Büchi R-114 rotary evaporator with Büchi Waterbath thermoregulator bath B-480 at a temperature of 60º C. The sample was treated with hot water to remove chlorophyll and then it was dried. A fraction was used for chromatographic analysis. 28.6 g of this extract was suspended in 100 mL of ethyl acetate providing in this way a solution of 0.286 g/mL, which was used for biological tests.

Antimicrobial Assays
To evaluate the antibacterial activity, the hole-plate agar diffusion method was implemented (Van den Berghe and Vlietinck, 1991) 4-5 colonies were taken from the microorganisms studied. The colonies were re-suspended in physiological saline solution, in duplicate, to 0.5 Mac-Farland concentration (10^8 CFU/mL). The bacterial suspension was uniformly spread by using sterile cotton swab on a sterile Petri dish MH agar. After letting it dry for 10-15 minutes, 6 mm diameter holes were cut in the agar at 24 mm distance from each other. 20 μL of serial dilutions obtained from the initial extract were placed in each well. These dilutions had concentrations ranging from 7200 μg/mL to 0.72 μg/mL. Plates were incubated overnight at 35º C, under aerobic conditions. After incubation, confluent bacterial growth was observed. The inhibition zone around the hole was measured with a caliper. Minimum inhibition concentration (MIC) was defined as the lowest concentration that inhibited visible growth.
Essential oil was assayed to each microorganism in the same way that ethyl acetate extract. 20 µL of serial dilutions in n-hexane 1:10; 1:100; 1:1000; 1:10000 were assayed. Negative controls were performed with pure solvent. All the experiments were repeated five times.

Inhibition assays were performed by adding antibiotic clinical samples as described in NCCLS 2003 and CLSI, 2009 in order to compare with the results obtained after addition of ethyl acetate extract and essential oil from bark. 20 µL of either 70 µg/mL gentamicin, amikacin and ciprofloxacine clinical samples were added.

**Antibiotics assay**

To determine the sensitivity of microorganism to antibiotics (MICs) of amikacin (AKN), gentamicin (GEN) and ciprofloxacine were obtained (CIPRO) in vitro through the inhibition zone determination method, as stated by microdilution NCCLS guidelines (National Committee for Clinical Laboratory Standards) and CLSI (Clinical and Laboratory Standards Institute) Salmonella sp. 108,109; Serratia marcescens 1332-2; Pseudomonas aeruginosa 1020-1,1020-2,1048-2; Acinetobacter baumannii 999, Staphylococcus aureus 2312. Isolates from clinical samples and the ATCC species Staphylococcus aureus ATCC29583, Pseudomonas aeruginosa ATCC 27853, Bacillus subtilis ATCC6633. It has been reported that these bacteria are sensitive to these antibiotics (Fernández-Cuenca et al., 2004 and Zambrano et al., 2004; Simberkoff et al., 1986).

**Chromatographic analysis**

The essential oil and ethyl acetate extract were diluted with n-hexane, 1 µL was sampled for the gas chromatography and analyzed in a chromatograph HP 6890 equipped with a programmable temperature that vaporizes inlet (PTV). In addition, it was coupled to an HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA, USA) in scan mode in order to determine the composition of extracts fractions. The injector temperature was maintained at 280º C in a pulsed splitless mode. A GC program temperature ramp was set at 70º C for 3 min. and then at a rate of 10 ºC/min. up to 300º C was used to afford the best separation by using a capillary HP-5 MS column. The transfer line was maintained at 300º C. Mass spectra was obtained at 70 eV and compared through direct matching by using a NIST Mass Spectral Search Program and a NIST/EPA/NIH Mass Spectral Library (NIST, 1998).

**Data analysis**

Results were expressed as mean value ± standard error of growth inhibition halo diameters were obtained with ethyl acetate extract and essential oil of Laurelia sempervirens bark. To determine significant differences among inhibitory activity of Antibiotic and essential oil or antibiotic and ethyl acetate extract, a one way ANOVA was carried out using GraphPad Prism V5 software. P values less than 0.05 (p < 0.05) were considered as significant.

**RESULTS AND DISCUSSION**

**Composition analysis of the ethyl acetate extract and essential oil by gas chromatography coupled to a mass selective detector**

Tables I and II show the data obtained from gas chromatographic analysis of the ethyl acetate extract and essential oil of Laurelia sempervirens bark. According to Cespedes et al., (2006) and Zapata (2010), the high antimicrobial activity of the essential oil and ethyl acetate extract from bark of L. sempervirens are not that surprising. This is because safrole (63.03 and 65.03% respectively) was one of the main metabolites in these samples. Safrole is also the main constituent (74.29%) present in the essential oil of Piper auritum. It has shown high Antimicrobial activity when was tested against Xanthomonas albilineans and Acidovorax avenae subsp. avenae (Sánchez et al., 2009) The other component of the ethyl acetate extract is (E)-5-prop-1-etyl benzo [d] [1,3] dioxolan, better known as isosafrole (12.94%), a carcinogenic aromatic compound (Tulp and Bohlin, 2004; Delaforge et al., 1982). The isosafrole has two types of isomers and it is the precursor to the MDMA drug of abuse (known as ecstasy) and Spathulenol (6.60%) this sesquiterpene presents activity against Escherichia coli, Salmonella typhimurium and Staphylococcus aureus (de Souza et al., 2011). In essential oils we found 3-Carene and β-Caryophyllene, described in essential oils that present antimicrobial activity (Pichette et al., 2006; Oztürk et al., 2009). α-Gurjunene was identified in the essential oil of tubers of Cyperus rotundus Linn. (Bisht et al., 2011), and showed antifungal activity against the pathogenic Trichophyton mentagrophytes (Flach et al., 2002). Finally, we note the presence of 3-Allyl-6-
methoxyphenol, compound that has Antioxidant activity (Phutdhawong et al., 2007).

### Table I
Volatile compounds identified in ethyl acetate extract from bark of *Laurelia sempervirens*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RI</th>
<th>Area (%)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isosafrole</td>
<td>1177.5</td>
<td>12.94</td>
<td>RI, MS, Co-I</td>
</tr>
<tr>
<td>Safrole</td>
<td>1292.6</td>
<td>63.03</td>
<td>RI, MS, Co-I</td>
</tr>
<tr>
<td>Spathulenol</td>
<td>1457.2</td>
<td>6.60</td>
<td>RI, MS</td>
</tr>
</tbody>
</table>

### Table II
Major compounds identified in essential oil from bark of *Laurelia sempervirens*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RI</th>
<th>Area (%)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>941.11</td>
<td>1.48</td>
<td>RI, MS</td>
</tr>
<tr>
<td>Camphene</td>
<td>957.37</td>
<td>0.11</td>
<td>RI, MS</td>
</tr>
<tr>
<td>α-Phellandrene</td>
<td>1009.80</td>
<td>0.33</td>
<td>RI, MS</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>1031.75</td>
<td>0.12</td>
<td>RI, MS</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>1037.66</td>
<td>1.08</td>
<td>RI, MS</td>
</tr>
<tr>
<td>3-Carene</td>
<td>1070.4</td>
<td>0.42</td>
<td>RI, MS</td>
</tr>
<tr>
<td>Isosafrole</td>
<td>1177.5</td>
<td>11.90</td>
<td>RI, MS, Co-I</td>
</tr>
<tr>
<td>Safrole</td>
<td>1292.6</td>
<td>65.03</td>
<td>RI, MS, Co-I</td>
</tr>
<tr>
<td>Piperonal</td>
<td>1358.2</td>
<td>0.37</td>
<td>RI, MS</td>
</tr>
<tr>
<td>3- Allyl-6-methoxyphenol</td>
<td>1359.6</td>
<td>1.13</td>
<td>RI, MS</td>
</tr>
<tr>
<td>β-Caryophyllene</td>
<td>1384.9</td>
<td>0.74</td>
<td>RI, MS</td>
</tr>
<tr>
<td>α-Gurjunene</td>
<td>1396.6</td>
<td>0.24</td>
<td>RI, MS</td>
</tr>
<tr>
<td>α-Humelene</td>
<td>1440.9</td>
<td>0.35</td>
<td>RI, MS</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>1444.8</td>
<td>2.33</td>
<td>RI, MS</td>
</tr>
<tr>
<td>Spathulenol</td>
<td>1457.2</td>
<td>11.16</td>
<td>RI, MS</td>
</tr>
<tr>
<td>α-Cadinol</td>
<td>1615.0</td>
<td>1.10</td>
<td>RI, MS</td>
</tr>
</tbody>
</table>


**Bacterial Development Inhibition**

The ethyl acetate extract and essential oil from bark of *Laurelia sempervirens* show strong and efficient antimicrobial activity against different types of bacteria. As shown in table III and IV, the extract and essential oil were tested against *Acinetobacter baumanii, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella spp, Bacillus subtilis ATCC 9799 and Serratia marcesens*, High efficiency against these resistant and persistent microorganisms causing nosocomial infections was observed. The hole plate method was chosen because it is a suitable diffusion technique for testing suspensions of plants extracts. In this method, the presence of particulate matter in the sample is much less likely to interfere with the diffusion of the antimicrobial substance into the agar than in the filter paper disc (Valgas et al., 2007).

**Minimum Inhibitory concentration (MIC)**

Previously defined as the minimum inhibitory concentration as the lowest concentration of pure compounds or greater dilution of essential oil that inhibited visible bacterial growth using as control the pure solvent. In all cases we considered as bacterial growth inhibition when the inhibition halo was 1 mm in diameter at least. The results are shown in tables III and IV.
Gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, and gram negatives: *Salmonella* spp, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. An extensive literature describes the structural and physiological differences between gram (-) and gram (+) bacteria. However, *Staphylococcus aureus*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, represents a challenge for healthcare as a cause of hospital acquired infections, difficult to treat because they represent opportunistic pathogens multidrug resistant. In the hospital, they may be isolated from equipment and accessories, air conditioner ducts as well as the skin of workers and rarely cause infection in healthy persons (Harbarth *et al.*, 2008; McGowan, 2006; Uduman *et al.*, 2002; Hejazi and Falkiner, 1997).

Multi-resistance to methicillin and other antibiotics by *Staphylococcus aureus* as reported, representing a high risk of infection in surgical patients (Harbarth *et al.*, 2008).

### Table III

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration (µg/ml)</th>
<th>Average of inhibition halo (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> sp</td>
<td>72</td>
<td>12</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 1020</td>
<td>72</td>
<td>23</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>7200</td>
<td>14</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0.72</td>
<td>25</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> 2312</td>
<td>72</td>
<td>17</td>
</tr>
</tbody>
</table>

*Serratia marcescens*, classified as a member of the enterobacteriaceae, has been recognized because of resistance to a variety of antibiotics, including ampicillin, cephalosporins, quinolones and aminoglycosides (Hejazi and Falkiner, 1997) and is involved in various types of infection such as respiratory tract infections, urinary tract infections, neonatal sepsis and meningitis (Uduman *et al.*, 2002). The high resistance to fluoroquinolones (ciprofloxacin) that nosocomial *Pseudomonas* shows is a worldwide problem in many hospitals and health services as reported by Van Eldere 2003. The high resistance to fluoroquinolones (ciprofloxacin) that nosocomial *Pseudomonas* shows is a worldwide problem in many hospitals and health services as reported by Van Eldere 2003.

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Acinetobacter is a multidrug resistant bacteria (Akers *et al.*, 2010) able to develop microcolonies and microbial biofilms on materials for medical use such as catheters being the cause of bloodstream infections (Storti *et al.*, 2005).

In the last two decades *Acinetobacter baumannii* has emerged as a major relevant world nosocomial pathogen can be a causal agent of diseases like pneumonia, bacteremia, meningitis, soft tissue and urinary tract infections, associating to high mortality. (Diomedi, 2005).
Table IV
Minimum Inhibitory concentration of essential oil from bark of *Laurelia sempervirens*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dilution in n-hexane</th>
<th>Average of inhibition halo (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella sp</em></td>
<td>1/10000</td>
<td>12</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>1/10000</td>
<td>25</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 1020</td>
<td>1/10000</td>
<td>23</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>1/10000</td>
<td>16</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>1/10000</td>
<td>17</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> 2312</td>
<td>1/10000</td>
<td>14</td>
</tr>
</tbody>
</table>

The minimum inhibitory concentration of ethyl acetate extract among bacteria could be due to the strains origin. Those with greater susceptibility are those with greater possibility of presenting multi-resistance activity due to its nosocomial origin.

We can not compare the results of bacterial growth inhibition among ethyl acetate extract and essential oil, it is not possible because we could not determine the minimum inhibitory concentration for essential oil since in all cases inhibition was achieved with the maximum dilution tested 1/10000 (Table IV).

However, we compare these results with the inhibitory capacity of clinically used antibiotics tested (Table V), calculating the ratios among the inhibition halos of ethyl acetate extract, essential oil and antibiotics (Graphic 1).

From these results, highlight the activity of ethyl acetate extract (ratio inhibition halo/antibiotic inhibition halo = 4.7) and essential oil (ratio inhibition halo/antibiotic inhibition halo = 4.2) from bark of *Laurelia sempervirens* against *Acinetobacter baumannii* (Graphic 1). Results based on *Salmonella spp.* and *Bacillus subtilis* are significant but these are minor clinic significance.

Our results show that the phytochemical study of native plants and subsequent biological tests are a way to get products with antimicrobial activity. It has been possible to characterize components of the essential oil through the method of GC-MS compounds, but this is not possible in ethyl acetate extract of *Laurelia sempervirens*, because GC-MS technique used for the volatile and nonvolatile be retained in the column and/or decomposed in injection. Biological activity of essential oil and ethyl acetate extract depends on their chemical composition and interaction among structural components. Even minor compounds can have a critical function (Zapata et al., 2010), therefore we are not in a position to explain the different mechanisms of action. However, antimicrobial activity of essential oil and ethyl acetate extract obtained from *Laurelia sempervirens* bark was demonstrated against a multidrug resistant bacteria isolated from clinical specimens, nevertheless, toxic effects to humans should be evaluated in future studies.
Table V
The susceptibility of the microorganisms to selected antibiotics.

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration (µg/ml)</th>
<th>Average of inhibition halo (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella sp</em> (CIPRO)*</td>
<td>70</td>
<td>14</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>70</td>
<td>6</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 1020</td>
<td>(GEN)c</td>
<td>70</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>(CIPRO)*</td>
<td>70</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>(GEN)c</td>
<td>70</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> 2312</td>
<td>(GEN)c</td>
<td>70</td>
</tr>
</tbody>
</table>

a; Ciprofloxacin, b; Amikacin, c; Gentamicin

* The inhibition rate was calculated dividing the ethyl acetate extract and essential oil inhibition halo (mm) with respect to the antibiotic inhibition halo (mm) (table V).
CONCLUSIONS
Antibiotics are an essential part of medicine. However, bacterial resistance emerges and reduce efficacy of antibiotics in the human population. At this point, the search of new compounds with potential effects against pathogenic bacteria is required (Morales et al., 2003). Chilean flora is a potential source of bioactive compounds, including some with antimicrobial activity.

These preliminary studies are highly interesting as they open new pathways for further studies, which would allow the validation of the traditional use of plants in the treatment of infections. The next step in our research is the isolation of pure compounds to determine their antibiotic activity and their mechanisms of action. Therefore, our study serves as a basis for new targets on molecular mechanisms of action for natural compounds. Our findings provide with new data to fight this pathogen and with new drugs that can be developed from natural products.

ACKNOWLEDGMENTS
This work was supported by DI-Universidad Andres Bello, Grant N° DI-19-08/R and DID UACH-s-2005-41. PIIC 2009-2010, PAC 2011-2012 and DGIP of Universidad Técnica Federico. Santa María for financial support.

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