ANTIMICROBIAL ACTIVITY AND CHEMICAL CHARACTERIZATION OF LAUREL ESSENTIAL OIL

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ABSTRACT: The factors that arouse interest in the study of essential oils as biocidal agents are numerous, such as the fact that they have antibacterial, antifungal, insecticidal, antioxidant, anti-inflammatory and larvicidal properties. The objective of this work was to evaluate the antimicrobial activity, in vitro, of the laurel (*Laurus nobilis* L) essential oil on the growth of pathogenic bacteria *Salmonella enterica* serovar Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 25923, at different exposure times, as well as to perform the chemical characterization. Twenty compounds were identified and quantified, representing 96.57% of the total composition. The class of oxygenated monoterpenes represented the majority class of the essential oil, with 1,8-cineol (33.8%) as the substance found in greater quantity, followed by linalool (17.79%). The third constituent in greater quantity was sabinene (12.23%), belonging to the group of monoterpene hydrocarbons. Terpinyl acetate (9.41%) was also considered to be quantitatively representative. Laurel essential oil showed bacteriostatic activity against *S.* Typhimurium ATCC 14028 and *S. aureus* ATCC 25923.

KEYWORDS: Antibacterial. *Salmonella* Typhimurium. *Staphylococcus aureus.*

ATIVIDADE ANTIMICROBIANA E CARACTERIZAÇÃO QUÍMICA DO ÓLEO ESSENCIAL DE LOURO

RESUMO: Os fatores que despertam interesse no estudo dos óleos essenciais como agentes biocidas são inúmeros, como o fato de possuírem propriedades antibacteriana, antifúngica, inseticida, antioxidante, antiinflamatória e larvicida. O objetivo deste trabalho foi avaliar a atividade antimicrobiana, in vitro, do óleo essencial de louro (*Laurus nobilis* L) sobre o crescimento das bactérias patogênicas *Salmonella enterica* sorovar Typhimurium ATCC 14028 e *Staphylococcus aureus* ATCC 25923, em diferentes tempos de exposição, assim como realizar a caracterização química do óleo. Vinte compostos foram identificados e quantificados, representando 96,57% da composição total. A classe dos monoterpenos oxigenados representou a classe majoritária do óleo essencial, sendo o 1,8-cineol (33,8%) a substância encontrada em maior quantidade, seguido do linalol (17,79%). O terceiro constituinte em maior quantidade foi o sabineno (12,23%), pertencente ao grupo dos hidrocarbonetos monoterpênicos. O acetato de terpinila (9,41%) também foi considerado quantitativamente representativo. O óleo essencial de louro apresentou atividade bacteriostática contra *S.* Typhimurium ATCC 14028 e *S.* aureus ATCC 25923.

PALAVRAS CHAVE: Antibacteriano. *Salmonella* Typhimurium. *Staphylococcus aureus.*

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ACTIVIDAD ANTIMICROBIANA Y CARACTERIZACIÓN QUÍMICA DEL ACEITE ESENCIAL DE LAUREL

RESUMEN: Los factores que despiertan interés en el estudio de los aceites esenciales como agentes biocidas son innumerables, como el hecho de que tienen propiedades antibacterianas, anti fúngicas, insecticidas, antioxidantes, antiinflamatorias y larvicidas. El objetivo de este trabajo ha sido evaluar la actividad antimicrobiana, in vitro, del aceite esencial de laurel (*Laurus nobilis L*) sobre el crecimiento de bacterias patógenas *Salmonella enterica serovar* Typhimurium ATCC 14028 y *Staphylococcus aureus* ATCC 25923, en diferentes momentos de exposición, así como realizar la caracterización química del aceite. Se identificaron y cuantificaron veinte compuestos, que representan el 96,57% de la composición total. La clase de mono terpenos oxigenados representó la clase principal de aceite esencial, siendo el 1,8-cineol (33,8%) la sustancia que se encuentra en mayor cantidad, seguida del linalol (17,79%). El tercer constituyente en mayor cantidad fue el sabineno (12,23%), perteneciente al grupo de los hidrocarburos monoterpénicos. El acetato de terpinilo (9,41%) también se consideró cuantitativamente representativo. El aceite esencial de laurel mostró actividad bacteriostática contra *S.* Typhimurium ATCC 14028 y *S. aureus* ATCC 25923.

PALABRAS CLAVE: Antibacteriano. *Salmonella* Typhimurium. *Staphylococcus aureus.*

Introduction

Aromatic and medicinal plants have been used for thousands of years in different cultures around the world, especially because they contain essential oils, formed from secondary metabolic pathways and defined as complex mixtures of volatile, lipophilic, usually odoriferous and liquid substances (SIMÕES *et al.,* 2004). In general, these oils contain about 20 to 60 components in different concentrations, characterized by two or three major compounds in high concentrations (20-70%) compared to other components present in minimal amounts (BAKKALI *et al.*, 2008).

Among the plants producing essential oils, the laurel, (*Laurus nobilis* L), belonging to the Lauraceae family, stands out, a tree native to the southern Mediterranean region, grown mainly in Europe and the USA, as an ornamental and medicinal plant (CAPUTO *et al.*, 2017). Phytochemical investigations with this oil revealed the main presence of sesquiterpenes, phenolic compounds and flavonoids, which showed cytotoxic activities and inhibitory effects on alcohol absorption (CHEN *et al*., 2014).

The factors that arouse interest in the study of essential oils as biocidal agents are numerous, such as the fact that they have antibacterial, antifungal, insecticidal, antioxidant, anti-inflammatory and larvicidal pharmacological properties (ALEXOPOULOS *et al*., 2011; GUIMARÃES *et al*., 2011; PAULIQUEVIS; FAVERO, 2015; VALERIANO *et al*., 2012). These oils emerged as a control alternative to pathogenic strains resistant to conventional antibiotics and causing serious public health problems worldwide (ARAUJO; LONGO*,* 2016; HENSEL, 2004; VALERIANO *et al*., 2012). Among the main biological agents causing these pathologies are bacteria of the genus *Salmonella* spp. and *Staphylococcus aureus*, both transmitted by food and direct contact to humans and animals, causing several gastrointestinal infections (HENSEL, 2004).

Bacteria of the genus *Salmonella* are classified as Gram-negative, have a lipopolysaccharide component on their outer membrane and a cell wall composed of a thin layer of peptidoglycans. Most of its serotypes are pathogenic to man, as in the case of *Salmonella enterica* Enteretidis and *Salmonella enterica* Typhimurium, the main serovars responsible for causing salmonellosis; one of the main zoonoses for public health worldwide, generating a significant cost to society, due to the high endemicity, morbidity and difficulty in its control (SHINOHARA *et al*., 2008).

Bacteria of the species *S. aureus* integrate part of the human microbiota and are classified as Gram-positive, do not contain lipopolysaccharide, but in compensation, have a thick layer of peptidoglycans. The infection by this pathogen presents a high risk to humans, due to the combination of antibiotic resistance and its virulence factors. When the infection happens associated with the weakened host's immune system, this bacterium is responsible for serious clinical pathologies, which can be fatal (OTTO, 2010). In addition, *S. aureus* produces thermostable toxins that can remain in the food even after cooking; among the food poisoning of bacterial origin in the world, about 45% are related to *S. aureus* (ARAUJO; LONGO, 2016).

The objective of this work was to carry out the chemical characterization of the laurel essential oil and to evaluate the antimicrobial activity, *in vitro*, on the growth of microorganisms *S.* Typhimurium ATCC 14028 and *S. aureus* ATCC 25923, at different times of exposure.

Material and Methods

Location of the experiment

The chemical characterization of the laurel essential oil was carried out at EMBRAPA Swine and Poultry in Concordia and microbiological analyzes were conducted at the Biology Laboratory, at the Federal Catarinense Institute (IFC) – Concordia.

Collection of the material and exsiccate

The plant material was collected in the Concórdia/SC. The collection took place at 7 am on April 10, 2017 and only the leaves of laurel were collected. Later, the dry vegetable was assembled in an exsiccate, which will be conserved at the Herbarium Padre Balduíno Rambo of URI in Erechim/RS, HPBR 12.087 *Laurus nobilis* L. (laurel), carrying a label with all the data of the collection form.

Maintenance of bacteria and inoculum preparation

The microorganisms used were *Salmonella enterica* serovar Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 25923. The stock cultures were kept in a freezing medium, consisting of glycerol (DYNAMIC, BR) and *Brain Heart Infusion* (BHI) (BACTO ™, USA), in the proportion

1:1 at -70ºC. The strains were reactivated in BHI (BACTO ™, USA) broth with incubation at 37ºC for 24 h. The inoculum were standardized at 10^8 CFU mL^{-1} after elaboration of growth curves ($D.O._{550nm}$) in BHI (BACTO ™, USA), plating in Tryptic Soy Agar (TSA) (NEOGEN, USA) and incubation at 37°C for 24 h.

Essential Oil Extraction

To extract the laurel essential oil, the steam distillation method was used, in a pilot scale distiller made of stainless steel. About 5 kg of fresh vegetable material (aerial parts) were stored in the extraction system. The steam was generated in a boiler and conducted through the plant material to release the aromatic content. After condensation in a refrigerated coil, the aqueous phase was continuously transferred to a decanting funnel, where the essential oil was obtained by spontaneous separation of the phases. After two hours of extraction, the crude essential oil was transferred to a 50mL tube containing 0.5g of anhydrous Na_2SO_4 for total removal of the water particles. The sample was vortexed for 30 seconds and then centrifuged at 2000g for 10 minutes at 5°C. The clear essential oil was transferred to an amber glass bottle and stored at -20°C. The sample of essential oil (100mg) was dissolved in dichloromethane (10mL) and the resulting solution was analyzed by gas chromatography (GC-FID and GC-MS) (CUNHA *et al*., 2013).

Gas chromatography with flame ionization detection (GC-FID)

The analysis of laurel essential oil was performed in an Agilent 7820A gas chromatograph (AGILENT TECHNOLOGIES, CN) equipped with a *split/splitless* injector, a flame ionization detector (FID) and an Agilent auto injector 7693A. The sample solution (1µl) was injected in *split* mode at a ratio of 1:10. The analyses were performed with an Agilent J&W HP-5 capillary column (30m x 0.25mm i.d., film with a thickness of 1µm, stationary phase consisting of 5% diphenyl/95% dimethylpolysiloxane). The oven temperature was maintained at 50°C for two minutes, increased from 50 to 220°C at a rate of 2°C/min and maintained at 220°C for three minutes. Nitrogen 5.0 was used as carrier gas at a flow rate of 1.2mL/min. The injector and detector temperatures were set at 240°C and 280°C, respectively. The air flows, H_2 and N_2 in the detector were 300, 30 and 30mL/min. The OpenLAB CDS software was used for equipment management and data processing. The composition of the essential oil was expressed as a percentage of normalized area (ADAMS, 2001; CUNHA *et al*., 2013).

Gas chromatography coupled to mass spectrometry (CG-MS)

The essential oil was analyzed in a gas chromatograph coupled to a Shimadzu GCMS-QP2010 mass spectrometer equipped with a *split/splitless* injector. The sample solution (1µl) was injected in *split* mode at a ratio of 1:10. The analyses were performed with a Supelco Equity-5 capillary column (30m x 0.2mm i.d., film with a thickness of 0.2µm, stationary phase consisting of 5% diphenyl/95% dimethylpolysiloxane). The oven temperature was maintained at 50°C for 2 minutes, increased from 50 to 220°C at a rate of 2°C/min and maintained at 220°C for 3 minutes. Helium 5.0 was used as carrier gas at a flow rate of 1.2 ml/min (constant linear speed of 39.2 cm/s). The injector temperature was fixed at 240°C. The interface and ion source temperatures were set at 220 and 200°C, respectively. The quadrupole mass spectrometer was operated in 70V electronic impact mode with scanning in the range of *m/z* 35-350 in 0.5s cycles. The GCMS solution software was used for equipment management and data processing. The compounds were identified by searching the NIST 05 mass spectrum library and comparing their retention indices for the C7-C30 alkane series with those found in the literature (ADAMS, 2001; CUNHA *et al*., 2013).

Essential oil-based solutions

The 2.5% essential oil stock solution was prepared using as diluent 0.16µL of 2% absolute ethyl alcohol PA (LABIMPEX, BR), 7.68mL of 0.85% saline water with addition of tween 80 to 0.5% (v/v) (DINAMICA, BR) and 0.2µL of laurel essential oil (MILLEZI *et al*., 2016). From the stock solution, working solutions were prepared in different concentrations, being 0.4% (1.28mL of stock solution and 2.72mL of BHI (BACTO ™, USA), 0.8% (2.56mL of stock solution and 1.44 mL of BHI and 1.0% (3.2mL of stock solution and 0.8 mL of BHI.

Bacterial death curve

To evaluate the influence of the different concentrations of the laurel essential oil on the strains of *S. aureus* ATCC 25923 and *S.* Typhimurium ATCC 14028, the bacterial inoculum standardized at approximately 10^8 CFU/mL was transferred to 12 sterile falcon tubes, containing the different concentrations of essential oil and the control (without the essential oil, containing only the standardized inoculum in BHI medium (BACTO ™, USA). Then, they were incubated in an orbital shaker (TECNAL, BR) at 37°C, stirring at 80 rpm for 24 h. In addition, controls were made containing sterile distilled water to replace the corresponding aliquots of essential oil of each concentration, submitted to the same procedure. After 0.5; 1; 2; 4; 6; 8; 10 and 12 h of exposure at a temperature of 37°C, aliquots of 100µL of the samples were transferred to microtubes containing 900µL of distilled water, with serial dilutions and subsequent plating in TSA medium (NEOGEN, USA). The quantification of Colony Forming Units (CFU) was performed after 18-24 h. of incubation at 37°C.

Experimental design and statistical analysis

The experimental design used was completely randomized (DIC). The data obtained were analyzed using generalized linear models (GLM) and the means compared by the Tukey test at 5% probability. The analyzes were performed in the R program, using the agricultural package and lme4 (BATES *et al*., 2015).The experimental tests were performed in triplicate with three repetitions.

Results

The characterization of laurel essential oil identified 20 compounds, totaling 96.57% of the composition (Table 1). Among the identified compounds, there was a predominance of the terpenoid class (92.47%), comprising oxygenated monoterpenes (64.99%), monoterpene hydrocarbons (26.22%) and sesquiterpene hydrocarbons (1.26%). Additionally, compounds of the class of phenylpropanoids (4.1%) were identified. Among oxygenated monoterpenes, 1,8-cineol (33.8%) stood out as the substance found in greater quantity, followed by linalool (17.79%). The third constituent in greater quantity was sabinene (12.23%), belonging to the group of monoterpene hydrocarbons. Terpinyl acetate (9.41%) was also considered to be quantitatively representative.

Table 1: Chemical composition (% in normalized area) and yield of laurel essential oil

Compounds[a]	R.I.[b]	Area (%)
α-Thujene	924	0.51
α-Pinene	929	4.53
Camphene	943	0.1
Sabinene	969	12.23
β-Pinene	972	4.15
β-Mircene	990	1.26
δ -3-Carene	995	0.42
δ -4-Carene	1014	0.34
(+)-Sylvestrene	1025	2.51
1.8-Cineol	1029	33.8
(E)-β-Ocimene	1046	0.17
γ-Terpineno	1055	0.71
Linalool	1101	17.79
4-Terpineol	1174	1.0
α-Terpineol	1188	2.28
terpinyl acetate	1315	9.41
Eugenol	1355	0.83
β-Elemene	1388	0.5
Eugenol methyl ether	1405	3.27
β-Caryophyllene	1407	0.76
Monoterpene Hydrocarbons		26.22
Oxygenated monoterpenes		64.99
Sesquiterpene hydrocarbons		1.26
Phenylpropanoids		4.1
Total identified (%)		96.57

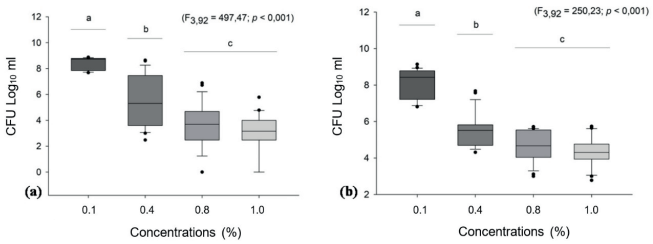
[a]The compounds are listed in order of elution on a Supelco Equity-5 column.

[b]Retention index experimentally determined on a Supelco Equity-5 column using a homologous series of aliphatic hydrocarbons (C7-C30)

The concentrations of laurel essential oil tested against *S.* Typhimurium ATCC 14028 bacteria showed a significant reduction in the bacterial population compared to the control (Figure 1a). Comparing the inhibitory action of concentrations, it is observed that 0.8 and 1.0% were more effective in reducing the number of bacteria cells than the 0.4% concentration (Figure 1a).

Similarly, cells of *S. aureus* ATCC 25923 exposed to different concentrations of laurel essential oil showed a reduction in bacterial population when compared to the control. There was a significant difference between treatments, with concentrations of 0.8 and 1.0% performing better than concentrations of 0.4% (Figure 1b).

Figure 1: Box plot indicating the effect of laurel essential oil concentrations on (a) *Salmonella enterica* ser Typhimurium ATCC 14028 and (b) *Staphylococcus aureus* ATCC 25923 cells.



Means followed by the same letter do not differ, using the Tukey test (p> 0.05).

Analyzing time only, the laurel essential oil significantly reduced the population of *S.* Typhimurium ATCC 14028 by up to 2 h of experiment, with a mean of less than 0.5 h (Table 2). The reduction in the microbial load of *S. aureus* occurred up to 8 h, with a lower mean at 4 h. (Table 2).

Table 2: Effect of time in reducing the mean number of Colony Forming Units (CFU) (± EP).

Time (h)	*Salmonella enterica* ser Typhimurium ATCC 14028 (CFU $\log_{10}$ mL)	*Staphylococcus aureus* ATCC 25923 (CFU $\log_{10}$ mL)
0.5	3.09 ± 0.89 f	5.91 ± 0.19 a b c
1	4.08 ± 0.72 d e f	5.52 ± 0.26 a b c
2	3.95 ± 0.77 e f	5.31 ± 0.46 b c
4	5.03 ± 0.70 c d e	5.03 ± 0.63 c
6	5.25 ± 0.68 b c d	5.17 ± 0.61 b c
8	6.03 ± 0.60 a b c	5.58 ± 0.55 a b c
10	6.39 ± 0.60 a b	6.16 ± 0.53 a b
12	7.08 ± 0.51 a	6.48 ± 0.52 a
F	172	9.2
p	< 0.001 ***	0.003 **

Means followed by the same letter do not differ, using the *Tukey* test (p> 0.05).3

Discussion

The yield and chemical composition of essential oils can vary significantly, according to a number of factors, such as the stage of the plant's vegetative cycle, the plant's chemotype (chemical race), environmental factors such as altitude, temperature, relative humidity, time of sun exposure, presence of micronutrients and even the wind regime to which the plant is submitted (FIDAN *et al*., 2019; OUSSALAH *et al*., 2007). In addition, variations can also occur depending on the part of the plant that is used for extraction (KOVACEVIC *et al*., 2007).

In our study, we found as major components the compounds 1.8-cineol (33.8%), linalool (17.79%), sabinene (12.23%) and terpinyl acetate (9.41%). These compounds belong to the class of terpenes, reported as efficient antimicrobials, are likely to interfere with cell wall biosynthesis and ionic permeability of the membrane (PEIXOTO *et al.*, 2017). In the analysis of the laurel fresh leaves oil, Al-Kalaldeh, Abu-Dahab and Afifi (2010) argued that 1,8-cineol (eucalyptol) (40.91%) was the main component. Additionally, Guenane *et al*. (2017) also described 1,8-cineol (44.13%) as a major compound, in addition to α-terpinyl acetate (17.33%) and methyl eugenol (6.53%). Caputo *et al*. (2017), identified 1,8-cineol (31.9%), sabinene (12.2%) and linalool (10.2%) as constituents in greater abundance.

The reduction in the bacterial population caused by the laurel essential oil can be attributed to the presence of these oxygenated monoterpenes, such as 1,8-cineol and linalool, present in greater quantities in this study, which have been shown to increase the antimicrobial activity of essential oils and antiseptics (BELLAVER *et al*, 2022; HERMAN *et al*., 2016; SIMSEK; DUMAN, 2017). Constituents of this class have demonstrated antibacterial activity against Gram-positive and Gram-negative (ROCHA *et al*., 2018; ROJAS *et al*., 2010). In addition, minority compounds can also contribute significantly to the antimicrobial activity of essential oils, since *in vitro* tests demonstrate that isolated compounds have less activity compared to essential oil (BURT, 2004; MILLEZI *et al*., 2019).

The use of essential oils against bacteria is justified due to its cytotoxic activity (BAKKALI *et al*., 2008). This effect is dependent not only on the chemical composition, but also on the state of division of the pathogenic organism (BAKKALI *et al*., 2008). Furthermore, because they are lipophilic compounds, these constituents act: crossing the cell wall and membrane, causing its permeability; as a consequence, it is observed the extraction of cellular materials such as ATP and nucleic acids; inducing coagulation of the cytoplasm; and damaging the lipids and proteins, leading to cell lysis (BAKKALI *et al*., 2008; RAUT; KARUPPAYIL, 2014).

The essential oil showed considerable activity against the bacteria studied, occurring to the reduction of bacterial cells, however, over 12 h, there is a resumption of bacterial growth, which can be attributed to the volatile properties present in the essential oil, in addition to the solution pH, treatment temperature and concentrations and type of active components Burt (2004). In addition, it is known that the range of action of essential oils against bacteria can reach values that result in a decline in the number of bacterial cells (bactericidal) or that inhibit only bacterial growth (bacteriostatic). The bactericidal action has a permanent effect, even after the neutralization of the agent, the microbial cells are not able to grow and reproduce; in contrast, the bacteriostatic has a reversible character, because after the neutralization of the agent, the microbial cells recover their reproductive capacity (FALEIRO, 2011). It is believed that in the present study, the action was bacteriostatic, as the cells resumed their growth after a certain time.

In the literature, similar results were found, which showed that essential oils are effective in inhibiting both Gram-positive and Gram-negative bacteria (DORMAN; DEANS, 2000; MILLEZI *et al*. 2019; SZCZEPANSKI; LIPSKI, 2013; ZHANG *et al*., 2018). For Siriken *et al.* (2018), the strong antibacterial activity of laurel against Gram-negative and Gram-positive, was intensified with the use of zinc oxide nanoparticles (Ln-ZnO NPs). For Burt (2004), Gram-positive bacteria have been less or equally sensitive to Gram-negative bacteria. However, Oussalah *et al*. (2007) report that Gram-positive bacteria are more sensitive to essential oils than Gram-negative bacteria. Corroborating this study, Caputo *et al*. (2017) show that the laurel essential oil showed bacteriostatic activity against *S. aureus* and in bacteria classified as Gram-negative.

The concentrations tested despite inhibiting the growth of *S.* Typhimurium ATCC 14028 and *S. aureus* ATCC 25923, did not fully reduce the Colony Forming Units (CFU/mL) over the 12 h of experiment. According to the literature, meat contamination levels around 10^6 CFU/cm may indicate the beginning of the deterioration process with the production of unpleasant odor and compromised shelf life (FRANCO; LANDGRAF, 2005). In the case of *S. aureus*, the interval considered significant to cause food poisoning is 10^5-10^6 CFU/mL; unsatisfactory hygienic-sanitary conditions are one of the most likely hypotheses for verifying contamination (SIMEÃO *et al*., 2002). Data from ICMSF (1998) corroborate showing that concentrations of this microorganism above 10^5 CFU/ml of product are considered sufficient for the production of staphylococcal toxins at levels propitious to food poisoning. In the case of *Salmonella* spp., the infective dose to healthy humans ranges from 10^6 to 10^8 CFU, although food salmonellosis at much lower doses has been reported Humphrey (2004).

Conclusion

Based on the exposed herein, the concentrations of 0.8 and 1.0% showed a significant reduction in the bacterial population, suggesting that they can be used for more in-depth tests, with other methodologies. There is the possibility of using the studied essential oil, in the production of chemical detergents or disinfectants, alternative controls to microorganisms, use in dairy products, in the cleaning of milking, pens and drinking fountains used in animal feed. Also, essential oils are natural products, they do not harm the environment.

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