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Chemical composition and antimicrobial activity of the essential oil from the leaves and flowers of *Aloysia gratissima*

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ABSTRACT: Volatile oils from leaves and flowers of *Aloysia gratissima* were investigated for their chemical composition and antimicrobial activity against the bacteria *Bacilus subtilis, Staphylococcus aureus, Salmonella choleraesuis, Pseudomonas aeruginosa, Streptococcus pneumoniae* and the *Candida albicans* yeast. The minimum inhibitory concentrations (MIC) of the oils were determined by the micro-dilution method, while the chemical composition was determined by GC-MS (gas chromatography mass spectrometry). The fresh leaves and inflorescence were subjected to hydrodistillation for 120 min using a Clevenger-type apparatus, and the essential oil was tested against microorganisms. High concentrations of sesquiterpenes were observed for the inflorescence, and monoterpenes were observed for the leaves. The main compounds of the inflorescence essential oil were *E*-caryophyllene, germacrene B, guaiol and bulnesol, while in the leaves the main compounds were *trans*-pinocamphone, *trans*-pinocarveyl acetate, and guaiol. The essential oil from the leaves showed an effect against *P. aeruginosa* and *S. pneumonia,* and the essential oil of the inflorescence showed an effect against *P. aeruginosa, S. pneumonia,* and *Candida albicans*.

Keywords: Verbenaceae, medicinal plant, gas chromatography

RESUMO: **Composição química e atividade antimicrobiana do óleo essencial de folhas e flores de *Aloysia gratissima.*** O óleo essencial de folhas e de flores de *Aloysia gratissima* foi avaliado quanto à composição química e ação antimicrobiana contra as bactérias *Bacilus subtilis, Staphylococcus aureus, Salmonella choleraesuis, Pseudomonas aeruginosa, Streptococcus pneumoniae,* e a levedura *Candida albicans*. A concentração mínima inibitória (MIC) dos óleos essenciais foi determinada pelo método da microdiluição e a composição química determinada por CG-EM (Cromatografia Gasosa acoplada a Espectrômetro de Massas). Folhas e inflorescências frescas foram hidrodestiladas por 120 minutos em aparelho Clevenger sendo o óleo essencial testado contra microorganismos. Para as flores foi observada maior concentração de sesquiterpenos, enquanto que as folhas apresentaram maior concentração de monoterpenos. Os principais constituintes do óleo essencial da flor foram: *E*-cariofileno, germacreno B, guaiol e bulnesol; e das folhas foram: *trans*-pinocamfona, acetato de *trans*-pinocarveol e guaiol. O óleo essencial da folha mostrou atividade contra *P. aeruginosa* e *S. pneumoniae,* e o óleo essencial da flor mostrou atividade contra *P. aeruginosa, S. pneumoniae* e *Candida albicans*.

Palavras-chaves: Verbenaceae, planta medicinal, cromatografia gasosa.

INTRODUCTION

In the last decades, essential oils have been of great interest as natural products. They have been screened for their potential uses as alternatives in the treatment of many infectious diseases (Kelen and Tepe, 2008).

Aloysia is an aromatic genus with approximately 40 species, spread in tropical and subtropical areas, descending probably from South America, growing in Brazil, Argentina and Uruguay. The species *Aloysia gratissima* (Gillies and Hook) Tronc. is also known as *Verbena gratissima* Gill et Hook, *Aloysia lycioides* Cham e *Lippia lycioides*

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(Cham) Steudel (Santos et al. 2011*).* It is a bush used in folk medicine for the treatment of bronchial infections, lung diseases, bladder disorders, and as an antispasmodic. It is also used as an antimicrobial agent and as a flavoring for infusions and meat (Pinto et al., 2007; Santos et al., 2009).

It grows spontaneously and is commonly pollinated by insects, especially bees, during its flowering. It is used in folk medicine for the treatment of bronchial infections, lung diseases, bladder disorders, and as an antispasmodic. It is further used as an astringent, antimicrobial agent, stimulant and tranquilizer, as well as in the treatment of headaches, respiratory infection (inhalation), indigestion, earache and colic in babies. Due to its aromatic properties, it is also used as a flavoring for infusions and meat, and pot-pourri (Silva et al.,2007).

Compounds of *A. gratissima* oil have been subjected to a few studies which have shown a difference in its constituents depending on the region of cultivation, and there have been some variations in the constituents from different countries. It has been found that oil from A. gratissima leaves oil from Argentina contains pulegone (65.8%), and is also the main component of A. gratissima flower oil (Zygadlo, 1995); and α-thujone (26.0%) and β-caryophyllene (27.0%) (Ricciardi et al.,2000). The Brazilian variety contains isopinocamphone (cis-3-pinanone) (25.4%), limonene (15.1%), and guaiol (12.7%) (Trovali et al.,2009) and *trans*-pinocamphone (10.86%), *trans*-pinocarveyl acetate (9.27%) (Sartoratto e Augusto, 2003).

Studies have demonstrated antibacterial activity of the crude extracts and oil from the leaves of *Aloysia* species (Vandresen et al.,2010). There is no report that investigated the antimicrobial activity of volatile compounds of this plant. The aim of this study was to identify the chemical composition and evaluate the antimicrobial activity of leaves and inflorescence oils of *A. gratissima* from southeast of Brazil against different microorganisms.

MATERIAL AND METHODS

Plant material

Aloysia gratissima (Gillies et Hook) Tronc. fresh leaves and inflorescence were collected at Lavras, Minas Gerais state, Brazil. The county of Lavras is situated at 918.87 m altitude, 21º14´S latitude and 45 º 00´W longitudes. The plant was identified based on morphological features, and the voucher specimen (number 19810) has been deposited in the herbarium of the Department of Biology at the Federal University of Lavras (UFLA).

Essential oil extraction

The fresh leaves and inflorescence parts (120g and 60g, respectively) of *A. gratissima* were subjected to hydrodistillation for 120 min using a Clevenger-type apparatus. The essential oil was dried over anhydrous magnesium sulphate ($MgSO_4$) and preserved in a sealed vial at 4ºC for further analysis.

Gas chromatography (GC-FID) and Gas chromatography-mass spectrometry (GC-MS) analysis

The chemical analysis of the essential oil was performed using Agilent Technologies CG-6890A and MS5975 with quadruple detector operating at 70eV in electron ionization mode, equipped with HP-5 fused silica capillary column (300 × 0.25 mm i.d.; 0.25 µm film thickness). The carrier gas helium (He) was used at a constant flow rate of 1.0 mL min^{-1}. The thermal program was 60-240°C at a rate of 3°C per min. The injected volume was 1,0 µL in split ratio 30:1. The injector and detector temperatures were kept at 220ºC and 250ºC, respectively. The amount of each compound was expressed as a relative percentage of the total area of the chromatograms.

Identification procedure

The identification of the oil components was established from their GC retention indices, relative to C_8–C_{32} n-alkanes (Sigma, St Louis, MO, USA), by comparison of their MS spectra with those reported in the literature (Adams, 2007), and by computer matching with the Agilent Chemstation mass spectra and NIST/EPA/NHI (NIST, 2010) libraries.

Microorganisms

Antimicrobial activity was investigated using a panel which included laboratory control strains from the American Type Culture Collection (ATCC) and Tropical Culture Collection (CCT), André Tosello Foundation, Campinas, São Paulo State, Brazil: Gram-positive bacteria *Bacillus subtilis* CCT 2576, *Staphylococcus aureus* CCT 2740 and *Streptococcus pneumoniae* ATCC 11733; Gram-negative bacteria *Salmonella choleraesuis* CCT 4296 and *Pseudomonas aeruginosa* ATCC 13388, and a yeast *Candida albicans* ATCC 10231.

Antimicrobial assay

The antimicrobial assays were carried out at the Microbiology Division of CPQBA UNICAMP, Campinas, São Paulo State, Brazil. Culture media for preservation and activity tests with bacteria and *C. albicans* were, respectively, Muller-Hinton broth and RPMI-1640. Prior to the assays, microorganism strains were grown overnight at 36ºC in the

respective media. Inocula for the assays were prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometrical reading at 600 nm. Cell suspensions were finally diluted to 10^4UFC mL^{-1} for use in the activity assays. Minimum Inhibitory Concentration (MIC) tests were carried out according to CLSI (2002 e 2005), on a tissue culture test plate (96 wells). Stock solutions from the essential oils were prepared in 0.1% Tween 80 in distilled water at 4 mg mL^{-1}. An aliquot of 100 µL was transferred into the first well, and serial dilutions were performed so that concentrations in the range of 1.0 – 0.0156 mg mL^{-1} were obtained. Each oil was tested in duplicate. Chloramphenicol and nystatin (Merck) were used as the reference antimicrobial control.

The inoculum was added to all wells and the plates were incubated at 36 ºC for 24-48h. Antibacterial activity was detected by adding 20 µL of 0.5% TTC (triphenyl tetrazoluim chloride, Merck) aqueous solution. MIC was defined as the lowest concentration of the compounds that inhibited visible growth, as indicated by TTC staining (dead cells are not stained by TTC). After the incubation period, changes in the RPMI-1640 medium color were verified from pink (original color) to yellow, for anti-*C-albicans* activity evaluation. The change indicates a medium acidification by the yeast growth.

RESULTS AND DISCUSSION

Chemical compositions

The extraction yields of essential oil shows that the inflorescence (0.56%) of *A. gratissima* contained more oil than their leaves (0.35%). The essential oil from inflorescence is viscous, white and becomes solid in low temperatures. Otherwise, essential oil from leaves is limpid yellow and liquid.

The essential oils were obtained by hydrodistillation from fresh parts of *A. gratissima* and subsequently analyzed by GC-MS. The identified compounds with their relative percentage are reported in Table 1. In total, 29 compounds were identified in the leaves (97.3% of the total oil) and 21 compounds in the inflorescence (86.6% of the total oil). There were 15 monoterpenes compounds on leaves (56.0%) and seven monoterpenes on inflorescences (11.7%). The leaves' essential oil presented more monoterpenes, while the essential oil from inflorescence was dominated mainly by sesquiterpenes, being 30.4% non-oxygenated and 44.5% oxygenated, which corresponds to 74.9% of total sesquiterpenes. On the other hand, there is 41.3% of total sesquiterpenes on the leaves, 18.7% non-oxygenated and 22.6% oxygenated (Figure 1 and Table 1). The difference amount between mono and sesquiterpenes can be seen in *Eucalyptus oleosa*. The observed difference may be due to the species, seasonal variations and organ (Marzouf et al., 2011).

The major compounds derived from the essential oil of *A. gratissima* leaves were *trans*-pinocarveyl acetate (17.6%), *trans*-pinocamphone (16.3%) and guaiol (11.5%) totaling 45.4% of identified compounds. On inflorescences, there is a higher content of guaiol (19.5), germacrene B (10.5%), bulnesol (10.0%), *E*-caryophyllene (8.9%)

TABLE 1. Chemical composition (%) of *A. gratissima* leaves and inflorescence essential oil, plants were cultivated on field.

Analyte	RI[a]	Leaves (%)	Inflorescence (%)
Oxygenated monoterpene		56.0	11.7
Trans sabinene hydrate	1098	1.5	0.3
Linalol	1100	1.0	nd
Camphenolw	1106	0.4	nd
α-campholenal	1125	0.4	nd
Sabinol (trans)	1138	4.5	0.6
cis-pinal - 2- ol	1140	0.6	nd
Trans verbenol	1144	2.5	1.0
Pinocamphone (trans)	1161	16.3	2.1
Pinocamphone (cis)	1173	4.9	0.8
Terpin-4-ol	1176	0.5	nd
Cimen-8-ol (para)	1184	0.5	nd
Mirtenol	1195	3.7	0.7
trans-carveyl	1217	0.6	nd
Bornyl acetate	1284	1.0	nd
Trans pinocarveyl acetate	1300	17.6	6.2
Sesquiterpene		18.7	30.4
β- bourbonene	1382	0.5	nd
β-elemene	1390	0.5	0.9
E-caryophyllene	1417	3.1	8.9
γ- elemene	1431	nd	0.7
α-humulene	1450	1.1	2.4
γ-muurolene	1479	4.6	2.1
Bicyclogermacrene	1494	2.8	4.2
Bulneseno (α)	1502	0.5	0.7
Germacrene (B)	1554	5.6	10.5
Oxygenated sesquiterpene		22.6	44.5
Cubebol	1512	1.0	1.4
Sphathulenol	1575	2.6	3.8
Guaiol	1597	11.5	19.5
Caryophyllene oxide	1580	3.3	8.2
10- epi γ eudesmol	1618	0.5	1.6
Bulnesol	1665	3.7	10.0
TOTAL		97.3	86.6

[a] Retention indices calculated against *n*-alkane series on HP-5MS column.
nd: not detected.

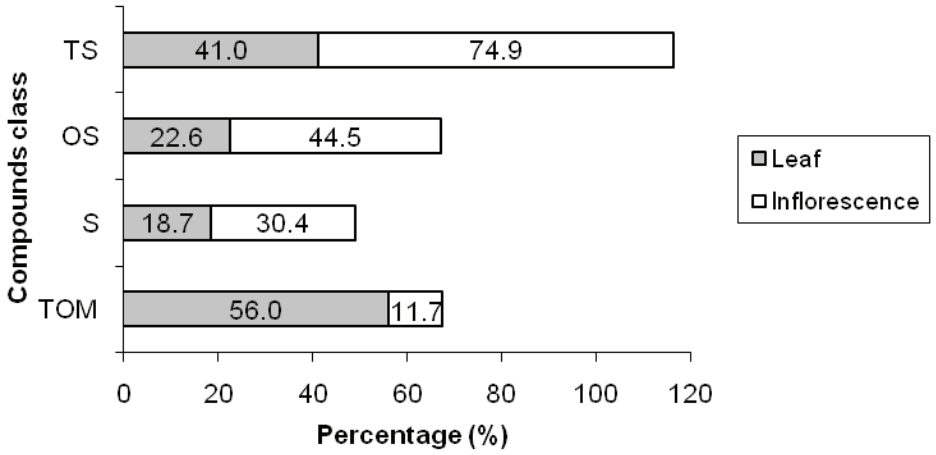

FIGURE 1. Percentage of compounds in essential oil from leaves and inflorescence of *Aloysia gratissima*– Total Sesquiterpene total (TS); Oxygenated sesquiterpene (OS); sesquiterpene (S); total oxygenated monoterpene (TOM).

and caryophyllene oxide (8.24%), which corresponds to 57.2% of the identified compounds.

The results on chemical composition of the essential oil from the leaves of *A. gratissima* differed partially from those reported previously in other literature (Zygadlo, 1995; Ricciardi et al., 2000; Trovati et al., 2009). On the other hand, our findings were in accordance to those obtained by (Sartoratto and Augusto, 2003), who also identified the major presence of *trans*-pinocarveyl acetate (9.3%) and *trans*-pinocamphone (10.9%), though in a smaller quantity than the ones obtained in this current research.

The major compounds of the essential oil of *A. gratissima* leaves were pinocarveyl acetate (17.6%), *trans*-pinocamphone (16.3%) and guaiol (11.5%) totaling 45.4% of identified compounds. On inflorescences, there is a higher concentration of guaiol (19,5%), germacrene B (10.5%), bulnesol (10.0%) amounting to 40.0% of identified compounds. It is necessary to point out that part of the plant and genetic differences strongly influence the chemical composition of the essential oil. The literature shows differences for oils from different plant parts (Torabbeigi et al., 2012; Shafaghat, 2011; Shafaghat and Shafaghatlonbar, 2011; Marzoug et al., 2011; Tosun et al., 2011; Wang and Liu, 2010; Safaei-Ghomi and Batooli, 2010).

Antimicrobial activity

The essential oils showed variable activities against tested bacteria. The results on antimicrobial activity oils against different microorganisms are summarized in Table 2. The essential oil from leaves showed activity against *P. aeruginosa* (MIC 0.8 mg mL^{-1}) and *S. pneumoniae* (MIC 0.6 mg mL^{-1}), similar to the reference antibiotic used in the assays. While, the essential oil from inflorescence showed activity against *P. aeruginosa* (MIC 0.15 mg mL^{-1}), *S. pneumoniae* (MIC 0.025 mg mL^{-1}) and *Candida albicans* (0.02 mg mL^{-1}).

The essential oil activity of the flower was more effective than of the leaf, and was especially pronounced against gram-negative bacterium *P. aeruginosa,* gram-positive bacterium *S. pneumonia*, and the yeast *C. albicans*. The positive controls used in this assay were chloramphenicol and nystatin for the bacteria and yeast respectively. Our results showed that flower oil had antimicrobial activity higher than the positive antibacterial standard chloramphenicol and nystatin. The inflorescence oil was five times more effective against *P. aeruginosa* (MIC 0.15 mg mL^{-1}) than chloramphenicol, which needed 0.85 mg mL^{-1} to suppress the bacterial growth. When we evaluated it for *S. pneumoniae,* the MIC from inflorescence oil with the smallest inhibitory dose was 0.02 mg mL^{-1}, which means that its inhibition is three times more effective than the reference antibiotic concentration (0.06 mg mL^{-1}). For the yeast *C. albicans,* there was an inhibition 2 ½ times lower, with MIC 0.02 mg mL^{-1}, with 0.05 mg/mL necessary for nystatin. The main compounds of flower oil were *E*-caryophyllene, germacrene B, guaiol and bulnesol (49.0%). The antimicrobial activity of an essential oil is attributed mainly to its major compounds; however the synergistic or antagonistic effect of a minor component of the complex mixture has to be considered. Others studies showed antimicrobial activity with oil containing at least one of these major oil compounds as the principal component (Hisham et al., 2006; Mevya et al., 2007; Asfaha et al., 2008; Ho et al., 2009). The results showed that Gram-positive bacteria *S. pneumoniae* was more sensitive than Gram-negative

TABLE 2. Minimum inhibitory concentration (MIC – mg/mL) of *Aloysia gratissima* leaves and inflorescence essential oil, plants were cultivated on field.

Samples	Microorganisms					
	Gram-positive bacteria			Gram-negative bacteria		Yeast
	Bacillus subtilis	*Staphylococcus aureus*	*Streptococcus pneumoniae*	*Salmonella choleraesuis*	*Pseudomonas aeruginosa*	*Candida albicans*
Leaves	*	*	0.60	*	0.80	0.90
Flowers	0.15	0.25	0.02	*	0.15	0.02
Control	0.02	0.02	0.06	0.06	0.85	0.05

* concentration >1.0 mg mL^{-1}

P. aeruginosa and *S. choleraesuis* probably because they possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering. Without this barrier, the membrane in gram-positive bacteria can be permeated more easily (Marzoug et al., 2011).

Otherwise, the essential oil from leaves showed an activity similar to chloramphenicol (0.85 mg mL^{-1}) against *P. aeruginosa* (0.80 mg mL^{-1}) only. It showed no activity against the other five microorganisms, when compared to the reference antimicrobials values. The essential oils of flowers had better antimicrobial activities than that of leaves. This is probably because the essential oils of flowers were full of sesquiterpenes. It´s known that sesquiterpenes usually possess antimicrobial activity (Chen et al., 2011).

These results corroborate the importance of ethnopharmacology on studies that are guided by folk culture, such as the use of *A. gratissima* for treatments against pneumonia and bronchial affections. Nevertheless, pharmacological research and specific clinics are still necessary for making feasible the use of the essential oil of *A. gratisima* as a phytotherapeutic.

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