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Antimicrobial activity and chemical composition of essential oil of *Pelargonium odoratissimum*

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Abstract: The chemical composition of the essential oil from the leaves of *Pelargonium odoratissimum* (L.) L'Hér., Geraniaceae, was determined and the antimicrobial activities against the *Aspergillus flavus* CML 1816, *Aspergillus carbonarius* CML1815 and *Aspergillus parasiticus* CMLA 817 fungi, as well the *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25 992 bacteria were evaluated. The essential oil was isolated by steam distillation using a modified Clevenger apparatus, and its constituents were identified and quantified by GC/MS and GC-FID analyses. *In vitro* bioanalytical testing was performed using a completely randomized design. The concentrations of essential oil employed ranged from 0.1 to 2 $\mu L.mL^{-1}$ (in dimethyl sulfoxide) for the fungus species and from 1 to 500 $\mu L.mL^{-1}$ for the bacteria. The diameters of the inhibition zones formed for bacteria and the mean diameters of mycelial growth in perpendicular directions for fungi were measured, followed by calculation of the percentage of inhibition. The essential oil from the leaves of *P. odoratissimum* furnished methyleugenol (96.80%), a phenylpropanoid. This essential oil inhibited the growth of fungi (100% inhibition) and exhibited a small effect on the bacteria at the concentrations tested.

Article

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Introduction

In recent decades, most countries have been increasingly concerned about the adequacy of food supplies. The World Health Organization has warned about the need to prevent the contamination of food by biological agents that may present health risks. Foods are excellent substrates for numerous species and varieties of microorganisms because of several environmental factors. Because of its important role in the global economy, food must be produced using sanitation standards that insure good health and physical, chemical, and biological safety (Forsythe, 2005).

The causative agent must initially be present in the food for food-borne diseases to occur, and intrinsic and extrinsic factors must be favorable to its development. Such contamination can be caused by poor manipulator hygiene and inadequate locations for manufacturing and storage. As soon as the microorganisms come in contact with the food, they multiply and can produce enough toxins to cause food poisoning (Pereira et al., 2008).

Many studies have been undertaken to discover less aggressive alternatives for the control of microorganisms that cause deterioration of foods and/or are pathogenic to humans. Essential oils can be considered as an important alternative to the use of synthetic additives in the control of these microorganisms. However, it is fundamental that the chemical and biological characterization of the plant species be performed to obtain information on the chemical constitution of these oils (Jay, 2005).

The essential oils have an important role in the pharmaceutical, food, perfume and cosmetic industries. Brazilian exports of essential oils and their derivatives are increasing. From January 2005 to October 2008, shipments of these products earned US$ 309.5 million for 119772 tons of oil, 95% being citrus oils (Bizzo et al., 2009).

Many of the species belonging to the Geraniaceae family and the *Pelargonium* genus are

aromatic; *P. capitatum, P. graveolens* and *P. radens* are used in cultivation programs for the ennoblement of geranium oil. Studies on *Pelargonium* have focused on the chemical composition of the essential oils (Williams & Harborne, 2002). Rana et al. (2000) determined the presence of thirty compounds in the essential oil from *P. graveolens*, accounting for 99.1% of the oil. The main components identified were citronellol (33.6%), geraniol (26.8%), linalool (10.5%), citronellyl formate (9.7%), and p-menthone (6.0%).

Previous reports have documented the antimicrobial activity of the extracts of *Pelargonium* and their constituents against several bacterial and fungal pathogens (Mativandlela et al., 2006). Lalli et al. (2008) determined the minimum inhibitory concentrations of the essential oil of *P. graveolens* against the bacterial pathogens *S. aureus* (4000 mg mL^{-1}), *Bacillus cereus* (2000 $mg.mL^{-1}$), and *K. pneumoniae* (2000 $mg.mL^{-1}$) and the *Candida albicans* (3300 $mg.mL^{-1}$) strain of fungus.

Pelargonium odoratissimum (L.) L'Hér. is a plant for which little technical and scientific knowledge exists. It belongs to the Geraniaceae family, and its leaves are popularly used as a flavoring; as an insect repellent; in perfumery; and in aromatherapy for the treatment of gastrointestinal diseases, throat infections, and bleeding.

The studies that have been performed have furnished little information as to the identity of the chemical constituents in the essential oil of this species or its biological activities. Lis-Balchin & Roth (2000) reported methyleugenol, limonene and fenchone as main components of the essential oil of P. odoratissimum. There are no reports of studies on the antifungal activity of this essential oil or of extracts of this plant genus. Lis-Balchin et al. (1998) evaluated the antibacterial activity of the essential oil of *P. odoratissimum* against *S. aureus, Proteus vulgaris, Bacillus cereus* and *Staphylococcus epidermidis* and observed inhibition of bacterial growth. Thus, the purposes of this study were to analyze the essential oil of *P. odoratissimum* and to evaluate its biological effect on toxigenic fungi and pathogenic bacteria.

Materials and Methods

Essential oil

The leaves of *Pelargonium odoratissimum* (L.) L'Hér., Geraniaceae, were collected in the morning hours during June 2007 at the Medicinal Plants Garden of the Federal University of Lavras, Lavras-MG, Brazil. The species collected was properly identified and recorded in the ESAL Herbarium located in the Department of Biology at the Federal University of Lavras, with registration number 22.278.

The leaves of *P. odoratissimum* were steam distilled using a modified Clevenger-type apparatus. The essential oil was isolated with dichloromethane, which was completely evaporated under vacuum on a rotary evaporator and stored in sealed glass vials in a refrigerator prior to analysis (Guimarães et al., 2008).

Chromatographic analysis of the essential oil

The analysis of the volatile compounds was performed on a Shimadzu model GC-17 A GC-MS instrument under the following conditions: a fused silica capillary column (30 m x 0.25 mm) containing DB50.(film thickness, 25 µm), temperature program, 60-240 ºC (3 ºC/min); injector temperature, 220 ºC; carrier gas, helium, adjusted to a linear velocity of 32 cm/s (measured at 100 ºC); injection type, splitless (2 µL of a 1:1000 dilution in *n*-hexane); the split flow was adjusted to give a 20:1 ratio; septum sweep was a constant 10 mL/min; EIMS: electron energy, 70 eV; ion source temperature and connecting parts: 180 ºC. The quantitative data for the oils were obtained by peak area normalization using a Shimadzu GC 17 A gas chromatograph equipped with a flame ionization detector (FID) operated under the same conditions as the GC-MS. The individual components of oils were identified by comparison of the mass spectrum and the GC retention data with those of authentic compounds previously analyzed and stored in the data system. Additional identifications were achieved by comparison of the mass spectra with those existing in the data system libraries and/or cited in the literature (Adams, 2007; NIST, 2005). The retention index was calculated for all the volatile constituents using a homologous series of normal alkanes.

Biological activity of the essential oil

Inhibitory effect against fungus species

The assay was conducted separately for each fungus (*Aspergillus flavus* CML 1816, *Aspergillus carbonarius* CML 1815, and *Aspergillus parasiticus* CMLA 817). The effects of different concentrations (2, 1, 0.5, 0.25, and 0.10 $\mu L.mL^{-1}$) of the essential oil diluted in dimethylsulfoxide (DMSO) on the mycelial growth of a fungus culture were evaluated using the in vitro bioanalytical method. The plates containing the essential oil were compared with those of the absolute control (a plate containing only CZAPEK-DOX) and the relative control (a plate containing CZAPEK-DOX and DMSO to verify the effect of solvent on the mycelial growth). The essential oil dissolved in DMSO was added to the CZAPEK-DOX culture medium,

previously autoclaved and semi-liquefied, in an aseptic, laminar-flow hood. The culture medium was transferred to nine-centimeter Petri dishes, and the microorganism was inoculated in the center of the plate. The plates were incubated in a B.O.D. incubator at a temperature of 25 ºC, the optimum temperature for growth and production of mycotoxin (Pitt & Hocking, 1997).

The assessments were performed seven days after initiating the experiment by determining the averages of the diameters of the mycelial growth, measured in perpendicular directions. The percentage inhibition of mycelial growth was calculated using the formula proposed by Lindsey & Standen (2004).

Inhibition of bacterial growth

Strains of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25992 were employed for the evaluation of the *in vitro* inhibitory effect of the essential oil. The microorganisms were maintained in slants of tryptic soy agar (TSA) under refrigeration (4 ^{o}C), transferred to a brain and heart (BHI) infusion broth, and incubated at 37 ^{o}C for 24 h for activation of the culture. *E. coli* and *S. aureus* cultures were prepared in the eosin-methylene blue-agar (EMB) selective media and the standard counting agar (SCA), respectively, and incubated at 37 ^{o}C for 24 h. Three colonies of the same morphological type were selected from these plates and transferred to a tube with 5 mL of tryptic soy broth (TSB). The tubes were incubated at 37 °C until they reached or exceeded the turbidity of a McFarland 0.5 standard solution, resulting in a suspension containing 10^{8} CFU mL^{-1}. The turbidity readings were obtained at a wavelength of 625 nm using a Shimadzu UV-1601 PC spectrophotometer. Subsequently, the suspension of bacterial cells was transferred to a Mueller-Hinton culture medium, which was the medium used in agar diffusion. The Mueller-Hinton agar was inoculated with the cultures (*S. aureus* and *E. coli*) and deposited on a layer of the same agar, where wells had been prepared with the aid of glass beads. These wells were filled with 10 µL of DMSO containing different concentrations: 1, 5, 10, 50, 100, 200, 300, 400 and 500 $\mu L.mL^{-1}$ of the essential oil. The plates were incubated at 37 °C in a B.O.D. incubator for 24 h, and the diameters of the inhibition halos were measured. A relative control to which 10 µL of DMSO was applied and an absolute control without solvent were similarly incubated (NCCLS, 2005; Ogunwande et al., 2005).

Statistical analysis

A fully randomized block design with the bacteria factor as a block was used for the statistical analysis, in which the factorial scheme was 2 (9 x 2) (one essential oil, two repetitions, nine concentrations, and two bacteria). A completely randomized block design was used to study the inhibitory effect of fungi, with the fungi factor as a block in which the factorial scheme was 3 (6 x 3) (an oil, three repetitions, six concentrations and three fungi). The statistical program used was SISVAR (Ferreira, 2003). The treatments were submitted to the Scott-Knott test at 5% of significance for the analysis of variance.

Results and Discussion

The main component encountered in the essential oil was methyleugenol, with a retention time of 30.85 min. This compound is a phenylpropanoid present in the volatile fraction of a variety of herbs (De Vincenzi et al., 2000). It composed 96.80% of the oil. The other constituents in the essential oil and their proportions are listed in Table 1.

Table 1. Constituents of the essential oil of *Pelargonium odoratissimum* (L.) L'Hér., Geraniaceae, identified by GC/MS and quantified by gas chromatography.

RIc*	RIt**	Constituent	Abundance (%)
1405	1403	methyleugenol	96,8
1492	1492	methylisoeugenol	1,7
1495	1500	bicyclogermancrene	0,9
1557	1561	germancrene B	0,3

* RIc: Calculated Rentetion indices; **RIt: Tabulated Rentetion indices.

Data reported in the literature present few studies with respect to the chemical constitution of the essential oil of *P. odoratissimum* Lis-Balchin & Roth (2001) observed that the essential oil from the leaves of this plant contained methyleugenol as the major component, an observation that agrees with the result obtained in this work. However, the presence of other constituents found by those authors, such as limonene, fenchone, α-thujone, isomenthone, and *p*-cymene, was not confirmed in this work.

The effect of various concentrations of the essential oil from the leaves of *P. odoratissimum* on the mycelial growth of *A. flavus, A. carbonarius*, and *A. parasiticus* can be seen in Table 2. A significant inhibition of mycelial growth with increasing doses of the essential oil was observed for the three toxigenic fungi. The inhibitory activity of the essential oil was more efficient against *A. parasiticus* and *A. carbonarius*. Seven days after inoculation, 100% inhibition with the 0.5 $\mu L.mL^{-1}$ concentration was observed. *A. flavus* also presented little growth at this concentration. There are no reports of studies of the fungitoxic potential of the essential oil or

extracts of this plant against genera of toxigenic fungi. The essential oil presented a significant biological activity against *A. flavus, A. parasiticus*, and *A. carbonarius*.

Ruitón et al. (1999) found that the major component of the essential oil from *Ocimum micranthum* W was methyleugenol (52.02%). When they assessed the biological potential against the *Kleibsiella pneumonae, S. aureus, E. coli, Bacillus cereus, Shiguella* sp., *A. niger, A. flavus, A. fumigatus,* and *Penicillum* sp. microorganisms, they observed that a 1000 $\mu L.L^{-1}$ concentration of the essential oil inhibited the growth of *A. flavus*.

Lee (2007) found that methyleugenol (34.18%) and methylisoeugenol (4.9%) were the major components of the *n*-hexane fraction of the extract of *Acorus gramineus* rhizomes. At the concentration of 1000 $mg.L^{-1}$, this worker also observed activity against phytopathogenic fungi, with 77% inhibition of the growth of *Rhizoctonia solani* and 100% inhibition of *Phytophthora* weeds.

The inhibition halos of *S. aureus* ATCC 25923 and E. coli ATCC 25992 induced by the essential oil from leaves of *P. odoratissimum* were measured and demonstrated the existence of a small inhibitory effect that can be seen in Table 3. The growth of *E. coli* was inhibited by the essential oil from *P. odoratissimum* at a concentration of 100 $\mu L\ mL^{-1}$ (Table 3). The greatest inhibition occurred at the concentration of 300 $\mu L\ mL^{-1}$. The essential oil showed no inhibitory effect against *S. aureus* at concentrations of 1.0, 5.0, 10.0, 50.0 and 100 $\mu L\ mL^{-1}$, but it presented some inhibition at concentrations greater than 200 $\mu L\ mL^{-1}$, and the greatest inhibition occurred at a concentration of 500 $\mu L.mL^{-1}$.

When the essential oil from *P. odoratissimum* was assessed for activity against *S. aureus, Proteus vulgaris, Bacillus cereus,* and *S. epidermidis*, Lis-Balchin et al. (1998) observed a 0.8 cm inhibition halo for S. aureus at a 20% concentration of the oil in methanol. This result differs from the results obtained in the present study, since this level of inhibition for *S. aureus* was achieved at the 50% concentration of the oil in DMSO. However, there was no description of the quantity of microorganisms inoculated onto the plate in the above-mentioned study, and the quantity has a significant influence on the results.

The essential oil from *P. graveolens* was tested on 25 species of bacteria by Dorman & Deans (2000), who found that this oil did not present any activity against *E. coli.* An inhibition zone of 13.6 mm was observed when 15 µL of oil was employed, and the plates were inoculated with $1x10^5$ CFU of *S. aureus*. Although this type of microorganism belongs to the same genus, the results differ from those obtained in the present work. This fact can be explained by differences in the composition of the essential oil of *P. graveolens*, which presents geraniol as the major component.

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Table 2. Means for mycelial growth of *Aspergillus flavus, Aspergillus carbonarius* and *Aspergillus parasiticus* and means of the growth inhibition halos of the bacteria *Staphylococcus aureus* and *Escherichia coli* with the different treatments.

Concentration of the essential oil ($\mu L.mL^{-1}$)	Mycelial growth (cm)			Inhibition halo (cm)	
	A. flavus	*A. carbonarius*	*A. parasiticus*	*S. aureus*	*E. coli*
0	7.20 a	8.90 a	8.10 a	0.00 a	0.00 a
DMSO	7.15 a	8.80 b	8.10 a	0.00 a	0.00 a
0.10	3.90 b	1.80 c	2.50 b	-	-
0.25	2.15 c	1.10 d	1.65 c	-	-
0.50	0.30 d	0.00 e	0.00 d	-	-
1.00	0.00 e	0.00 e	0.00 d	-	-
2.00	0.00 e	0.00 e	0.00 d	-	-
50	-	-	-	0.00 a	0.00 a
100	-	-	-	0.00 a	0.65 b
200	-	-	-	0.50 b	0.75 b
300	-	-	-	0.50b	0.85 c
400	-	-	-	0.60 b	0.90 c
500	-	-	-	0.80 c	0.95 c

*Means followed by the same letter are equal by the Scott-Knott test ($\alpha = 5\%$). **Inhibition halo including the well's diameter. Well's diameter, 0,4 cm.

Table 3. Means of the growth inhibition halos of the bacteria *Staphylococcus aureus* and *Escherichia coli* with different treatments.

Concentration of the essential oil (μL/mL)	Inhibition halo (cm)	
	Staphylococcus aureus	*Escherichia coli*
0	0.00 a	0.00 a
DMSO	0.00 a	0.00 a
1.0	0.00 a	0.00 a
5.0	0.00 a	0.00 a
10	0.00 a	0.00 a
50	0.00 a	0.00 a
100	0.00 a	0.65 b
200	0.50 b	0.75 b
300	0.50b	0.85 c
400	0.60 b	0.90 c
500	0.80 c	0.95 c

*Means followed by the same letter are equal by the Scott-Knott test (α = 5%).
**Inhibition halo including the well's diameter. Well's diameter, 0,4 cm.

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