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Antimicrobial Activity of Essential Oils from *Pimenta pseudocaryophyllus* and *Tynanthus micranthus*

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ABSTRACT

The present study describes the chemical composition and antimicrobial activity of essential oils obtained by hydrodistillation from the leaves of Pimenta pseudocaryophyllus *(1.0% w/w) and* Tynanthus micranthus *(1.1% w/w). GC and GC/MS analysis demonstrated that eugenol was the only component in the* T. micranthus *essential oil (99.9%) and the major component in the* P. pseudocaryophyllus *essential oil (92.59%), which also presented methyleugenol, terpinen-4-ol, o-cymene and (E)-caryophyllene, among others. Both the oils presented antimicrobial activity against bacteria, yeast and filamentous fungi tested.The Bioautography test revealed that eugenol was the bioactive component in both the oils against* Cladosporium herbarum*. This is the first report about the* T. micranthus *essential oil, and the antifungal activity of* P. pseudocaryophyllus*. The results confirmed the potential of eugenol-rich essential oils not only as a source of flavor compounds, but also of use as antimicrobial agent in agriculture and in pharmaceutical and food products.*

Key words: *Pimenta pseudocaryophyllus*, *Tynanthus micranthus*, essential oil, antimicrobial activity

INTRODUCTION

Synthetic pesticides have been used for decades to prevent, control or eradicate the plant diseases. In spite of their efficiency, these compounds may disrupt the equilibrium of ecosystems leading to health and environmental problems. The adverse environmental impact caused by the first developed fungicides has resulted in the development of alternative methods for the pest and disease control in agriculture and forestry (Lee et al., 2008). Such issues have created a significant market opportunity for natural products, including the essential oils. These substances, obtained from the aromatic plants by hydrodistillation, have been traditionally used to protect the grains and vegetables during the storage (Murray, 2000), and their potential use as alternative medicine for the treatment of several infectious diseases have also been studied (Prabuseenivasan et al., 2006). Several studies have been carried out on the application of aromatic plants, not only in food

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manufacturing but also in pharmaceutical and cosmetic formulations (Guillén and Manzanos, 1996).

Eugenol, 4-allyl-2-methoxy-phenol, is found in the essential oils from the aromatic plants such as *Eugenia caryophillis* (clove), *Cinnamomum verum* (cinnamon) and *Ocimum gratissimum* (*alfavacão*) (Ali et al., 2005), and many studies have discussed their activities against several pathogenic bacteria and fungi (Shapiro et al.,, 1994; Ali et al., 2005; Faria et al., 2006). Recently, the applications of natural and synthetic eugenol and essential oils from *E. caryophyllata, Pimenta officinalis and Cinamomum zeylanicum* on the prevention and treatment of animal diseases caused by bacteria, fungi, and parasites have been patented (Vojin, 2005). In addition to these activities, eugenol has nematicide, insecticide and allelopathic effects, which inhibits the germination of various species (Mazzafera, 2003).

Eugenol has been used commercially as analgesic and skin disinfectants in dentistry, in the manufacturing of toothpastes, perfume and soaps, in histological clarification techniques, and as raw material to obtain vanillin, a compound widely used by the food industry. This compound has also been studied as raw material for the synthesis of biologically active natural products (Costa, 2000).

P. pseudocaryophyllus (Gomes) L. R. Landrum and *Tynanthus micranthus* Corr.Méllo ex K.Schum. are native species of the Brazilian flora with a clove-like taste. *T. micranthus* (Bignoniaceae) is used by popular medicine in the production of tea used in the healing process of colds, but there are no studies of this species. Data on *Tynanthus* genus are scarce; however, a study on the bark of *T. panurensis* reported the occurrence of verbascoside, isoverbascoside, leucosceptoside, and flavonoid, namely katchimoside and phenylpropanoid glycoside (Plaza et al.,2005), while a phytochemical investigation on *T. fasciculatus* led to the isolation of β-sitosterol-β-D-glucoside (Vilegas et al.,1993), coumarin, stigmasterol and melilotic acid derivative (Vilegas et al.,1995). The *Pimenta* genus (Myrtaceae) has been widely studied due to its biological properties, which include antimicrobial, anti-inflammatory, antinociceptive and hypotensive activities, among others (Fernández et al.,2001; García et al., 2004; Suárez et al.,1997). *P. pseudocaryphyllus* is used as flavor enhancer in the cooking and in the preparation of tea. The species presents an essential oil in the leaves whose composition seems to vary depending of the origin of the specimen (Lima et al., 2006; Sakita et al., 1994).

In continuation to the search of essential oils rich in eugenol, in order to test its antimicrobial effects, the essential oils of *Pimenta pseudocaryophyllus* and *Tynanthus micranthus* collected in São Jerônimo da Serra and Londrina, respectively, were analyzed. The Essential oils extracted from the fresh leaves of the plants were evaluated according to yield, chemical composition and activity against pathogenic bacteria and fungi, some of which were phytopathogenic and bringing losses to the agriculture.

MATERIALS AND METHODS

Plant material

The leaves of *P. pseudocaryophyllus* were collected in the county of São Jerônimo da Serra (Paraná, Brazil) in December/2007. The leaves of *T. micranthus* were collected in April/2008 at Universidade Estadual de Londrina Department of Agronomy – UEL (Londrina, Paraná, Brazil). Voucher specimens of *P. pseudocaryophyllus* and *T. micranthus* were prepared, identified, and deposited at the Universidade Estadual de Londrina Herbarium (FUEL 43025 and FUEL 45005, respectively).

Essential oil extraction

The essential oils were obtained from the fresh leaves (80 g) of both the species by hydrodistillation in a Clevenger apparatus for two hours. The distillate was extracted with dichloromethane (4 x 70 mL). The organic phase was dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. The method yielded 0.8 g of *P. pseudocaryophyllus* oil and 0.9 g of *T. micranthus* oil. The two oils were solubilized in dichloromethane for gas chromatography and mass spectrometry analysis.

GC and GC/MS analysis

The analysis of the volatile compounds were carried out on a Shimadzu gas chromatography model GC-17A, equipped with a flame ionization detector (FID) and a DB-5 (JandW Scientific) capillary column (30 m long, 0.25 mm internal diameter, 0.25 µm film thickness). The programmed temperature began at 60 ^{o}C and increased by 7 ^{o}C/ min up to 320 ^{o}C (5 min).

Injector and detector temperatures were 220 and 300 °C, respectively. Nitrogen was used as carrier, at a flow rate of 1.2 mL/min. The diluted samples (1:10 in dichloromethane, v/v) of 2.0 µL were injected and the split ratio was 1:20. The area of the GC peak was used for quantitative determination.

GC/MS-capillary gas chromatography was performed in a Shimadzu chromatography coupled to a Shimadzu GC/MS-QP5000 using a DB-1 capillary column (30 m x 0.25 mm) 0.25 µm film thickness. Injector and detector temperatures were 300 and 250 °C, respectively. The carrier gas was helium at a flow rate of 1.2 mL/min, and the programmed temperature began at 60 °C and increased by 7 °C/ min up to 320 °C (5 min). Mass spectra (electron impact) of the compounds were obtained using an ionization chamber at a temperature of 250 °C.

Identification of the oils constituents

Oil components were identified by comparing their mass spectra with NIST98 (National Institute of Standards and Technology, Gaithersburg) mass spectral database and by comparing their GC retention indexes (RI), related to the retention time of a series of *n*-alkanes with linear interpolation, with those of literature data (Adams, 2007). Eugenol mass spectrum and retention time were compared with data from an authentic sample. Relative amount of individual component was performed on the basis of their GC peak areas.

Antimicrobial activity

Microbial strains

The microorganisms used in the antimicrobial and antifungal tests were: (i) three clinically isolated yeast from the UEL School Hospital: *Candida albicans*, *Candida krusei* and *Candida tropicalis*; (ii) four referenced strains: two Gram-positive bacteria: *Bacillus subtilis* (ATCC 8272) and *Staphyloccocus aureus* (25923), two Gram-negative bacteria: *Pseudomonas aeruginosa* (27853) and *Escherichia coli* (25922) and (iii) five filamentous fungi cultures from the Biochemistry Laboratory collection of the Universidade Estadual de Londrina, Brazil: *Botryosphaeria rhodina*, *Botryosphaeria ribis*, *Claudosporium herbarum*, *Fusarium verticillioides* and *Lasiodiplodia theobromeae*. The strains were grown on a Mueller-Hinton agar (MHA) for bacteria, a Sabouraud Dextrose Agar (SDA) for yeasts and a Potato Dextrose Agar for moulds.

Preparation of inocula

Bacteria

The strains preserved in the Müeler-Hinton agar at 4 °C were revived in Müeller-Hinton solution and incubated at 37± 2 °C during 18-24 h. The inoculums for the tests consisted from a suspension of the cells (10^8 UFC/ mL using the Mc Farland scale).

Yeast

The strains preserved in the Sabouraud agar at 4 °C were revived in Sabouraud solution and incubated at 30±1 °C for 24-48 h. The inoculums for the tests consisted from a suspension of cells containing 10^8 UFC/ mL.

Filamentous fungi

The filamentous fungi preserved in the Potato Dextrose Agar at 4 °C were revived in Potato Dextrose Agar and incubated at 28± 2 °C for 5-10 days. The plugs of 7 mm diameter were used as inocula.

Antimicrobial screening

Three techniques were used to test the microbial activity for the two oils: the TLC bioautography (Homans and Funchs, 1970), the agar diffusion (Quiroga et al.,2001) and the microdilution test (NCCLS document M7-A6, 2003).

TLC bioautography

The essential oils were applied (0.25 mg/ spot) on the teo TLC plates and developed in a hexane/ dichloromethane mixture (50%). One plate was used as the reference chromatogram, and the other for the bioautography. A spore suspension of *C. herbarum* was sprayed over the developed TLC plate, which was incubated at 28 °C under humid conditions for three days (Homans and Funchs, 1970). The observed inhibitory zones were then correlated with the spots seen on the TLC plate used as the reference chromatogram and visualized under the UV light at 254 nm.

Agar dilution

The *in vitro* biological activity of the oils against the filamentous fungi was assessed according to their hyphal radial growth rate. Different volumes of both the oils were diluted in ethyl acetate and poured into Erlenmeyer flasks containing 16 mL hot sterilized growth medium (PDA). Afterwards it was thoroughly mixed and poured into Petri

dishes (60 x 15 mm). For control tests, the plates were prepared with ethyl acetate as solvent control, a plate with Captan (N-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide), as negative growth control, and a plate containing only PDA as positive growth control. The assay was performed by placing a 7 mm diameter plug of growing media onto the centre of a Petri dish containing the oil in the medium. This plug was taken from 5-10 days of culture. The plates were incubated for 5-10 days, to confirm the growth in the positive growth control plate.

Determination of the minimum inhibitory concentration (MIC)

A dilution agar method was used to determine the MIC. A 96-well plastic microplate was used. Each well contained 100 µL of Mueller-Hinton for the bacteria and Sabouraud for the yeasts, plus a fixed volume of serially diluted oil plants, with concentrations of 0.5 to 66 µL/ mL. Each well was incubated with a 100µL aliquot of the inoculum. Incubations were performed at 30 °C for 24 h for the bacteria and 25 °C for 48 h for the yeast. Next, 50 µL of aqueous solution of TTC (Triphenyl Tetrazolium Chloride) at 0.5% was added and the microplate was incubated for 3 h at the aforementioned temperature. The MIC was defined as the lowest concentration of oil that inhibited the microorganism growth.

RESULTS AND DISCUSSION

Chemical composition of oils

Essential oils yields were of 1.0 and 1.1% from *P. pseudocaryophyllus* and *T. micranthus*, respectively. The analysis revealed eugenol as the main constituent in *P. pseudocaryophyllus* oil (92.59%) and as the only component in *T. micranthus* oil (99.9%). The identity of the substance was confirmed through the comparison of its mass spectrum and retention index with an eugenol authentic sample. Results obtained in this first report on the composition of *T. micranthus* essential oil was similar to that obtained for *T. panurensis* cortex, which presented eugenol as the only constituent (99.9%) (Leclercq et al., 2000). The *P. pseudocaryophyllus* essential oil presented other components such as methyleugenol, terpinen-4-ol, *o*-cymene and (E)-caryophyllene, identified by the comparison of its mass spectrum and the Kowats Index with literature data (Adams, 2007).

Studies on the *P. pseudocaryophyllus* essential oil revealed different oil compositions. The analysis of the *P. pseudocaryophyllus* oil collected in Campos do Jordão, São Paulo state, showed the geranial (34.26%) and neral (27.85%) as main constituents, among others (Sakita et al.,1994). However, different results were obtained in the studies with the two specimens collected from other locations in the state of São Paulo (Lima et al., 2006). The essential oil in a specimen collected from Cardoso Island also presented eugenol as its main component (71.9%), while the essential oil of the specimen collected from Paranapiacaba county presented the 4-methyleugenol (94.6%) as its main component.

Other species of the *Pimenta* also contain essential oils. *P. dioica* leaves provided an essential oil with eugenol as its main constituent (77.9%) (Marongiu et al., 2005). The *P. racemosa* var. *terebinthina* essential oil presented α- terpineol acetate (37%), α- terpineol (20%) and methoxyeugenol (12.6%), as constituents, differently from *P. racemosa* var. *grisea* essential oil, which contains 4-methoxyisoeugenol (75.2%) and 4-methoxyeugenol (4.5%) (Garcia et al.,2002).

Antimicrobial activity

In the TLC bioautography, both the essential oils presented a spot (Rf= 0.29) with strong activity against *C. herbarum*. The active compounds of both the oils were isolated by the preparative thin-layer chromatography and identified as eugenol by GC/MS and by comparison with authentic sample.

Although the two oils presented the same main compound (eugenol) results were different in the tests. In the dilution agar test with filamentous fungi, the *T. micranthus* oil acted better than the *P. pseudocaryophyllus* oil. Both the oils showed good inhibition at the highest concentration. The *T. micranthus* oil inhibited more than 80% of the mycelial growth for *B. ribis* and 40 to 80% of the growth of other filamentous fungi tested. The *P. pseudocaryophyllus* oil presented an inhibition of 40 to 80% of the micelial growth for *B. rhodina* and *F. verticillioides* and up to 40% of inhibition against *B. ribis* and *L. theobrome* (Table 1). However, the microdilution test with bacteria and yeasts showed different results. The *P. pseudocaryophyllus* oil showed an equal or lower MIC than the *T. micranthus* oil for all the bacteria and yeasts tested (Table 2).

Table 1 - Percentage of inhibition promoted by *P. pseudocaryophyllus* and *T. micranthus* essential oils on the growth of fungi. The inhibition was reported as (-) <10% growth inhibition, (±) between 10 and 20%, (+) between 20 and 40%, (++) between 40 and 80%, and (+++) >80%.

Filamentous fungi	***P. pseudocaryophyllus* oil**						***T. micranthus* oil**					
	Concentrations (mg/plate)						**Concentrations (mg/plate)**					
	4.0	2.0	1.5	1.0	0.5	0.25	4.0	2.0	1.5	1.0	0.5	0.25
Botryosphaeria rhodina	++	+	+	+	-	-	++	+	±	-	-	-
Botryosphaeria ribis	±	-	-	-	-	-	+++	+	-	-	-	-
Fusarium verticillioides	++	+	+	±	-	-	++	+	+	+	-	-
Lasiodiplodia theobromeae	+	-	-	-	-	-	++	±	±	-	-	-

Table 2 - Minimum inhibitory concentration (MIC) of *P. pseudocaryophyllus* and *T. micranthus* essential oils.

Microrganism	MIC (μL/mL) *P. pseudocaryophyllus*	MIC (μL/mL) *T. micranthus*
Bacillus subtilis	17	17
Staphylococcus aureus	17	33
Pseudomonas aeruginosa	66	66
Escherichia coli	17	17
Candida albicans	4	17
Cândida krusei	4	17

Among the tested bacteria, *P. aeruginosa* was the most resistant and the MIC for both the oils showed high concentration on the microplate. This concentration was bactericidal for the *P. pseudocaryophyllus* oil and bacteriostatic for the *T. micranthus*. The same MIC was found for the *B. subtilis* and *E. coli*, and was bactericidal for the former and bacteriostatic for the latter for both the oils. For *S. aureus*, the *P. pseudocaryophyllus* oil presented better activity than the *T. micranthus* oil, and both the oils presented bactericidal activity.

Yeast tests showed that the MICs for both the oils were fungicidal; however, the *P. pseudocaryophyllus* oil presented the lower MICs than the *T. micranthus* oil, showing good activity against the yeasts tested.

There are no reports in the literature on the antifungal activity of *P. pseudocaryophyllus*. However, Lima et al. (2006) have determined the antibacterial activity of the essential oils in two *P. pseudocaryophyllus*, collected from the Cardoso Island and in Paranapiacaba, using the microdilution method. The eugenol-rich specimen from Cardoso Island presented the best results against *E. coli* while against *C. albicans*, the best result came from a specimen collected from the Paranapicaba County, rich in 4-methyleugenol. Both the specimens showed similar results against *P. aeruginosa* and *S. aureus*. The differences in the sensitivity observed were attributed to the different chemical composition: the presence of 4-methyleugenol increased its toxic effects against *S. aureus* and *C. albicans* and decreased the toxicity against *E. coli*. The presence of estragole might have contributed to the results by synergism with 4-methyleugenol.

There are reports that the *P. racemosa var. grisea* essential oil shows a more pronounced activity than the *P. racemosa* var. *terebinthina* oil against Gram (+) and Gram (-) bacteria. The antibacterial effect exhibited by *P. racemosa var. grisea* could be attributed to the presence of 4-methoxyeugenol (75.23%) and 4-methoxyeugenol (4.52%) in the oil. The antibacterial effect presented by the essential oil from the *P. racemosa* var. *terebinthina*, compared to the standards used or with other volatile oils as pine, cinnamon and thyme, was attributed to the terpene nature of the main components, α-terpineol acetate (27%) and α-terpineol (20%), of this oil (Saenz et al.,2004).

In the present study, the highest activity of *T. micranthus* essential oil against filamentous fungi could be attributed to the high concentration of eugenol present in this oil (99.9%), when compared to the content of eugenol present in the *P. pseudocaryophyllus* essential oil (92.59%). Furthermore, the slight differences of activity between the two oils tested suggested that these activities must also be related to other components present in the *P. pseudocaryophyllus* essential oil than in eugenol.

CONCLUSIONS

In this study, the chemical composition and antimicrobial activity of the essential oils from *T. micranthus* and *P. pseudocaryophyllus* were investigated. Both the oils were obtained in good yield (~ 1%). The GC and GC/MS analysis demonstrated that eugenol was the only component in the *T. micranthus* essential oil (99.9%) and the major component in the *P. pseudocaryophyllus* essential oil (92.59%), which also presented methyleugenol, terpinen-4-ol, *o*-cymene and (*E*)-caryophyllene, among others. The chemical composition of *P. pseudocaryophyllus* essential oil differed from those reported earlier. The pronounced activity against the bacteria, yeast and moulds demonstrated the antimicrobial potential of eugenol-rich essential oils and suggested the possibility of using these oils as natural food preservative and *in vivo* studies to develop the antimicrobials for human, animal and plants pathogens.

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RESUMO

O presente trabalho descreve a análise da composição química e a avaliação da atividade antimicrobiana dos óleos essenciais obtidos por hidrodestilação das folhas de *Pimenta pseudocaryophyllus* (1.0% m/m) e de *Tynanthus micranthus* (1.1% m/m). As análises por CG e CG-EM demonstraram que o óleo essencial de *P. pseudocaryophyllus* apresenta o eugenol como componente principal (92.6%), além de outros constituintes como methyleugenol, tepinen-4-ol, O-cimeno e (E)-cariofileno. O óleo de *T. micranthus* contém o eugenol como único constituinte (99.9%). Ambos os óleos apresentaram atividade contra bactérias, leveduras e fungos filamentosos. O teste de bioautografia revelou o eugenol como a substância responsável pela atividade contra o *Cladosporium herbarum* dos óleos das duas espécies. Este é o primeiro estudo sobre o óleo essencial de *T. micranthus* e o primeiro relato sobre a atividade antifúngica do óleo essencial de *P. pseudocaryophyllus*. Os resultados obtidos confirmaram o potencial de óleos essenciais ricos em eugenol para uso como agentes antimicrobianos na agricultura e na preparação de produtos alimentícios e farmacêuticos.

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