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Chemical composition and antibacterial activity of *Cymbopogon citratus* and *Cymbopogon flexuosus* essential oils

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Abstract

Plant secondary metabolites have attracted considerable attention from the industry as consumers are increasing their interest for natural products over chemically synthesized reagents to be used as additives in food, cosmetics or pharmaceuticals. Some plant essential oils show significant antimicrobial properties and this could be exploited to produce new food preservatives or novel antimicrobial formulations. Here we describe the extraction, chemical analysis and antimicrobial properties of grass lemon Cymbopogon citratus *and* Cymbopogon flexuosus *essential oils. Essential oils were extracted from dried leaves using hydrodestilation and their composition was established by gas chromatography coupled to mass spectrometry. Agar diffusion assays indicated that* C. citratus *and* C. flexuosus *essential oils act as antimicrobial agents against both gram negative and gram positive model organisms. These data support that lemon grass essential oils can be used as an alternative for microbiological control.*

Key words: *Antimicrobial. Essential oil. Natural product*

1 Introduction

Plant secondary metabolites constitute a range of different chemical compounds most of which are members of the phenylpropanoids and terpenoids family (OLIVEIRA et al., 2011). These molecules may act as chemical connections between the plant and the environment, playing roles in plant survival, defense and adaptation (GEROMINI et al. 2012; OOTONI et al. 2013).

The use of plant metabolites is attracting considerable market attention because consumers tend to opt for natural products due to their health benefits and low environmental impacts (Pereira et al. 2008). Plant essential oils are plant secondary metabolites that can be used in food, cosmetic, pharmaceutical and perfumery industry (GEROMINI et al. 2012; EKPENYONG END AKPAN, 2015; LEITE et al. 2016).

Plant essential oils can also be applied in the biocontrol of phytopathogens such as insects, fungi and bacteria. Hence, these molecules may be a sustainable alternative for agrochemicals (OOTONI et al. 2013; FONSECA et al. 2015).

Despite having a huge and diverse local flora, many exotic plant species have been introduced in Brazil since colonization (DUARTE, 2008), including the species *Cymbopogon citratus* and *Cymbopogon flexuosus* belonging to the Poaceae family. *C. citratus* originated from West India and *C. flexuosus* from east India. These two species are known as lemongrass and produce a chemical compound named citral which is an isomeric mixture of neral and geranial and gives these two plants a typical flavor (AKHILA, 2009; NISHIJIMA et al. 2014; COBOS, 2015).

The emergence of bacterial strains that are resistance to multiple antibiotics and chemicals is common treat to human health (SANTOS AND CUNHA, 2007). Antimicrobial compounds are defined as chemicals that have the ability to cause death or inhibition in microbial growth by diverse mechanisms (SOUZA AND RODRIGUES, 2012). One alternative for the substitution of classical antibiotics is the use plant natural products (Duarte, 2008).

Here we report the extraction, chemical composition and antimicrobial activities of essential oils produced by *C. citratus* and *C. flexuosus* that were cultivated in South Brazil. The oils produced by both plant species were able to inhibit the growth of both Gram-negative and Gram-positive model organisms.

2 Materials and methods

2.1 Botanical material

Plants were collected at the Experimental Farm of Canguiri, Federal University of Paraná, Pinhais, PR, Brazil on June, 2015. Plants were transported to the Herbarium of the Municipal Botanical Museum in Curitiba, PR, where they were herborized (LAWRENCE, 1951; IBGE, 1992), and registered in the collection. The species of the study were identified by depositing the exsicata of *C. citratus* in the herbarium of the Municipal Botanical Museum of Curitiba with the number MBM 389414 and the specimen of *C. flexuosus* in the herbarium Royal Botanic Gardens, Kew with the number H2014/02284.

2.2 Extraction of essential oils

The extraction of the essential oil was accomplished by hydrodistillation for 2.5 hours in a Clevenger type graduated apparatus using 100 g of fresh leaves in 1L of distilled water with 3 replicates (WASICKY, 1963). After the extraction, the samples were collected with precision pipette and conditioned in a freezer where they remained until the moment of the analysis.

2.3 Determination of the chemical composition of essential oils

Identification of the chemical constituents was performed by gas chromatography coupled to mass spectrometry (GC-MS). The essential oils were diluted in dichloromethane at the ratio of 1% and 1µl was injected into a gas chromatograph 6890 (Agilent) coupled to a mass detector 5973N. The injector was maintained at 250°C. The separation of the constituents was performed using a capillary column HP-5MS (5%-phenyl-95%-dimethylpolysiloxane, 30 m x 0,25 mm x 0,25 µm) and helium as carrier gas (1,0 mL min-1). The oven temperature was programmed from 60 to 240 ° C at a rate of 3°C min-1. The mass detector was operated in the electronic ionization mode (70 eV), at a rate of 3.15 scans s-1 and range of 40 a 450 u. The transfer line was maintained at 260º C, the ion source at 230º C and the quadrupole analyzer at 150º C.

The identification of the chemical constituents was obtained by comparing their mass spectra with a library (WILEY, 1994; NIST, 2016), and also by their linear retention rates, calculated from the injection of a homologous series of hydrocarbons (C_7 - C_{26}) and compared with data from the literature (ADAMS, 2007).

For quantification, the diluted samples were injected into an Agilent 7890A chromatograph equipped with a flame ionization detector (DIC), operated at 280°C. The same column and analytical conditions described above were employed except for the carrier gas used, which was hydrogen, at a flow rate of 1,5 mL min-1. The percentage composition was obtained by electronic

integration of the DIC signal by dividing the area of each component by the total area (area %).

2.4 Antibacterial activity assay

The bacterial species used in this work were *Escherichia coli* NCM 3722 (SOUPENE et al. 2003), and *Bacillus thuringiensis israelensis* HD-500 (ZEIGLER, 1990). The culture media used were prepared according to Sambrook (1989). Isolated colonies were cultured in LB medium and incubated on a rotating shaker at 30° C for *B. thuringiensis* and 37° C for *E. coli* for 24 hours. After this period a 1:5 dilution was performed in fresh LB to obtain an suspension containing approximately 2.10^8 cells/mL ($D.O_{600nm}$=0,3). An aliquot of 100µL of this dilution was evenly distributed on the surface of the solid medium in petri dishes to form the carpet of cells over the agar. Assays using *B. thuringiensis* were performed in LB-agar and the assays with *E. coli* were performed using M9-agar.

The antibacterial activity of the essential oils was determined using the agar diffusion technique. After letting dry the bacterial sample over the plate, 6 mm diameter wells were drilled with a sterile perforator, these wells received 60µl of the essential oils to be tested. Mineral oil was used as a negative control and kanamycin 200µg/mL was used as a positive control. The plates were incubated at 30°C for *B. thuringiensis* and at 37°C for *E. coli*. After 24 hours the antibacterial activity was evaluated by measuring the halo of the inhibition zone of microbial growth around the wells. Analyses were performed in duplicates and results are presented as average ± standard deviation.

3 Results and discussion

Dried leaves of *Cymbopogon citratus* and *Cymbopogon flexuosus* were subjected to essential oil extraction by hydrodestilation and its composition was established by gas chromatography coupled to mass spectrometry (Table 1). The essential oils from both species had very similar composition; the major component was citral, which is an isomer mixture of neral and geranial. Other components present in both species were linalol, geraniol and geranyl acetate. B-mircene was only identified in *C. citratus* composing up to 14% of the essential oil extracted from this species (Table 1). The composition of the oil of C. citratus is very similar to previously reported in the literature, the headline that some variations are justified by the different types of climate, soil composition, season extraction, among others (PEREIRA et al. 2008; SACCHETTI et al. 2005; ONAWUNMI et al. 1984).

Table 1 - Chemical composition (%) of essential oil extracted from leaves of Cymbopogon citratus and Cymbopogon flexuosus.

Compounds	IRa	IRb	Cymbopogon citratus	Cymbopogon flexuosus
β-Mircene	990	998	14.1	ND
Linalool	1099	1095	0.8	0.7
Neral *	1241	1235	29.6	31.4
Geraniol	1254	1249	5.7	3.9
Geranial *	1272	1264	39.7	46.4
Geranyl Acetate *	1384	1381	1.8	3.3
Others			5.9	10.9
Total identified			97.6	96.6
Citral*			69.3	77.8

(*): monoterpenes that make up the Citral. IRa: Calculated retention index. IRb: retention index of the literature. ND – Not detected.

The antibacterial effects of these essential oils we tested against two unrelated bacterial species: *E. coli,* used as gram negative model organism and *B. thuringiensis,* was used as a gram positive model organism. Bacterial cells were replicated in LB medium overnight and cultured in solid medium for 24 hr; antimicrobial activity was determined by the Agar diffusion method. The inhibitory effect was assessed by measuring the inhibition zone surrounding the wells containing the essential oil. Kanamycin at 200µg.ml^{-1} was used as a positive control, this concentration of kanamycin is 10x above the concentration normally employed to inhibit the growth of *E. coli*. Hence, oils capable of generating inhibition zones larger than the positive control may be considered good candidates to be employed as antimicrobial agents.

Oils from both species, *C. citratus* and *C. flexuosus*, were effective against *E. coli* and *B. thuringiensis*. The results reported in Table 2 indicate that the inhibition of growth from these oils were higher than that of the positive control kanamycin, the only exception being the *C. citratus* oil against *E. coli* (Table 2).

The Gram-positive bacterium *B. thuringiensis* showed greater sensitivity than *E. coli* not only to the action of the oils but also to the action of the control antibiotic kanamycin (Table 2), this may be explained by the differences in the cell structures and/or metabolism between these two bacteria. Alternatively, these differences may be just related to the differences in doubling times between *E. coli* and *B. thuringiensis* as the size of the growth inhibition zones will also dependent on this variant. Antibacterial activity has already been described for *C. citratus* essential oil and in as observed here, increased efficiency was observed against gram positive bacteria (PEREIRA et al. 2008; SACCHETTI et al. 2005; ONAWUNMI et al. 1984; NAIK et al. 2010; MACHADO et al2015; LUCENA et al. 2013).

Table 2 - Antibacterial activity of essential olis.

Essencial oil	E. coli Inhibition zone (mm)	B. thuringiensis Inhibition zone (mm)
Cymbopogon citratus	2.7 ± 0.5	20.0 ± 0.5
Cymbopogon flexuosus	4.5 ± 1.0	27.0 ± 0.5
Kanamycin *	5.0 ± 1.0	10.0 ± 1.0
Mineral oil**	-	-

The values represent the mean inhibition zone ± DPM (Standard deviation of the mean) in mm from two independent replicates. * Positive control; ** Negative Control; - no inhibition zone detected. Dilutions of the essential oils were also evaluated using mineral oil as solvent; however, no significant inhibition of bacterial growth could be detected from 10% (v/v) dilution.

The *C. flexuosus* oil showed higher activity against both gram positive and gram negative Bacteria in comparison to the oil extracted from *C. citratus* (Table 2). The reason for these differences are unknown but is tempting to speculate that the higher citral content in the oil from *C. flexuosus* is responsible for its high antibacterial activity. Indeed, previous analysis using fractionated *C. citratus* oil indicated that citral is the major component responsible for the antibacterial activity of the essential oil, on the other hand, purified β-mircene had no significant antibacterial activity in isolation (ONAWUNMI, 1984).

Alternatively, other minor components not chemically assigned in the GC-MS analysis could be responsible for the differences in the antibacterial activities between the *C. flexuosus* and *C. citratus* oils (Table 1).

Essential oils have complex composition; hence, it is difficult to assign the inhibitory effect to a certain compound. Furthermore, the mechanism of bacterial growth inhibitory is not understood but is probably related to the hydrophobic interaction between oil components with the cell membrane causing its membrane disruption (DORMAN AND DEANS, 2000; FERREIRA et al. 2014). Some authors suggested that the anti-bacterial activities of essential oils are related to the major components present in the oil (AKHILA, 2009; SACCHETTI et al. 2005; LUCENA et al. 2013).

In contrast, other studies showed that the inhibitory effect can be attributed to a synergic action between major and minor components in the essential oil mix (AKHILA, 2009). Further studies will be necessary to determine the molecular basis for the different antibacterial activities of *C. flexuosus* and *C. citratus* essential oils.

4 Conclusion

The essential oils from *C. flexuosus* and *C. citratus* aromatic plants had citral as major component with β-mircene being found only in *C. citratus*. Both oils were able to inhibit growth of gram negative and gram positive bacteria with *C. flexuosus* presenting higher antibacterial activity. The results suggest that lemon grass oils can be used as a sustainable alternative in microbiological control.

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