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Chemical composition and antibacterial activity of the essential oil from *Eucalyptus pellita* F. Muell.

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The essential oil from leaves of *Eucalyptus pellita* F. Muell. was obtained by the hydrodistillation method and forty-three compounds in quantities higher than 0.02% were detected by gas chromatography/mass spectrometry analysis. The α-pinene (27.19% of the total amount) was the major component, followed by limonene (23.84%) and 1,8-cineole (19.01%), and considerable quantities of *p*-cymene, α-terpineol and γ-terpinene were identified too. Compounds such as 1,8-cineole and α-terpineol had not been previously reported in this essential oil. The antibacterial activity of the essential oil on *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* ATCC strains was evaluated by the agar disc diffusion method. The essential oil was active against all tested microorganisms with inhibition zone diameters from 8 to 24 mm; although a high activity (effective) against Gram-positive strains was observed.

Key words: Essential oil, chemical composition, antibacterial activity, *Eucalyptus pellita*.

INTRODUCTION

Natural plant products have been composed of phyto-medicine throughout human history and in the past 200 years, plants have become an important source for novel pharmaceutical compounds (Vaghasiya et al., 2008). In this sense, natural medicine has given special importance to the search for antimicrobial agents as a consequence of the increasing resistance of bacterias to commercial antibiotics (Warnke et al., 2009).

Bacterias are responsible for well known diseases as tuberculosis, pest, syphilis, tetanus, cholera and many types of pneumonia. It has been verified that 93% of wound infections are caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In addition, *S. aureus* has been isolated from the 40% of the skin and mucous infections while *P. aeruginosa* has been responsible for the 12% (Kirby et al., 2002). In other cases, micro-organisms taken from bacteriemias, gastroenteritis and urinary tract infections are almost all caused by *Escherichia coli*, a pathogen widely dispersed in nature that produces the major part of child dearreas (Gransden et al., 1990).

Many researchers have appealed to the plant kingdom looking for biological active compounds, such as those contained in the *Eucalyptus* genus essential oils, with a lot of biological activity reported and traditionally used by population with medicinal ends. They are extremely effective as respiratory decongestants, anti-inflammatory, analgesic, antibacterial, antifungal, anti-tumor and anti-diabetic. Also, they are common ingredients in over-the-counter cold remedies today and have been long used as

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an effective treatment for colds, flu, sore throats, bronchitis, pneumonia and headache (Vaghasiya et al., 2008).

Additionally, some essential oil components from the *Eucalyptus* genus have proved biological activity, for example: the 1,8-cineole has shown ovicidal and larvicidal activity on *Haemonchus contortus* (Macedo et al., 2009) and to *p*-cymene, a repellent activity on *Aedes aegypti* mosquitos and on *Leptotrombidium chiggers* larvas has been reported (Lucia et al., 2009). Compounds such as α-pinene and globulol have antibacterial properties and to citronellal, an important anticaries activity has been reported (Ben Marzoug et al., 2010).

Despite this, the residual foliage from the forest exploitation of *Eucalyptus* species is generally trashed in Cuba. This foliage has good percentages of essential oils and some of the previously mentioned active compounds; which have been less studied as antibacterial agents (Álvarez et al., 2007; García et al., 2004). So the aim of the present work was to determine the chemical composition of the *Eucalyptus pellita* F. Muell. essential oil and to evaluate its antibacterial activity against some medically-important bacterial strains.

MATERIALS AND METHODS

Plant

Fresh leaves of *E. pellita* F. Muell. were collected in October 2010 from a few 6 years old trees and about 8 m of high, in the forest of the Granma University, Bayamo, Granma, Cuba. Specimens were authenticated by Dr. C. Rolando Quert Álvarez from the Higher Institute of Technologies and Applied Science (specialist in the botanic of the *Eucalyptus* genus).

Essential oil extraction

The plant was chopped into small pieces and subjected to simple hydrodistillation for 3 h using a Clevenger type apparatus. The obtained essential oil was dried with anhydrous Na_2SO_4, weighed and stored in a sealed dark glass under refrigeration conditions. The average yield of essential oil (ratio final volume of essential oil/weight of fresh vegetal material) from four ended distillations (200 g each) was 0.89%.

Gas chromatography/mass spectrometry (GC/MS) analyses

The essential oil was submitted to GC/MS analysis (0.2 µl of the sample) in a Shimadzu GC/MS model QP2010 gas chromatograph equipped with a mass spectrometer and a flame ionization detector. An apolar DB-5 fused silica capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness) was used. Detector and injector temperatures were set to 250°C and the last one operated in split mode at ratio 30:1. Helium was used as carrier gas at flow rate of 1 ml/min. The oven temperature program was 2 min at 60°C, increase to 100°C at 4°C min^{-1}, stand for 5 min and heat at 10°C min^{-1} until 200°C and finally held under isothermal conditions by 10 min at this temperature. Mass spectrums were recorded from 30 to 400 m/z.

These conditions were maintained for all analyzed essential oil samples with 25 min as the run-time in all cases. Individual components were identified by comparing retention times, retention indices (by Kovats) and mass spectrums with those from authentic compounds previously analyzed and reported in NIST Library (Massada, 1976), described by Adams (1995) or existing in literature (Cunico et al., 2007; Simionatto et al., 2005). Retention indices were determined after analysing a linear C_9-C_{17} hydrocarbon series in the GC/MS, at same conditions of the essential oil samples.

Microbial strains

The microorganisms were supplied by the Study Center of Vegetal Biotechnology of the Granma University. Four bacterial strains included in the American Type Culture Collection were used for the assay: *S. aureus* (ATCC 15008), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 113-3) and *Bacillus subtilis* (ATCC 6633). These bacterias were selected as they are frequently reported in human diseases and have shown a high resistance to commercial antibiotics (Warnke et al., 2009).

Preparation of inoculums

Four colonies of each bacterial culture were taken for inoculum preparation. They were prepared by growing cells in 10 ml of Muller-Hinton broth (BioLife) and incubating at 35°C during 6 h under agitation conditions at 100 rpm (MLW, Germany). The McFarland equivalent concentration of inoculums was about 1.5×10^8 CFU/ml.

Antibacterial activity test

The disc diffusion method adopted by the Cilincal and Laboratory Standards Institute (CLSI, 2007) was used for evaluating the antibacterial activity of the essential oil. Petri plates (Alumbra, Chinese, 90 mm diameter) containing 20 ml of Muller-Hinton agar medium were allowed to solidify and then were aseptically covered with 1 ml of inoculums prepared before. Sterile filter papers (PB-SA, Brazil) chopped in 6 mm of diameter discs were placed in the Petri plates and they were impregnated with 6 µl of the sample.

Pure essential oil and two dilutions at 50 and 25% in dimethylsulphoxide (DMSO) were tested. For each experiment 6 µl of DMSO were used as negative control. Amikacin, Gentamycin and Chloramphenicol (30 µg, Sensi-DiscTM, France) were used as positive controls. The plates were incubated during 18 h at 37°C and finally the antibacterial activity was estimated as the average of the circular inhibition zone diameter in millimeters, measured from triplicate tests.

Statistical analyses

All experiments were statistically analyzed by determining the Least Significant Difference at $p < 0.05$ with the Sheffe test, included in the analysis of variance (ANOVA) techniques of the Statistica® Software Package (StatSoft, 2007).

RESULTS

The percentage yield of essential oil obtained from the hydrodistillation process of the fresh leaves of *E. pellita* F. Muell. was 0.89 ± 0.03% (v/w).

A total of 43 peaks (with base line at 0.02%) were detected from the GC/MS analysis of the EO, while only 28 of them with percentage concentrations higher than

Table 1. Chemical composition of the *E. pellita* F. Muell essential oil.

Peak	[a] Compounds	Formula	MW (g/mol)	[b]Rt (min)	[c]Ri	Essential oil (%)
01	Isovaleric aldehyde	$C_5H_{10}O$	86	1.966	-	2.86
02	Isoamyl acetate	$C_7H_{14}O_2$	130	4.658	-	0.16
03	α-pinene	$C_{10}H_{16}$	136	6.081	940	27.19
04	camphene	$C_{10}H_{16}$	136	6.461	954	0.82
05	α-pinene	$C_{10}H_{16}$	136	7.287	980	0.52
07	α-phellandrene pelandreno	$C_{10}H_{16}$	136	8.219	1005	0.21
08	α-terpinene	$C_{10}H_{16}$	136	8.477	1018	0.23
09	*p*-cymene	$C_{10}H_{14}$	134	9.018	1029	7.69
10	limonene	$C_{10}H_{16}$	136	9.231	1031	23.84
11	1,8-cineole	$C_{10}H_{18}O$	154	9.321	1034	19.01
12	β-ocimene	$C_{10}H_{16}$	136	9.540	1039	0.22
13	γ-terpinene	$C_{10}H_{16}$	136	10.360	1060	3.32
14	terpinolene	$C_{10}H_{16}$	136	11.401	1086	0.42
15	linalool	$C_{10}H_{18}O$	154	11.740	1096	0.17
17	fenchol	$C_{10}H_{18}O$	154	12.197	1112	0.59
18	α-campholenal	$C_{10}H_{16}O$	152	12.582	1120	0.40
19	*trans*-pinocarveol	$C_{10}H_{16}O$	152	12.971	1144	0.52
20	borneol	$C_{10}H_{18}O$	154	13.740	1167	1.36
21	4-terpineol	$C_{10}H_{18}O$	154	14.041	1175	1.05
24	α-terpineol	$C_{10}H_{18}O$	154	14.401	1190	3.86
26	*trans*-carveol	$C_{10}H_{16}O$	152	15.318	1209	0.14
31	α-terpineol acetate	$C_{12}H_{20}O_2$	200	18.012	1252	1.18
34	*trans*-β-caryophillene	$C_{15}H_{24}$	204	19.797	1426	0.28
35	spathulenol	$C_{15}H_{24}O$	220	23.871	1564	0.73
36	globulol	$C_{15}H_{26}O$	222	23.959	1572	0.58
37	*epi*globulol	$C_{15}H_{26}O$	222	24.064	1589	0.25
40	epi-γ-eudesmol	$C_{15}H_{26}O$	222	24.422	1618	0.23
42	α-cadinol	$C_{15}H_{26}O$	222	24.551	1640	0.15
Total of identified compounds			-	-	-	97.98

[a]Identified compounds (per cent > 0.1%) listed by their elution order from a DB-5 apolar capillary column; [b]Retention times in the chromatographic column; [c]Retention indices according to Kovats and determined with the C_9-C_{17} linear hydrocarbons.

0.1% were identified (consisting the 97.98% of total chromatographic area). Table 1 shows the main results obtained from the qualitative-quantitative analysis of these identified compounds.

Chemical composition of the essential oil shows some difference while comparing to that reported in literature (Miranda et al., 1986; Orwa et al., 2009). Major components were α-pinene (27.19%), limonene (23.84%), 1,8-cineole (19.01%), *p*-cymene (7.69%), α-terpineol (3.86%), γ-terpinene (3.86%) and isovaleric aldehyde (2.86%), representing the 87.77% of the total essential oil.

The bacterial growth of all tested microorganisms was inhibited by the pure essential oil samples and a decrease of this antibacterial activity was observed when diluting the oil in DMSO (at 25 and 50%). Results from the antibacterial activity assay while testing the pure EO and Amikacin, Gentamycin and Chloramphenicol as positive controls under the four bacterial strains used in experiments, are shown in Figure 1.

According to susceptibility classification tables for microorganisms published by the CLSI, *S. aureus* (ATCC 15008) and *E. coli* (ATCC 113-3) strains were susceptible to all positive controls used in the assay, with inhibition zone diameters ranging from 16 to 24 mm (Figure 1). *P. aeruginosa* (ATCC 27853) was susceptible to Amikacin (19 mm), showed intermediate susceptibility to Chloramphenicol (17 mm) and was resistant to Gentamycin.

The inhibition zone diameter of the essential oil against the *S. aureus* strain did not differ significantly ($p < 0.05$) when compared with those obtained from Gentamycin and Chloramphenicol (Figure 1). Although a higher inhibition activity than the obtained one with the Amikacin control was observed.

The inhibition activity of the essential oil against the *B. subtilis* strain was similar to that observed for Amikacin and Gentamycin, but it was considerably higher than the resulting one for Chloramphenicol. *B. subtilis* is not a

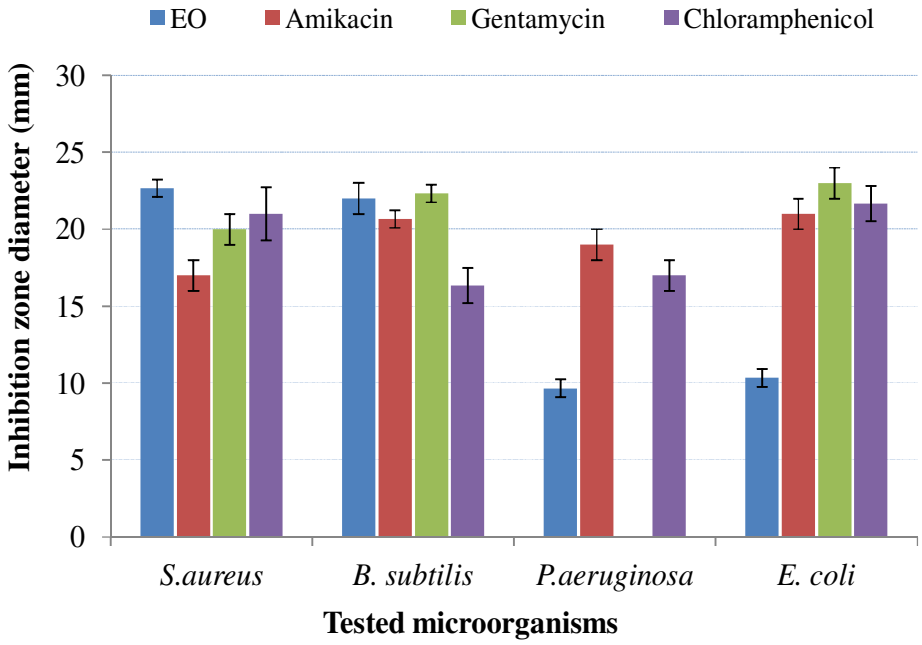

Figure 1. Antibacterial activity assay of essential oil (vertical lines above bars represent the standard deviation of three measured values).

pathogen interest bacteria, but it was tested as biological reference model for *S. aureus* (both are Gram-positive bacterias). In fact, the inhibition zone diameters obtained for these two bacterial strains were not statistically different at a confidence level of 95% ($p < 0.05$).

The inhibition zone diameters of the essential oil on the Gram-negative strains were not as higher as for the Gram-positive ones (Figure 1). It must be consider that the concentration difference between commercial antibiotics and active components of essential oil (none in more than 30%) is relevant. Nevertheless an inhibitory activity of the essential oil over the *P. aeruginosa* strain was observed, while this pathogen shows an extreme resistance to Gentamycin with no evidence of inhibition zone.

DISCUSSION

The essential oil yield was higher than that previously reported for the *E. pellita* using the same extraction method (0.1 to 0.5%, v/w) (Miranda et al., 1986). This difference could be associated to the fact of having gathered the foliage in October, when the content of volatile metabolites in *Eucalyptus* spp. is higher. Also, it can be related to the hybridization of *E. pellita* with *Eucalyptus citriodora* Hook and *Eucalyptus saligna* Smith in the gathering area. These species have shown high yields of essential oil from its leaves (3 to 6%) according to Lastra (1979).

The presence of α-pinene, limonene, *p*-cymene and γ-terpinene in this essential oil has been recently reported in the Agroforestry tree reference database (Orwa et al., 2009). The determined percentage concentrations for those compounds are agreed with the ranges informed in that reference. However, to our knowledge, this is the first study reporting the presence of 1,8-cineole and α-terpineol for the *E. pellita* F. Muell. Essential oil. It was suggest that the variability of its chemical composition depends closely on the recollection date, hybridization and growth conditions.

From preliminary antibacterial activity studies was concluded that the pure DMSO (negative control) has no biological effects on the evaluated microorganisms. As a result, was acceptable to assume that the inhibitory activity perceived for essential oil during antibacterial assays is closely related to the presences of biological active compounds on it. These results agreed with those obtained in others related research, which has been verified that for higher concentrations of the diluted essential oil samples, higher inhibition zones diameters are obtained (Ávila et al., 2006).

High inhibition zone diameters for essential oil in front Gram-positive strains could be useful for pharmaceutical uses, as they suggest the possibility to employ this oil for the treatment of several diseases caused by *S. aureus* and *B. subtilis*, with equal or better results to those obtained using commercial antibiotics. Besides, an anti-

bacterial formulation based on components from the studied essential oil may be an unknown active principle for these pathogens, and well could decrease their resistance (Mulligan et al., 1993).

Comparing the obtained results with those from others similar studies (evaluation of biological activity of essential oil), we consider that the *E. pellita* F. Muell. essential oil has a high potential for antibacterial uses. We found reports of antibacterial activity for two essential oils from the Myrtaceae family against *P. aeruginosa* and *S. aureus*, but they were inactive against some resistant *E. coli* strains (Apel et al., 2006). Essential oil under study showed a considerable activity in front of these three microorganisms. Additionally, against *S. aureus*, the *E. pellita* essential oil shows inhibition zone diameters higher than the obtained ones for essential oil of *Eucalyptus robusta* and *Eucalyptus saligna* (Sartorelli et al., 2007).

Conclusions

Finally, it is concluded that the *E. pellita* F. Muell. essential oil is of monoterpenoide type, containing α-pinene, limonene, 1,8-cineole, *p*-cymene, α-terpineol and γ-terpinene as major compounds. This oil has antibacterial activity against bacterial strains such as *P. aeruginosa*, *E. coli*, *S. aureus* and *B. subtilis*, which could play an important role to control several diseases caused by these microorganisms, particularly from the Gram-positive ones.

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ABBREVIATIONS

GC, Gas chromatography; **MS,** mass spectrometry; **ATCC,** American type culture collection; **EO,** essential oil(s); **CLSI,** Clinical Laboratory Standard Institute; **DMSO,** dimethylsulphoxide.

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