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Antifungal activity and gas chromatography coupled with mass spectrometry (GC-MS) leaf oil analysis of essential oils extracted from *Eucalyptus globulus* (Myrtaceae) of north centre region of Morocco

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Essential oil from the leaves of *Eucalyptus globulus* collected in north centre region of Morocco obtained by hydro-distillation were analyzed by gas chromatography equipped with flame ionisation detector (GC-FID) and gas chromatography coupled with mass spectrometry (GC-MS). To evaluate the antifungal activities of these aromatic extracts, their *in vitro* antifungal activities were determined by disk diffusion testing to find out minimum inhibitory concentration (MIC). *Penicillium citrinum* was used as test fungal strains. The results of the study revealed that essential oil yields and the total oil of *E. globulus* were 1.21 and 63.96%, respectively. 54 compounds were identified in the essential oils and the main constituents of the essential oils were: 1.8-cineole (22.35%), limonene, (7.01%), solanol (6.05%), β-pinene (5.20%), trans-verbenol (4.02%), terpinen-4-ol (3.10%), aristolene (2.35%), terpinyl acetate (2.10%), isosativene (1.85%), sabinene (1.49%), α-myrcene (1.15%) and α-terpineol (1.10%). The essential oil of *E. globulus* exhibited the activity against, *P. citrinum* exerting the minimum inhibitory concentration values (MIC) ranging from 3.07 to 96.14 µl/ml, respectively. These results showed that extracts could be considered as a natural antifungal source that can be used for production of natural antifungal agents.

Key words: *Eucalyptus globulus*, essential oil, gas chromatography coupled with mass spectrometry (GC-MS), antifungal activity, 1.8-cineole.

INTRODUCTION

The essential oils which were utilised centuries ago in cosmetics usually show interesting biological features. Essential oils were used in ancient Rome, Greece and Egypt and throughout the Middle and Far East as perfumes, food flavours, deodorants and pharmaceuticals (Baris et al., 2006). Medicinal plants have been used as a source of remedies since ancient times and the ancient Egyptians were familiar with many medicinal herbs and were aware of their usefulness in treatment of various diseases (Abu-Shanab et al., 2004). Until recently, essential oils have been studied most from the viewpoint of their flavour and fragrance chemistry only for flavouring foods, drinks and other goods. Actually, however, essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey et al., 2001; Gianni et al., 2005; Sawamura,

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2000). Plant essential oils and their components have been known to exhibit biological activities, especially antimicrobial, since ancient time. With the growing interest in the use of either essential oils or plant extracts in the food and pharmaceutical industries, screening of plant extracts for these properties is of increasing importance (Amvam et al., 1998). The World Health Organization has recommended and encouraged the use of chewing sticks (Almas and Al Lafi, 1995). *Eucalyptus* belongs to the family Myrtaceae, and is a globally distributed genus important as one of the two most-extensively planted pulpwood plantation species (Zobel, 1988). Many species of the genus *Eucalyptus* are used in many parts of the world for the treatment of a wide variety of diseases including microbial infections (Benarfa et al., 2007). Several studies have been reported on the chemical composition of the essential oils of *Eucalyptus* species belonging to different regions in the world (Benayache et al., 2007; Chalchat et al., 1997; Menut et al., 1992).

Morocco is blessed with a rich source of aromatic plants, many of which have not been previously investigated for their chemical constituents and biological potentials. *Eucalyptus globulus* is a plant that belongs to the family Myraceae, which grows in Morocco region and is a potential source of essential oils

The aim of this study was to elucidate the chemical constituents and evaluate the antifungal activity of the essential oil of the leaves of *E. globulus* collected in Atlas mean (Tichoukt), a mountainous region of Morocco.

MATERIALS AND METHODS

Chemicals and standards

All solvent were of analytical grade, unless otherwise specified. Hexane solution, anhydrous sodium sulfate, fungal strains**,** series of alkanes (C_4 - C_{28}) standards were obtained from, Faculty of Sciences, Sidi Mohamed Ben Abdellah University, Fez, Morocco.

Plant material

The leaves of *E. globulus* were collected in April 2010 at Skoura (Tichoukt) near Boulmane, 90 km in the south east of Fez. The climate is semi-humid with strong continental influence with an annual average temperature of 20°C. The plants were then isolated from the other specimen and conserved for extraction.

Essential oil extraction

The leaves of *E. globulus* were shade dried (25 days) at room temperature and immediately hydro-distilled (500 g) for 3.5 h using a modified Clevenger-type apparatus. The oil was extracted from the distillate with hexane and then dried over anhydrous sodium sulfate. After filtration, the solvent was removed by distillation under reduced pressure in a rotary evaporator at 35°C and the pure oil kept at 4°C in the dark, until the moment of analysis.

Gas chromatography analysis (GC/FID)

The quantitative analysis of essential oil of *E. globulus* was done with the help of a chromatographer in gas phase equipped with flame ionisation detector (GC-FID, Trace GC ULTRA S/N 20062969, Thermo Fischer), Varian capillary column (5% poly diphenyl 95% dimethylsiloxane, TR5- CPSIL- 5CB; 50 m length, 0.32 mm of diameter and film thickness 1.25 µm). The column temperature was programmed from 40 to 280°C for 5°C/min and finally held at that temperature for 10 min. The temperature of the injector was fixed to 250°C and the one of the detector (FID) to 260°C. The debit of gas vector (azoth) was fixed to 1 ml/min and split injection with split ratio 1:40. The volume injected was 1 µl of diluted oil in hexane solution (10%). The percentage of each constituent in the oil was determined by area peaks.

Gas chromatography-mass spectrometry

The identification of different chemical constituents was done by gas phase chromatography (Ultra GC Trace) coupled with spectrometer (PolarisQ/ S/N 210729, Thermo Fischer); with ionisation energy of 70 ev. The utilised column was; Varian capillary column (TR5- CPSIL- 5CB; 50 m length, 0.32 mm of diameter and film thickness 1.25 µm). The column temperature was programmed from 40 to 280°C for 3°C/min. The temperature of the injector was fixed to 260°C and the one of the detector (PolarisQ) to 200°C. The debit of gas vector (Helium) was fixed to 1 ml/min. The volume of injected specimen was 1 µl of diluted oil in hexane. The constituents of essential oils were identified in comparison with their Kovats index, calculated in relation to the retention time of a series of lineary alkanes (C_4- C_{28}) with those of reference products and in comparison with their Kovats index with those of the chemical constituents gathered by Adams (2001) and in comparison with their spectres of mass with those gathered in a library of (NIST-MS) type and with those reported in the literature (Derwich et al., 2011).

Antifungal assay

In the last few years, there has been target interest in biologically active compounds, isolated from plant species for the elimination of pathogenic microorganisms, because of the resistance that microorganisms have built against antibiotics (Essawi and Srour, 2000) or because they are ecologically safe compounds (Lee et al., 2005). The antifungal activity of the extracts from *E. globulus* was measured according to the procedure described by Adiguzel et al. (2002) with some modifications.

The dried plant extracts were dissolved in the same solvent (ethanol) to a final concentration of 30 mg/ml and sterilised by filtration through 0.45 µm Millipore filters. Antifungal tests were then carried out by the disc diffusion method (Murray et al., 1995) using 50 µl of suspension containing 52 spore/ml of fungi spread on nutrient agar (NA), sabouraud dextrose agar (SDA), and potato dextrose agar (PDA) mediums, respectively. The discs (6 mm in diameter) were impregnated with 10 µl of essential oil or 30 mg/ml extracts (300 µg/disc) placed on the inoculated agar. Negative controls were prepared using the same solvents as that employed to dissolve the plant extracts. Ofloxacin (10 µg per disc), sulbactam (30 µg)+ cefoperazona (75 µg) (105 µg/disc) and/or netilmicin (30 µg/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 27 for 72 h with fungi isolates. Antifungal activity was evaluated by measuring the zone of inhibition against the test organisms and the minimal inhibition concentration (MIC) values were evaluated according to published procedures (Celikel and Kavas, 2008; Bounatirou et al., 2007; Baratta et al, 1998). Each assay in this experiment was repeated twice.

RESULTS AND DISCUSSION

Phytochemical content of the leaf essential oils

Results obtained for the yields, compositions, contents, and identification of the leaf essential oils of *E. Globules* oils have been shown in Table 1. Yields of leaf essential oils from the hydro-distillation of *E. globulus* were 1.21%. In this study of the leaf essential oil of *E. globulus*, 54 compounds were identified, which made up 63.96% of the total essential oil and the major constituents was: 1.8-cineole (22.35%), other components present in appreciable contents were limonene, (7.01%), solanol (6.05%), β-pinene (5.20%), trans-verbenol (4.02%), terpinen-4-ol (3.10%), aristolene (2.35%), terpinyl acetate (2.10%), isosativene (1.85%), sabinène (1.49%), α-myrcène (1.15%) and α-terpinéol (1.10%).

The chemical compositions of the leaf oils of Eucalyptus from various parts of the world have been reported. 1.8-Cineole was identified as the major component in samples growing in Taiwan (Yu-Chang et al., 2006), Uruguay (Dellacassa et al., 1990), Algeria (Benayache et al., 2001), Burundi (Dethier et al., 1994), Congo (Cimanga et al., 2002), Mozambique (Pagula et al., 2000), Greece (Tsiri et al., 2003), Australia (Brophy et al., 1991), Tunisia (Bendaoud et al., 2009), Italy (Gianni et al., 2005), Nigeria (Islaka et al., 2003) and Turkey (Azcan et al., 1995). Also, 1.8-cineole was identified as the major component in others plants: *Laurus Nobilis* (Derwich et al., 2009; Ozcan and Chalchat, 2005); *Origanum minutiflorum* (Dadalioglu and Evrendilek, 2004); *Eucalyptus smitii* and *Callistemon speicosus* (Ntezurubanza, 2000). Previous studies of the leaf oil compositions of *Eucalyptus* species used commercially as a natural source of 1.8- cineole have been reported (Dethier et al., 1994; Boland et al., 1991).

The essential oils composition obtained in this study showed a relatively similar pattern to those published for other geographical regions: 1.8-cineole (84.7%), α-pinene (4.4%), trans-pinocarveol (2.2%), were reported as the major component in the essential oil of *Eucalyptus viridis* and 1,8-cineole (89.4%), β-pinene (1.2%) and α-pinene (1%) of *Eucalyptus oleosa* from Iran (Jaimand et al., 2009), oxygenated monoterpene: 1,8-Cineole (69.53%) and the monoterpene hydrocarbon: α-pinene (11.94%) from Tunisia (Bendaoud et al., 2009). Also it is different to the chemical composition of essential oil of leaves of *Eucalyptus robusta* and *Eucalyptus saligna* reported in Brazilian study in which the major component were *α*-pinene (73.0%) and *p*-cymene (54.2%) respectively (Patrícia et al., 2007) and they are different to those found in *Eucalyptus tessellaris* oil in Australia (Bignell et al., 1997) and Nigeria (Siaka et al., 2005), in which the major component was α-pinene (0.1-64.4%) and (46.60%) respectively,. Intense studies on Genus *Eucalyptus* essential oil composition have been published already (Nair et al., 2008; Gamal et al., 2007).

In this study, the yields of the oils obtained from the hydro-distillation of the leaves of *E. globulus* was 1.21%; it is relatively lower than other plants as a source of essential oils: *Myrtus communis* (1.75%) (Derwich et al., 2011), (*Eucalyptus microtheca* (2.3%), *Eucalyptus tereticornis* (3.4%) and *Eucalyptus grandis* (4.7%) (Islaka et al., 2003) and it is higher to the yield of essential oil isolated by hydro-distillation of the needles with twigs of *Pseudosuga menziesii* which was found to be 0.67% based on fresh material (Tesevici et al., 2009). Also it is higher to the yield of essential oil extracted of *Mentha piperita* from Morocco by Derwich et al. (2010) which is 1.02%. The yield and chemical composition of the leaf oil vary widely between species, individual trees as well as with the growing environment (Robbins, 1983; Penfold and Willis, 1961).

Antifungal activity

The essential oil extracted from the flowers of *E. globulus* was used in the present study to investigate its antifungal potential. *Penicillium citrinum* was used. The results obtained and screening of antifungal activity of essential oil of *E. globulus* are presented in Table 2.

With the agar disc diffusion assay, oils were found to be active against *P. citrinum* at a minimal inhibitory concentration (MIC) of 96.14 µl/ml. The data indicated that *P. citrinum* was the most sensitive strain tested to the oil of *Eucalyptus globulus* with the strongest inhibition zone (11.87mm). Modest activities were observed with minimal inhibitory concentration (MIC) of 3.07 µl/ml. These results are similar to those found by Chang et al. (2008).

The component of this oil, 1.8- cineole, has been known to exhibit antimicrobial activity (Sivropoulou et al., 1997). The antimicrobial activities, in general have been mainly explained through terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active sites of the target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils (Belletti et al., 2004). Pinene-type monoterpene hydrocarbons (α-pinene and β-pinene) are well known chemicals having antimicrobial potentials (Dorman and Deans, 2000). The difference in antifungal efficacy is a result of higher concentrations of the same chemical or a result of different chemicals composition between plants.

Several studies have been have been conducted to understand the mechanism of action of plant extracts and essential oils, however it is still unclear. Omidbeygi et al. (2007) suggested that components of the essential oils and extracts cross the cell membrane interact with the enzymes and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and ultimately their death. Cristani et al. (2007) reported that the antimicrobial activity is related to ability of terpenes to affect not only permeability but

Table 1. Constituents of the oil of *E. globulus* from Morocco.

Compound	*KI	**Air(%)	Method of identification
A-Thujone	1062	0.10	KI, GC//MS
Humulene	1579	0.31	KI, GC//MS
3-Carene	948	0.10	KI, GC//MS
A-Terpinene	998	0.11	KI, GC//MS
A -Pinene	948	0.20	KI, GC//MS
Camphene	943	0.20	KI, GC//MS
A- Elemene	1410	0.30	KI, GC//MS
A-Cubebene	1344	0.30	KI, GC//MS
Gama-Cadinene	1440	0.30	KI, GC//MS
B-Caryophyllene	1494	0.10	KI, GC//MS
Ocimene	958	0.10	KI, GC//MS
Epizonarene	1469	0.10	KI, GC//MS
Cis-Ocimene	976	0.10	KI, GC//MS
B-Pinene	943	5.20	KI, GC//MS
Isocaryophillene	1494	0.10	KI, GC//MS
Isoledene	1419	0.10	KI, GC//MS
Seychellene	1275	0.16	KI, GC//MS
Copaene	1221	0.20	KI, GC//MS
Ylangene	1221	0.10	KI, GC//MS
Patchoulene	1432	0.10	KI, GC//MS
Sabinene	983	1.49	KI, GC//MS
Isosativene	1339	1.85	KI, GC//MS
Aristolene	1403	2.35	KI, GC/MS
Solanone	1296	6.05	KI, GC/MS
B-Phellandrene	964	0.18	KI, GC/MS
Myrcene	940	1.15	KI, GC/MS
Terpene Hydrochlorite	1116	0.21	KI, GC/MS
Cymene	1042	0.25	KI, GC/MS
Terpenyl Formate	1330	0.20	KI, GC/MS
1,8-Cineole	1059	22.35	KI, GC/MS
Limonene	1018	7.01	KI, GC/MS
Bornyl acetate	1277	0.05	KI, GC/MS
Terpinyl acetate	1333	2.10	KI, GC/MS
Neryl acetate	1352	0.10	KI, GC/MS
A -Eudesmol	1598	0.10	KI, GC/MS
Terpinolene	1052	0.11	KI, GC/MS
Trans-verbenol	1136	4.02	KI, GC/MS
4- Caranol	1125	0.19	KI, GC/MS
Terpinen-4-Ol	1137	3.10	KI, GC/MS
A-Terpineol	1174	1.10	KI, GC/MS
P-Meth-1-En-4-Ol cis	1201	0.15	KI, GC/MS
1-Octen-3-Ol	969	0.17	KI, GC/MS
Geranyl acetate	1352	0.18	KI, GC/MS
Linalyl acetate	1272	0.10	KI, GC/MS
Geraniol	1228	0.19	KI, GC/MS
Linalool	1082	0.21	KI, GC/MS
Carvacrol	1262	0.04	KI, GC/MS
Panasone	2942	0.09	KI, GC/MS
Piperitone	1158	0.14	KI, GC/MS
mentha, 4-8 diene	990	0.19	KI, GC/MS
borneol	1138	0.05	KI, GC/MS

Table 1. Contd.

cis-linalool oxide	1164	0.13		KI, GC/MS
terpinyl isovalerate	1567	0.08		KI, GC/MS
Total Identified Compounds (%)			63.96	
Yields (%)			1.21	

*KI: Kovats Index was determined by GC-FID on a TR5- CPSIL- 5CB column. **Air was determined by mass spectrometry (PlarisQ).

Table 2. Antifungal activity of leaves essential oils of *E. globulus* from Morocco.

Micro-organism	*MIC (µl /ml)	**Disc diffusion assay (inhibition zone mm)
Penicillium citrinum	3.07	4.87
	8.21	5.15
	15.02	5.45
	31.34	6.03
	43.56	9.80
	62.01	9.96
	75.87	11.03
	96.14	11.87

*MIC: Minimal inhibitory concentration, concentration range: 3.07 to 96.14 µl/ml; **Disc diameter 6 mm average of two consecutive trials

also other functions of cell membranes; these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites.

Conclusion

This study revealed a high level of chemical composition of the essential oils of *E. globulus* originated from localities in Atlas median from Morocco. The leaf oil obtained from *E. globulus* was characterized by GC-MS, GC-FID and 54 volatile compounds were identified which made up 63.96% of the total essential oil. The essential oil yields of the studies were 1.21%. The main constituents were 1.8-cineole (22.35%), limonene, (7.01%), solanol (6.05%), β-pinene (5.20%), trans-verbenol (4.02%), terpinen-4-ol (3.10%), aristolene (2.35%), terpinyl acetate (2.10%), isosativene (1.85%), sabinene (1.49%), α-myrcene (1.15%) and α-terpineol (1.10%). The fungal strains *P. citrinum* tested were found to be sensitive to essential oils studied and showed a very effective fungicidal activity with minimum inhibitory concentrations (MIC) ranging from 3.07 to 96.14 µl/ml respectively. These results showed that extracts could be considered suitable alternatives to chemical additives for the control of fungal diseases in plants.

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