Advances in Natural and Applied Sciences, 3(3): 305-313, 2009
ISSN 1995-0772

ORIGINAL ARTICLE

GC/MS Analysis of Volatile Constituents and Antibacterial Activity of the Essential Oil of the Leaves of *Eucalyptus globulus* in Atlas Median from Morocco

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E.derwich, Z.Benziane and A.Boukir; GC/MS Analysis of Volatile Constituents and Antibacterial Activity of the Essential Oil of the Leaves of Eucalyptus globulus in Atlas Median from Morocco; *Adv. in Nat. Appl. Sci., 3(3): 305-313, 2009*.

ABSTRACT

The chemical compounds of the essential oil obtained from the leaves of *Eucalyptus globulus* in the Atlas median, mountain region of Morocco was determined by hydro-distillation method, analysed by gas chromatography equipped with flame ionisation detector (GC-FID) and gas chromatography coupled with mass spectrometry (GC-MS). The antimicrobial activities were tested in vitro by in a bioassay on three bacterial against gram-negative: *Escherichia coli* and gram-positive: *Staphylococcus aureus, Staphylococcus intermedius* and were evaluated using two different methods; agar disc diffusion and minimum inhibitory concentration (MIC). The results of the study revealed that essential oil yield and the total oil of *Eucalyptus globulus* were 1.21% and 63.96% respectively. 54 compounds were identified in the essential oil and the main constituents of the essential oil 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%). Essential oil extracted from Eucalyptus globulus showed an excellent activity against *Escherichia coil,* than that of *Staphylococcus aureus and Staphylococcus intermedius*, with the strongest inhibition zone 48.15, 13.50 and 10.26mm respectively.

Key words: *Eucalyptus globulus*, essential oil, GC/MS, 1.8-Cineole, Antibacterial activity.

Introduction

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 and Coutiere, 2001; Sawamura, 2000; Gianni *et al*, 2005). For thousands of years, plant products and their modified derivatives have been rich sources for clinically useful drugs. Even today, about 80% of the world's population relies predominantly on plants and plant extracts for health care (Jennifer *et al*, 2007). Essential oils and their components are widely used in medicine as constituents of different medical products, in the food industry as flavouring additives and also in cosmetics as fragrances (Cowan, 1999).

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

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herbs and were aware of their usefulness in treatment of various diseases (Abu-Shanab *et al*, 2004).Plant essential oils and their components have been known to exhibit biological activities, especially antimicrobial, since ancient time. With the growing interest of the use of either essential oils or plant extracts in the food and pharmaceutical industries, screening of plant extracts for these properties has become 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 (Ben Arfa *et al*, 2007).The genus *Eucalyptus* comprises well-known plants of over 600 species of trees (Boland *et al*, 1991).The essential oil of leaves of *Eucalyptus* species has been the object of several studies antibacterial, antioxidant, Antihyperglycemic and antifungal activity (Ghalem and Benali, 2008; Bendaoud *et al*, 2009; Dellacassa *et al*, 1989; El-Ghorab *et al*, 2003; Oyedeji *et al*, 1999; Hajji *et al*, 1993; Kumar *et al*, 1988; Ogunwande *et al*, 2003; Faouzia *et al*, 1993; Okamura *et al*, 1993; Gray and Flat, 2001; Hammouchi *et al*, 1990; Yu-Chang Su *et al*, 2006).

Multiple studies have been reported on the chemical composition of the essential oils of *Eucalyptus* species belonging to different regions in the world (Azcan *et al*, 1995; Dunlop *et al*, 1999; Benayache *et al*, 2001; Menut *et al*, 1992; Chalchat, 1997; Bignell, 2001; Boland *et al*, 1991; Islaka *et al*, 2003).

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 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 Antibacterial Activity of the essential oil of the leaves of Eucalyptus globulus collected in Atlas mean (Tichoukt), a mountainous region from Morocco.

Materials and methods

- *Plant material and essential oil extraction*

The leaves of *Eucalyptus globulus* were collected in March 2009 at Skoura (Tichoukt) near Boulmane (90 km in the south east of Fez. The coordinates: latitude: 35 ° 42 '21 " longitude: 4 ° 32' 31"; altitude: 3200 m). 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. The leaves of *Eucalyptus globulus* were shade dried (25 days) at room temperature and immediately hydro-distilled (500g) 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.

- *Chromatographic (GC/MS and GC-FID) analysis*

Essential oil extracted of the leaves of *Eucalyptus globulus* were analysed by chromatography techniques in gas phase led by an flame ionisation detector (GC-FID) and chromatography in gas phase coupled with Mass spectrometry (GC/MS, Trace GC ULTRA S/N 20062969/PolarisQ S/N 210729, Thermo Fischer) in the light of the following experimental protocol:

The quantitative analysis was done with the help of a chromatography in gas phase equipped with flame ionisation detector (GC-FID), Varian capillary column (5% poly diphenyl 95% dimethylsiloxane, TR5- CPSIL-5CB; 50m length, 0.32mm 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 1mL/min and split injection with split ratio 1:40.The volume of injected was 1µL of diluted oil in hexane solution (10%). The percentage of each constituent in the oil was determined by area peaks.

The identification of different chemical constituents was done by gas phase chromatography (Ultra GC Trace) coupled with spectrometer (PolarisQ); with ionisation energy of 70ev. The utilised column was; Varian capillary column (TR5- CPSIL- 5CB; 50m length, 0.32mm 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 1mL/min.The volume of injected specimen was 1µL 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 covets 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 (Woerdenbag *et al*, 1993; Palá-Paúl *et al*, 1999).

- *Antibacterial tests*

The selected essential oils were screened against four: bacteria gram-negative: *Escherichia* coli and Gram-positive*: Staphylococcus aureus* and *Staphylococcus intermedius.* The minimal inhibition concentration (MIC) values were evaluated according to published procedures (Guven and Uysal, 2005; Iscan and Baser, 2002; Koneman, 1997; Demirci *et al*, 2008).The minimal inhibitory concentration (MIC) was determined only with micro-organisms that displayed inhibitory zones. MIC was determined by dilution of the essential oils in dimethyl sulfoxide (DMSO) and pipetting 0.01 mL of each dilution into a filter paper disc. Dilutions of the oils within a concentration range of 0.15- 1.08mg/mL were also carried out. MIC was defined as the lowest concentration that inhibited the visible bacterial growth (N C C L S, 2006). The bacterial plates were incubated at 37°C and the zone of inhibition measured in mm after 24h, 48h and 72h of growth. A control experiment was set up by using an equal amount of sterile distilled water in place of different extract concentrations. Many screening reports, using disc diffusion and dilution techniques, have established an antimicrobial activity of Eucalyptus extracts from various species against a number of pathogens including Inouye *et al* (2001) (*Haemophilus influenzae, Streptococcus pneumoniae* and *Staphylococcus aureus*), Ghalem and Benali (2008) (*Staphylococcus aureus* and *Escherichia coli),* Takarada *et al* (2003) (Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Streptococcus mutans, and Streptococcus sobrinus), Wilkinson and Cavanagh (2005) (*Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis)*, Mounchid *et al* (2005) (*Escherichia coli)* and Latha *et al* (2009) (*Escherichia coli, Staphylococcu Peudomonas aeruginosa, Enterococcus faecalis, Salmonella typhi* and *Bacillus cereus*).

Results and discussion

- *Yield, compositions, contents and identification of the leaf essential oil*

The constituents of leaves essential oil of *Eucalyptus globulus* from Morocco are listed in order of their elution on the TR5- CPSIL- 5CB column, figure 1.

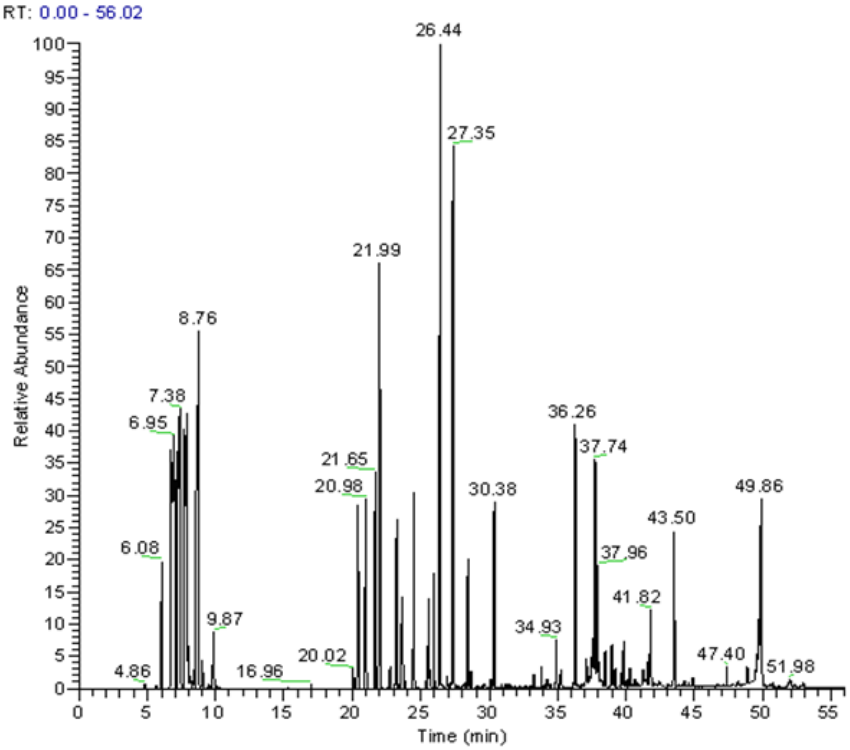

Fig. 1: Chromatogram of *Eucalyptus globulus from Morocco*

Results obtained for the Yields, compositions, contents, and identification of the leaf essential oils of *eucalyptus globulus* oils have been shown in Table 1. Yields of leaf essential oils from the hydro-distillation of *Eucalyptus globulus* were 1.21 %. In this study, the leaf essential oil of *Eucalyptus 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%).

Table 1: Chemical composition of the leaf oils of Eucalyptus globulus from Morocco

Peak	Compounds	*RT (min)	**KI	Air(%)	***Largest peaks(m/z)
1	α-Thujone	4.86	1062	0.10	(152),110,81,95,67,68,41,69,109,55,70
2	Humulene	4.92	1579	0.31	(204),93,80,41,121,92,43,55,67,91,147
3	3-Carene	5.95	948	0.10	(136),93,91,79,77,92,121,80,136,94,105
4	α-Terpinene	5.98	998	0.11	(136),93,91,136,121,77,92,79,43,41,105
5	α -Pinene	6.08	948	0.20	(136),93,91,39,121,77,92,79,43,41,105
6	Camphene	6.50	943	0.20	(136),93,79,91,77,41,121,67,27,107,39
7	α- Elemene	6.95	1410	0.30	(204),161,119,204,41,105,189,91,121,93,133
8	α-Cubebene	7.38	1344	0.30	(204),161,105,119,41,81,91,120,93,55,204
9	Gama-Cadinene	7.50	1440	0.30	(204),161,189,204,41,105,91,119,133,27,55
10	β-caryophyllene	7.95	1494	0.10	(204),93,133,91,41;79,69,105,107,120,77
11	Ocimene	8.01	958	0.10	(136),93,41,27,39,79,80,77,43,29,91
12	Epizonarene	8.04	1469	0.10	(204),161,204,81,189,105,119,162,133,205,93
13	Cis-ocimene	8.50	976	0.10	(136),93,41,79,39,91,77,92,27,80,53
14	β-Pinene	8.76	943	5.20	(136),93,91,69,39,77,92,79,53,41,27
15	Isocaryophillene	8.92	1494	0.10	(204),93,69,41,133,161,79,91,105,81,107
16	Isoledene	9.01	1419	0.10	(204),161,105,119,41,91,204,133,55,93,81
17	Seychellene	9.12	1275	0.16	(204),41,91,105,161,93,204,79,121,77,107
18	Copaene	9.87	1221	0.20	(204),161,119,105,93,41,91,92,81,120,204
19	Ylangene	16.96	1221	0.10	(204),105,119,93,120,161,41,91,92,107,55
20	Patchoulene	20.02	1432	0.10	(204),161,204,41,121,91,81,107,105,189,93
21	Sabinene	20.56	983	1.49	(136),93,41,91,77,79,39,27,69,94,43
22	Isosativene	20.98	1339	1.85	(204),94,91,41,105,79,93,204,119,39,77
23	Aristolene	21.65	1403	2.35	(204),105,161,91,41,147,119,133,204,189,107
24	Solanone	21.99	1296	6.05	(194),43,93,136,121,41,79,81,91,77,39
25	β-Phellandrene	22.80	964	0.18	(136),93,77,91,136,79,94,41,80,92,39
26	α -Myrcene	23.02	940	1.15	(136),41,93,69,39,27,53,79,77,67,91
27	Terpene hydrochlorite	23.45	1116	0.21	(172),95,93,121,136,41,67,79,91,77,81
28	Cymene	23.50	1042	0.25	(134),119,134,91,120,117,41,77,39,65,115
29	Terpenyl formate	25.72	1330	0.20	(182),59,93,121,136,43,111,94,137,81,107
30	1,8-Cineole	26.44	1059	22.35	(154),43,93,81,71,69,84,68,108,41,55
31	Limonene	27.35	1018	7.01	(136),68,93,39,67,41,27,53,79,94,92
32	Bornyl acetate	29.52	1277	0.05	(196),95,43,93,436,121,41,80,55,108,69
33	terpinyl acetate	30.38	1333	2.10	(196),43,121,93,136,68,41,59,67,81,79
34	Neryl acetate	31.01	1352	0.10	(196),69,41,43,68,93,80,121,136,67,39
35	α -Eudesmol	35.05	1598	0.10	(222),59,149,161,189,204,107,109,93,41,81
36	Terpinolene	35.64	1052	0.11	(136),93,121,91,136,79,77,105,39,41,107
37	Trans-verbenol	36.26	1136	4.02	(152),109,41,94,81,39,69,55,91,43,57
38	4- Caranol	37.50	1125	0.19	(154),93,136,121,81,43,55,41,107,96,69
39	Terpinen-4-ol	37.74	1137	3.10	(154),71,111,93,43,86,41,69,55,68,154
40	α-terpineol	37.96	1174	1.10	(154),59,93,121,136,81,43,68,95,67,41
41	p-Meth-1-en-4-ol cis	38.45	1201	0.15	(154),93,67,81,79,121,41,77,123,43,55
42	1-Octen-3-ol	39.15	969	0.17	(128),57,72,29,41,55,27,85,58,39,43
43	Geranyl acetate	40.12	1352	0.18	(196),69,43,41,68,93,136,67,121,80,39
44	Linalyl acetate	40.50	1272	0.10	(196),93,43,41,69,80,121,68,55,71,79
45	Geraniol	41.20	1228	0.19	(154),69,41,68,29,93,123,67,70,84,55
46	Geraniol	41.21	1228	0.19	(154),69,41,68,29,93,123,67,70,84,55
47	Linalool	41.82	1082	0.21	(136),71,41,43,93,55,69,80,39,121,27
48	Carvacrol	42.50	1262	0.04	(150),135,150,91,136,77,107,117,115,79,105
49	Panasone	44.10	2942	0.09	(434),121,122,43,315,147,135,414,223,333,91
50	Piperitone	45.01	1158	0.14	(152),82,110,39,41,27,95,137,109,54,152
51	m- Mentha, 4-8 diene	47.40	990	0.19	(136),93,136,79,91,121,107,92,39,41,77
52	Borneol	48.50	1138	0.05	(154),95,41,110,93,55,67,139,121,96,69
53	Cis-linalool oxide	49.12	1164	0.13	(170),59,43,41,68,55,67,94,93,111,81
54	Terpinyl isovalerate	51.98	1567	0.08	(238),136,121,93,85,41,57,60,81,137,68
	Total				63.96
	Yields (%)				1.21

*RT: Retention time obtained by chromatogram (Fig1).
**KI: Kovats Index was determined by GC-FID on a TR5- CPSIL- 5CB column.
***Largest peaks (m/z) were determined by mass spectrometry (PlarisQ).

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 from samples growing in Taiwan. (Yu-Chang Su *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 from others plants: *Laurus Nobilis* (Derwich *et al*, 2009; Ozcan *et al*, 2005; Dadalioglu *et al*, 2004; Kilic *et al*, 2005; Zheng-kui *et al*, 1990; Politeo *et al*, 2007; Simic *et al*, 2004); *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 (Boland *et al*, 1991; Dethier *et al*, 1994).

The essential oil composition of *Eucalyptus globulus* obtained of 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's different to the chemical composition of essential oil of leaves of *Eucalyptus robusta* and *Eucalyptus saligna* study in Brazil which the major component were *α*-pinene (73.0%) and *p*-cymene (54.2%) respectively (Patrícia *et al*, 2007) and it are different to those found in *Eucalyptus tessellaris* oil in Australia (Bignell *et al*, 1997) and Nigeria (Isiaka *et al*, 2005), 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 and Sabrin, 2007; Batista-Pereira *et al*, 2006; Sartorelli *et al*, 2006; Hedges and Wilkins, 1991; Bignell *et al*, 1998).

In this study, the yields of the oils obtained from the hydro-distillation of the leaves of *Eucalyptus globulus* was 1.21%, it's relatively lower than other plants as a source of essential oil: *Eucalyptus microtheca* (2.3%), *Eucalyptus tereticornis* (3.4%) and *Eucalyptus grandis* (4.7%) (Islaka *et al*, 2003) and it is higher the yield of essential oil isolated by hydro-distillation of the needles with twigs of *Pseudosuga menziesii* was found to be 0.67 % based on fresh material *(*Tesevic *et al*, 2009). 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; Coppen and Hone, 1992).

- *Antibacterial activity*

Table 2: Antibacterial activity of leaves essential oils of *Eucalyptus globulus* from Morocco.

Essential oils	Micro-organisms		
	Escherichia coli	*Staphylococcus aureus*	*Staphylococcus intermedius*
*Disc diffusion assay (inhibition zone mm)	48.15	13.50	10.26
**MIC (mg/mL)	0.15	0.75	1.08

*Disc diameter 6 mm average of two consecutive trials

**MIC: Minimal Inhibitory Concentration, concentration range: 0.15-1.08 mg/ml.

Results obtained in the antibacterial activity study of the essential oils are shown on Table 2. With the agar disc diffusion assay, oils were found to be active against *Escherichia coli* at a minimal inhibitory concentration (MIC) of 0.15mg/mL. Against *Staphylococcus aureus and Staphylococcus intermedius*, the oil from the leaves was found to be more active; the oils showed MIC values of 0.75 and 10.8mg/mL respectively. The data indicated that *Escherichia coli* were the most sensitive strain tested to the oil of *Eucalyptus globulus* with the strongest inhibition zone (48.15mm). The *Staphylococcus aureus* was, in general, found to be more sensitive among bacteria with inhibition zone of 13.50mm. Modest activities were observed against *Staphylococcus intermedius*, with inhibition zones of 10.26mm. These results are similar to those found by (Trivedi and Hotchandani, 2004; Ghalem and Benali, 2008; Gamal and Sabrin, 2007; Nair *et al*, 2008).The major component of this oil, 1.8- cineole, has been known to exhibit antimicrobial activity against the bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus typhi, Staphylococcus aureus, Staphylococcus intermedius,* and *Bacillus subtilis*) (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 wellknown chemicals having

antimicrobial potentials (Dorman *et al*, 2000). On the other hand, enantiomers of α-pinene, β-pinene, limonene and linalool have a strong antibacterial activity (Magiatis *et al*, 1999; Filipowicz *et al*, 2003; Koji *et al*, 2004). The antimicrobial activity of essential oils is known to be beneficial in the treatment of different diseases. Our experiments proved the antibacterial activity of *Eucalyptus globulus* oil and its main constituent, 1.8-cineole, which means that 1.8-cineole-containing substances are potential agents that could eliminate of bacteria.

Conclusion

This study revealed a high level of chemical composition of the essential oils of *Eucalyptus globulus* originated from localities in Atlas median from Morocco. The leaf oil obtained from *Eucalyptus 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 bacterial strains gram-negative: *Escherichia coli* and gram-positive: *Staphylococcus aureus and Staphylococcus intermedius* tested were found to be sensitive to essential oils studied and showed a very effective bactericidal activity with minimum inhibitory concentrations (MIC) ranging from 0.15 to 1.08 mg/ml.

Acknowledgements

The authors would like to acknowledge the Regional Center of Interface, University Sidi Mohamed Ben Abdellah, Fez, Morocco for the gas chromatography coupled with mass spectrometry (GC/MS) and gas chromatography with flame ionization detection (GC-FID) analysis.

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