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ESSENTIAL OIL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF *ARTEMISIA HERBA-ALBA* ASSO GROWN IN MOROCCO

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Abstract: Volatile components of *Artemisia herba-alba* Asso essential oil obtained by hydrodistillation growing in eastern Morocco (Taforalt) were investigated by GC/FID and GC-MS. The major components were found to be camphor (43.07%), camphene (7.2%), 1,8-cineole (7.08%), filifolone (7.04%), borneol (4.88%), and bornyl acetate (3,79%).The essential oil has been tested for antimicrobial activity against Gram-negative and Gram-positive bacteria. Inhibition of growth was tested by the agar diffusion method. The minimal Inhibitory Concentration (MIC) was determined by the method of microtitration assay.

Keywords: Essential oil, Artemisia herba-alba, antimicrobial activity, Morocco.

Introduction

Since ancient times the crude herbal extracts of aromatic plants have been in use for different purposes such as food, drugs and perfumery [HEATH, 1981] . The essential oils are considered among the most important antimicrobial agents present in these plants, and may also have antioxidant and anti-inflammatory activities. In the recent decades, antimicrobial plant products have gained special interest because of the resistance to antibiotics that some micro organisms have acquired [ESSAWI, 2000]. Besides, the increasing popular concern about the safety of food and the potential impact of synthetic additives on health has highlighted their importance [REISCHE, 1998].

Many members of the genus *Artemisia* (Asteraceae) are important medicinal plants. For example, *A. vulgaris* (mugwort), native of Britain and Europe, has been used as a tonic, febrifuge, anthelmintic, women's menstrual troubles and infertility, anti-nervous disorders, against complaints of the gastrointestinal tract (e.g. stomach ulcers and indigestion) [GRUENWALD, 2000].

The essential oil shows the antimicrobial activity and contains 1,8-cineole, camphor and thujone as major constituents [GRUENWALD, 2000]. Similarly, *A. absinthium* (wormwood) [BRUNETON, 1999] has been used in Europe and *A. mexicana* [MORTON ,1981] in Mexico to stimulate the appetite and aid digestion.

The species *Artemisia herba alba* which is a medicinal and aromatic dwarf shrub that grows wild in arid areas of Mediterranean basin, extending into north-western Himalayas [VERNIN, 1995]. The plant is widely used for the treatment of gastric disturbances such as diarrhoea, abdominal cramps and for healing external wounds [TAECKHOLM 1939; BAILEY, 1981].

Over the last 30 years, this species has been extensively studied. Previous investigation on the non- volatile constituents of *A. herba-alba* revealed the presence of sesquiterpene lactones [MARCO, 1994; VERNIN, 2001] and flavonoids [SALEH, 1987; SALAH, 2005] in the aerial parts. Numerous studies in the literature have reported the composition of *A. herba-alba* essential oils from different parts of the world [VERNIN,1995; HURABIELLE, 1981; FEUERSTEIN, 1986; FEUERSTEIN, 1988; BOUTEKEDJIRET, 1992; SALIDO, 2001; BENJILALI, 1980; BENJILALI, 1982; LAWRENCE,1989; LAWRENCE,1993; LAWRENCE,1995; FLEISHER, 2002; SALIDO,2004]. Antibacterial and antileishmanian properties have been reported for this oil [YASHPHE, 1979; HATIMI, 2001].

The aim of the present investigation was to study the antibacterial effect of *A.herba alba* oil on different bacterial species.

This was done through tow techniques: the agar diffusion assay as well as the micro plate bioassay (micro dilution) was used for determination of minimum inhibitory concentration (MIC). In addition, the compositions of volatile compounds were determined to use these data to deduce which components are likely to contribute to the activities of the whole oils and to determine any structural relationships between the components and their antibacterial activity.

Material and methods

2.1. Plant material

The dried aerial parts were submitted to Hydrodistillation for 3h using Clevenger type apparatus, according to the European Pharmacopoeia (1996).The essential oil was collected, dried over anhydrous sodium sulphate and stored at 4° C until used.

2.2. Gas Chromatography

Essential oil samples of 0.1 µl were injected neat (directly) into an HP 6890 gas chromatography equipped with a flame ionisation detector (FID) and a 30 m x 0.25 mm HP-5 (cross-linked Phynel-Methyl Siloxane) column with 0.25 µm film thickness (Agilent), was used for the study. Helium was used as carrier gas, the flow through the column was 1.4 ml/min, and the splitless mode was used. The column was maintained at 40°C for 5 min, increased to 230°C at rate of 10°C/min and finally raised from 230 to 280 at rate of 30°C/min.

2.3. Mass spectrometry analysis

The oil was analysed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 6890 mass selective detector coupled with a Hewlett Packard 6890 gas chromatograph equipped with a 30 m x 0.25 mm HP-5 (cross-linked Phynel-Methyl Siloxane) column with 0.25 µm film thickness (Agilent), Helium was used as carrier gas, the flow through the column was 1,4 ml/min, and the splitless mode was used. The column temperature was programmed from 35 to 85 °C at 20°C/min, increased from 85 to 300°C at rate of 5°C/min and finally held for 10 min. The MS operating parameters were as follows: ionisation potential, 70 eV; ionisation current, 2 A; ion source temperature, 200°C, resolution, 1000. Mass unit were monitored from 30 to 450 m/z. Identification of components in the oil was based on retention indices relatives to n-alkanes and computer matching with the WILLEY 275.L library, as well as by comparison of the fragmentation patterns of mass spectra with those reported in the literature [ADAMS, 1995].

2.4. Antimicrobial Activity

The essential oils were individually tested against Gram+ bacteria and Gram- bacteria. Some Bacterial strains used in this study were obtained from American type culture collection (ATCC), USA; National Institute of Healthy (NIH): *Pseudomonas aeruginosa* ATCC12228, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC125922. The other bacterial strains were isolated from urine and feces of patients in the National Institute of Health (NIH) Rabat, Morocco, and of animals and poultry in Veterinary Cabinet, Zaio, Morocco.

All bacteria were stored in trypticase soy (Sanofi Diagnostic Pasteur, France) broth containing 25% (v/v) glycerol (Sigma-Aldrich) at -20°C. Prior to use, the culture were propagated twice in the appropriate media as mentioned above to make them physiologically active.

2.4.1. Antibacterial screening

Two techniques were used to test the microbial activity of *Artmisia herba-alba* oil. A 16-h culture was diluted with sterile physiological saline solution with reference to the MC Farland 0.5 standard to achieve an inoculum of approximately 10^6 CFU/ ml. A suspension was swabbed in three directions on 4 mm thick Mueller Hinton agar (MHA) (Oxoid, England) with a cotton swap. We made holes with sterile, 6 mm diameter stainless steel cylinders on plates of MHA. In these holes we would put the E.O later on. In each hole, 100µl of filter sterilised test E.O (dilution with tween 80 at 2%) were added. The plates were then incubated for 24h at 37°C. The results recorded by measuring the zones of growth inhibition surrounding the cylinders.

2.4.2. Micro plate bioassay

In addition to the solid medium diffusion procedure, the micro plate bioassay (micro dilution) was used, as recommended by NCCLS, for determination of minimum inhibitory concentration (MIC) [30]. The MIC

was defined as lowest concentration of *A. herba-alba oil* inhibiting visible bacterial growth after incubation for 20h at 37°C.
Into each well 100µl of Brain Heart Infusion broth (BHI, Difco Laboratories, Detroit, MI, USA) inoculated with the bacteria inoculum prior to the essay. An aliquot (100µl) of the essential oil was added in first well. Geometric dilutions ranging from 0.041mg/ml to 21mg/ml of the essential oils were prepared in a 96 well micro titre plate, including one growth control (BHI+Tween 80)and one sterility control (BHI + Tween 80 + test oil). The contents of the wells were mixed and micro plates were incubated at 37°C for 24h. The MIC was determined by quantitative tetrazolium based colorimetric method.10 microliters of 4mg/ml solution of 3-(4.5-dimethylthiazo-2-yl)-2.5-diphenyl tetrazolium bromide (MTT;Biochemika, Fluca) in distillated water were added to each well; the plates were incubated at 37°C.Ater a few minutes at room temperature; the plates were read. A colour change from blue to mauve was indicative of bacterial growth.

Results

Hydrodistillation of the aerial parts of *A. herba-alba* yielded yellow liquid oil with a strong penetrating pleasant herbaceous odor characteristic of the plant. The oil yield was 1.0 %. The chemical composition of the oil was investigated using GC/ FID and GC/MS techniques.
Table I lists the components, identified in the oil of *A. herba-alba*. Forty-tree compounds were identified, representing 87.97% of the oil. Monoterpenes hydrocarbons and their derivatives dominated the chemical composition of the investigated oils. The sesquiterpenes are present in smaller quantities (α.-amorphene 0.23%), germacrene-D (1.37%), germacrene-B (0.55%), β-cubebene (0.15%), γ-elemene (0.12%), α-copaene (0.26%), and σ-cadinene (0.22%). Other significant constituents were thymol (1%), β-thujone (1.74%), sabinene kitone (1.07%), terpinen-4-ol (1.07%), sabinene (0.97%), camphene (7.2%) and 1,8-cineol (7.08%). Other important compounds were the oxygenated monoterpenes, camphor (43.07%), Borneol L(4.88%), bornyl acetate(3.79%) and filifolone (7.04%).

The principal component of the oil was camphor (43.07%), what also occurs on *A. herba-alba* from different origins; Algeria (Bou Saada, Batna, Sidi Aissa, Djelfa and Kenchela) [VERNIN,1995], Spain (Hills near Aranjuez) [FEUERSTEIN, 1988] and Israel (Southern Negev-type 2) [FLEISHER, 2002]. On the other hand, the major component of this oil differed from that reported by several authors for A. *herba-alba* oils, in which α- and β-thujone [BOUTEKEDJIRET, 1992; FLEISHER, 2002], davanone [BENJILALI, 1982; SALIDO, 2001; FLEISHER, 2002; SALIDO, 2004], chrysanthenone [HURABIELLE, 1981; BOUTEKEDJIRET, 1992], 1,8-cineole[FEUERSTEIN,1986, SALIDO, 2004, cis-chrysanthenol [FEUERSTEIN, 1986; FLEISHER, 2002] and cis-chrysanthenyl acetate [FEUERSTEIN, 1986; BOUTEKEDJIRET, 1992; FLEISHER, 2002] were found to be the most abundant components.

The chemical composition of essential oils of *A. herba-alba* shows a large interspecies variability and, within the same species, it seems to depend on the genetic characteristics of the plant and on the conditions under which it has grown.

Table1.
Percentage composition of essential oil of *Artemisia herba alba (%)*

RI [a]	Compound	R.T	%
903	Terpinolene	4,37	0,60
923	Tricyclene	4,64	0,37
934	α-pinene	4,78	0,61
950	Camphene	5,01	7,20
974	Sabinene	5,32	0,97
990	1-octen-3-ol	5,54	0,27
1007	L-Phellandrene	5,79	0,41
1017	α-terpipene	5,99	0,26
1025	p-Cymene	6,12	0,48
1033	1,8-cineole	6,26	7,08
1058	γ-Terpinene	6,70	0,30
1091	α-Terpinolene	7,28	0,26
1107	Filifolone	7,59	7,04
1119	β-Thujone	7,86	1,74
1124	Chrysanthenone	7,96	0,40
1153	Camphor	8,60	43,07
1156	Sabina ketone	8,67	1,07
1169	Borneol l	8,94	4,88
1178	Terpinene-4-ol	9,14	1,07
1206	Verbenone	9,76	0,23
1229	Cis-carveol	10,26	0,25
1237	E-ocimenone	10,44	0,34
1244	Carvone	10,58	0,15
1275	Bornyl acetate	11,28	3,79
1287	Thymol	11,55	1.00

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2010, I(2),

1295	Cuminol	11,72	tr[b]
1298	γ- Elemene	11,81	0,12
1313	Bicycloelemene	12,14	0,17
1316	Eugenol	12,23	0,16
1322	α.-Copaene	12,35	0,26
1339	Verbenone	12,75	0,52
1355	β- Cubebene	13,12	0,15
1389	α.-Amorphene	13,93	0,23
1393	Germacrene D	14,02	1.37
1398	α-Patchoulene	14,13	0,17
1418	Germacrene B	14,61	0,55
1444	Bicyclogermacrene	15,19	0,11
1457	Delta-cadinene	15,51	0,22
1465	Spathulenol	15,69	tr
1467	Virdifloral	15,72	tr
1489	Kaurene	16,23	tr
1496	9-octadecenamide	16,41	0,10

[a] RI: retention indices on HP-5 capillary column.
[b]tr: trace<0.09%

The in vitro antimicrobial activity of *A. herba-alba* essential oil against the microorganisms employed and its activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters and MIC values.

Table 2 reports the inhibition zone of essential oil determined for 24 of Gram positive or Gram negative bacteria using the diffusion technique on solid media.

The data indicated that Gram-positive *L. monocytogenes* was the most sensitive strain tested to the oil of *A. herba-alba* with the strongest inhibition zone (70.00±0.11mm). The *streptococcus* group was found to be a sensitive among Gram-positive bacteria with inhibition zone of 23.07± 0.11mm *for S. pneumoniae*.

Among these, Gram-negative strains also displayed variable degree of susceptibility against investigated oil. Maximum activity was observed against *N. meningitidis* (60.10±0.17 mm), followed by *H.influezae* (34.00±0– 49.83±0.76 mm) and *K. pneumoniae* (16.00±0.1- 40.00±0). Modest activities were observed against *E. coli*, with inhibition zones of (12±0 - 17.23±0.25 mm), *E. cloacae* with inhibition zone of (15.07±0.20mm). *P. aeruginosa* was considered resistant since no inhibition zone was observed. It is known to have high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a combination of a very restrictive outer membrane barrier which is highly resistant even to synthetic drugs.

Table 2.

Antibacterial activity of essential oil as determined by diffusion technique on solid media

Microorganisms	**Essential oil zone inhibition (mm)***
Neisseria meningitidis(1)	60.1 ± 0.17
Neisseria meningitidis(2)	26 ± 0
Enterobacter cloacae	15.07 ± 0.2
Klebsiella pneumoniae (1)	16 ±0.1
Klebsiella pneumoniae (2)	40 ± 0
Heamophilus influezae (1)	34 ± 0
Heamophilus influezae (2)	49.83 ± 0.76
Pseudomonas aeruginosa ATCC12228	0
Pantoea sp	30 ± 0.5
Escherichia coli ATCC125922	17.23 ± 0.25
Escherichia coli	12 ± 0
Proteus mirabilis-	17.9 ± 0.36
Staphylococcus aureus ATCC 25923	28.17 ± 0.28
Staphylococcus aureus	0
Streptococcus pneumoniae	23.07 ± 0.11
Listeria monocytogenes	70 ± 0.11

*Data are presented as mean values ± SD
*difference between Microorganisms very significant ($p < 0.0001$)
(1) and (2): means that each bacteria is taken from two deferens patients
Experiments were done in triplicate and results are mean values

The in vitro activity of *A. herba-alba* essential oil was evaluated by a broth micro dilution method using a panel of micro organisms. Antimicrobial activity was expressed as minimum inhibitory concentration (MIC). The results of the MIC are in Table 3. The data indicate that the oil exhibited varying levels of antimicrobial activity against the investigated food pathogens. The inhibitory properties of the oil were observed within a range of concentrations from 0.041 to 21.00 mg/ml. In liquid medium the essential oil was active against all the test strains. The Gram-negative *P. aeruginosa* seemed to be resistant to the investigated oil with a MIC of 21.00 mg/ml. Maximum activity was observed against the *K. pneumoniae*, *N. meningitidis* and *H. influezae* with MIC of 0.041mg/ml to the oil. The oil exhibited the highest inhibitory effect against Gram positive bacteria. *S. aureus* with MIC of 0.016mg/ml and *S. epidermidis* (0.33mg/ml). The *E. coli*(1) strains tested showed an MIC of 0.66±0.01 mg/ ml). On the other hand, *P. mirabilis* and *S. pneumoniae* were the last sensitive with an MIC of 1.33±0.01 mg/ml.

The essential oil was evaluated for antimicrobial activity against pathogenic strains of Gram positive (*L. monocytogenes, S. pneumoniae, S. aureus, S. epidermidis*.) and Gram negative (*N. meningitides, H. influezae* , *E. coli*, *P. aeruginosa*, *K. pneumoniae*, Proteus mirabilis, *E. cloacae*, *Pantoea sp*) bacteria (table 2 and 3). It was found to be active against all the bacterial strains except for *P. aeruginosa* (0 mm). Among the Gram negative bacteria, the oil was very active against *N. meningitides* (26 ± 0 - 60.1 ± 0.17mm) with MIC of (0.04±0.01 mg/ml), *H. influezae* (49.83 ± 0.76 mm) with MIC of 0.04-0.33 mg/ml, *K. pneumoniae* (16.00±0.1 – 40 ± 0 mm) with MIC of (0.04mg/ml).

P. aeruginosa was considered resistant to the essential oil and even to the reference antibiotic Chloramphenicol, since no inhibition zone was observed. This bacterium has shown resistance to antimicrobial agents and diterpenes present in Salvia species [MATASYOH, 2007]. The essential oils evaluated in this work have a great variety of phytochemicals that could be considered as responsible for a larger or smaller part of the antimicrobial activity. Although they usually occur as complex mixtures, their activity can generally be accounted for in terms of their major monoterpenoid components. Research into the antimicrobial actions of monoterpenes suggests that they diffuse into and damage cell membrane structures [SIKKEMA, 1995]. Besides, the most abundant component in essential oil of *A.herba alb*, camphor, has been reported to exhibit bacteriostatic activity against *P.aeruginosa* [TIRILLINI, 1996], and this compound is a major constituent in a number of antibacterial essential oils [HAMMERSCHMIDT, 1993; MAGIATIS, 2002].

The antimicrobial activities of *A.herba alba* can be attributed to the presence of camphor, 1,8-cineole and thujone , three monoterpenes with well documented antibacterial and antifungic potential [JALSENJAK, 1987; SIVROPOULOU, 1997] . In addition, other minor components such as borneol (4.88%) have been also reported to have antimicrobial potential [KNOBLOCH, 1989]. Indeed, Mourey and Canillac [MOUREY, 2002] also found that the borneol shows bacteriostatic activity against *L. monocytogenes* at concentration of less than 0.62µl/ml. In other studies, 1,8-cineole, has been known to exhibit antimicrobial activity against the bacterial strains (*E. coli, P. aeruginosa, S. typhi, S. aureus, rhizobium leguminosarum, bacillus subtilis*).

In fact, the synergistic effects of the diversity of major camphor (43.07%) camphene (7.02%), 1-8 cineol (7.08%) filifolone (7.04%) and minor (eugenol (0.16%), thymol (1%), terpene-4-ol(1.07%) and α-pinene (0.61%) constituents present in the essential oils should be taken into consideration to account for their biological activity. In general, the antimicrobial activity of the essential oils tested was more pronounced against Gram-positive than against Gram-negative bacteria, a general observation derived from studies with essential oils from many other plant species [NOSTRO, 2000; OUATTARA, 1997].This generally higher resistance among Gram-negative bacteria could be ascribed to the presence of their outer phospholipidic membrane, almost impermeable to lipophilic compounds [NIKAIDO,1985].The absence of this barrier in Gram-positive bacteria allows the direct contact of the essential oils hydrophobic constituents with the phospholipid bilayer of

the cell membrane, where they bring about their effect, causing either an increase of ion permeability and leakage of vital intracellular constituents, or impairment of the bacterial enzyme systems [COWAN ,1999; WENDAKOON, 1995].

Table 3.

Minimal inhibitory concentration (MIC) of essential oil from A. herba alba.

Microorganisms	**Essential oil**
	MIC (mg/ml)*
Staphylococcus aureus ATCC25923	0.16
Staphylococcus epidermidis ATCC12228	0.33
Streptococcus pneumoniae	1.33 ± 0.01
Heamophilus influezae (1)	0.04
Heamophilus influezae(2)	0.33 ± 0.01
Pseudomonas aeruginosa ATCC27853	21
Escherichia coli (1)	0.66 ± 0.01
Proteus mirabilis	1.33
Neisseria meningitides (1)	0.04
Neisseria meningitides (2)	0.04 ± 0.01
Klebsiella pneumoniae	0.04
Pantoea sp	0.33

Data are presented as mean values ± SD.
Experiments were done in triplicate and results are mean values
(1) and (2): means that each bacteria is taken from two deferens patients
*difference between Microorganisms very significant ($p < 0.004$)

Zaika [ZAIKA,1988] proposed that Gram positive bacteria are more resistant then Gram-negative bacteria to the antibacterial properties of plant volatile oils which is in contrast to the hypothesis proposed by Deans that the susceptibility of bacteria to plant volatile oils and the Gram reaction appears to have little influence on growth inhibition [DEANS, 1987; DEANS, 1995].

Although the antibacterial activity of essential oils from many plant species has been extensively surveyed, their antimicrobial mechanism has not been reported in great detail. Since the active antimicrobial compounds of essential oils are phenolics and terpenes, it seems reasonable that their mode of action might be similar to that of other phenolic compounds. Most of the studies on the mechanism of phenolic compounds focused on their effects on cellular membranes, altering its response to antimicrobial challenge. These effects may develop as a result of membrane depolarization by altered ion transport or through changes in the membrane structure, inhibition of energy (ATP) generation by interference with glucose uptake or inhibition of enzymes involved in oxidative or substrate level phosphorylation. Increases in cytoplasmic membrane permeability appear to be a consequence of the loss of the cellular pH gradient, decreased ATP levels and, loss of the proton motive force, which lead to cell death [SKOČIBUŠIĆ, 2006].

Conclusions

The composition of the essential oil of *A. herba-alba* growing in Morocco has been analysed and its antimicrobial activity investigated. The results indicate that the oil may be used in the treatment of diseases caused by the micro organisms tested. Further toxicological and clinical studies are required to prove the safety of the oil as a medicine.

Acknowledgements

This work was financed by the cooperation between Morocco and Belgium UMP-CUD (university Mohamed I - the university commission for the developpement).

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Received: September 12, 2010
Accepted: November 21, 2010