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Full Length Research Paper

Essential oil composition and antimicrobial activities of aerial parts from Tunisian *Anacyclus clavatus* (Desf.)

Saoussen Hammami[1], Abdelkader Ben Salem[1], Maha Mastouri[2], Danilo Falconieri[3,4], Mohamed Gorcii[5], Mohamed Farouk M'henni[1], Bruno Marongiu[3] and Zine Mighri[1]*

[1]Unit Recherche12-04, Applied Chemistry and Environment, Faculty of Sciences of Monastir, 5000 Monastir, Tunisia.
[2]Laboratory of Bacteriology, University Hospital F. Bourguiba, 5000 Monastir, Tunisia.
[3]Department of Chemistry, University of Cagliari, Cittadella Universitaria of Montserrat, 09042, Cagliari, Italy.
[4]M. Technical Institute Giua, via Monte Cassino, Cagliari, Italy.
[5] Laboratory of Parasitology-Mycology, University Hospital F. Bourguiba, 5000 Monastir, Tunisia.

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Volatiles composition of aerial parts from medicinal *Anacyclus clavatus* (Asteraceae) was determined by gas chromatography-flame ionization detector (GC/FID) and gas chromatography-mass spectrometry (GC/MS) analysis. Altogether, 46 compounds were identified representing 81.2% of the total volatiles. The main components were *Trans*-Chrysanthenyl acetate (12.3%), *Cis*-Thujone (9.8%), Chrysanthenone (8.2%) and *Trans*-Thujone (7.3%). The volatile oil was screened for antibacterial and anti-*Candida* activities using dilution and disc diffusion methods. Thus, the studied fraction showed moderate activities against four *Candida* species and was most effective against Gram-negative *Pseudomonas aeruginosa*.

Key words: *Anacyclus clavatus* (Desf.), aerial parts, steam distillation, gas chromatography-mass spectrometry (GC/MS), antimicrobial effects.

INTRODUCTION

In recent year, essential oils are considered to be very useful in every day human life as food flavorings and as constituents of some cosmetics and perfumes. Some of them were useful as biocides and insect repellents. Many research works were focused on detailed studies of antimicrobial potentials of natural plants essential oils. Significant antimicrobial effects of various plant extracts against some pathogenic micro-organisms were reported (Hüsnü and Buchbaur, 2010; Bakkali et al., 2008). Many reports on the antifungal activity of the essential oils from aromatic plants belonging to Asteraceae family have been cited in the literature (Rahman, 2007). *Anacyclus clavatus* (Desf.) Pers. growing in Tunisia belongs to Asteraceae family and represents one from three indicated species of same genus widely distributed in Tunisia (Pottier, 1981). It is an annual polymorphous plant having 20 to 50 cm high with erect or decumbent stems and fairly divaricated branches. The leaves are simple and bipennate, the flowers are heterogamous gathered in rayed terminal capitula 2.5 to 3 cm. This specie is widespread throughout the world in the Mediterranean basin. In Tunisia, it grows especially in Kroumirie, Medjerda valley, in the North-East, the Cap Bon, in Tunisian central ridge and central Tunisia. In traditional folk medicine, *A. clavatus* flowers are known to be used for gastric ulcer treatment (The Floc'h E). *Pyrethrum* specie, belonging to same genus, has been studied for its medicinal uses and was reported to possess stimulant, cordial and rubefacient effects. The roots of *A. pyrethrum* have been included in many pharmaceutical preparations, based upon Indian medicine for the treatment of sciatica, paralysis, hemiplegia and amenorrhoea (Gautam et al., 2011). Previous biological studies demonstrated that roots have antibacterial, anti-inflammatory, antioxidant, immuno-stimulating and aphrodisiac activities (Sujith et al., 2011).

*Corresponding author. E-mail: zinemighri@yahoo.fr.

Evaluation of antibacterial activities of *A. pyrethrum* against some oral bacteria showed that methanol extract produced little antibacterial effects against *Staphylococcus aureus* and *Staphylococcus sanguis* (Jalayer et al., 2012). Anacycline has been reported as a natural insecticide in *A. pyrethrum* (Bergaoui et al., 2008a). To our knowledge, there are no previous studies related to *A. clavatus* essential oil chemical composition and biological effects. However, Bergaoui et al. (2008b) studied the chemical composition of *Anacyclus cyrtolepidioides* essential oil and signaled that monoterpene hydrocarbons constituted the major fraction represented by α-pinene as its chemotype. Flower heads from *A. cyrtolepidioides* essential oil showed a phagostimulant activity against *Tribolium confusum* larveae. The present study was carried out to investigate for the first time, the chemical composition and the anti-microbial effects of *A. clavatus* volatile fraction searching for alternative materials to synthetic antimicrobial drugs.

MATERIALS AND METHODS

Volatiles extraction

A. clavatus (Desf.) subject of this study was collected in March 2011 from Chott Meriam region, Sousse, Tunisia. Voucher specimens (A_1) have been deposited at Chemistry Department, Faculty of Science, Monastir, Tunisia. Aerial parts volatile fraction (1 kg) was prepared by steam distillation during 3 h. The oil (90 mg) was extracted with dichloromethane and dried over anhydrous sodium sulfate.

GC and GC/MS analysis

Analyses of the volatile extracts were carried out by gas chromatography (GC) and by gas chromatography-mass spectrometry (GC-MS). Analytical GC was carried out in a gas chromatograph (Agilent, Model 7890A, Palo Alto, CA), equipped with a flame ionization detector (FID), an autosampler (Agilent, Model 7683B), Agilent HP5 fused silica column (5% phenyl-methylpolysiloxane), 30 m × 0.25 mm i.d., film thickness 0.25 μm, and a Agilent ChemStation software system. Oven temperature was settled at 60°C, raising at 3°C min^{-1} to 246°C and then held 20 min at 246°C; injector temperature, 250°C; carrier gas, helium at 1.0 ml min^{-1}; splitting ratio, 1:10; detectors temperature, 300°C.

GC-MS analysis was carried out using a gas chromatograph (Agilent, Model 6890N, Palo Alto, CA, USA) equipped with a split-splitless injector, an Agilent model 7683 autosampler and an Agilent HP5-MS fused silica column (5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 μm). The GC conditions included programmed heating from 60 to 246°C at 3°C min^{-1}, followed by 20 min under isothermal conditions. The injector was maintained at 250°C. Helium was the carrier gas at 1.0 ml min^{-1}. Samples were run diluted in hexane with a dilution ratio of 1:100 and (1 μl) were injected in the split mode (1:20). The GC was fitted with a quadrupole mass spectrometer with an Agilent model 5973 detector. The MS conditions were as follows: ionization energy, 70 eV; electronic impact ion source temperature, 200°C; quadrupole temperature, 150°C; scan rate, 3.2 scan s^{-1}; mass range, 30 to 480 amu.

The software that was used to handle and analyse the mass spectra and chromatograms was an Agilent MSD ChemStation E.01.00.237. The linear retention indices (RIs) for all of the compounds were determined by injection of a hexane solution containing the homologous series of C_8-C_{26} n-alkanes (Van Den Dool and Kratz, 1963). The identification of the essential oil constituents was accomplished by comparison of their retention indices and their mass spectra with the literature data and the mass spectra databases, including HPCH2205 (Adams, 2007) and W8N05ST (NIST/EPA/NIH, 2005). Table 1 shows the chromatographic results, expressed as area percentages (GC) calculated without any response factor.

Antimicrobial activities

Test micro-organisms

Antimicrobial screening was performed using Gram-positive bacteria *S. aureus* (ATCC 27853) and *Enterococcus foecalis* (ATCC 29212), Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 25923) and the fungi *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) provided from the Laboratory of Parasitology-Mycology and the Laboratory of Bacteriology, CHU. F. BOURGUIBA, Monastir, TUNISIA.

Determination of minimum inhibitory concentration

10 mg of essential oil were dissolved in 1 ml of 10% DMSO solution. Overnight broth cultures were adjusted to yield approximately 1 × 10^6 CFU/ml of bacteria. The Minimal Inhibitory Concentrations (MIC) was determined on the basis of the broth microdilution assay (CLSI, 2008; Mousavi and Raftos, 2012) using liquid cultures in 96-well microplates for measuring bacterial growth. Two hundred microliters of the essential oil were added to four wells of the column 1 of each plate and then serially diluted with DMSO (10%) solution as doubling dilutions up to the well number eight of first column dilution factor (1:1). Each well was then inoculated with 50 mL of inocula. Four wells of one column from each plate were inoculated just with conidial suspension without any essential oil (positive control). Broth medium was used as a negative control. The micro plates were incubated for 24 h at 37°C

Antifungal activity assay

Disc diffusion method was employed during the preliminary antifungal screening of the volatile oil. Test strains suspension of 1 Mc Farland was prepared from fresh cultures. Plates were aseptically streaked with the tested micro-organism and allowed to dry for a few minutes. The sample (20 mg) was dissolved in 1 ml of methanol. Sterile filter paper Whatman discs (6 mm of diameter), were impregnated with 20 µl of *A. clavatus* essential oil sample, the discs were then aseptically placed on the inoculated sabouraud chloramphenicol plates. The plates were therefore incubated during 24 h at a temperature of 37°C. Tests were carried out in triplicates. The presence of a clear circular zone around the sample impregnated disc was used as an indicator of antifungal activity. The results were recorded by measuring diameter inhibition zones in mm. Disc impregnated with the solvent was used as negative control. For comparative purposes, standard drug fluconazole (40 µg/disc) was used as a positive control (Lutta et al., 2008).

RESULTS AND DISCUSSION

The GC-MS analysis of the volatile fraction was

Table 1. Retention Index calculated (RI) and Chromatographic area percentage of compounds found in the volatile fraction from aerial parts of *A. clavatus.*

RI_{litt}[a]	RI	Compound	% Area	Identification[b]
1026	1030	1,8-Cineole	0.2	MS, IR
1026	1033	Benzyl alcohol	1.2	MS, IR
1054	1057	γ Terpinene	0.8	MS, IR
1056	1059	Artemisia Ketone	0.4	MS, IR
1065	1065	*Cis*-sabinene hydrate	0.9	MS, IR
1098	1098	*Trans*-Sabinene hydrate	0.7	MS, IR
1103	1104	Filifolone	1.2	MS, IR
1101	1105	*Cis*-Thujone	9.8	MS, IR
1112	1115	*Trans*-Thujone	7.3	MS, IR
1118	1121	*Cis*-*β*-Menth-2-en-1-ol	0.7	MS, IR
1124	1124	Chrysanthenone	8.2	MS, IR
1137	1135	*Cis*-Verbenol	0.8	MS, IR
1137	1139	*Trans*-Sabinol	1.1	MS, IR
1146	1142	Eucarvone	2.2	MS, IR
1154	1156	*β*–Pinene oxide	0.2	MS, IR
1174	1176	Terpinen-4-ol	4.4	MS, IR
1186	1187	α-Terpineol	0.8	MS, IR
1194	1193	*Trans*-3(10)-Caren-2-ol	1.9	MS, IR
1215	1209	*Trans*-Carveol	0.6	MS, IR
1235	1235	*Trans*-Chrysanthenyl acetate	12.3	MS, IR
1272	1271	Isopiperitenone	0.3	MS, IR
1388	1392	1-Tetradecene	0.6	MS, IR
1484	1479	Germacrene D	2.0	MS, IR
1500	1495	Bicyclogermacrene	1.7	MS, IR
1577	1575	Spathulenol	1.2	MS, IR
1588	1593	1-Hexadecene	0.9	MS, IR
1627	1627	1-epi Cubenol	0.8	MS, IR
1635	1632	*Cis*-Cadin-4-en-7-ol	2.7	MS, IR
1647	1645	Himachalol	0.2	MS, IR
1649	1648	*β*-Eudesmol	0.7	MS, IR
1652	1653	α–Cadinol	0.5	MS, IR
1718	1714	Nonylphenol	0.4	MS, IR
1717	1718	*(Z)*-*α*-Atlantone	0.3	MS, IR
1789	1793	1-Octadecene	1.2	MS, IR
1987	1993	1-Eicosene	0.8	MS, IR
2035	2029	Z-Falcarinol	3.0	MS, IR
2077	2082	n-Octadecanol	0.8	MS, IR
2085	2085	dehydro-Juvibione	1.0	MS, IR
2114	2112	E-Phytol	0.5	MS, IR
2189	2194	1-Docosene	0.9	MS, IR
2300	2300	Tricosane	0.4	MS, IR
2395	2394	1-Tetracosene	0.6	MS, IR
2500	2500	Pentacosane	0.9	MS, IR
2589	2595	Cyclotetracosane	0.5	MS, IR
2700	2700	Heptacosane	1.2	MS, IR
2900	2900	Nonacosane	1.4	MS, IR
		Total identified	81.2	

Compounds are listed in order to their elution on the HP5-MS column; [a]linear retention indices (IR_{litt}) from literature (Adams, 2007 and NIST web book); [b]dentification has been realised by comparing mass spectra (MS) and linear retention indices (IR).

Table 2. Antifungal effects of *A. clavatus* volatile fraction (20 mg.mL^{-1})

Fungus	Inhibition zones diameter (mm)	
	***A. clavatus* volatile fraction**	**Fluconazole**
Candida albicans ATCC 90028	8±0	10
Candida parapsilosis ATCC 22019	8±0	30
Candida glabrata ATCC 90030	9±0	15
Candida Krusei ATCC 6258	7±0	15

Table 3. Minimum Inhibitory Concentrations of *A. clavatus* volatile oil against different pathogens using the Browth-dilution method.

Gram+/-	Bacteria	MIC (mg.ml^{-1})
Gram +	*Staphylococcus aureus* (ATCC 27853)	1.8
	Enterococcus foecalis (ATCC 29212)	3.5
Gram -	*Escherichia coli* (ATCC 25922)	1.8
	Pseudomonas aeruginosa (ATCC 25923)	0.62

allowed to identify 46 components representing 81.2% of the total oil constituents. Table 1 summarizes the identification, the retention indices as well as, the percentage of each compound. Fourteen hydrocarbons (13.9%), twenty one alcohols (24.2%), eight ketones (29.7%), two esters (13.3%), one carboxylic acid (0.3%), one ether (0.2%) and one epoxide (0.2%) were identified in *A. clavatus* aerial parts essential oil. We noticed that terpenes (68.44%) represents the main class of the oil constituents (63.94% of oxygenated terpenoïds and 4.5% are terpenes hydrocarbons). Oxygenated monoterpenoids predominate with 54.03% from the whole volatiles. Trans chrysanthenylacetate (12.3%) represented the chemotype followed by cis-thujone, chrysanhtenone and trans-thujone (9.8, 8.2 and 7.3%, respectively) as the most abundant monoterpenes in this fraction. Sesquiterpenoids were present in the volatile oil with a percentage of 13.07% where 9.35% from which are oxygenated.

We noticed that these results differ from those reported in the literature and is related to *A. cyrtolepidioïdes* growing in Tunisia, since essential oil from *cyrtolepidioïdes* specie was rich in monoterpene hydrocarbons dominated by α-pinene as a chemotype of the essential oil, (Zardi-Bergaoui et al., 2008a, b). However, chemical composition of *A. clavatus* oil was quite similar to that of *Tanacetum santolinoïdes* collected in Egypt and belonging to same Asteraceae family, *T. santolinoïdes* oil consisted mainly of oxygenated monoterpene compounds rich in thymol (17.9%), *Trans*-Thujone (17.5%) and *Trans*-Chrysanthenyl acetate (13.2%) (El-Shazly et al., 2002).

Antifungal properties of *A. clavatus* oil were evaluated for the first time against four *Candida* species: *Candida albicans*, *Candida parapsilosis*, *Candida glabrata* and *Candida Krusei*; the observed results are recorded in Table 2, thus, the volatile oil exhibited a broad spectrum of fungitoxic behavior against four *Candida* species exhibiting weak inhibition zones of 7 to 9 mm. *C. glabrata* was the most sensitive fungus (9 mm). According to literature, phenolic components usually contribute to antimicrobial activity of essential oils. A luck of potent antifungal volatiles like eugenol, camphor and linalool in *A. clavatus* volatile oil (Ashour et al., 2009) and the few amount of 1,8-cineole, could be the reason for its weak antifungal activities.

On the other hand, antibacterial activity of *A. clavatus* volatile oil against four gram positive and Gram negative bacteria was assessed by determining the MIC values as given in Table 3. These results showed that the tested oil have some significant antibacterial activities. The bacterial effects varied according to the type of pathogens. It is worthy to note that the volatile oil exhibited the greatest significant activity against Gram-negative *P. aeruginosa* bacterial strains (MIC=0.62 mg mL^{-1}). Previous studies signaled that essentials oils are generally more effective against Gram-positive than Gram-negative bacteria. *P. aeruginosa* is notorious for its involvement in nosocomial infections and frequent resistance to antibiotics. It was classified as the most highly resistant bacteria to essential oils. The resistance is the result of an external impermeable membrane to essential oil molecules and the presence of efflux mechanisms and porine dependant inhibition (Longbottom et al., 2004; Mayaud et al., 2008).

Conclusions

A. clavatus volatile oil dominated by terpenoids showed

significant antibacterial effects. *Trans*-Chrysanthenyl acetate (12.3%), *Cis*-Thujone (9.8%), Chrysanthenone (8.2%) and *Trans*-Thujone (7.3%) were the major constituents. These results suggest that the studied oil may possess some compounds which can be used as antimicrobial agents in new drugs for treatment of infection diseases. However, further tests are required to evaluate the antimicrobial effects of major phyto-chemicals, in particular *Trans*-Chrysanthenyl acetate.

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