Essential Oil Composition and Antimicrobial Activity of *Diplotaenia damavandica*

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Z. Naturforsch. **60 c**, 821–825 (2005); received March 21/April 27, 2005

Antimicrobial activity of the essential oils obtained from leaves, root and the seeds of *Diplotaenia damavandica* Mozaffarian, Hedge & Lamond, an endemic plant to Iran, was determined against 10 microorganisms using the disk susceptibility test as well as measuring minimum inhibitory concentrations. The results showed that all three oils had antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis* and *Escherichia coli*. The essential oil from the leaves had the highest antimicrobial activity against all test microorganisms including the fungal strains. The essential oils compositions were analyzed and determined by GC and GC-MS. The oils analyses resulted in the identification of 16, 17 and 20 compounds representing 94.2%, 96.4% and 95.1% of the total oils, respectively. The main components of the leaf essential oils were (*Z*)-β-ocimene (21.6%), α-phellandrene (21.3%) and terpinolene (20%). Dill apiol (30.1%) and γ-terpinene (16.2%) were the main components of the root and seed essential oils, respectively.

Key words: Antimicrobial Activity, Essential Oil Compositions, *Diplotaenia damavandica*

Introduction

Essential oils are rich sources of biologically active compounds. Recently, there has been a profound interest in the antimicrobial properties of the aromatic plants, particularly in their essential oils (Lis-Balchin and Deans, 1997; Pattnaik *et al*., 1997; Knobloch *et al*., 1989). Members of the parsley family (Apiaceae) are well known with regard to their diversity of essential oils (Yassa *et al*., 2003). This family is well represented in the Iranian flora with at least 112 genera and 316 species of which 75 are endemic (Hedge *et al*., 1987). *Diplotaenia damavandica* Mozaffarian, Hedge & Lamond is a perennial wild herb which grows exclusively in central Alborz Mountains around Tar Lake, Damavand, Iran (Hedge *et al*., 1987). The plant is locally called "kozal" and upon contact with skin followed by exposure to sunlight causes photosensitization (Aynechi *et al*., 1999). The extracts obtained from the aerial parts of the plant have been reported to have antifungul activity and contain furanocoumarins (Aynechi *et al*., 1999; Sardari *et al*., 2000).

As far as our literature survey could ascertain, composition of the essential oils from *D. damavandica* and its antimicrobial activity have not been reported previously. Here, we report the antimicrobial activity of the essential oils isolated from leaves, root and the seeds, as well as their compositions.

Material and Methods

Plant material

Leaves, root and the seeds of *Diplotaenia damavandica* were collected from the Tar Lake area, Damavand, Iran, at an altitude of 2200 m around June–September 2003. A voucher specimen (No. 85055) is deposited at the Herbarium of Biology Department, Shahid Beheshti University.

Essential oil isolation

The powdered plant parts (250 g) of *D. damavandica* were hydrodistilled using a Clevenger type apparatus for 3 h. The resulting essential oils

0939–5075/2005/1100–0821 $ 06.00

were dried over anhydrous sodium sulfate and stored at −4 °C until analyzed and tested.

Essential oil analysis

GC analyses of the essential oils were conducted using a Thermoquest gas chromatograph instrument equipped with a fused silica capillary DB-1 column (30 m × 0.32 mm i.d., film thickness, 0.25 μm). Nitrogen was used as the carrier gas at the constant flow rate of 1.1 ml/min. The oven temperature was held at 60 °C for 10 min, then programmed to 250 °C at a rate of 4 °C/min, and then held for 10 min. The injector and detector (FID) temperatures were kept at 250 °C and 280 °C, respectively. GC-MS analysis was carried out on a Thermoquest-Finnigan Trace GC-MS instrument equipped with a fused silica capillary DB-1 column (60 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was raised from 60 °C to 250 °C at a rate of 5 °C/min, held at 250 °C for 10 min; transfer line temperature was 250 °C. Helium was used as the carrier gas at a flow rate of 1.1 ml/min; split ratio was 1/50. The quadruple mass spectrometer was scanned over the 45–465 amu with an ionizing voltage of 70 eV and an ionization current of 150 μA. The constituents of the oils were identified by calculation of their retention indices under programmed temperature conditions for *n*-alkanes (C_6–C_{24}) and the oil on a DB-1 column under the same conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those reported in the literature (Adams, 1995; Davies, 1987; Shibamoto, 1987). Quantitative data was obtained from FID area percentages without the use of correction factors.

Microbial strains

Ten microbial reference strains were used which included *Bacillus subtilis* (ATCC 465), *Enterococcus faecalis* (ATCC 29737), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 85327), *Aspergillus niger* (ATCC 16404), *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763).

Antimicrobial screening by the disk diffusion method

The antimicrobial activities of essential oils were determined by the disk diffusion method (NCCLS, 1997). Briefly, 0.1 ml of a suspension of the test microorganism (10^8 cells/ml) was spread on Mueller-Hinton agar plates for bacteria and Sabouraud agar for the fungi. Sterile 6 mm disks, each containing 20 μl of essential oil, were placed on the microbial lawns. Disks containing dill apiol, ocimene, terpinolene, γ-terpinene and *p*-cymene (Fluka Chemicals, Taufkirchen, Germany) were used to study the antimicrobial activity of the major oils components at 10 μg/disk for bacteria and 30 μg/disk for the fungal strains. The plates were incubated at 37 °C for 24 h for bacteria and 30 °C for 48 h for fungi. The diameters of the zones of inhibition were measured and reported in mm. Triplicate tests were carried out for each oil.

Minimum inhibitory concentrations (MIC)

MIC values were determined by the broth microdilution assay recommended by the NCCLS (1999). Serial two-fold dilutions of the essential oils were made in Mueller-Hinton broth containing 0.5% Tween 80 for bacteria and Sabouraud dextrose broth with 0.5% Tween 80 for fungi within the range of 28.6 to 0.9 mg/ml in 96-well microtiter plates. Fresh microbial suspensions prepared from overnight grown cultures in the same media were added to give a final concentration of 5×10^5 organisms/ml. Controls of medium with microorganisms or the essential oil alone were included. The microplates were incubated at 37 °C for 24 h for bacteria and 30 °C for 48 h for fungi. The first dilution with no microbial growth was recorded as MIC.

Results and Discussion

Essential oils composition

The constituents of the essential oils obtained from leaves, root and the seeds of *D. damavandica* are presented in Table I, where the compounds are listed in order of their elution from a DB-1 column. The essential oils analyses resulted in identification of 16, 17 and 20 compounds representing

Table I. Composition of essential oils from leaves, root and seeds of *Diplotaenia damavandica*.

Compound	RI[a]	% of the oils		
		Leaf	Root	Seed
α-Pinene	0933	2.0	3.1	1.9
Sabinene	0967	0.7	0.4	1.8
Myrcene	0979	1.5	0.9	1.2
α-Phellandrene	1001	21.3	13.0	14.8
Δ-3-Carene	1007	–	0.4	–
p-Cymene	1013	2.5	4.0	15.9
β-Phellandrene	1024	11.2	–	–
(*Z*)-β-Ocimene	1027	21.6	7.1	8.5
(*E*)-β-Ocimene	1036	1.8	–	1.5
γ-Terpinene	1050	0.8	0.5	16.2
6-Camphenone	1079	–	9.2	–
Terpinolene	1083	20.0	14.0	4.7
allo-Ocimene	1117	0.7	–	–
p-Cymene-8-ol	1160	2.2	–	0.6
Estragol	1175	–	–	0.8
6-Camphenyl acetate	1225	–	7.3	–
Sabinyl acetate	1310	–	0.7	–
α-Terpinyl acetate	1338	–	–	2.0
Geranyl acetate	1358	–	0.4	–
β-Caryophyllene	1426	–	1.5	1.5
γ-Elemene	1431	–	–	2.1
γ-Curcumene	1474	–	–	1.6
Germacrene-D	1483	–	–	1.2
Elemicin	1519	2.8	–	–
Kessane	1533	0.6	2.8	8.5
Elemol	1541	–	0.9	–
Germacrene-B	1562	0.5	–	2.7
Spathulenol	1573	–	–	4.7
Dill apiol	1599	4.0	30.1	2.9
Monoterpene hydrocarbons		84.1	52.6	66.5
Oxygenated monoterpenes		2.2	8.4	3.4
Sesquiterpene hydrocarbons		3.9	4.4	17.6
Oxygenated sesquiterpenes		4.0	40.0	7.6
Total		94.2	96.4	95.1

Compounds listed in order of their elution from a DB-1 column.
[a]RI, retention index relative to *n*-alkanes (C_6–C_{24}).

94.2%, 96.4% and 95.1% of the total oils, respectively. Ten compounds were common in all three oils, of which (*Z*)-β-ocimene (21.6%), α-phellandrene (21.3%) and terpinolene (20.0%) had higher quantities in the leaf oil compared to the root and seed oils. Dill apiol (30.1%) was the major component of the root oil and *p*-cymene (15.9%), γ-terpinene (16.2%) and kessane (8.5%) were mostly found in the seed oil. β-Phellandrene (11.2%) and elemicin (2.8%) were two constituents found only in the leaf oil whereas

6-camphenone (9.2%) and 6-camphenyl acetate (7.3%) were found exclusively in the root oil. The latter two compounds were also reported to be present in the root oil of *D. cachrydifolia* (Harkis and Salehy-Surmaghy, 1987). Spathulenol (4.7%) was one of the five major components found specifically in the seed oil. Overall, monoterpene hydrocarbons were found to be the major compounds with 84.1%, 52.6% and 66.5% in leaf, root and seed oils, respectively. Oxygenated sesquiterpenes were found mainly in root oil (40.0%) with dill apiol (30.1%) as the main component. Sesquiterpene hydrocarbons (17.6%) were mostly found in seed oil with kessane (8.5%) as the second major compound.

Biological activity

All essential oils showed antibacterial activity by the disk diffusion assay. However, the best results were obtained with the leaf oil which was active not only against the bacterial strains but also produced good zones of inhibition against the fungal test organisms (Table II). The most susceptible bacteria were: *Bacillus subtilis* and *Staphylococcus aureus* (MIC values of 3.6 mg/ml) followed by *Staphylococcus epidermidis* and *Escherichia coli* (MIC 7.2 mg/ml), and *Enterococcus faecalis* (MIC 14.3 mg/ml). *Klebsiella pneumoniae* showed little susceptibility and *Pseudomonas aeruginosa* was resistant to all essential oils. There was a correlation between the MIC results with the disk sensitivity profile as shown in Table II. With inhibition zones of 19–21 mm, the MIC values were 3.6 mg/ml. Inhibition zones of 15–19 mm corresponded with MIC values of 7.2 mg/ml, and zones of 14 mm or less yielded higher MIC values (14.3–28.6 mg/ml). The MIC for the leaf oil against *Saccharomyces cerevisiae* with an inhibition zone of 25 mm was 1.8 mg/ml. Overall, the leaf oil had the highest activity against all microorganisms used. We believe that this is the first report on the antimicrobial activity of *D. damavandica.*

Table III shows the antimicrobial activity of the five commercial oils tested. Among these, terpinolene had the highest antimicrobial activity against all test organisms. The MIC values obtained for terpinolene were generally lower than the results obtained for the leaf oil but were comparable for *Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli*. Antimicrobial activity of the other commercial oils varied from weak to acceptable. Terpinolene is a monoterpene hydrocarbone which was shown to have antifungal, antibacterial and insecticidal properties

Table II. Antimicrobial activity of essential oils from *Diplotaenia damavandica*.

Microorganism	Leaf oil		Root oil		Seed oil		Standard antibiotics	
	DD[a]	MIC[b]	DD	MIC	DD	MIC	Ampicillin[c]	Nystatine[d]
Bacillus subtilis	20 ± 0.2	3.6 ± 0.5	19 ± 0.4	3.6 ± 0.4	18 ± 0.4	7.2 ± 0.4	14 ± 0.4	nt
Enterococcus faecalis	13 ± 0.2	14.3 ± 0.3	10 ± 0.1	28.6 ± 0.6	–	nt	11 ± 0.3	nt
Staphylococcus aureus	19 ± 0.4	3.6 ± 0.2	15 ± 0.2	7.2 ± 0.3	14 ± 0.3	28.6 ± 0.5	13 ± 0.3	nt
Staphylococcus epidermidis	17 ± 0.3	7.2 ± 0.4	16 ± 0.3	7.2 ± 0.6	14 ± 0.5	28.6 ± 0.3	19 ± 0.5	nt
Escherichia coli	17 ± 0.2	7.2 ± 0.2	–	nt	12 ± 0.2	28.6 ± 0.4	12 ± 0.2	nt
Klebsiella pneumoniae	11 ± 0.3	28.6 ± 0.4	–	nt	–	nt	–	nt
Pseudomonas aeruginosa	–	nt	–	nt	–	nt	9.7 ± 0.2	nt
Aspergillus niger	19 ± 0.4	7.2 ± 0.4	–	nt	–	nt	nt	16 ± 0.4
Candida albicans	21 ± 0.3	3.6 ± 0.3	–	nt	–	nt	nt	18 ± 0.5
Saccharomyces cerevisiae	25 ± 0.6	1.8 ± 0.4	–	nt	–	nt	nt	18 ± 0.2

Values given as mean ± standard deviation of triplicate tests.
[a] Zone of inhibition includes diameter of disc (6 mm).
[b] Minimum inhibitory concentration values in mg/ml.
[c] Tested at 10 μg/disc.
[d] Tested at 30 μg/disc.
Inactive (–); moderately active (7–13); active (> 14); nt, not tested.

Table III. Antimicrobial activity of the main compounds of the essential oils from *Diplotaenia damavandica*.

Microorganism	Dill apiol		Ocimene		Terpinolene		γ-Terpinene		*p*-Cymene	
	DD[a]	MIC[b]	DD	MIC	DD	MIC	DD	MIC	DD	MIC
B. subtilis	15 ± 0.6	7.2 (32.4) ± 0.2	14 ± 0.4	7.5 (55.0) ± 0.4	22 ± 0.8	3.6 (26.4) ± 0.3	19 ± 0.6	3.75 (27.5) ± 0.2	17 ± 0.6	3.75 (27.9) ± 0.4
E. faecalis	9 ± 0.1	> 14.3 (> 64.3) ± 0.2	8 ± 0.1	> 15 (110.1) ±0.3	16 ± 0.6	7.2 (52.8) ± 0.3	12 ± 0.4	7.5 (55.1) ± 0.2	–	nt
S. aureus	12 ± 0.4	14.3 (64.3) ± 0.4	9 ± 0.2	15 (110.1) ± 0.2	23 ± 0.8	3.6 (26.4) ± 0.2	9 ± 0.1	> 15 (110.1) ± 0.3	10 ± 0.2	15 (55.9) ± 0.3
S. epidermidis	14 ± 0.5	7.2 (32.4) ± 0.3	12 ± 0.3	7.5 (55.0) ± 0.4	25 ± 0.5	1.8 (13.2) ± 0.7	14 ± 0.2	7.5 (55.1) ± 0.4	9 ± 0.1	15 (55.9) ± 0.2
E. coli	10 ± 0.1	> 14.3 (> 64.3) ± 0.4	9 ± 0.1	> 15 (> 110.1) ± 0.3	19 ± 0.7	7.2 (52.8) ± 0.3	12 ± 0.1	7.5 (55.1) ± 0.5	12 ± 0.3	15 (55.9) ± 0.4
K. pneumoniae	–	nt	–	nt	17 ± 0.4	7.2 (52.8) ± 0.4	–	nt	11 ± 0.2	> 15 (> 55.9) ± 0.3
P. aeruginosa	–	nt	–	nt	–	nt	–	nt	–	nt
A. niger	11± 0.1	> 14.3 (> 64.3) ± 0.6	–	nt	25 ± 0.6	1.8 (13.2) ± 0.3	12 ± 0.3	> 15 (> 110.1) ± 0.4	–	nt
C. albicans	12 ± 0.2	14.3 (64.3) ± 0.5	–	nt	32 ± 0.8	0.9 (6.6) ± 0.4	15 ± 0.5	7.5 (55.1) ± 0.2	12 ± 0.2	> 15 (> 55.9) ± 0.3
S. cerevisiae	10 ± 0.1	> 14.3 (> 64.3) ± 0.4	–	nt	30 ± 0.7	0.9 (6.6) ± 0.4	13 ± 0.4	> 15 (> 110.1) ± 0.4	12 ± 0.1	> 15 (> 15 (55.9) ± 0.4

Values given as mean ± standard deviation of triplicate tests.
[a] Zone of inhibition includes diameter of disc (6 mm).
[b] Minimum inhibitory concentration values in mg/ml (millimolar).
Main compounds tested at 10 μl/disc on bacteria and 30 μl/disc on fungi.
Inactive (–); moderately active (7–13); active (> 14); nt, not tested.

(Faroog *et al*., 2002; Masten, 1999). The antifungal activity has been observed against various pathogens as well as the spores of *Diplodia pinea*. We also found that terpinolene had good antifungal activity (Table III). Antibacterial properties of terpinolene have been reported including activity against *Prpionibacterium* which causes acne. Weak antibacterial activity of α-pinene, sabinene and *p*-cymene has also been reported (Sokmen *et al*., 2003; Dorman and Deans, 2000; Bougatsos *et al*., 2003). The antimicrobial activity of the essential oils from *D. damavandica* may well be due to the presence of synergy between terpinolene and other constituents of the oil with various degrees of antimicrobial activity. Considering the fact that the leaf oil contained 20% terpinolene, the results obtained may be quite significant.

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