

The Bioactive Essential Oil of *Heracleum sphondylium* L. subsp. *ternatum* (Velen.) Brummitt[#]

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Z. Naturforsch. **58c**, 195–200 (2003); received August 22/October 15, 2002

The essential oil of *Heracleum sphondylium* L. subsp. *ternatum* (Velen.) Brummit (Umbelliferae) was isolated from crushed seeds by means of hydrodistillation and analyzed by GC and GC/MS. Major components were identified as 1-octanol (50.3%), octyl butyrate (24.6%), and octyl acetate (7.3%). Furthermore, antimicrobial activity of the oil was evaluated using microdilution broth and agar diffusion methods. The bioactive constituent of the essential oil was determined as 1-octanol by using a bioautography assay.

Key words: *Heracleum sphondylium* subsp. *ternatum*, Essential Oil, Antimicrobial Activity

Introduction

The genus *Heracleum* of the Umbelliferae family includes more than 70 species all around the world (Evans, 1996), and is represented in Turkey by 14 species. *H. sphondylium* which is known as “cow parsnip” in Europe is used against diarrhoea. It is known as tavşancılotu and used against dysentery in Turkey (Evans, 1996; Baytop, 1999). The use of this species for a liqueur preparation in France and as food or food additive in some Asian countries have been reported (French, 1971). Although some investigations on the biological activity of this species have been reported previously (Abel *et al.*, 1985; Uğur *et al.*, 1998; Weimark *et al.*, 1980; Amoros *et al.*, 1977), antimicrobial activity and the identification of the bioactive compounds of the essential oil have not been investigated. *Heracleum sphondylium* L. subsp. *ternatum* (Velen.) Brummitt grows in North-west Turkey (Davis, 1972).

As continuation of our ongoing work on bioactive Umbelliferae essential oils (Başer, 2000; Demirci *et al.*, 2000; Işcan, 2002), the essential oil of *H. sphondylium* subsp. *ternatum* was obtained by hydrodistillation. It was later evaluated against various human and plant pathogenic bacteria,

fungi and yeast. After determination of the antimicrobial activity by using the agar diffusion and microdilution broth methods, the active component of the essential oil was spotted using the thin layer chromatography (TLC)-bioautography assay (Işcan *et al.*, 2002). The chemical composition of the essential oil was revealed using both gas chromatography (GC) and gas chromatography/mass spectroscopy (GC/MS).

Methods and Materials

Plant material and isolation of the essential oil

The plant material was collected from Denizli, Elmayın Village (Turkey) in July 1999. Voucher specimens (ESSE 12874) are kept at the herbarium of Faculty of Pharmacy of Anadolu University, Turkey. The essential oil was obtained by hydrodistillation using a Clevenger-type apparatus for 3 h, from air dried and crushed fruits of *Heracleum sphondylium* subsp. *ternatum*. The essential oil yield was calculated on dry weight basis as 3.7%.

The essential oil and its constituent was analysed both by GC and GC/MS at the conditions:

Gas chromatography (GC)

GC analysis was carried out using a Shimadzu GC-17A system. CP-Sil 5CB column (25 m × 0.25 mm inner diameter, 0.4 µm film thickness)

[#] This work was presented at the 33rd Symposium on Essential Oils (33rd ISEO) on 4–7 September, 2002 in Lisbon, Portugal.

was used with nitrogen as carrier gas (1 ml/min). The oven temperature was kept at 60 °C and programmed to 260 °C at a rate of 5 °C/min, and then kept constant at 260 °C for 40 min. Split flow was adjusted at 50 ml/min. The injector and FID detector temperatures were at 250 °C. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Gas chromatography-mass spectrometry (GC/MS)

A Shimadzu GC/MS-QP5050A system, with CP-Sil 5CB column (25 m × 0.25 mm Ø) was used with helium as carrier gas. GC oven temperature was kept at 60 °C and programmed to 260 °C for at a rate of 5 °C/min, and then kept constant at 260 °C for 40 min. Split flow was adjusted to 50 ml/min. The injector temperature was at 250 °C. Mass spectra were recorded at 70 eV and the mass range was between *m/z* 30 to 425. Library search was carried out using the in-house "TBAM Library of Essential Oil Constituents".

Biological evaluation: antimicrobial assays

Microorganisms were stored at + 4 °C on agar slants. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (NRRL B-123), *Enterobacter aerogenes* (NRRL 3567), *Salmonella typhimurium* (NRRL B-4420), *Candida albicans* (Clinical Isolate, Osmangazi University, Faculty of Medicine, Eskişehir, Turkey), *Klebsiella pneumoniae*, *Yersinia enterocolitica*, *Listeria monocytogenes* (Ankara University, Department of Biology, Ankara, Turkey), *Bacillus cereus* (NRRL B-3711), *Staphylococcus epidermidis* (ATCC 12228), *Xanthomonas campestris* pv. *phaseoli* (Ankara Ziraat Araştırma Merkezi, Kemal Benlioğlu, Turkish Plant Pathogenic Bacteria: TPPB 5001), *Pseudomonas syringae* pv. *phaseolicola* (TPPB 4101), *Pseudomonas syringae* pv. *tomato* (TPPB 4212), *Pseudomonas syringae* pv. *syringae* (TPPB 4250) *Xanthomonas campestris* pv. *campestris* (NRRL B-1459), were used as test bacteria in the microdilution broth assay.

In the agar diffusion assay *Aspergillus flavus* (ATCC 9807), *Aspergillus parasiticus* (NRRL 2999), *Fusarium solani* (ATCC 12820), and *Sclerotium rolfsii* (Çukurova University, Faculty of Agriculture, Adana, Turkey) were used as the test

fungi to determine the antifungal activity of the essential oil. The bacteria and yeast were refreshed in Mueller Hinton Broth (Merck) at 35–37 °C, and inoculated on Mueller Hinton Agar (Mast Diagnostics, Merseyside, U. K.) media for examination of the strains. Filamentous fungi were refreshed on Sabouraud Dextrose Agar (Acumedia, Baltimore, Maryland, USA) slants or plates and were grown at 28 °C.

Microdilution broth method

Microdilution broth susceptibility assay (Konecman *et al.*, 1997; Vanden Berhge *et al.*, 1991; Zgoda *et al.*, 2001) was used for the antimicrobial evaluation of the oil and the main compound (1-octanol). Stock solution of essential oil and pure compound were prepared in dimethylsulfoxide (DMSO, Carlo-Erba, France). Dilution series using sterile distilled water were prepared from 4 mg/ml to 0.007 mg/ml in micro-test tubes (Eppendorf), which were transferred to 96-well microtiter plates. Overnight grown bacterial and *C. albicans* suspensions in double-strength Mueller-Hinton broth were standardised to 10⁸ CFU/ml using McFarland No: 0.5 standard solution. 100 µl of each microorganism suspension was then added into the wells. The last well-chain without microorganism was used as a negative control. Sterile distilled water and the medium served as a positive growth control. After incubation at 37 °C for 18–24 h the first well without turbidity was determined as the minimal inhibitory concentration (MIC). Chloramphenicol was used as standard antibacterial agent whereas ketoconazole was used as antifungal.

Agar diffusion method

This method (Janssen *et al.*, 1987; Vanden Berhge *et al.*, 1991) was used for screening the antifungal activity. Stock solutions of the essential oil and 1-octanol were arranged in the same way as described above. Spore solutions were prepared using Tween-80 (10% v/v, Aldrich). A Thoma counting chamber (Hawskley, London, U. K.) was used to determine the amount of spores (10⁶ spore/ml) in Tween-80. 25 ml of Sabouraud Dextrose Agar was poured into the Petri dishes having 120 mm-diameter, under sterile conditions. The spore solution (1 ml) was transferred and dis-

persed with a sterile Drigalsky spatula on the surface of the agar medium. Using a sterile cork-borer 9 mm-diameter wells were punched onto the medium. Each well was filled with 50 µl of the oil suspensions or other test samples where Ketocanazole was used as standard antifungal agent. Plates were incubated at 28 °C for 24–48 h.

Bioautography method

This technique was used to determine the active constituent of the essential oil (Janssen *et al.*, 1986; Rahalison *et al.*, 1991, 1994; Hostettmann, 1999). TLC was run for the essential oil followed by detection of the active constituent applying the tetrazolium salt. The compound was consequently isolated by scraping on eluting the appropriate zone.

Thin layer chromatography (TLC)

Precoated silica gel 60F 254 (0.2 mm, Riedel de Haën) plates, cut to appropriate size, on aluminium supports were used. The samples (1 µl) were applied using Drummond micro-capillaries onto two TLC plates and developed (90:10 v/v, *n*-hexane/ethyl acetate). In one plate, the separated compounds were visualized under UV light (365 and 254 nm) and sprayed with anisaldehyde/H₂SO₄ reagent followed by heating to 110 °C. The other plate was used for the bioautography assay, to determine the separated active components.

Determination of activity

For the formation of an agar base Nutrient Agar (15 ml, Difco) was poured in to the Petri dishes (120 mm-diameter). One of the developed TLC plates was then located on the agar base. The optical density at 600 nm (OD₆₀₀) of the *C. albicans* culture which was previously incubated at 37 °C for 8 h in Mueller Hinton Broth, was measured with a UV-spectrophotometer (OD₆₀₀ = 1 corresponds to approximately 10⁷ cells/ml). Sterilized Mueller Hinton Broth in addition of agar (7.5%) was used in molten form and kept at 45 ± 1 °C water bath. Pre-grown *C. albicans* was transferred into the molten agar to obtain a final concentration of 10⁵ cells/ml. Finally, the inoculated medium was poured onto the TLC plate and incubated at 37 °C for 24 h. The plate was sprayed using a 1% (w/v, EtOH) tetrazolium violet reagent (2,5-diphe-

nyl-3 [α-naphthyl] tetrazolium chloride, Sigma) and incubated at 37 °C for further 3 h. After incubation, inhibition zones were visualized against the coloured background.

Isolation of the active component

After identification of the active zone on the bioautographic TLC plate, prep-TLC was performed. The essential oil (approx. 20 mg) was applied onto plate (20 × 20 cm) and was developed in *n*-hexane/ethyl acetate (90:10 v/v) solvent system. The separated compounds were visualized using first UV light (365 and 254 nm). The isolation was carried out by scraping off the detected zone (R_f 0.3) with a spatula and transferred into a small percolator. The substance was then set free from silica by elution with acetone, which was then evaporated to yield 2 mg 1-octanol. The isolated substance (*n*-hexane/ethyl acetate, 90:10 v/v) was tested and compared for its purity using authentic 1-octanol (Alltech Inc., Deerfield, IL, USA) sample, TLC and GC/MS.

Results and Discussion

Heracleum sphondylium L. subsp. *ternatum* (Velen.) Brummitt essential oil was analyzed both by GC and GC/MS to determine its main constituents (Table I), and furthermore it was investigated for its antimicrobial properties against 21 human and plant pathogenic microorganisms (Table II). The composition and relative percentages of individual components of the oil was determined by chromatographic and spectroscopic means. As a result of GC and GC/MS analyses, 27 components were identified representing 92.6% of the total oil. 1-octanol (50.3%) and octyl butyrate (24.6%) and octyl acetate (7.3%) were characterized as main constituents. These results are in agreement with our recent essential oil work on this species (Özek *et al.*, 2002).

The essential oil showed, using the microdilution broth assay, an average inhibitory effect on human pathogenic bacteria from 0.125 to 1.0 mg/ml. However, the oil showed stronger inhibition (MIC 0.0312 mg/ml) against the plant pathogenic microorganisms *Pseudomonas syringae* pv. *syringae*, *Xanthomonas campestris* pv. *phaseoli* and *X. campestris* using the same test system. The yeast *Candida albicans* was also inhibited moderately

Table I. The GC analyses of *Heracleum sphondylium* subsp. *ternatum* essential oil.

Compound	%
3-methyl-2-buten-1-ol	0.1
Hexanol	0.4
Octanal	1.2
Butyl-2-methyl butyrate	0.1
Butyl-3-methyl butyrate	0.1
(Z)-3-octen-1-ol	2.6
1-Octanol	50.3
Nonanal	0.1
Isoamyl isovalerate	0.1
Amyl isovalerate	0.1
Hexyl butyrate	0.3
Decanal	0.1
Octyl acetate	7.3
Hexyl-2-methyl butyrate	0.3
Hexyl-3-methyl butyrate	0.6
Decanol	0.4
Carvacrol	0.1
Octyl isobutyrate	0.1
(Z)-4-Octenyl butyrate	1.1
Octyl butyrate	24.6
Decyl acetate	0.1
Octyl-2-methyl butyrate	0.4
Octyl-3-methyl butyrate	0.5
Myristicin	2.4
β-Sesquiphellandrene	0.2
Octyl hexanoate	1.2
Decyl butyrate	0.2

with a MIC value of 0.5 mg/ml, lower than that of the standard drug ketoconazole (MIC = 0.062 mg/ml). The antibacterial and anticandidal effects of the oil, against the standard antimicrobial agents

are given in detail Table II. Furthermore, the essential oil displayed a weak antifungal activity (with a concentration of 4 mg/ml) against *Aspergillus parasiticus*, *A. fumigatus*, *Fusarium solani* and *Sclerotium rolfsii* when tested using the agar dilution assay.

The antimicrobial activity observed in the assays applied above encouraged the application of the oil on a bioautography assay system. Bioautography is a very appropriate and simple technique for evaluating plant extracts and mixtures for their effects on human and plant pathogenic microorganisms. In particular, the agar-overlay assay (a bioautographic method) is useful for the examination of plant materials against yeasts such as *Candida albicans* (Hostettman, 1999). This technique enables to trace (in some cases, related to their polarity and absorption on the agar) active constituents. Consequently, it helps to visualize the inhibition zone after the incubation period. This compound was isolated and identified as a 1-octanol by GC/MS. The purity was also checked in comparison with a commercially available authentic sample. For the confirmation of antimicrobial effects of 1-octanol, a microdilution broth assay was also evaluated against all test microorganisms, where it showed moderate inhibitory effects ranging from 0.125 to 1.0 mg/ml (Table II).

Although the essential oil showed stronger inhibition (MIC 31.25 µg/ml) against *Pseudomonas syringae* pv. *syringae*, *Xanthomonas campestris* pv.

Microorganisms	Ess. Oil ^a	1-Octanol	ST
<i>Bacillus cereus</i>	125	125	15.6
<i>Enterobacter aerogenes</i>	500	500	62.5
<i>Escherichia coli</i>	500	500	31.25
<i>Klebsiella pneumoniae</i>	125	500	1.9
<i>Listeria monocytogenes</i>	125	125	1.95
<i>P. syringae</i> pv. <i>phaseolicola</i>	125	250	15.6
<i>P. syringae</i> pv. <i>syringae</i>	31.25	250	15.6
<i>P. syringae</i> pv. <i>tomato</i>	500	500	1.95
<i>Proteus vulgaris</i>	500	1000	15.6
<i>Pseudomonas aeruginosa</i>	1000	1000	62.5
<i>Salmonella typhimurium</i>	500	500	31.25
<i>Staphylococcus aureus</i>	500	500	3.9
<i>Staphylococcus epidermidis</i>	250	250	3.9
<i>X. campestris</i> pv. <i>phaseoli</i>	31.25	1000	3.9
<i>Xanthomonas campestris</i>	31.25	1000	3.9
<i>Yersinia enterocolitica</i>	125	500	1.9
<i>Candida albicans</i>	500	500	62.5 ^b

Table II. The MIC results of Essential oil and its main component (µg/ml).

^a Ess. Oil: *Heracleum sphondylium* subsp. *ternatum* essential oil.
St: Standard antimicrobial agent: chloramphenicol,
^b Ketoconazole (for yeast and fungi).

phaseoli and *X. campestris*, the pure compound 1-octanol showed moderate effects (MIC 250–1000 µg/ml) against the same tested microorganisms suggesting a possible synergistic effect.

Previous reports on antibacterial effects of 1-octanol against some plant pathogenic bacteria were consistent with our results. Maruzzella *et al.* (1963) reported that, using vapor phase test, 1-octanol showed moderate activity against *Pseudomonas syringae* pv. *striafaciens*, and no inhibition on *P. syringae* pv. *glycinea* (Maruzzella *et al.*, 1963). 1-octanol was also previously evaluated against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Propionibacterium acnes*, *Enterobacter aerogenes*, *E. coli* and *C. utilis*. Close to our results, they were inhibited with MIC values of 0.8, > 0.8, 0.4, 0.2, >0.8, 0.4, 0.2 mg/ml respectively. (Kubo *et al.*, 1995) In other studies, using serial dilution methods, *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus cereus* and *Candida albicans* were inhibited having MIC values of 600 (Pauli, 1994),

400 (Kubo *et al.*, 1995), 2000, 500, 500 (Kato *et al.*, 1980) and 500 ppm (µg/ml) (Maruzzella, 1962), respectively. Pathogens are economically damaging in agriculture and food industry. Therefore, there is a need to screen essential oils and plant extracts against plant pathogenic bacteria and fungi (Hadacek *et al.*, 2000). In this study, the examined essential oil showed significant inhibitory effect against some plant pathogenic bacteria.

In conclusion, the microdilution broth and bioautography assays in combination can give some information about bioactivity of plant products like essential oils and can help to identify the bioactive components in essential oils.

Acknowledgements

This study was supported by the Research Fund of Anadolu University (AÜAF-1048). The authors would like to thank Dr. Alexander Pauli for his AMICBASE-Database search of the antimicrobial constituent 1-octanol.

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