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

Gökalp İşcan[a], Fatih Demirci[a], Mine Kürkçüoğlu[a], Merih Kıvanç[b], and K. Hüsnü Can Başer[a,*]

[a] Medicinal and Aromatic Plant and Drug Research Centre (TBAM), Anadolu University, 26470-Eskişehir, Turkey. Fax: +90 222 335 01 27. E-mail: khcbaser@anadolu.edu.tr
[b] Faculty of Sciences, Department of Biology, Anadolu University, 26470-Eskişehir, Turkey

* Author for correspondence and reprint requests

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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; İşcan, 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 (İşcan *et al.*, 2002). The chemical composition of the essential oil was revealed using both gas chromatography (GC) and gas chromatography/mass spectroscopy (GC/MS).

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Methods and Materials

Plant material and isolation of the essential oil

The plant material was collected from Denizli, Elmayanı 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)

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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 Zirai 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 (Koneman *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 10^8 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 (10^6 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 corkborer 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 Ketoconazole 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/H_2SO_4 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 (OD_{600}) 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 (OD_{600} = 1 corresponds to approximately 10^7 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 10^5 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-diphenyl-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.

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

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

[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.

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Abel G., Erdelmeier C., Meier B., and Sticher O. (1985), Isopimpinellin, a furocoumarin from *Heracleum sphondylium* with chromosome damaging activity. Planta Med. **3**, 250–252.

Amoros M., Fauconnier B., and Girre L. (1977), Antiviral effects of some plant extracts. Ann. Pharm. Fr. **35**, 371–376.

Başer K. H. C. (2000), Recent Advances on the Umbelliferae Essential oils of Turkey, 8th International Symposium on Natural Product Chemistry, Karachi, Pakistan.

Baytop T. (1999), Türkiye'de Bitkiler ile Tedavi Geçmişten Bugüne (Therapy with Medicinal Plants in Turkey-Past and Present), 2nd Ed., Nobel Tip Basımevi, İstanbul, 373.

Davis P. H. (1972), Flora of Turkey and East Aegean Islands, University Press, Vol. **4**, Edinburgh, 265.

Demirci F., İşcan G., Güven K., Kırımer N., Demirci B., and Başer K. H. C. (2000), Antimicrobial activities of *Ferulago* essential oils. Z. Naturforsch. **55 c**, 886–889.

Evans W. C. (1996), Trease and Evans Pharmacognosy, 14th Edition, University of Nottingham, WB. Sanders Comp, Notthingam, UK.

French D. H. (1971), Ethnobotany of the Umbelliferae, The Biology and Chemistry of the Umbelliferae (Heywood V. H., ed.). Academic Press, London, England, pp. 385–402.

Hadacek F., and Greger H. (2000), Testing Of Antifungal Natural Products: Methodologies, Comparability of Results and Assay Choice. Phytochem. Anal. **11**, 137–147.

Hostettmann K. (1999), Strategy for the biological evaluation of plant extracts. Pure App. Chem. **70**, 1109–1113.

İşcan G. (2002), Antimicrobial Activity of Essential Oils from Some Umbelliferae Species, MSc Thesis, Graduate School of Natural and Applied Sciences, Anadolu University, Eskişehir, Turkey.

İşcan G., Kırımer N., Kürkçüoğlu M., Başer K. H. C., and Demirci F. (2002), Antimicrobial screening of *Mentha piperita* essential oils. J. Agric. Food Chem. **50**, 3943–3946.

Janssen A. M., Chin N. L. J., Scheffer J. J. C., and Baerheim S. A. (1986), Screening for antimicrobial activity of some essential oils by the agar overlay technique, Pharm. Weekbl. **8**, 289–292

Janssen A. M., Scheffer J. J., and Baerheim S. A. (1987), Antimicrobial activity of essential oils a literature review (1976–1986). Aspects of the test methods, Planta Med. **53**, 395–398.

Kato N. and Shibasaki I. (1980), The antimicrobial characteristics of 1-alkanols. Bokin-Bobai- Japan **8**, 1–7.

Koneman E. W., Allen S. D., Janda W. M., Schreckenberger P. C., and Winn W. C. (1997), Color Atlas and Textbook of Diagnostic Microbiology, Lippincott-Raven Publ., Philadelphia, USA, pp. 785–856.

Kubo I., Muroi H., and Kubo A. (1995), Structural functions of antimicrobial long-chain alcohols and phenols. Bioorganic Medic. Chem. **3**, 873–80.

Maruzzella J. C. (1962), The germicidal properties of perfume oils and perfumery chemicals. Amer. Perfumer Cosmetics **77**, 67–70.

Maruzzella J. C., Kleinberg C. C., and Urso C. J. (1963), Effects of vapors of aromatic chemicals on phytopathogenic bacteria. Plant Dis. Rep. **47**, 1067–70.

Özek T., Demirci B., and Başer K. H. C. (2002), Comparative study of the essential oils of *Heracleum sphondylium* ssp. *ternatum* obtained by micro- and hydro-distillation methods. Chem. Nat. Comp. **38**, 48–50.

Pauli A. (1994), Chemische, physikalische und antimikrobielle Eigenschaften von in ätherischen Ölen vorkommenden Phenylpropanen. Dissertation, Würzburg, Germany.

Rahalison L., Hamburger M., Hostettmann K., Monod M., and Frenk E. A. (1991), Bioautographic agar overlay method for the detection of antifungal compounds from higher plants. Phytochem. Anal. **2**, 199–203.

Rahalison L., Hamburger M., Monod M., Hostettmann K., and Frenk E. (1994), Antifungal tests in phytochemical investigations: comparsion of bioautographic methods using phytopathogenic and human pathogenic fungi. Planta Med. **60**, 41–44.

Uğur M. S., Gürkan E., Köksal E. P., and Tuzlacı E. (1998), Cytotoxicity assay and fibrinolytic evaluation of *Heracleum sphondylium* and *Ferulago thirkeana*. Fıtoterapia **69**, 378–379.

Vanden Berhge D. A. and Vlietinck A. J. (1991), Screening Methods for Antimicrobial and Antiviral Agents from Higher Plants. In: Methods in Plant Biochemistry (Harborne J. B. and Dey P. M., eds.). Academic Press, London, England, pp. 37–53.

Weimark G. and Nilsson E. (1980), Photoxicity in *Heracleum sphondylium*. Planta Med. **38**, 97–98.

Zgoda J. R. and Porter J. R. (2001), A convenient microdilution method for screening natural products against bacteria and fungi, Pharm. Microbiol. **39**, 221–225.