J Food Sci Technol (July 2017) 54(8):2193–2202
DOI 10.1007/s13197-017-2610-z

ORIGINAL ARTICLE

Edible wild plant *Heracleum pyrenaicum* subsp. *orsinii* as a potential new source of bioactive essential oils

Ljuboš Ušjak[1] · Silvana Petrović[1] · Milica Drobac[1] · Marina Soković[2] · Tatjana Stanojković[3] · Ana Ćirić[2] · Marjan Niketić[4]

Revised: 25 December 2016 / Accepted: 24 March 2017 / Published online: 16 June 2017

Abstract Many *Heracleum* L. taxa (Apiaceae) are used as food and spices, and in traditional medicine. In this work, the chemical composition of *Heracleum pyrenaicum* subsp. *orsinii* (Guss.) F. Pedrotti and Pignatti root, leaf and fruit essential oils, their antimicrobial activity and cytotoxic effect on malignant and normal cells were investigated for the first time. The composition of the oils was analyzed by GC and GC–MS. Monoterpenes prevailed in the root oil, with *β*-pinene (38.6%) being dominant, while in the leaf oil, sesquiterpenes, mostly (*E*)-nerolidol (20.5%) and (*E*)-caryophyllene (17.0%), were the most abundant constituents. The fruit oil contained the majority of aliphatic esters, mainly octyl acetate (36.8%) and octyl hexanoate (22.1%). The antimicrobial activity was determined by microdilution method against eight bacteria and eight fungi (standard strains, clinical or food isolates). The best antibacterial activity, better than the activity of ampicillin, was shown by the root oil against *Salmonella typhimurium*, *Escherichia coli* and *Pseudomonas aeruginosa*. The strongest antifungal activity, stronger than the activity of ketoconazole, was exhibited by the leaf and root oils against *Trichoderma viride*, and by the root oil against *Aspergillus ochraceus*. The cytotoxic effect of the oils, determined by MTT test, was prominent against malignant HeLa, LS174 and A549 cells (IC_{50} = 6.49–14.56 μg/mL). On the other hand, the oils did not show toxicity against normal MRC-5 cells at tested concentrations (IC_{50} > 200.00 μg/mL). It can be concluded that investigated *H. pyrenaicum* subsp. *orsinii* oils represent potential new raw materials for food and pharmaceutical industry.

Keywords *Heracleum pyrenaicum* subsp. *orsinii* · Essential oils · Chemical composition · Antimicrobial activity · Cytotoxic effect

Introduction

The genus *Heracleum* L. (Apiaceae) comprises about 125 aromatic taxa predominantly distributed in the temperate Northern Hemisphere, mainly in Eurasia (Pimenov and Leonov 2004). *Heracleum pyrenaicum* Lam. is edible wild plant that is often mentioned in survival handbooks in Serbia. The different parts of this plant are consumed as food. The young leaves are used to prepare salad, soup, stew and mash, their petioles are eaten after peeling and marinating, young stems are consumed fried, while roots, which contain significant amounts of sugar and starch, are used to make syrup and flour (Rašić 2002; Vračarić et al. 1977). The roots and aerial parts of this plant are also applied in folk medicine for the curing of intestinal catarrh and as aperitifs and antidiarrheal drugs (Rašić 2002). Similarly, in many other countries, several *Heracleum* taxa are used as food or traditional herbal medicines. For example, in India, *H. candicans* Wall. ex DC. fruits are added to food as spice and are known to be agents for the

✉ Silvana Petrović
silvana.petrovic@pharmacy.bg.ac.rs

[1] Department of Pharmacognosy, University of Belgrade - Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia

[2] Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia

[3] Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Serbia

[4] Natural History Museum, Njegoševa 51, 11000 Belgrade, Serbia

treatment of indigestion, sexual weakness and "nervous" disorders (Chauhan et al. 2014). In Iran, the fruits of *H. persicum* Desf. are used as carminative and spice for food, as well as pain killer herbal drug, and the young stems of this plant are consumed pickled (Hajhashemi et al. 2009).

The chemical composition and biological activities of *Heracleum* essential oils have been intensively investigated in the last decade (Chauhan et al. 2014; Hajhashemi et al. 2009; Karuppusamy and Muthuraja 2011; Tkachenko 2009). *Heracleum pyrenaicum* subsp. *orsinii* (Guss.) F. Pedrotti and Pignatti (Pignatti 1982) is a perennial plant, with simple, 5–7 lobed leaves and greenish flowers. This taxon inhabits the limestone screes of the mountains of the Balkan Peninsula, as well as Central and South Apennines (Brummitt 1968; Pignatti 1982). It belongs to widely circumscribed *H. sphondylium* L. group (Tonascia 1992). In this work, the chemical composition of *H. pyrenaicum* subsp. *orsinii* root, leaf and fruit essential oils, their antimicrobial activity against standard strains, clinical and food isolates, as well as cytotoxic activity on malignant and normal cell lines were investigated for the first time.

Materials and methods

Plant material

Plant material was collected on Mt. Durmitor in the northwestern part of Montenegro. The fruits were collected in August 2011, and the roots and leaves in August 2013. Voucher specimen is deposited in the Herbarium of the Natural History Museum, Belgrade (BEO) under collector number 20110804/BEO. The plant was identified by Dr. Marjan Niketić, curator/botanist of the BEO.

Isolation of the essential oils

Air-dried material was powdered (roots and fruits) or crashed (leaves) and hydrodistilled using Clevenger-type apparatus for 2.5 h. Collecting solvent was *n*-hexane. The oils were dried over anhydrous sodium sulfate and kept at 4 °C until analysis.

Essential oils analysis

The chemical composition of isolated oils was analyzed by Gas chromatography (GC) and Gas chromatography-Mass spectrometry (GC–MS). GC analysis was carried out using an Agilent 6890N gas chromatograph (Agilent Technologies, USA), equipped with a split/splitless injector (200 °C), attached to a HP-5MS capillary column (Agilent Technologies; 30 m × 0.25 mm; film thickness 0.25 μm) and connected to a flame ionization detector (FID). The FID and transfer line temperatures were set at 300 and 250 °C, respectively. Split ratio was 1:10 and the injected volume was 1 μL of 3% solution of oil in 99.9% (v/v) ethanol. The carrier gas was He (1.0 mL/min). The thermal program was set from 60 to 280 °C at a rate of 3 °C/min. GC–MS analysis was performed on an Agilent 6890–5975 GC–MS system, operating in the electron ionization (EI) mode at 70 eV, equipped with a split/splitless injector (200 °C) and attached to a HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The analytical conditions were the same as that used for the GC analysis. The identification of the compounds was based on the comparison of their retention indices (RI), retention times (Rt) and mass spectra to those from the NIST/NBS 05, Wiley libraries 8th edition and the literature (Adams 2007; Nitz et al. 1990). The linear RIs were determined in relation to homologue series of *n*-alkanes (C_8–C_{40}) ran under the same operating conditions. Relative percentages of the compounds were calculated based on the peak areas from the FID data.

Antimicrobial activity

Microbial strains

The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Listeria monocytogenes* (NCTC 7973) and *Micrococcus flavus* (ATCC 10240), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311) and *Enterobacter cloacae* (human isolate) were used. The fungi *Aspergillus fumigatus* (human isolate), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *A. niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112) and *P. verrucosum* var. *cyclopium* (food isolate) were tested. The micromycetes were maintained on malt agar, the cultures stored at 4 °C and sub-cultured once a month.

Antibacterial activity

Minimum inhibitory and minimum bactericidal concentrations (MICs and MBCs) were determined by the microdilution method in 96-well microtitre plates (CLSI 2009; Tsukatani et al. 2012). Bacterial suspensions were adjusted with sterile saline to a concentration of 1.00×10^5 CFU/mL. The oils were dissolved in 5% dimethylsulfoxide (DMSO) solution that contained 0.10% Tween 80 (v/v) (10 mg/mL) and added to Tryptic Soy broth (TSB) medium (100 μL) with bacterial inoculum

(1.00×10^4 CFU per well), to achieve concentrations from 0.21 to 8.30 mg/mL. The MICs were defined as the lowest concentrations without visible bacterial growth (determined at binocular microscope). The MICs were also determined by the colorimetric microbial viability assay that is based on the reduction of *p*-iodonitrotetrazolium violet (INT) color. Results were compared to the positive controls. The MBCs were determined by serial sub-cultivations of 2 µL of tested oils (dissolved in medium and inoculated for 24 h) into microtitre plates that contained 100 µL of broth per well, after further incubation for 24 h. The lowest concentration without visible bacterial growth was defined as the MBC, indicating that 99.5% of the original inoculum was killed. The optical density of each well was measured by Microplate manager 4.0 (Bio-Rad Laboratories, USA) at the wavelength of 655 nm and compared to the blank and positive controls. Streptomycin, Sigma P 7794 (0.04–0.52 mg/mL) and ampicillin, Panfarma, Serbia (0.25–1.24 mg/mL) were used as the positive controls. 5% DMSO was used as the negative control.

Antifungal activity

In order to investigate the antifungal activity of the oils, modified microdilution technique was used (Espinel-Ingroff 2001; Hänel and Raether 1988). Fungal spores were washed off from the surface of agar plates with 0.85% sterile saline that contained 0.10% Tween 80 (v/v). Spore suspensions were adjusted with sterile saline to a concentration of 1.00×10^5 in the final volume of 100 µL per well. The oils were dissolved in 5% DMSO solution that contained 0.10% Tween 80 (v/v) (10 mg/mL) and added to broth Malt medium with the inoculum (to achieve concentrations 0.12–8.26 mg/mL). The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The minimum fungicidal concentrations (MFCs) were determined by serial sub-cultivations of a 2 µL of the tested oils (dissolved in medium and inoculated for 72 h) into microtitre plates that contained 100 µL of broth per well, after further incubation for 72 h at 28 °C. The MFC was defined as the lowest concentration without visible growth, indicating that 99.5% of the original inoculum was killed. Commercial fungicides bifonazole, Srbolek, Serbia (0.10–0.25 mg/mL) and ketoconazole, Zorkapharma, Serbia (0.20–3.50 mg/mL) were used as the positive controls. 5% DMSO was used as the negative control.

Statistical analysis

All the tests were carried out in triplicate. The results were expressed as mean values ± standard deviation (SD), and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD test with $\alpha = 0.05$, to determine whether there is a statistically significant difference between them. The analysis was carried out by Statistical Package for the Social Sciences (SPSS) version 18.0.

Cytotoxic activity

Cell cultures

Cervix adenocarcinoma HeLa, human colon carcinoma LS174, non-small cell lung carcinoma A549, as well as human normal fetal lung fibroblast MRC-5 cell lines (ATCC) were cultured as a monolayer in the RPMI 1640 nutrient medium, supplemented with heat inactivated (at 56 °C) 10% fetal bovine serum (FBS), 3 mmol/L of L-glutamine and antibiotics, at 37 °C, in a humidified air atmosphere with 5% CO_2.

Treatment of cell lines

In vitro assay for the cytotoxic activity of the oils was performed when the cells reached 70–80% of confluence. The stock solution (100 mg/mL) of each oil was dissolved in RPMI 1640 medium to obtain required concentrations. Neoplastic HeLa (2000 cells per well), LS174 (7000 cells per well), A549 (5000 cells per well) and normal MRC-5 cells (5000 cells per well) were seeded into 96-well microtitre plates and 24 h later, after the cell adhesion, five different, double diluted concentrations of the oils were added to the wells. The final concentrations of the oils were 12.5, 25, 50, 100 and 200 µg/mL. Control wells contained only nutrient medium that was made of RPMI 1640 medium, supplemented with 3 mmol/L L-glutamine, 100 mg/mL streptomycin, 100 IU/mL penicillin, 10% heat inactivated (56 °C) FBS and 25 mmol/L HEPES (2-[4-(2-hydroxyethyl)piperazinyl] ethanesulfonic acid). The pH of the medium was adjusted to 7.2 with bicarbonate solution. The cultures were incubated for 72 h.

Determination of cell survival (MTT test)

The effect of the oils on cell survival was determined by the MTT test (microculture tetrazolium test), according to Mosmann (1983) with modification by Ohno and Abe (1991), 72 h after the addition of the oils. Briefly, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL phosphate-buffered saline, PBS) was added to each well. The samples were incubated for further 4 h, at 37 °C, in 5% CO_2 humidified air atmosphere. During this period MTT dye was converted to insoluble product, formazan by viable cells. This precipitate was then dissolved by adding 100 µL of 10% sodium dodecylsulfate (SDS). The number of viable cells in each

well was proportional to the intensity of the light absorbance (A) that was measured 24 h later by an ELISA plate reader (Thermo Fisher Scientific Inc., Australia) at 570 nm. To calculate cell survival (%), the A of a sample with cells grown in the presence of various concentrations of the oils were divided with control optical density (the A of control cells grown only in nutrient medium) and multiplied by 100. The A of the blank was always subtracted from the A of the corresponding sample with target cells. The IC_{50} value was defined as the concentration of an agent that inhibits the survival of 50% cells, compared to the vehicle treated control. Cisplatin was used as the positive control. The IC_{50} values were expressed as mean values ± SD that were determined on the basis of the results of three independent experiments.

Results and discussion

Chemical composition of the essential oils

The hydrodistillation of *H. pyrenaicum* subsp. *orsinii* roots, leaves and fruits yielded 0.13, 0.20 and 0.61% (w/w) of the yellow essential oils, respectively. Seventy-five components were identified in the root oil, eighty-nine in the leaf oil and seventy-eight in the fruit oil (representing 91.3, 94.2 and 90.2% of the total oils, respectively) (Table 1).

The dominant monoterpene fraction of the root oil (70.2%) was characterized by non-oxygenated components (67.5%), with β-pinene being the most abundant (38.6%). Similarly, Tkachenko (2009) reported the prevalence of monoterpenes, mostly β-pinene (17.6–39.0%), in the root oils of seven other *Heracleum* taxa grown at experimental station of V. L. Komarov Botanical Institute in Leningrad Oblast′ (Russia), with the highest amounts being present in the root oils of Caucasian species, *H. wilhelmsii* Fisch. and C. A. Mey. and *H. ponticum* (Lipsky) Schischk. ex Grossh. The monoterpene fraction of *H. pyrenaicum* subsp. *orsinii* leaf oil was less abundant (20.3%), but qualitatively very similar to the monoterpene fraction of the root oil. The leaf oil was characterized by the high percentage of sesquiterpenes (65.4%), and among them oxygenated (33.6%) and non-oxygenated (31.8%) constituents were present in similar amounts, with (*E*)-nerolidol (20.5%) and (*E*)-caryophyllene (17.0%) being the most prominent. In the oil obtained by microdissection of widespread *H. sphondylium* L. subsp. *sphondylium* leaf companion canals, sesquiterpenes were also dominant; (*E*)-caryophyllene (28.0%) prevailed, while (*E*)-nerolidol was not identified (Bicchi et al. 1990). In contrast to *H. pyrenaicum* subsp. *orsinii* root and leaf oils, the fruit oil of this taxon contained significantly lower quantity of terpenes. This oil was mainly composed of aliphatic esters (78.7%), with octyl acetate (36.8%) and octyl hexanoate (22.1%) being the principal constituents. This is in accordance with the conclusion of Başer (2002) that aliphatic esters, e.g. octyl esters, can be considered as marker compounds of *Heracleum* fruit oils. For example, octyl acetate (65.3%) also dominated in the oil of *H. siamicum* Craib fruits, which are used as spice in Thailand (Kuljanabhagavad et al. 2011).

Antimicrobial activity of the essential oils

The antimicrobial activity of *H. pyrenaicum* subsp. *orsinii* essential oils was determined by microdilution method, and expressed as minimal inhibitory concentrations (MICs) and minimal bactericidal/fungicidal concentrations (MBCs/MFCs) (Tables 2, 3).

The leaf essential oil exhibited the best antibacterial activity against the clinical isolate of *B. cereus* (MIC = 0.21 mg/mL, MBC = 0.53 mg/mL). This effect was comparable to the effect of ampicillin. The best activity against all other tested bacteria was shown by the root oil. The effect of this oil against *S. typhimurium*, *E. coli* and *P. aeruginosa* (MICs = 0.23 mg/mL, MBCs = 0.47 mg/mL) was comparable with the activity of streptomycin and even better than the effect of ampicillin, while the effect against *Staphylococcus aureus* (MIC = 0.23 mg/mL, MBC = 0.47 mg/mL) was similar to the activity of ampicillin. These bacteria cause a wide variety of diseases. For example, *B. cereus*, *S. typhimurium*, *E. coli* and *S. aureus* are the source of foodborne diseases. Moreover, *E. coli* causes urinary infections, while *S. aureus* causes respiratory, urinary, skin and eye infections. They are both the significant source of hospital-acquired infections. Similarly, *P. aeruginosa* causes nosocomial respiratory, urinary and wound infections (Pommerville 2011).

The most significant antifungal activity was exhibited by *H. pyrenaicum* subsp. *orsinii* leaf (MIC = 0.12 mg/mL, MFC = 0.25 mg/mL) and root (MIC = 0.23 mg/mL, MFC = 0.46 mg/mL) oils against *T. viride*, and the root oil (MIC = 0.46 mg/mL, MFC = 1.88 mg/mL) against *Aspergillus ochraceus*. These effects were more pronounced than the effects of ketoconazole. Additionally, the activity of the leaf oil against *T. viride* was comparable with the activity of bifonazole. Although the members of *Trichoderma* genus rarely infect humans, some species can cause infections in immunocompromised patients (De Miguel et al. 2005). *Aspergillus ochraceus*, on the other hand is a food contaminant that produces nephrotoxic, hepatotoxic, teratogenic and immunosuppressive ochratoxin A (Basílico and Basílico 1999).

The effect comparable with the effect of ketoconazole and slightly weaker than the effect of bifonazole was shown by *H. pyrenaicum* subsp. *orsinii* root oil

Table 1 Chemical composition of *H. pyrenaicum* subsp. *orsinii* essential oils (%)

RI exp[a]	RI lit[b]	Compound	Root oil	Leaf oil	Fruit oil
865	–	2-Methyl octane	0.5[c]	tr[d]	–
870	863	*n*-Hexanol	0.1	–	tr
886	880	Isopropyl 2-methyl butanoate	–	–	0.4
894	–	Isopropyl isovalerate	–	–	0.3
901	900	*n*-Nonane	0.6	tr	tr
904	901	Heptanal	1.4	tr	tr
916	908	Isobutyl isobutanoate	–	tr	tr
932	924	α-Thujene	tr	tr	tr
940	932	α-Pinene	7.4	3.4	1.9
955	946	Camphene	0.7	0.6	0.4
961	953	Thuja-2,4(10)-diene	tr	tr	tr
979	969	Sabinene	tr	0.3	tr
986	974	*β*-Pinene	38.6	5.5	0.3
994	988	Myrcene	2.1	1.7	tr
1004	–	Isobutyl 2-methyl butanoate	–	0.3	1.6
1006	998	*n*-Octanal	3.5	–	tr
1008	–	Isobutyl isovalerate	–	0.2	0.4
1015	1007	Isoamyl isobutanoate	–	tr	tr
1018	–	2-Methyl butyl isobutanoate	–	0.2	0.5
1029	1020	*p*-Cymene	tr	1.2	tr
1034	1024	Limonene	4.4	2.4	0.9
1043	1032	(*Z*)-*β*-Ocimene	11.2	1.0	tr
1045	–	Butyl 2-methyl butanoate	–	tr	tr
1049	–	Butyl isovalerate	–	tr	tr
1052	1044	(*E*)-*β*-Ocimene	tr	0.3	tr
1057	–	(4*Z*)-2-Methyl-4-decene	0.4	–	–
1062	1049	(2*E*)-Octen-1-al	tr	–	–
1063	1054	γ-Terpinene	tr	0.4	–
1066	1047	(3*Z*)-Octen-1-ol	–	–	0.5
1067	–	2-Methyl decane	0.3	tr	–
1073	1063	*n*-Octanol	tr	tr	3.0
1092	1086	Terpinolene	3.2	tr	tr
1094	–	(*E*)-4-Undecene	0.2	tr	tr
1099	1095	6-Camphenone	tr	–	tr
1100	1100	*n*-Undecane	tr	–	tr
1101	1100	Isopentyl 2-methyl butanoate	–	0.4	tr
1105	1100	*n*-Nonanal	0.4	–	–
1106	1100	2-Methyl butyl 2-methyl butanoate	–	1.4	1.3
1108	1102	Isopentyl isovalerate	–	tr	tr
1110	1103	2-Methyl butyl isovalerate	tr	1.2	0.6
1125	1118	*cis*-*p*-Menth-2-en-1-ol	tr	0.3	tr
1130	1122	α-Campholenal	tr	tr	tr
1132	1128	*allo*-Ocimene	tr	tr	–
1135	1128	(*Z*)-Epoxy-ocimene	tr	tr	tr
1142	1135	Nopinone	tr	tr	–
1144	1136	*trans*-*p*-Menth-2-en-1-ol	–	0.2	–
1144	1137	*trans*-Sabinol	0.4	–	tr
1150	–	4,8-Epoxy-*p*-menth-1-ene	0.3	–	–
1150	1140	*trans*-Verbenol	–	0.5	–
1152	1147	Hexyl isobutanoate	–	tr	0.5

Table 1 continued

RI exp[a]	RI lit[b]	Compound	Root oil	Leaf oil	Fruit oil
1167	1160	Pinocarvone	tr	tr	tr
1170	1165	Borneol	–	tr	tr
1181	1174	Terpinen-4-ol	0.5	tr	tr
1187	–	1-Methyl butyl 3-methyl 2-butenoate	tr	tr	tr
1188	1179	*p*-Cymen-8-ol	tr	tr	–
1193	1186	α-Terpineol	0.4	tr	–
1197	1193	(4*Z*)-Decenal	–	–	tr
1200	1195	Myrtenal	0.1	0.2	–
1200	1195	Methyl chavicol	0.8	1.1	–
1210	1201	*n*-Decanal	–	–	2.1
1223	1211	Octyl acetate	–	–	36.8
1242	1233	Hexyl 2-methyl butanoate	–	tr	0.7
1247	1241	Hexyl isovalerate	tr	tr	0.6
1265	1260	(2*E*)-Decenal	1.6	tr	–
1288	1287	Bornyl acetate	tr	1.0	0.5
1294	1292	(2*E*,4*Z*)-Decadienal	tr	–	–
1299	1300	*n*-Tridecane	tr	tr	–
1318	1315	(2*E*,4*E*)-Decadienal	0.6	tr	tr
1349	–	Octyl isobutanoate	–	tr	0.8
1387	1387	β-Bourbonene	–	0.2	–
1393	–	Octyl butanoate	–	–	3.6
1395	1389	β-Elemene	tr	3.0	–
1396	–	1-Butenylidene-cyclohexane	–	–	1.0
1397	1393	Phenyl ethyl isobutanoate	–	tr	–
1405	1403	Methyl eugenol	0.3	tr	–
1412	1407	Decyl acetate	–	tr	0.6
1412	1408	Dodecanal	–	–	0.9
1417	–	Bornyl isobutanoate	0.4	0.7	tr
1428	1417	(*E*)-Caryophyllene	–	17.0	tr
1437	–	Octyl 2-methyl butanoate	–	–	0.7
1438	1432	α-*trans*-Bergamotene	tr	tr	tr
1442	–	Octyl isovalerate	tr	–	0.5
1458	1452	α-Humulene	–	2.1	tr
1458	1454	(*E*)-β-Farnesene	tr	–	–
1485	1484	Germacrene D	–	2.5	–
1489	1486	Phenyl ethyl 2-methyl butanoate	–	0.9	tr
1491	1499	4-*epi*-*cis*-Dihydroagarofuran	0.5	–	–
1494	1490	Phenyl ethyl isovalerate	–	0.5	tr
1499	1500	Bicyclogermacrene	tr	1.9	–
1500	1500	Isodaucene	tr	–	–
1510	1505	β-Bisabolene	0.6	3.2	–
1519	–	Bornyl isovalerate	0.6	0.5	tr
1526	1521	β-Sesquiphellandrene	tr	0.3	–
1531	1529	Kessane	2.2	tr	–
1534	1529	(*E*)-γ-Bisabolene	0.3	tr	–
1545	1545	Selina-3,7(11)-diene	tr	tr	–
1561	1555	Elemicin	tr	–	tr
1561	1559	Germacrene B	–	1.5	–
1565	1561	(*E*)-Nerolidol	0.4	20.5	tr

Table 1 continued

RI exp[a]	RI lit[b]	Compound	Root oil	Leaf oil	Fruit oil
1580	1568	(*Z*)-Isoelemicin	–	0.1	–
1580	1577	Spathulenol	0.3	4.8	–
1584	–	Octyl hexanoate	–	–	22.1
1590	1582	Caryophyllene oxide	tr	6.5	–
1633	1631	(*E*)-Sesquilavandulol	0.4	–	–
1637	1639	Caryophylla-4(14),8(15)-dien-5*β*-ol	–	0.3	–
1641	1639	Caryophylla-4(14),8(15)-dien-5α-ol	–	1.1	tr
1662	1665	Intermedeol	0.8	–	–
1689	1685	Germacra-4(15),5,10(14)-trien-1-α-ol	–	0.5	–
1782	–	Octyl octanoate	–	–	6.7
1837	–	Neophytadiene	–	0.3	tr
1844	–	Hexahydrofarnesyl acetone	–	0.3	tr
1898	1900	*n*-Nonadecane	–	–	tr
1963	1959	Hexadecanoic acid	1.0	0.5	–
2024	2033	Isobergapten	tr	–	tr
2034	2035	(*Z*)-Falcarinol	1.9	–	tr
2054	2056	Bergapten	tr	–	tr
2095	2100	*n*-Heneicosane	–	–	tr
2099	–	*γ*-Palmitolactone	–	0.3	–
2109	–	Phytol isomer	–	0.6	–
2122	–	Pimpinellin	0.4	–	tr
2194	–	Falcarindiol	1.4	–	–
2232	2237	Isopimpinellin	tr	–	–
2293	2300	*n*-Tricosane	–	tr	tr
2492	2500	*n*-Pentacosane	–	tr	–
2691	2700	*n*-Heptacosane	–	0.2	–
2889	2900	*n*-Nonacosane	–	0.2	–
Monoterpene hydrocarbons			67.5	16.8	3.5
Oxygenated monoterpenes			2.7	3.5	0.5
Sesquiterpene hydrocarbons			0.9	31.8	tr
Oxygenated sesquiterpenes			4.6	33.6	tr
Aliphatic esters			tr	3.6	78.7
Others			15.6	4.9	7.5
Total identified			91.3	94.2	90.2

[a] RI exp—retention indices on HP-5MS column relative to C_8–C_{40} *n*-alkanes

[b] RI lit—retention indices obtained from the literature (Adams 2007)

[c] Relative area percentage of the compounds obtained from FID area percent data

[d] tr—trace (<0.1%)

(MIC = 0.23 mg/mL, MFC = 0.46 mg/mL) against the human isolate of *A. fumigatus*. This result is particularly interesting because *A. fumigatus* is an airborne pathogen that causes a usually fatal invasive aspergillosis in immunosuppressed hosts (Pommerville 2011).

Among analyzed essential oils, the fruit oil had the weakest antimicrobial activity. This is in accordance with its chemical composition, i.e. the fruit oil was dominated by aliphatic esters, compounds with lower antimicrobial potential than terpenic constituents identified in the root and leaf oils (Maggi et al. 2014). Demonstrated activity of the root and leaf oils can be at least partly explained by the presence of terpenes, antimicrobial potential of which was established previously. Among them are not only the major components of these oils [namely *β*-pinene in the root oil, and (*E*)-nerolidol and (*E*)-caryophyllene in the leaf oil], but also some of their minor compounds (such as α-pinene, caryophyllene oxide, limonene, germacrene D and α-humulene) (Setzer et al. 2006; Soković et al. 2010; Tao et al. 2013). Stronger antimicrobial activity of the root oil in contrast to the leaf oil could be justified by its different chemical composition and appropriate synergism between

Table 2 Antibacterial activity of *H. pyrenaicum* subsp. *orsinii* essential oils and antibiotics (mg/mL)

Bacteria	Root oil MIC* MBC	Leaf oil MIC MBC	Fruit oil MIC MBC	Streptomycin MIC MBC	Ampicillin MIC MBC
Staphylococcus aureus	0.23 ± 0.03^{b}	1.08 ± 0.02^{c}	2.59 ± 0.01^{d}	0.04 ± 0.00^{a}	0.25 ± 0.02^{b}
	0.47 ± 0.06^{c}	2.15 ± 0.01^{d}	4.15 ± 0.03^{e}	0.09 ± 0.00^{a}	0.37 ± 0.01^{b}
Bacillus cereus	0.94 ± 0.00^{c}	0.21 ± 0.01^{b}	2.59 ± 0.03^{d}	0.09 ± 0.30^{a}	0.25 ± 0.00^{b}
	1.88 ± 0.06^{c}	0.53 ± 0.00^{b}	4.15 ± 0.00^{d}	0.17 ± 0.07^{a}	0.37 ± 0.01^{b}
Micrococcus flavus	0.94 ± 0.03^{b}	2.15 ± 0.03^{c}	2.59 ± 0.03^{c}	0.17 ± 0.01^{a}	0.25 ± 0.02^{c}
	1.88 ± 0.01^{b}	4.13 ± 0.06^{c}	4.15 ± 0.03^{c}	0.34 ± 0.00^{a}	0.37 ± 0.00^{a}
Listeria monocytogenes	1.88 ± 0.00^{c}	2.15 ± 0.06^{d}	2.09 ± 0.06^{d}	0.17 ± 0.01^{a}	0.37 ± 0.00^{b}
	3.75 ± 0.06^{b}	4.13 ± 0.02^{c}	4.15 ± 0.00^{c}	0.34 ± 0.00^{a}	0.49 ± 0.03^{a}
Pseudomonas aeruginosa	0.23 ± 0.03^{a}	1.08 ± 0.01^{c}	2.09 ± 0.03^{d}	0.17 ± 0.01^{a}	0.74 ± 0.03^{b}
	0.47 ± 0.03^{b}	4.13 ± 0.00^{d}	4.15 ± 0.06^{d}	0.34 ± 0.00^{a}	1.24 ± 0.00^{c}
Salmonella typhimurium	0.23 ± 0.03^{b}	2.15 ± 0.03^{c}	2.09 ± 0.03^{c}	0.17 ± 0.01^{a}	0.37 ± 0.01^{b}
	0.47 ± 0.01^{b}	4.13 ± 0.06^{c}	4.15 ± 0.06^{c}	0.34 ± 0.00^{a}	0.49 ± 0.03^{b}
Escherichia coli	0.23 ± 0.06^{b}	2.15 ± 0.06^{c}	2.59 ± 0.03^{c}	0.17 ± 0.00^{a}	0.25 ± 0.02^{b}
	0.47 ± 0.03^{b}	4.13 ± 0.03^{c}	4.15 ± 0.01^{c}	0.34 ± 0.03^{a}	0.49 ± 0.01^{b}
Enterobacter	1.88 ± 0.03^{c}	2.15 ± 0.02^{d}	4.15 ± 0.02^{e}	0.26 ± 0.00^{a}	0.37 ± 0.01^{b}
cloacae	3.75 ± 0.03^{b}	4.13 ± 0.01^{c}	8.30 ± 0.06^{d}	0.52 ± 0.01^{a}	0.74 ± 0.01^{a}

* MICs and MBCs are expressed as the mean ± SD determined from the results obtained in three independent experiments

[a–e] Different letters in superscript indicate significant differences between the mean values of MICs or MBCs ($p < 0.05$)

Table 3 Antifungal activity of *H. pyrenaicum* subsp. *orsinii* essential oils and antibiotics (mg/mL)

Fungi	Root oil MIC* MFC	Leaf oil MIC MFC	Fruit oil MIC MFC	Bifonazole MIC MFC	Ketoconazole MIC MFC
Aspergillus fumigatus	0.23 ± 0.00^{b}	1.08 ± 0.03^{c}	2.09 ± 0.01^{d}	0.15 ± 0.00^{a}	0.20 ± 0.01^{b}
	0.46 ± 0.02^{b}	2.15 ± 0.03^{c}	4.15 ± 0.03^{d}	0.20 ± 0.01^{a}	0.50 ± 0.00^{b}
Aspergillus versicolor	0.46 ± 0.03^{c}	1.08 ± 0.01^{e}	0.52 ± 0.03^{d}	0.10 ± 0.01^{a}	0.20 ± 0.00^{b}
	1.88 ± 0.03^{c}	2.15 ± 0.01^{d}	1.04 ± 0.03^{c}	0.20 ± 0.02^{a}	0.50 ± 0.02^{b}
Aspergillus ochraceus	0.46 ± 0.03^{b}	4.15 ± 0.01^{d}	1.04 ± 0.01^{c}	0.15 ± 0.02^{a}	1.50 ± 0.07^{c}
	1.88 ± 0.01^{b}	8.26 ± 0.00^{d}	2.09 ± 0.00^{c}	0.20 ± 0.03^{a}	2.00 ± 0.10^{c}
Aspergillus niger	0.94 ± 0.06^{b}	1.08 ± 0.00^{c}	2.09 ± 0.02^{d}	0.15 ± 0.00^{a}	0.20 ± 0.01^{a}
	1.89 ± 0.06^{c}	2.15 ± 0.06^{d}	4.15 ± 0.02^{e}	0.20 ± 0.02^{a}	0.50 ± 0.02^{b}
Trichderma viride	0.23 ± 0.00^{b}	0.12 ± 0.03^{a}	1.04 ± 0.03^{c}	0.15 ± 0.01^{a}	1.00 ± 0.01^{c}
	0.46 ± 0.01^{b}	0.25 ± 0.06^{a}	2.09 ± 0.06^{d}	0.20 ± 0.02^{a}	1.00 ± 0.00^{c}
Penicillium funiculosum	0.46 ± 0.03^{b}	1.08 ± 0.06^{c}	1.04 ± 0.06^{c}	0.20 ± 0.02^{a}	0.20 ± 0.00^{a}
	1.88 ± 0.00^{c}	2.15 ± 0.01^{d}	1.52 ± 0.03^{c}	0.25 ± 0.02^{a}	0.50 ± 0.02^{b}
Penicillium ochrochloron	0.94 ± 0.03^{b}	1.08 ± 0.03^{c}	1.04 ± 0.00^{c}	0.20 ± 0.00^{a}	2.50 ± 0.07^{d}
	1.88 ± 0.03^{b}	2.15 ± 0.06^{c}	2.09 ± 0.06^{c}	0.25 ± 0.00^{a}	3.50 ± 0.03^{d}
Penicillium verrucosum	0.94 ± 0.01^{a}	1.08 ± 0.03^{c}	1.04 ± 0.06^{c}	0.10 ± 0.00^{a}	0.20 ± 0.01^{b}
	1.88 ± 0.01^{c}	2.15 ± 0.06^{d}	2.09 ± 0.06^{d}	0.20 ± 0.01^{a}	0.30 ± 0.00^{b}

* MICs and MFCs are expressed as the mean ± SD determined from the results obtained in three independent experiments

[a–e] Different letters in superscript indicate significant differences between the mean values of MICs or MFCs ($p < 0.05$)

Table 4 Cytotoxic activity of *H. pyrenaicum* subsp. *orsinii* essential oils and cisplatin (μg/mL)

Essential oils	IC_{50}^{a}			
	Malignant cells			Normal cells
	HeLa	LS174	A549	MRC-5
Root	13.92 ± 0.78	12.67 ± 0.75	14.56 ± 0.72	>200
Leaf	10.96 ± 0.59	11.64 ± 1.01	12.85 ± 0.43	>200
Fruit	9.08 ± 0.44	7.42 ± 0.53	6.49 ± 0.84	>200
Cisplatin	0.84 ± 0.11	2.82 ± 0.13	4.16 ± 0.69	15.22 ± 0.12

[a] IC_{50} values are expressed as the mean ± SD determined from the results of MTT assay in three independent experiments

its components—a phenomenon described previously for different essential oils (Bakkali et al. 2008; Burt 2004).

Cytotoxic activity of the essential oils

The cytotoxic activity of isolated *H. pyrenaicum* subsp. *orsinii* essential oils was determined by MTT test and expressed as the concentrations of the oils that inhibited the growth of 50% cells (IC_{50}). Analyzed oils showed significant effect against all the tested malignant cells (Table 4): human malignant cervix adenocarcinoma HeLa, colon carcinoma LS174 and non-small cell lung carcinoma A549 cells (IC_{50} = 6.49–14.56 μg/mL), satisfying the criterion of the National Cancer Institute (NCI) for cytotoxicity (IC_{50} < 30.00 μg/mL) (Suffness and Pezzuto 1991). The strongest effect was exhibited by the fruit oil against A549 cells. The effect of cisplatin, used as positive control, was more prominent (IC_{50} = 0.84–4.16 μg/mL). However, cisplatin also exhibited strong toxicity against human normal fetal lung fibroblast MRC-5 cells (IC_{50} = 15.22 μg/mL), in contrast to all the investigated *H. pyrenaicum* subsp. *orsinii* oils, which were not toxic against these normal cells at tested concentration (IC_{50} > 200.00 μg/mL).

As in the case of antimicrobial activity, exhibited cytotoxic effect of analyzed *H. pyrenaicum* subsp. *orsinii* essential oils can be at least partly explained by the presence of some dominant and minor components. For example, cytotoxicity of *β*-pinene, (*E*)-nerolidol, (*E*)-caryophyllene, α-pinene, caryophyllene oxide, *n*-octanol and α-humulene against the some of the malignant cells used in the present study was previously shown (Bourgou et al. 2010; Da Silva et al. 2007; Jun et al. 2011; Kubo and Morimitsu 1995).

Conclusion

The present work reveals *H. pyrenaicum* subsp. *orsinii* root, leaf and fruit essential oils composition for the first time, and thus contributes to a better knowledge of the volatile constituents of *Heracleum* taxa. Demonstrated biological activity represents a good starting point for further investigations of these oils as potential new herbal raw materials. Namely, against sixteen pathogens including food contaminants, the oils exhibited antimicrobial activity, which was in some cases comparable or even better than the activity of the standard antibiotics. Also, all the oils showed significant cytotoxic activity that was weaker but more selective than the effect of cisplatin.

Acknowledgements This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia under Grants Nos. 173021, 173032 and 175011.

References

Adams RP (2007) Identification of essential oil components by gas chromatography/mass spectroscopy, 4th edn. Allured Publishing Corporation, Carol Stream

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils—a review. Food Chem Toxicol 46:446–475

Başer HCK (2002) Aromatic biodiversity among the flowering plant taxa of Turkey. Pure Appl Chem 74:527–545

Basílico MZ, Basílico JC (1999) Inhibitory effects of some spice essential oils on *Aspergillus ochraceus* NRRL 3174 growth and ochratoxin A production. Lett Appl Microbiol 29:238–241

Bicchi C, D'Amato A, Frattini C, Cappelletti EM, Caniato R, Filippini R (1990) Chemical diversity of the contents from the secretory structures of *Heracleum sphondylium* subsp. *sphondylium*. Phytochemistry 29:1883–1887

Bourgou S, Pichette A, Marzouk B, Legault J (2010) Bioactivities of black cumin essential oil and its main terpenes from Tunisia. S Afr J Bot 76:210–216

Brummitt RK (1968) *Heracleum* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA (eds) Flora Europaea, vol 2. Cambridge University Press, London, pp 364–366

Burt S (2004) Essential oils: their antibacterial properties and potential applications in foods—a review. Int J Food Microbiol 94:223–253

Chauhan RS, Nautiyal MC, Tava A, Cecotti R (2014) Essential oil composition from leaves of *Heracleum candicans* Wall.: a sustainable method for extraction. J Essent Oil Res 26:130–132

CLSI (2009) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 8th edn. CLSI publication M07-A8. Clinical and Laboratory Standards Institute, Wayne

Da Silva SL, Figueiredo PM, Yano T (2007) Cytotoxic evaluation of essential oil from *Zanthoxylum rhoifolium* Lam. leaves. Acta Amazonica 37:281–286

De Miguel D, Gómez P, González R, García-Suárez J, Cuadros JA, Bañas MH, Romanyk J, Burgaleta C (2005) Nonfatal pulmonary *Trichoderma viride* infection in an adult patient with acute myeloid leukemia: report of one case and review of the literature. Diagn Microbiol Infect Dis 53:33–37

Espinel-Ingroff A (2001) Comparation of the E-test with the NCCLS M38-P method for antifungal susceptibility testing of common and emerging pathogenic filamentous fungi. J Clin Microbiol 39:1360–1367

Hajhashemi V, Sajjadi SE, Heshmati M (2009) Anti-inflammatory and analgesic properties of *Heracleum persicum* essential oil and hydroalcoholic extract in animal models. J Ethnopharmacol 124:475–480

Hänel H, Raether W (1988) A more sophisticated method of determining the fungicidal effect of water-insoluble preparations with a cell harvester, using miconazole as an example. Mycoses 31:148–154

Jun NJ, Mosaddik A, Moon JY, Jang K, Lee D, Ahn KS, Cho SK (2011) Cytotoxic activity of β-caryophyllene oxide isolated from jeju guava (*Psidium cattleianum* Sabine) leaf. Rec Nat Prod 5:242–246

Karuppusamy S, Muthuraja G (2011) Chemical composition and antioxidant activity of *Heracleum sprengelianum* (Wight and Arnott) essential oils growing wild in peninsular India. Iran J Pharm Res 10:769–775

Kubo I, Morimitsu Y (1995) Cytotoxicity of green tea flavor compounds against two solid tumor cells. J Agric Food Chem 43:1626–1628

Kuljanabhagavad T, Sriubolmas N, Ruangrungsi N (2011) Chemical composition, antibacterial and antifungal activities of essential oil from *Heracleum siamicum* Craib. Pharm Chem J 45:178–182

Maggi F, Quassinti L, Bramucci M, Lupidi G, Petrelli D, Vitali LA, Papa F, Vittori S (2014) Composition and biological activities of hogweed [*Heracleum sphondylium* L. subsp. *ternatum* (Velen.) Brummitt] essential oil and its main components octyl acetate and octyl butyrate. Nat Prod Res 28:1354–1363

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Nitz S, Spraul MH, Drawert F (1990) C_{17} polyacetylenic alcohols as the major constituents in roots of *Petroselinum crispum* Mill. ssp. *tuberosum*. J Agric Food Chem 38:1445–1447

Ohno M, Abe T (1991) Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). J Immunol Methods 145:199–203

Pignatti S (1982) Flora d'Italia, vol 2. Edagricole, Bologna

Pimenov MG, Leonov MV (2004) The Asian Umbelliferae biodiversity database (ASIUM) with particular reference to South-West Asian taxa. Turk J Bot 28:139–145

Pommerville JC (2011) Alcamo's fundamentals of microbiology. Jones and Bartlett Publishers, Sudbury

Rašić A (2002) Preživeti u prirodi. Kolor pres, Lapovo

Setzer WN, Schmidt JM, Noletto JA, Vogler B (2006) Leaf oil compositions and bioactivities of Abaco bush medicines. Pharmacologyonline 3:794–802

Soković M, Glamočlija J, Marin PD, Brkić D, Van Griensven LJLD (2010) Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. Molecules 15:7532–7546

Suffness M, Pezzuto JM (1991) Assay related to cancer drug discovery. In: Hostettmann K (ed) Methods in plant biochemistry. Assays for bioactivity, vol 6. Academic Press, London

Tao R, Wang C, Kong Z (2013) Antibacterial/antifungal activity and synergistic interactions between polyprenols and other lipids isolated from *Ginkgo biloba* L. leaves. Molecules 18:2166–2182

Tkachenko KG (2009) Essential oils from roots of certain *Heracleum* species. Chem Nat Compd 45:578–581

Tonascia N (1992) Biosystematische Untersuchungen an *Heracleum sphondylium* s.l. in der Schweiz. Ber Geobot Inst ETH 58:101–120

Tsukatani T, Suenaga H, Shiga M, Noguchi K, Ishiyama M, Ezoe T, Matsumoto K (2012) Comparison of the WST-8 colorimetric method and the CLSI broth microdilution method for susceptibility testing against drug-resistance bacteria. J Microbiol Methods 90:160–166

Vračarić B, Bakić J, Čolić D, Lintner V, Micković M, Rajšić R, Stevanović D, Uvalin M (1977) Ishrana u prirodi. Vojnoizdavački zavod, Narodna knjiga, Belgrade