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Chemical Composition and Bioactivity of the Essential Oils of *Heracleum pyrenaicum* subsp. *pollinianum* and *Heracleum orphanidis*

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The objective of this research was to analyze the chemical composition, antimicrobial and cytotoxic activity of *Heracleum pyrenaicum* subsp. *pollinianum* (Bertol.) F. Pedrotti & Pignatti (HPP) and *H. orphanidis* Boiss. (HO) essential oils. The composition of the oils was analyzed by GC and GC-MS. *β*-Pinene (35.1%) was the most abundant compound in HPP root oil, while (*Z*)-falcarinol (80.0%) dominated in HO root oil. (*E*)-Nerolidol (28.5%) was the main constituent in HPP leaf oil. HPP fruit oil, as well as HO leaf and fruit oils mainly contained aliphatic esters, mostly octyl acetate (50.5-84.5%). Antimicrobial screening was performed by microdilution method against eight bacterial and eight fungal strains. The strongest antibacterial activity was shown by both root oils (MICs 0.02-0.60 mg/mL and MBCs 0.04-2.50 mg/mL for HPP, and MICs 0.02-1.25 mg/mL and MBCs 0.04-2.50 mg/mL for HO), while the best antifungal potential was exhibited by HPP fruit oil (MICs 0.30-0.60 mg/mL and MFCs 0.60-1.25 mg/mL) and HO leaf oil (MICs 0.15-0.63 mg/mL and MFCs 0.30-1.25 mg/mL). The tested root and fruit oils exhibited strong cytotoxic effect, which was determined by MTT test against HeLa (IC_{50} 7.53-21.07 μg/mL) and LS174 (IC_{50} 24.16-58.86 μg/mL) cell lines.

Keywords: *Heracleum pyrenaicum* subsp. *pollinianum*, *Heracleum orphanidis*, Essential oils, Chemical composition, Antimicrobial activity, Cytotoxic effect.

Essential oils are alternatives for synthetic chemical products in pharmaceutical and food industry. Because of their antimicrobial or other medicinal properties, essential oils are used as antiseptic, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthesic remedies, as well as in the preservation of food [1].

Heracleum L. species (Apiaceae) are aromatic plants and some of them are used as traditional herbal medicines or food in the different parts of the world. *Heracleum pyrenaicum* subsp. *pollinianum* (Bertol.) F. Pedrotti & Pignatti (HPP) is distributed in the Eastern Alps and mountainous areas of the Balkan Peninsula [2-4]. In the countries of the Balkan Peninsula, the roots, aerial parts and fruits of this plant are traditionally used for the treatment of epilepsy and intestinal catarrh, and as aperitifs and antidiarrheal drugs. The roots, peeled petioles, young leaves and stems are consumed as food [5]. Evergetis *et al*. [6] analyzed the chemical composition and larvicidal activity of HPP (under the name *H. sphondylium* subsp. *pyrenaicum* (Lam.) Bonnier & Layens) flowering aerial parts essential oil.

Heracleum orphanidis Boiss. (HO) is a central Balkan (FYR Macedonia, northern Greece and southern Serbia) mountainous endemic species which inhabits Macedonian pine (*Pinus peuce* Griseb.) woodland and meadows on a silicate substrate [3,4,7].

In this work the chemical composition of the root, leaf and fruit oils of HPP and HO, the antimicrobial activity of these oils, and the cytotoxic effect of the root and fruit oils of both taxa were investigated for the first time.

The roots of HPP afforded 0.25% (w/w) of pale yellow essential oil, the leaves 0.22% (w/w) of greenish oil, and the fruits 1.66% (w/w) of colorless oil. The GC and GC-MS analysis revealed fifty-one components in HPP root oil, seventy-one in the leaf oil, and fifty-six in the fruit oil (representing 90.3, 94.9 and 89.0% of the total oils, respectively). HO roots yielded 0.10% (w/w) of yellow oil, the leaves 0.31% (w/w) of pale yellow oil and the fruits 2.57% (w/w) of colorless oil. The total of forty-four components were identified by GC and GC-MS in HO root oil, forty-nine in the leaf oil, and nineteen in the fruit oil (representing 96.4, 97.0 and 99.4% of the total oils, respectively). The results of the chemical analysis of HPP and HO oils are given in Tables 1 and 2, respectively.

HPP and HO fruit oils were characterized by high amounts of aliphatic esters (81.8 and 96.2%, respectively), mostly octyl acetate (50.5 and 84.5%, respectively). Aliphatic esters also dominated in the most of previously investigated *Heracleum* fruit oils. For example, octyl acetate was the main constituent in the fruit oils of *H. platytaenium* Boiss. (87.6%) and *H. crenatifolium* Boiss. (93.7%) [8]. On the contrary, in the fruit oil of *H. rigens* Wall. ex DC., monoterpenes, mostly bornyl acetate (51.2%) and *α*-pinene (22.6%), were the most prominent components [9].

HO leaf oil was also dominated by aliphatic esters (85.1%), with octyl acetate (83.5%) being the most abundant compound. Similarly, octyl acetate was identified as the main constituent in *H. stevenii* Manden. and *H. antasiaticum* Manden. leaf oils [10,11]. HPP leaf oil was characterized by sesquiterpenes (60.6%), with (*E*)- nerolidol (28.5%) as dominant, followed by germacrene D (10.8%).

Table 1: Chemical composition of *Heracleum pyrenaicum* subsp. *pollinianum* (HPP) essential oils.

RI exp[a]	RI lit[b]	Compound[c]	Root (%[d])	Leaf (%)	Fruit (%)
889	880	Isopropyl 2-methyl butanoate	-	-	tr[e]
901	900	*n*-Nonane	tr	tr	-
905	901	Heptanal	0.2	tr	tr
917	908	Isobutyl isobutanoate	-	tr	-
941	932	*α*-Pinene	2.7	1.1	0.5
956	946	Camphene	0.2	0.2	tr
979	969	Sabinene	tr	tr	tr
987	974	*β*-Pinene	35.1	4.7	0.1
995	988	Myrcene	2.1	1.0	tr
996	988	Dehydro-1,8-cineole	tr	0.2	-
1005	-	Isobutyl 2-methyl butanoate	-	0.7	1.2
1006	998	*n*-Octanal	0.9	-	tr
1009	-	Isobutyl isovalerate	-	0.5	0.2
1016	1007	Isoamyl isobutanoate	-	tr	tr
1019	-	2-Methyl butyl isobutanoate	-	0.7	0.2
1030	1020	*p*-Cymene	tr	0.2	-
1035	1024	Limonene	2.6	1.4	tr
1044	1032	(*Z*)-*β*-Ocimene	5.1	2.8	0.2
1053	1044	(*E*)-*β*-Ocimene	tr	3.2	tr
1064	1047	(3*Z*)-Octen-1-ol	-	-	0.4
1065	1054	*γ*-Terpinene	tr	tr	-
1074	1063	*n*-Octanol	0.3	tr	4.1
1095	1086	Terpinolene	9.6	tr	-
1103	1095	Linalool	-	tr	tr
1103	1100	Isopentyl 2-methyl butanoate	-	0.8	0.4
1108	1100	2-Methyl butyl 2-methyl butanoate	-	3.0	0.6
1111	1103	2-Methyl butyl isovalerate	tr	2.0	0.3
1120	-	(*E*)-4,8-Dimethyl-1,3,7-nonatriene	tr	0.5	-
1127	1118	*cis*-*p*-Menth-2-en-1-ol	0.3	0.5	-
1145	1136	*trans*-*p*-Menth-2-en-1-ol	0.4	0.2	-
1151	-	4,8-Epoxy-*p*-menth-1-ene	0.4	-	-
1153	1147	Hexyl isobutanoate	-	tr	0.4
1190	1179	*p*-Cymen-8-ol	0.8	tr	-
1195	1186	*α*-Terpineol	0.6	tr	-
1202	1195	Methyl chavicol	1.4	3.2	-
1225	1211	Octyl acetate	tr	0.5	50.5
1241	1232	(*Z*)-3-Hexenyl isovalerate	-	tr	-
1243	1233	Hexyl 2-methyl butanoate	-	tr	0.8
1247	1241	Hexyl isovalerate	-	tr	0.7
1262	1255	(4*Z*)-Decen-1-ol	-	tr	tr
1266	1260	(2*E*)-Decenal	0.5	-	-
1276	1266	*n*-Decanol	-	-	tr
1289	1287	Bornyl acetate	0.5	1.5	tr
1306	-	Octyl propanoate	-	-	0.6
1353	-	Octyl isobutanoate	-	0.5	8.0
1354	1346	*α*-Terpinyl acetate	0.4	0.5	-
1389	1387	*β*-Bourbonene	-	0.9	tr
1396	1389	*β*-Elemene	-	2.0	-
1396	-	1-Butenylidenecyclohexane	-	-	0.6
1398	1393	Phenyl ethyl isobutanoate	-	0.6	-
1404	-	(*Z*)-6-Decenyl acetate	-	0.3	0.8
1408	1403	Methyl eugenol	0.7	tr	-
1414	1407	Decyl acetate	-	tr	1.2
1419	-	Bornyl isobutanoate	tr	0.3	tr
1425	1417	(*E*)-Caryophyllene	-	2.9	-
1434	1430	*β*-Copaene	-	tr	tr
1441	-	Octyl 2-methyl butanoate	-	tr	7.5
1445	-	Octyl isovalerate	-	tr	3.5
1460	1452	*α*-Humulene	-	0.1	-
1464	1454	(*E*)-*β*-Farnesene	0.6	6.4	0.2
1486	1484	Germacrene D	tr	10.8	tr
1493	1486	Phenyl ethyl 2-methyl butanoate	-	1.3	tr
1496	1490	Phenyl ethyl isovalerate	-	1.3	tr
1496	1499	4-*epi*-*cis*-Dihydroagarofuran	1.2	-	-
1502	1500	Bicyclogermacrene	-	0.9	-
1502	1500	Isodaucene	0.6	0.8	-
1515	1505	*β*-Bisabolene	0.8	4.8	-
1521	-	Bornyl isovalerate	0.6	0.2	tr
1523	-	Octyl 3-methyl 2-butenoate	-	-	0.3
1533	1517	Myristicin	6.6	-	-
1537	1529	Kessane	7.9	0.3	tr
1563	1555	Elemicin	2.3	-	-
1569	1561	(*E*)-Nerolidol	1.8	28.5	0.9
1587	-	Octyl hexanoate	-	-	3.5
1588	1577	Spathulenol	-	0.8	-
1590	1582	Caryophyllene oxide	-	0.5	-
1620	1607	(*Z*)-Sesquilavandulol	0.5	-	-
1622	1607	Dodecyl acetate	0.5	-	-
1679	1671	*n*-Tetradecanol	0.3	-	-
1693	1687	Eudesma-4(15),7-dien-1-*β*-ol	-	0.9	tr
1781	-	Octyl octanoate	-	-	1.1
1820	-	1,13-Tetradecadiene	0.4	-	-
1881	1874	*n*-Hexadecanol	0.4	tr	tr
2029	2033	Isobergapten	tr	-	-
2038	2035	(*Z*)-Falcarinol	1.0	-	0.2
2057	2056	Bergapten	tr	tr	tr
2127	-	Pimpinellin	tr	-	tr
2207	-	(*Z*)-13-Octadecenyl acetate	tr	0.4	tr
2236	2237	Isopimpinellin	tr	-	-
2299	2300	*n*-Tricosane	-	tr	tr
2397	2400	*n*-Tetracosane	-	tr	-
2499	2500	*n*-Pentacosane	-	tr	tr
2698	2700	*n*-Heptacosane	-	tr	-
		Monoterpene hydrocarbons	57.4	14.6	0.8
		Oxygenated monoterpenes	4.0	3.4	tr
		Sesquiterpene hydrocarbons	2.0	29.6	0.2
		Oxygenated sesquiterpenes	11.4	31.0	0.9
		Phenylpropanoids	11.0	3.2	-
		Aliphatic esters	0.5	9.4	81.8
		Polyacetylenes	1.0	-	0.2
		Others	3.0	3.7	5.1
		Identified	90.3	94.9	89.0

[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 [31]; [c]Constituents listed in the order of elution on HP-5MS column; [d]% - Relative area percentage of the compounds obtained from FID area percent data; [e]tr - Trace (<0.1%).

Sesquiterpenes, mainly germacrene D (29.5%), were also the most abundant compounds in *H. candicans* Wall. ex DC. leaf oil [12]. Evergetis *et al*. [6] analyzed the chemical composition of the essential oil of HPP flowering aerial parts from Greece, and octyl acetate was the most abundant constituent (17.4%). In our research, HPP leaf oil also contained octyl acetate, but only in the quantity of 0.5%, in contrast to HPP fruit oil where it was dominant compound (50.5%).

The main compound of HO root oil was (*Z*)-falcarinol (80.0%). This polyacetylene was also isolated from the root of *H. moellendorffii* Hance [13]. On the other hand, previously investigated *Heracleum* root oils were dominated by aliphatic esters, monoterpenes, sesquiterpenes, or phenylpropanoids [10, 14-16]. HPP root oil was characterized by monoterpenes (61.4%), with *β*-pinene (35.1%) being the most abundant. Similarly, *β*-pinene was the most dominant compound in the root oils of *H. dulce* Fisch., *H. ponticum* (Lipsky) Schischk. ex Grossh., and *H. wilhelmsii* Fisch. & C. A. Mey. (33.9-39.0%) [14].

Table 2: Chemical composition of *Heracleum orphanidis* (HO) essential oils.

RI exp[a]	RI lit[b]	Compound[c]	Root (%[d])	Leaf (%)	Fruit (%)
902	900	*n*-Nonane	1.8	tr[e]	-
905	901	Heptanal	0.3	tr	-
940	932	*α*-Pinene	tr	0.1	tr
973	959	*n*-Heptanol	tr	tr	-
983	974	*β*-Pinene	tr	tr	tr
995	984	2-Pentyl furan	tr	tr	-
1007	998	*n*-Octanal	2.1	tr	0.9
1017	1007	Hexyl acetate	-	-	tr
1030	1020	*p*-Cymene	tr	tr	-
1035	1024	Limonene	0.2	tr	-
1036	1025	*β*-Phellandrene	0.3	tr	-
1041	1032	(*Z*)-*β*-Ocimene	0.4	tr	-
1053	1044	(*E*)-*β*-Ocimene	0.2	tr	-
1064	1054	*γ*-Terpinene	tr	tr	-
1075	1063	*n*-Octanol	0.8	4.7	2.3
1093	1086	Terpinolene	1.7	tr	-
1096	-	(*E*)-4-Undecene	0.3	-	-
1102	1100	*n*-Undecane	0.9	tr	-
1107	1100	*n*-Nonanal	0.3	0.4	-
1164	1157	(2*E*)-Nonen-1-al	0.2	-	-
1189	1179	*p*-Cymen-8-ol	0.1	-	-
1214	1211	Octyl acetate	tr	83.5	84.5
1249	1241	Hexyl isovalerate	-	-	tr
1266	1260	(2*E*)-Decenal	0.5	tr	-
1276	1266	*n*-Decanol	-	tr	tr
1290	1287	Bornyl acetate	-	tr	tr
1300	1300	*n*-Tridecane	tr	-	-
1310	1305	Undecanal	-	0.2	tr
1314	1311	*n*-Nonanyl acetate	-	tr	tr
1320	1315	(2*E*,4*E*)-Decadienal	0.2	tr	-
1389	1387	*β*-Bourbonene	-	0.2	-
1392	-	Octyl butanoate	-	0.1	0.6
1395	1389	*β*-Elemene	0.3	0.3	-
1407	1403	Methyl eugenol	tr	-	-
1411	1407	Decyl acetate	tr	0.5	1.0

1411	1408	Dodecanal	tr	0.6	tr
1426	1417	(*E*)-Caryophyllene	-	2.6	tr
1444	-	Octyl isovalerate	-	tr	0.1
1459	1452	*α*-Humulene	-	0.2	-
1486	1484	Germacrene D	tr	0.4	-
1490	1487	(*E*)-*β*-Ionone	-	0.2	-
1490	1489	*β*-Selinene	tr	-	-
1499	1498	*α*-Selinene	tr	-	-
1513	1505	*β*-Bisabolene	1.7	tr	-
1561	1555	Elemicin	tr	-	-
1567	1561	(*E*)-Nerolidol	tr	0.3	tr
1582	1577	Spathulenol	0.2	tr	-
1586	-	Octyl hexanoate	tr	1.0	9.7
1588	1582	Caryophyllene oxide	tr	0.3	-
1680	1671	*n*-Tetradecanol	1.4	-	-
1781	-	Octyl octanoate	-	tr	0.3
1840	-	Neophytadiene	-	0.2	-
1847	-	Hexahydrofarnesyl acetone	-	0.3	-
1881	1874	*n*-Hexadecanol	0.3	tr	-
2042	2035	(*Z*)-Falcarinol	80.0	tr	-
2074	-	(*Z*)-9-Octadecen-1-ol	0.6	tr	-
2082	-	(*E*)-9-Octadecen-1-ol	1.2	-	-
2083	2077	*n*-Octadecanol	-	0.3	-
2127	2115	7-Isoprenyl oxycoumarin	-	0.4	-
2135	-	Pimpinellin	0.4	-	-
2238	2237	Isopimpinellin	tr	-	-
2697	2700	*n*-Heptacosane	-	0.1	-
2896	2900	*n*-Nonacosane	-	0.1	-
		Monoterpene hydrocarbons	2.8	0.1	tr
		Oxygenated monoterpenes	0.1	tr	tr
		Sesquiterpene hydrocarbons	2.0	3.7	tr
		Oxygenated sesquiterpenes	0.2	0.6	tr
		Phenylpropanoids	tr	-	-
		Aliphatic esters	tr	85.1	96.2
		Polyacetylenes	80.0	tr	-
		Others	11.3	7.5	3.2
		Identified	96.4	97.0	99.4

[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 [31]; [c]Constituents listed in the order of elution on HP-5MS column; [d]% - Relative area percentage of the compounds obtained from FID area percent data; [e]tr - Trace (<0.1%).

The antibacterial activity of the essential oils isolated from HPP and HO roots, leaves and fruits is shown in Table 3. The antibacterial effect of HPP oils was decreasing in the following order: root oil (MICs 0.02-0.60 mg/mL and MBCs 0.04-2.50 mg/mL) > fruit oil (MICs 0.04-0.60 mg/mL and MBCs 0.08-2.50 mg/mL) > leaf oil (MICs 0.04-1.25 mg/mL and MBCs 0.08-2.50 mg/mL). Among HO oils, the root oil showed the best antibacterial potential (MICs 0.02-1.25 mg/mL and MBCs 0.04-2.50 mg/mL). HO leaf and fruit oils were less effective (MICs 1.25-2.50 mg/mL; MBCs 2.50-5.00 and 5.00-7.50 mg/mL, respectively). Overall, HPP oils possessed better antibacterial activity than HO oils, with the exception of HO root oil. The activity of some of the tested *Heracleum* oils was significant. HPP root oil against *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus flavus*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*, HPP leaf oil against *M. flavus* and *E. cloacae*, HPP fruit oil against *S. aureus*, *B. cereus* and *M. flavus*, and HO root oil against all the tested bacterial strains with the exception of *Listeria monocytogenes* were more effective than both antibiotics, streptomycin (MICs 0.04-0.26 mg/mL and MBCs 0.09-0.52 mg/mL) and ampicillin (MICs 0.25-0.74 mg/mL and MBCs 0.37-1.24 mg/mL). The antibacterial activity of HO leaf and fruit oils was similar to the activity of previously tested *H. sibiricum* L. aerial parts oil, which was also rich in aliphatic esters [17].

The antifungal effect of HPP and HO root, leaf and fruit oils is presented in Table 4. The antifungal potential of HPP oils decreased in the following order: fruit oil (MICs 0.30-0.60 mg/mL and MFCs 0.60-1.25 mg/mL) > root oil (MICs 0.15-1.25 mg/mL and MFCs 0.30-2.50 mg/mL) > leaf oil (MICs 0.60-2.50 mg/mL and MFCs 2.50-3.50 mg/mL). Among HO oils, the leaf oil had the best activity (MICs 0.15-0.63 mg/mL and MFCs 0.30-1.25 mg/mL), followed by the root oil (MICs 0.30-1.25 mg/mL and MFCs 0.40-2.50 mg/mL) and the fruit oil (MICs 0.20-1.65 mg/mL and MFCs 0.40-3.30 mg/mL). The antifungal effects of HPP root oil against *Aspergillus versicolor*, HO leaf oil against *A. ochraceus*, *Penicillium funiculosum*, *P. ochrochloron* and *P. verrucosum* var. *cyclopium*, and HO fruit oil against *P. funiculosum* and *P. ochrochloron* were comparable with those of bifonazole (MICs 0.10-0.20 mg/mL and MFCs 0.20-0.25 mg/mL) and higher than those of ketoconazole (MICs 0.20-2.50 mg/mL and MFCs 0.30-3.50 mg/mL).

It was previously shown that *β*-pinene, the most dominant compound of HPP root oil, inhibited the growth of *M. flavus*, *S. aureus*, *Salmonella typhimurium*, *Escherichia coli*, *L. monocytogenes*, *E. cloacae* and *P. aeruginosa* (MICs 0.005-0.01 mg/mL and MBCs 0.0055-0.013 mg/mL, respectively) [18]. (*Z*)-Falcarinol, the main constituent of HO root oil, was active against *S. aureus*, *P. aeruginosa* and *E. coli* (MICs 0.0031-0.00625 mg/mL) [19]. (*E*)-Nerolidol and germacrene D, the constituents of HPP leaf oil, also showed antimicrobial properties. The MIC values of (*E*)- nerolidol against *S. aureus* and *Aspergillus niger* were 0.0039 and 0.0156 mg/mL, respectively [20]. It potentiates the effect of antibiotics on *S. aureus* and *E. coli*, and enhances drug skin penetration [21]. The MIC value of germacrene D against *S. aureus* was 0.0625 mg/mL, against *L. monocytogenes*, *P. aeruginosa* and *E. coli* 0.125 mg/mL, against *B. cereus* 0.625 mg/mL and against *A. niger* 0.039 mg/mL [22,23]. Additionally, the antimicrobial activity against some of the same microorganisms tested in our study was previously confirmed for several compounds, like *α*-pinene, limonene, terpinolene and (*E*)-caryophyllene, that are present in HPP and HO oils in quantities below 10% [18,22-24].

Table 3: Antibacterial activity of tested *Heracleum* essential oils and antibiotics (mg/mL).

Bacteria	***Heracleum pyrenaicum* subsp. *pollinianum* (HPP)**			***Heracleum orphanidis* (HO)**			**Streptomycin**	**Ampicillin**
	root oil	**leaf oil**	**fruit oil**	**root oil**	**leaf oil**	**fruit oil**		
	MIC* **MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**	**MIC MBC**
Staphylococcus aureus	0.02±0.00[a] 0.04±0.00[a]	0.15±0.00[c] 0.30±0.02[d]	0.04±0.00[ab] 0.08±0.00[b]	0.02±0.00[c] 0.04±0.00[a]	1.25±0.02[e] 2.50±0.00[f]	2.50±0.07[f] 5.00±0.02[g]	0.04±0.00[ab] 0.09±0.00[b]	0.25±0.02[d] 0.37±0.01[e]
Bacillus cereus	0.02±0.00[a] 0.04±0.00[a]	0.15±0.02[ab] 0.30±0.00[c]	0.06±0.00[a] 0.08±0.00[ab]	0.02±0.00[a] 0.04±0.00[a]	1.25±0.01[c] 2.50±0.07[d]	2.50±0.07[d] 5.00±0.07[e]	0.09±0.00[b] 0.17±0.07[b]	0.25±0.00[ab] 0.37±0.01[c]
Micrococcus flavus	0.02±0.00[ab] 0.04±0.00[a]	0.04±0.00[ab] 0.08±0.00[a]	0.06±0.00[ab] 0.08±0.00[a]	0.02±0.00[a] 0.04±0.00[a]	2.50±0.07[d] 5.00±0.07[e]	2.50±0.10[e] 5.00±0.03[e]	0.17±0.01[bc] 0.34±0.00[b]	0.25±0.02[c] 0.37±0.00[b]
Listeria monocytogenes	0.08±0.00[a] 2.50±0.07[bc]	1.25±0.01[de] 2.50±0.00[bc]	0.30±0.02[bc] 2.50±0.20[b]	1.25±0.00[a] 2.50±0.08[d]	2.50±0.01[f] 5.00±0.07[d]	1.25±0.08[e] 7.50±0.07[e]	0.17±0.01[ab] 0.34±0.00[a]	0.37±0.00[c] 0.49±0.03[a]
Pseudomonas aeruginosa	0.15±0.01[a] 0.30±0.03[d]	0.60±0.03[e] 1.25±0.08[b]	0.30±0.02[b] 0.60±0.02[b]	0.15±0.00[b] 0.30±0.00[a]	2.50±0.01[f] 5.00±0.00[e]	1.25±0.07[e] 5.00±0.10[e]	0.17±0.01[a] 0.34±0.00[a]	0.74±0.03[d] 1.24±0.00[b]
Salmonella typhimurium	0.60±0.03[d] 1.25±0.02[e]	0.60±0.02[d] 2.50±0.10[d]	0.60±0.00[d] 1.25±0.00[e]	0.08±0.00[a] 0.15±0.00[a]	1.25±0.00[e] 2.50±0.10[d]	1.25±0.02[e] 5.00±0.10[e]	0.17±0.01[b] 0.34±0.00[ab]	0.37±0.01[c] 0.49±0.03[b]
Escherichia coli	0.30±0.00[a] 0.60±0.02[c]	1.00±0.07[e] 1.25±0.08[d]	0.30±0.02[ab] 0.60±0.03[c]	0.08±0.00[a] 0.15±0.01[a]	2.50±0.07[d] 5.00±0.00[f]	2.50±0.10[d] 5.00±0.10[g]	0.17±0.00[a] 0.34±0.03[b]	0.25±0.02[a] 0.49±0.01[bc]
Enterobacter cloacae	0.15±0.01[b] 0.30±0.03[b]	0.20±0.02[c] 0.30±0.02[b]	0.45±0.02[f] 0.60±0.00[d]	0.04±0.00[a] 0.08±0.00[a]	2.50±0.03[h] 5.00±0.07[f]	1.25±0.01[g] 5.00±0.00[f]	0.26±0.00[c] 0.52±0.01[c]	0.37±0.01[e] 0.74±0.01[e]

*MIC and MBC values are expressed as the mean ± SD determined from the results obtained in three independent experiments; [a-h]Different letters in superscript indicate significant differences between the mean values of MICs or MBCs ($p < 0.05$).

Table 4: Antifungal activity of tested *Heracleum* essential oils and antibiotics (mg/mL).

Fungi	*Heracleum pyrenaicum* subsp. *pollinianum* (HPP)			*Heracleum orphanidis* (HO)			Bifonazole	Ketoconazole
	root oil	leaf oil	fruit oil	root oil	leaf oil	fruit oil		
	MIC* MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC
Aspergillus fumigatus	0.30±0.02^{bc} 0.60±0.02^{b}	1.25±0.00^{f} 2.50±0.01^{d}	0.30±0.03^{c} 0.60±0.03^{b}	0.60±0.02^{d} 1.25±0.00^{c}	0.63±0.01^{d} 1.25±0.02^{c}	0.80±0.02^{e} 3.30±0.07^{e}	0.15±0.00^{a} 0.20±0.01^{a}	0.20±0.01^{ab} 0.50±0.00^{b}
Aspergillus versicolor	0.15±0.01^{a} 0.30±0.02^{c}	1.25±0.08^{f} 2.50±0.00^{h}	0.30±0.03^{cd} 0.60±0.03^{c}	0.30±0.03^{bc} 1.25±0.02^{f}	0.30±0.00^{c} 0.60±0.02^{c}	0.80±0.02^{e} 1.65±0.05^{g}	0.10±0.01^{a} 0.20±0.02^{b}	0.20±0.00^{ab} 0.50±0.02^{d}
Aspergillus ochraceus	0.60±0.00^{d} 1.25±0.00^{d}	1.25±0.08^{e} 2.50±0.03^{f}	0.30±0.02^{c} 0.60±0.00^{c}	0.60±0.03^{d} 1.25±0.02^{d}	0.15±0.01^{ab} 0.30±0.02^{b}	1.65±0.02^{f} 3.30±0.20^{g}	0.15±0.02^{b} 0.20±0.03^{ab}	1.50±0.07^{f} 2.00±0.10^{e}
Aspergillus niger	1.25±0.02^{e} 2.50±0.10^{d}	2.50±0.07^{f} 3.50±0.00^{e}	0.30±0.03^{d} 0.60±0.02^{b}	1.25±0.08^{e} 2.50±0.10^{d}	0.30±0.00^{cd} 0.60±0.00^{b}	0.40±0.00^{d} 0.80±0.03^{c}	0.15±0.00^{ab} 0.20±0.02^{a}	0.20±0.01^{bc} 0.50±0.02^{b}
Trichoderma viride	0.60±0.01^{d} 1.25±0.01^{c}	0.60±0.03^{d} 2.50±0.10^{f}	0.60±0.02^{d} 1.25±0.00^{e}	1.25±0.00^{f} 2.50±0.10^{g}	0.30±0.03^{c} 0.60±0.07^{b}	0.40±0.03^{c} 0.80±0.03^{c}	0.15±0.01^{b} 0.20±0.02^{a}	1.00±0.01^{e} 1.00±0.00^{d}
Penicillium funiculosum	0.60±0.00^{d} 1.25±0.02^{e}	1.25±0.02^{f} 2.50±0.03^{f}	0.30±0.00^{e} 0.60±0.03^{d}	0.30±0.03^{e} 0.40±0.00^{e}	0.15±0.02^{b} 0.30±0.02^{b}	0.20±0.00^{b} 0.40±0.03^{c}	0.20±0.02^{b} 0.25±0.02^{b}	0.20±0.00^{b} 0.50±0.02^{c}
Penicillium ochrochloron	1.25±0.00^{e} 2.50±0.20^{e}	1.25±0.05^{e} 2.50±0.10^{f}	0.30±0.07^{e} 0.60±0.00^{c}	0.60±0.00^{d} 1.25±0.02^{d}	0.15±0.02^{b} 0.30±0.03^{ab}	0.20±0.02^{b} 0.40±0.03^{bc}	0.20±0.00^{b} 0.25±0.00^{ab}	2.50±0.07^{f} 3.50±0.03^{g}
Penicillium verrucosum	0.60±0.00^{c} 1.25±0.00^{c}	1.25±0.02^{f} 2.50±0.10^{d}	0.60±0.02^{c} 1.25±0.02^{c}	0.60±0.03^{c} 1.25±0.01^{c}	0.15±0.02^{b} 0.30±0.00^{ab}	0.40±0.02^{d} 0.80±0.02^{b}	0.10±0.00^{ab} 0.20±0.01^{a}	0.20±0.01^{c} 0.30±0.00^{ab}

*MIC and MFC values are expressed as the mean ± SD determined from the results obtained in three independent experiments; $^{a-h}$Different letters in superscript indicate significant differences between the mean values of MICs or MFCs ($p < 0.05$).

The cytotoxic effect of the tested HPP and HO root and fruit oils is shown in Table 5. The IC_{50} values were in the range between 7.53-58.86 μg/mL. The activity of all the tested oils on cervix adenocarcinoma HeLa cell line, as well as HPP fruit oil and HO root oil on colon carcinoma LS174 cell line (IC_{50} < 30.00 μg/mL) meets the criterion of the National Cancer Institute (NCI) for cytotoxicity [25]. The strongest cytotoxic activity was shown by HPP fruit oil (IC_{50} against HeLa cell line 10.05 μg/mL and against LS174 cell line 24.16 μg/mL) and HO root oil (IC_{50} against HeLa cell line 7.53 μg/mL and against LS174 cell line 24.94 μg/mL). The effect of the oils against HeLa cell line was 2-3 times greater than the one observed for LS174 cell line. The IC_{50} values of cisplatin, which was used as positive control, were 0.72 μg/mL against HeLa cell line, and 2.30 μg/mL against LS174 cell line.

Table 5: Cytotoxic effect of tested *Heracleum* essential oils and cisplatin (μg/mL).

Essential oils	IC_{50}*	
	HeLa	LS174
H. pyrenaicum subsp. *pollinianum* root oil	21.07±1.01	40.13±0.66
H. pyrenaicum subsp. *pollinianum* fruit oil	10.05±0.22	24.16±0.73
H. orphanidis root oil	7.53±0.02	24.95±1.03
H. orphanidis fruit oil	16.55±2.34	58.86±1.91
Cisplatin	0.72±0.14	2.30±0.31

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

Investigated oils exhibited notably stronger cytotoxic activity against HeLa cell line than other previously tested *Heracleum* oils. The IC_{50} values of oils from the aerial parts of *H. transcaucasicum* Manden., *H. pastinacifolium* C. Koch, *H. persicum* Desf. and *H. rechingeri* Manden. were 594, 1398, >2000 and >2000 μg/mL, respectively [26].

Cytotoxic activity exhibited by HO root oil can be partly explained by the presence of (*Z*)-falcarinol. It inhibited the survival of HeLa cell line with the IC_{50} value of 8.58 μg/mL [27]. The cytotoxic activity of (*Z*)-falcarinol was also demonstrated against acute lymphoblastic leukemia cell line CEM-C7H2, human multiple myeloma cell line RPMI-8226, human histiocytic lymphoma cell line U937 and two cell lines of colorectal carcinoma HRT-18 and HT-2912 (IC_{50} 3.5-63.9 μmol/L). A medicinal usage of pure polyacetylenes is not feasible because of their pronounced chemical instability and their ability to induce allergic reactions. However, consumption of food containing polyacetylenes might have a chemopreventive benefit [28]. Regarding *β*-pinene, the main constituent of HPP root oil, its cytotoxic activity was previously shown against human colon carcinoma cell line HCT116, and human breast adenocarcinoma cell line MDA-MB 231 (IC_{50} 57.2 and 75.5 μg/mL, respectively) [29]. Some of the minor constituents of the tested oils, like (*E*)-nerolidol and *n*-octanol also exhibited cytotoxic activity against HeLa cell line [30].

This study is the first report of the chemical composition and antimicrobial activity of the root, leaf and fruit essential oils of *H. pyrenaicum* subsp. *pollinianum* and *H. orphanidis*, and the cytotoxic effect of the root and fruit oils of these taxa. It can be concluded that some of the most prominent compounds, such as (*Z*)-falcarinol, *β*-pinene, (*E*)-nerolidol and germacrene D, and also some minor constituents, at least partly contributed to the demonstrated bioactivity of the tested *Heracleum* oils. The chemical characterization of these oils is a good starting point for further investigations in order to identify other active compounds and modes of activity.

Experimental

Plant material: The material was collected in July 2012, in the southwestern part of FYR Macedonia: HPP on the limestone screes of Mt. Galičica, and HO in *Pinus peuce* wood on Mt. Baba Planina. Voucher specimens are deposited in the Herbarium of the Natural History Museum, Belgrade (BEO) under accession numbers 20120716/BEO for HPP and 20120706/BEO for HO, respectively. The plants were identified by Dr. Marjan Niketić, curator/botanist of the BEO.

Isolation of the essential oils: Air-dried plant material was powdered (roots and fruits) or crashed (leaves) and hydrodistilled for 2.5 h, using Clevenger-type apparatus. 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) alcohol. 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 [31]. 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: 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.

Minimum inhibitory and minimum bactericidal concentrations (MICs and MBCs) were determined by the microdilution method in 96-well microtitre plates [32,33]. 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 0.02 to 7.50 mg/mL. The MICs were defined as the lowest concentrations without visible bacterial growth (determined at binocular microscope). Additionally, the MICs were 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 control. 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 control. Streptomycin (0.04-0.52 mg/mL) and ampicillin (0.25-1.24 mg/mL) were used as the positive controls. 5% DMSO was used as the negative control.

In order to investigate the antifungal activity of the oils, modified microdilution technique was used [34,35]. 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 0.15-3.50 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 (0.10-0.25 mg/mL) and ketoconazole (0.20-3.50 mg/mL) were used as the positive controls. 5% DMSO was used as the negative control.

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 α=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 and human colon carcinoma LS174 cell lines (ATCC) were cultured as a monolayer in the RPMI 1640 nutrient medium, supplemented with heat inactivated (at 56 ºC) 10% fetal bovine serum (FBS), 3 mmol/L of L-glutamine and antibiotics, at 37 ºC, 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) and LS174 cells (7000 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 ºC) 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 cancer cell survival was determined by the MTT test (microculture tetrazolium test), according to Mosmann [36], with modification by Ohno and Abe [37], 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 ºC, 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.

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