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Short communication

CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF *GERANIUM ROBERTIANUM* L. ESSENTIAL OIL

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Geranium robertianum L. (*Geraniaceae*), is known as Herb Robert, Red Robin and Storksbill. This plant is native to Europe, Asia, North America and North Africa. *G. robertianum* is an annual or biennial plant, with the strong scent. In traditional herbalism Herb Robert was used for the treatment of wounds, ulcers and diarrhea and as a remedy for toothache and nosebleeds (1, 2). The active compounds identified in *Geranium robertianum* are tannins, flavonoids, phenolic acids, essential oil and vitamins (A, C). The essential oil showed antiplasmid effect, antioxidant activity, astringent, antibleeding, diuretic and antiseptic properties (1). Among 161 medicinal plants applied in ethnopharmacology, traditionally used in the South-West of Romania, infusion of *G. robertianum* aerial parts have antihypertensive, astringent, depurative, and antiseptic activity (3).

The aim of the study was to investigate the chemical composition of essential oil of *Geranium robertianum* and assessment its antibacterial properties. Microbiological activity was tested against human, animal, and plant pathogens.

EPERIMENTAL

Plant material

Plant samples utilized in this study were collected in June 2015 from the natural population in Malin, Lower Silesia, Poland (51°13'08"N 17°03'51"E). The analyzed plant material was dried at room temperature for two weeks to constant weight. Voucher specimens are deposited at the Department of Plant Protection, Wrocław University of Environmental and Life Sciences.

Extraction procedure of volatile compounds

Hydrodistillation-extraction applying Deryng apparatus was used for the extraction of the volatile compounds of *G. robertianum*. A suspension of 500 g of material was placed in a 2000-mL round flask together with 1000 mL of distilled water. The sample flask was heated for 2 h after reaching the boiling point. The vapors were condensed by means of a cold refrigerant. After 120 min of extraction to cyclohexane (1 mL), the essential oil was transferred into 2.5 mL vials and kept at -27°C until gas chromatography-mass spectrometry (GC-MS) analyses were performed. Analyses were run in triplicate.

Gas chromatography

The chemical composition of the volatiles was analyzed using a gas chromatograph (GC) coupled to a mass spectrometer (MS) detector (Shimadzu QP2010SE, Pennsylvania, USA) with ZB-5MSi (Phenomenex, CA, USA) column (30 m × 0.25 µm film × 0.25 mm i.d.). The analyses were carried out using helium as carrier gas at flow rate of 1.0 mL/min, in split 20 mode, and with the following program for the oven temperature: 60°C at the beginning and 3°C/min to 250°C with hold for 3

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min. The injector and detector was held at 220°C. The compounds were identified by using 3 independent analytical methods: retention indices (RI), retention times of authentic chemical standards, and comparison of mass spectra of compounds and NIST14 spectral library collection (4). The retention index standards used in this study consisted of a mixture of aliphatic hydrocarbons ranging from C-5 through C-30 dissolved in hexane. The quantification was carried out by gas chromatography analysis (GC, FID, carrier gas H_2) on Agilent Technologies 7890N (GC System, Santa Clara, CA, USA) with the ZB-5 column (30 m × 0.25 µm film × 0.25 mm i.d., CA, USA). The GC conditions were the same as those for GC-MS.

Antimicrobial activity

Test microorganisms

The compounds were tested against human and animals pathogens including Gram-positive cocci: *Staphylococcus aureus* PCM 2054, *S. pseudintermedius* KP-Spi1 (isolated from dog), *Streptococcus agalactiae* KP-Sag1 (isolated from dog), *S. canis* KP-Sac1 (isolated from dog), Gram-positive endospore forming rods: *Bacillus subtilis* PCM 1949, *B. cereus* PCM 1948; Gram-negative rods: *Escherichia coli* PCM 2057 as well as plant pathogens: *Pectobacterium carotovora* sbp. *carotovora* IOR-1815 and *P. carotovora* sbp. *carotovora* IOR-1822; *P. atrosepticum* IOR-1825, *P. atrosepticum* IOR-1826. These strains came from the following collections: KW - own collection (Department of Plant Protection, University of Environmental and Life Sciences, Wrocław, Poland), KP - Department of Pathology (University of Environmental and Life Sciences, Wrocław, Poland), PCM - Polish Collection of Microorganisms (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland), IOR - Culture Collection of Plant Pathogens at Institute of Plant Protection (Poznań, Poland). Cultures of strains were maintained on TSA (Tryptic Soy Agar, Fluka, Sigma-Aldrich) and stored on slants in the temperature of 4°C.

Antimicrobial screening

The minimum inhibitory concentration (MIC) was determined by serial dilution method in 48-well plates (Tissue Culture Plates VWR, Poland). Bacterial species were cultured at 37°C in Mueller-Hinton agar (MHA, Sigma-Aldrich, USA) for 20 h. After cultivation, bacterial suspensions were made in Mueller-Hinton broth (MHB, Sigma-Aldrich, USA) and their turbidity was standardized to 0.5 McFarland (spectrophotometer VIS-723G, Rayleigh, Beijing). Stock solutions of the essential oil *G. robertianum* were prepared in 10% DMSO, and then serial dilutions of the essential oil were made in a concentration range from 1.25 to 10 mg/mL. The bacterial inoculum was added to all wells, and the plates were incubated at 37°C during 24 h. In addition, 10% DMSO was used as control and tetracycline (30 µg/mL, Sigma-Aldrich) as positive control. The bacterial growth was visualized by adding 50 µL of rezasurin aqueous solution on well (pink color of medium indicates growth of microorganisms and blue means inhibition of growth). The resazurin sodium salt (300 mg, Sigma-Aldrich) was dissolved in 40 mL of sterile water.

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the tested chemical compounds that inhibited visible growth, while minimum bactericidal concentration (MBC) was defined as the lowest compound concentration that killed 99% of microorganisms cells. To determine MBC cultures were taken from each well without visible growth (blue color) and inoculated in MHA for 24 h at 37°C. The experiments were done in triplicate, in two series.

RESULTS AND DISCUSSION

Hydrodistillation is a standard method for extraction of essential oil. Hydrodistillation using Deryng apparatus of dry *Geranium robertianum* aerial parts provided an essential oil yield of 0.025%. This method was previously successfully used in different aromatic herbs, such as marjoram (5), thyme (6) and oregano (7). Table 1 shows total volatile components, their retention indices and relative percentages of *G. ronbertianum* essential oil. At this stage, it is worth mentioning that *G. robertianum* essential oil, contrary to the extracts resulting from solid-liquid extraction, is not well examined. Analysis using GC-MS led to identification of 117 of 122 detected compounds (97.8% of the total essential oil). The most abundant compounds were monoterpene alcohols such as linalool (13.4%), α-terpineol (2.7%) and geraniol (2.6%), sesquiterpenoids α-caryophyllene (3.4%), germacrene D (8.6%), diterpene phytol acetate (4.2%) and oxidized alkenes 2-(*E*)-hexenal (5.9%), 3-(*Z*)-hexen-1-ol (6.9%). The first study describing the composition of the essential oil of aerial part *G. robertianum* was carried out by Pedro et al., who identified 72 compounds and found that linalool, germacrene D, limonene and geraniol were major components (8).

Table 1. The percentage aroma composition of the essential oil of *G. robertianum*.

Compound	Area (%)[a]	Retention indices	
		Exp.[b]	Lit.[b]
3-(*E*)-Hexenol	0.20	855	853
2-(*E*)-Hexenal	6.09	861	855
3-(*Z*)-Hexen-1-ol	7.06	868	859
2-(*E*)-Hexenol	0.79	869	862
Hexanol	0.77	876	870
4-Heptanol	0.49	889	889
Heptanal	0.29	908	902
2,4-(*E*,*E*)-Hexadienal	0.33	913	909
Tricyclene	0.34	928	926
α-Pinene	0.24	940	939
2-(*E*)-Heptanal	0.23	952	954
Benzaldehyde	0.24	960	960
Heptanol	0.22	969	966
1-Octen-3-ol	0.31	981	979
Myrcene	0.53	992	990
2,4-(*E*,*E*)-Heptadienal	0.24	1001	1005
Octanal	0.26	1002	998
2-(*E*)-Hexenyl acetate	0.31	1011	1013
2,4-(*E*,*E*)-Heptadienol	0.25	1013	1016
α-Terpinene	0.23	1021	1017
p-Cymene	0.26	1028	1024
Limonene	0.74	1033	1029
1,8-Cineole	0.21	1036	1031
cis-β-Ocimene	0.33	1041	1037
Benzeneacetaldehyde	1.26	1050	1042
trans-β-Ocimene	0.80	1052	1050
γ-Terpinene	1.39	1062	1059
Octanol	0.58	1074	1068
trans-Linalool oxide	0.27	1083	1086
Terpinolene	0.46	1090	1088
Linalool	13.56	1099	1096
Nonanal	1.11	1099	1100
1-Terpineol	0.23	1138	1133
trans-Verbenol	0.22	1147	1144
2,6-(*E*,*Z*)-Nonadienal	0.23	1150	1154
2-(*E*)-Nonen-1-al	0.28	1158	1161
2-(*E*)-Nonenol	0.23	1166	1167
Borneol	0.23	1168	1169
Terpinen-4-ol	0.31	1180	1177
p-Cymen-8-ol	0.25	1189	1187
α-Terpineol	2.90	1192	1188
Methyl salicylate	0.22	1194	1191
Safranal	0.23	1201	1196

Table 1. Cont.

Compound	Area (%)[a]	Retention indices	
		Exp.[b]	Lit.[b]
Decanal	0.48	1206	1201
trans-Carveol	0.21	1217	1216
cis-Carveol	0.27	1225	1229
β-Cyclocitral	0.31	1225	1219
Nerol	1.03	1231	1229
Geraniol	2.81	1262	1252
2-(*E*)-Decanal	0.27	1262	1263
Decanol	0.31	1271	1269
Nonanoic acid	0.22	1271	1270
p-Menth-1-en-7-al	0.20	1275	1275
Bornyl acetate	0.20	1282	1285
1-Tridecene	0.24	1284	1290
Undecanal	0.27	1314	1306
2,4-(*E*,*E*)-Decadienal	0.32	1316	1316
2-(*E*)-Undecanal	0.26	1365	1360
α-Copaene	0.20	1377	1376
β-Bourbonene	0.27	1389	1388
β-Elemene	0.32	1392	1390
Tetradecane	0.25	1400	1400
Dodecanal	0.31	1410	1408
β-Caryophyllene	3.58	1420	1419
β-Copaene	0.20	1435	1432
trans-α-Bergamotene	0.37	1440	1434
trans-β-Farnesene	0.75	1459	1456
2-(*E*)-Dodecanal	0.29	1464	1464
Dauca-5,8-diene	0.23	1469	1472
γ-Muurolene	0.24	1480	1479
Germacrene D	8.77	1485	1481
trans-β-Ionone	0.68	1486	1488
β-Selinene	0.27	1498	1490
α-Muurolene	0.20	1506	1500
α-Farnesene	0.88	1509	1505
γ-Cadinene	0.26	1514	1513
trans-Cadina-1,4-diene	0.50	1532	1534
α-Cadinene	0.58	1538	1538
Unidentified	0.26	1548	
Germacrene B	0.56	1557	1561
Nerolidol	0.35	1563	1563
Hexenyl benzoate	0.24	1566	1566
Tridecanol	0.30	1578	1571
Caryophyllene oxide	0.27	1590	1583
Tetradecanal	0.31	1612	1612
Cedrene epoxide	0.35	1623	1622

Table 1. Cont.

Compound	Area (%)[a]	Retention indices	
		Exp.[b]	Lit.[b]
α-Muurolol	0.46	1646	1646
Cubenol	0.27	1646	1646
α-Eudesmol	0.43	1653	1653
α-Cadinol	0.69	1658	1654
β-Bisabolol	0.54	1668	1675
α-Bisabolol	0.23	1686	1685
Unidentified	0.34	1693	
2-Pentadecanone	0.23	1697	1697
Heptadecane	0.27	1700	1700
2,6-(*E*,*Z*)-Farnesal	0.23	1711	1713
Pentadecanal	0.31	1715	1715
Unidentified	0.27	1721	
2,6-(*E*,*E*)-Farnesal	0.62	1739	1741
α-Sinensal	0.26	1758	1756
1-Octadecene	0.27	1786	1790
Octadecane	0.38	1800	1800
Penadecanoic acid	0.23	1859	1867
Hexadecanol	0.24	1879	1875
Nonadecane	0.23	1900	1900
Unidentified	0.37	1921	
Phytol	0.33	1945	1943
Hexadecanoic acid	1.71	1966	1960
Eicosane	0.31	2000	2000
Octadecanol	0.27	2070	2077
Octadecanoic acid	0.35	2174	2170
Docosane	0.79	2200	2200
Phytol acetate	4.35	2222	2218
Unidentified	0.38	2289	
Heneicosanal	0.99	2329	2325
Tetracosane	1.60	2400	2400
Docosanal	0.30	2430	2427
Pentacosane	0.71	2500	2500
Tricosanal	0.45	2534	2529
Hexacosane	1.43	2600	2600
Unidentified	0.70	2658	
Heptacosane	1.30	2700	2700

[a] Average of the three replicates. [b] Exp. = experimental; Lit. = literature (22)

Radulović (9), identified 152 compounds from the aerial parts and 53 components from the underground parts of the plant. Fatty acids and fatty acid derivatives were dominated, constituting 49.2% of the aerial parts and 93.4% of the underground parts. This work showed that hexadecanoic acid, pentacosane, hexahydrofarnesyl acetone and caryophyllene oxide were the most abundant components in

Serbian *G. robertianum*. These differences might occur due to the influence of environmental factors and/or genetic variability of the investigated populations (9). These studies also showed very low yield of essential oil (< 0.1%).

Fatty acids were also found to be the predominant group of compounds in the *G. robertianum* essential oil also from the aerial parts collected in France (10). Among 32 compounds identified by GC-MS and GC-FID, hexadecanoic acid was the major component. Dodecanoic acid, tetradecanoic acid and 9,12-(*Z*,*Z*)-octadecadienoic acid were the other main constituents of the oil.

Antimicrobial activity of the essential oil from *G. robertianum* leaves was tested by use of serial dilution method determining MIC/MCB. Organisms used in this study are associated with various forms of human, animal and plant infections. Usually, microbial activity of essential oils is tested in the concentration range from 2 to 10 mg/mL. It depends on the qualitative and quantitative composition of the tested essential oil (11). In our research, the essential oil of Herb Robert showed antibacterial effectiveness in the range of concentration from 1.25 to 10 mg/mL. Results are shown in Table 2. The essential oil did not inhibit the growth of microorganisms at the level of the reference substance (tetracycline, 30 μg/mL). The Gram-positive cocci were much more sensitive to essential oil than the other tested microbes (Gram-positive bacilli, Gram-negative rods). The lowest minimal inhibitory concentration was found against *S. aureus* (MIC = 1.25 mg/mL) and animal pathogens *S. canis* and *S. pseudintermedius* (MIC = 2.5 mg/mL). However, the bactericidal effect against Gram-positive cocci was observed at 2-fold concentration (MBC = 5 mg/mL). The essential oil also inhibited the growth of endospore forming bacilli, but at the concentration MIC = 5 mg/mL. The tested Gram-negative bacteria (*E. coli* and *Pectobacterium* spp.) were also sensitive to Herb Robert essential oil. However, the minimum inhibitory concentration was observed at a dose of 10 mg/mL. The essential oil did not exhibit bactericidal activity to plant pathogens (*Pectobacterium* spp.) at the tested concentrations.

The obtained results showed that the *G. robertianum* essential oil from aerial part, possesses antimicrobial properties and can be used as a natural antimicrobial agent. The activity is due to the high content of monoterpenoids possessing hydroxyl group such as linalool and geraniol, α-terpineol as well as sesquiterpenes (germacrene D and B, β-caryophyllene) and monoterpenes (γ-terpinene) which account for over 30% of the ingredients of the essential oil from aerial part of Herb Robert. Similar results to our research was presented by Radulović et al. (9). They observed, that the essential oil exhibits inhibitory and bactericidal effect on *S. aureus*, *B. sub-*

Table 2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MCB) of essential oil from *G. robertianum* leaves.

	MIC [mg mL^{-1}]	MCB [mg mL^{-1}]
Gram-positive cocci		
S. aureus PCM 2054	1.25	5
S. pseudintermedius KP-Spi1	2.5	5
S. canis KP-Sac1	2,5	5
S. agalactiae KP-Sag1	5	5
Gram-positive endospore forming rods		
Bacillus subtilis PCM1949	5	10
Bacillus cereus PCM2019	5	10
Gram-negative rods		
E. coli PCM 2057	10	10
P. carotovora IOR-1815	10	< 10
P. carotovora IOR-1822	10	< 10
P. atrosepticum IOR-1825	10	< 10
P. atrosepticum IOR-1826	10	< 10

< 10 – not active in a range of assay doses.

tillis and *E. coli* at the concentration range from 5 to 10 mg/mL. Delaquis et al. (12) studied composition and microbial activity of essential oils from seeds and leaves of *Coriandrum sativum* L. Their research showed that essential oils contained, among others, linalool and γ-terpinene and were active against some Gram-negative rods (*Escherichia coli, Pseudomonas fragi*) and *S. aureus* in concentration range from 0.02 to 0.47 mg/mL. In diffusion tests, Herman et al. have demonstrated that the pure linalool and essential oils from some plants (e.g., *Pelargonium graveolens*, *Thymus vulgaris*, *Lavandula angustifolia*) possess antibacterial effects against *S. aureus*, *Pseudomonas aeruginosa* and *E. coli* (13). Moreover, Park et al. found that pure linalool and α-terpineol were active against periodontopathogens in concentration range from 0.1 – 0.8 mg/mL (14). These compounds also inhibited the growth of *S. mutants* (MIC ranged from 0.1 to 3.2 mg/mL). Zanetti et al. showed that pure geraniol inhibited growth of *S. aureus* and *E. coli* in concentrations 5 and 2.5 mg/mL, respectively (15). In our research, the differences between MIC and MCB values for tested microbes were more than 2-fold, suggesting that Gram-positive cocci are more sensitive to *G. robertianum* oil than Gram-negative. Mechanism of antimicrobial action of essential oils and their components has not been well studied. Hydrophobicity is an important property of essential oils and their components, which enables them to damage to cytoplasmic membrane causing degradation of the cell wall, and inhibiting bacterial enzyme activity (15).

CONCLUSION

In the present study, the essential oil from *G. robertianum* leaves was found to be effective against all tested microorganisms. The Gram-negative bacteria were more resistant to the essential oil than Gram-positive bacteria. The demonstration of microbiological activity of essential oil against both Gram-negative and Gram-positive bacteria (human, animal, plant) is an indication that the plant can be a source of bioactive substances that could possess broad spectrum of antibacterial activity. It is worth mentioning, that the microbial activity of the essential oil from *G. robertianum* against animals and plant pathogens is reported for the first time in this study.

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