

ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF ESSENTIAL OIL FROM THE AERIAL PARTS OF *PULICARIA DYSENTERICA* (L.) BERNH. (ASTERACEAE)

ORIGINAL SCIENTIFIC PAPER

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DOI: 10.51558/2232-7568.2023.16.2.81

RECEIVED
2024-02-08ACCEPTED
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ABSTRACT:

The chemical composition of *Pulicaria dysenterica* (L.) Bernh. aerial parts essential oil (EO), growing wild in Bosnia and Herzegovina, was presented in the study. In addition to the EO composition, antimicrobial and cytotoxic activities were also tested. The aerial parts of *P. dysenterica* contained 0.3% of yellow, liquid, fragrant EO. The 51 components identified accounted for 81.09% of the oil. The EO was characterized by the presence of a high concentration of oxygenated sesquiterpenes 51.83% while oxygenated monoterpenes constituted 15.57%, sesquiterpene hydrocarbons 9.32% and non-terpene compounds presented 4.37% of the EO. The dominant compounds were the sesquiterpenes caryophyllene oxide, (*E*)-nerolidol, β -caryophyllene and monoterpene nerol. The antimicrobial activity of EO was tested against selected ATCC strains of microorganisms, the bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, and the fungus *Candida albicans*. The results showed that the investigated EO inhibited the growth of all selected ATCC strains of microorganisms. The best result was obtained against *Escherichia coli* bacteria with MIC value of 1 mgmL⁻¹. The cytotoxicity of EO was measured against the HeLa cell line using the MTT method with IC₅₀ of 188.52 μ gmL⁻¹. This study has provided scientific baseline data on the therapeutic properties of *P. dysenterica*.

KEYWORDS: *Pulicaria dysenterica*, essential oil, antimicrobial activity, cytotoxicity

INTRODUCTION

Pulicaria dysenterica (L.) Bernh. syn. *Inula dysenterica* L. is a perennial plant growing up to 100 cm tall. The leaves are alternate. It is reproduced by roots. The flowers are yellow. It is widespread in humid areas of Europe, Anatolia, Iraq, Iran, Afghanistan, Pakistan and North Africa. *P. dysenterica* is found in the flora of Bosnia and Herzegovina [1]. The decoction of the aerial parts of this plant is used in traditional Iranian medicine for the treatment of diarrhoea and dysentery. The plant is also used to treat dysentery in the United Kingdom. It also has an insecticidal property [2], [3]. The chemical constituents of *P. dysenterica* include flavonoids,

acetylenes, sesquiterpene lactons, isocomene and essential oil [2].

Previous studies on the essential oil of the aerial parts of *P. dysenterica* are rare and related to the determination of its composition and content. Studies on the essential oil of *P. dysenterica* aerial parts from Greece and Iran confirmed the well-known fact that the chemical composition of the essential oil depends on various parameters such as environmental conditions [4].

The essential oils and extracts from genus *Pulicaria* have been found to possess considerable biological activities such as antioxidant, antimicrobial, cytotoxic, antitumor, insecticidal [5], [6], [7]. A

limited number of studies have been carried out on the biological activities of *P. dysenterica* extracts [4]. To the best of our knowledge, no studies have been carried out on the biological activities of the essential oil of *P. dysenterica* aerial parts.

The aim of this study is to present the chemical composition of *P. dysenterica* aerial parts essential oil from Bosnia and Herzegovina, its antimicrobial activity on selected ATCC strains of microorganisms and cytotoxic activity on HeLa cell line.

It has already been mentioned that essential oils from the genus *Pulicaria* have antimicrobial properties. Sesquiterpenes, among other compounds, contribute to their activity [8]. The mechanism of antimicrobial activity of the essential oils is not yet fully explained. Essential oils and their constituents have an important property of hydrophobicity, which allows them to be distributed between the lipids of the cell membrane and the mitochondria of the bacterial cell, thereby disrupting the structure and making them more permeable. A change in the permeability of the cell membrane also affects the control of osmotic loss of the cell, which is considered the basic principle of the antibacterial effect of the essential oils [9], [10].

The cytotoxicity of essential oils from the genus *Pulicaria* has also been described. In generally, the cytotoxicity of essential oils is mainly related to the presence of phenols, alcohols, and monoterpene aldehydes. As lipophilic mixtures, essential oils are able to cross the cell membrane and degrade and permeabilise the layers of polysaccharides, phospholipids and fatty acids. Cytotoxicity appears to involve such membrane damage. This permeabilization of the outer and inner membranes leads to cell death by apoptosis and necrosis [10].

EXPERIMENTAL

PLANT MATERIAL

Aerial parts of *P. dysenterica* were collected at the site Paklenik, Karaula, Olovo Municipality (Bosnia and Herzegovina) (N44°10'13.6" E18°39'07.2") during the flowering season in July 2020. The plant material was identified by the authors according to Flora Croatica (Domac, 1994). The voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Tuzla. The plant material was cleaned, cut, and air-dried.

REAGENTS AND CHEMICALS

All analyses were performed using analytical grade chemicals and reagents, with the exception of sample preparation solvents for GC-FID/MS analysis, which were MS grade. *P*-iodonitrotetrazolium

chloride and streptomycin were purchased from Sigma-Aldrich, while the antibiotic ampicillin was purchased from Panfarma, Belgrade, Serbia and ketoconazole was purchased from Zorka farma, Šabac, Serbia. Minimum Essential Medium Eagle (MEM), 2 mM glutamine, 1 % nonessential Amino Acids, 10% heat inactivated fetal bovine serum (HI FBS), penicillin and Thiazolyl Blue Tetrazolium Bromide (MTT) cell viability reagent were also purchased from Sigma-Aldrich. Double-distilled deionized water or culture medium were used for solution preparations and dilutions.

ISOLATION OF ESSENTIAL OIL

The dried aerial parts of *P. dysenterica* (200g) were crushed several times and subjected to hydrodistillation with 4 litres of distilled water for 3 h using a Clevenger-type apparatus. The essential oil obtained was separated, dried over anhydrous sodium sulfate, and stored at -20 °C until analysis.

GC-FID/MS ANALYSIS

Volatile compounds were determined by the GC-FID/MS analyses using an Agilent 6890N GC system coupled with an Agilent 5975 MSD, FID, and equipped with a HP-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). The oven temperature was programmed with a linear increase from 60 to 280°C at 3° min⁻¹ followed by isotherm at 280°C for 5 min; injector 200°C; FID 300°C; transfer-line 250°C; carrier gas He (1.0 mL min⁻¹, constant flow mode); injection volume 1 µL of essential oil dissolved in ethanol; split ratio 10:1. EI Mass spectra (70 eV) were acquired over the m/z range of 35-550. The identification of the individual compounds was based on the comparison of their retention times (tR), retention indices (RIs), and mass spectra with those obtained from authentic samples and/or listed in the NIST, Wiley mass spectral libraries, and the literature. The relative area percentages obtained by FID were used for quantification.

DETERMINATION OF ANTIMICROBIAL ACTIVITY

The antimicrobial activity (AMA) of *P. dysenterica* essential oil was tested against three strains of bacteria, i.e. Gram-positive *Staphylococcus aureus* (ATCC 6538) and Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 35210), as well as against the fungal strain *Candida albicans* (ATCC 10231). The microorganisms were obtained from the Mycology Laboratory, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia.

The antibacterial assay was performed by the microdilution method (Cazella et al., 2019) using 96-well microtiter plates to determine the minimum inhibitory concentration (MIC) and minimum bactericidal /fungicidal concentration (MBC/MFC). The inoculum was cultivated in a solid medium to verify the absence of contamination and for validation. The nutrient media used were Tryptic Soy Broth (TSB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungus. Microbial suspensions (inocula) were adjusted with sterile saline to a concentration of 1.0×10^5 CFU mL⁻¹. The inoculum was prepared daily and stored at 4 °C until use. The essential oil was added to the nutrient medium for the growth of microorganisms. The microbial inocula were then added and the plates were incubated for 24 h at 37°C for bacteria and 72 h at 28°C for fungus. The lowest concentration without visible growth of microbial biomass under a binocular magnifying glass was defined as the MIC. Determination of the absence of microbial growth, i.e. determination MBC/MFC, was performed by serial reinoculation of 10 µL of inoculated medium from wells with no microbial growth into 100 µL of sterile nutrient medium and reincubation for 24 h at 37°C for bacteria and for 72 h at 28°C for fungus. Results were confirmed by adding 40 µL of purple *p*-iodonitrotetrasolium chloride, microbial growth indicator solution (0.2 mg mL⁻¹ distilled water) to each well and incubating for 30 minutes at 37°C [11]. Comparison of color intensity was made with control wells in which microorganisms were allowed to grow unhindered, and commercial antimicrobial agents streptomycin, ampicillin, and ketoconazole were used as positive controls. Inoculated medium without added essential oil was used as the negative control. The antimicrobial tests were performed in triplicate.

DETERMINATION THE CYTOTOXICITY

In vitro culture of the cell lines

The HeLa (cervical cancer) cell line was cultured in MEM supplemented with 2 mM glutamine, 1% non-essential amino acids, 10% HI FBS and 1% penicillin/streptomycin antibiotics. Cells were maintained in humidified atmosphere containing 5% CO₂ at 37°C. For each experiment cells were grown to 80% confluence in cell culture flasks.

MTT Cell Proliferation Assay

The cytotoxic effects of *P. dysenterica* essential oil were assessed using the MTT assay. For each experiment cells were seeded (2×10^4 cells/well) in 96 well plates and incubated overnight. The next day, the cells were treated with increasing final concentrations

of *P. dysenterica* essential oil and incubated for further 48 h. After incubation of the cells, MTT solution 0.5 mgmL⁻¹ was added to each well, and the plates were incubated for another 4 hours at 37°C in a humidified atmosphere containing 5% CO₂. The MTT containing medium was then removed and the remaining MTT-formazan crystals were dissolved by adding 200 µL DMSO to each well with continuous gentle shaking for 15 minutes. The absorbance was read using a microplate reader at a wavelength of 570 nm.

The experiment was repeated three times and each experiment was performed in triplicate. Untreated cells were used as negative control and cells treated with 30% DMSO in culture medium were used as positive control. Samples were dissolved in 10% DMSO and diluted in culture medium. The final concentration of DMSO in treated samples did not exceed 0.1%. Prepared stock solutions of extracts were sterilized by filtration through 0.2 µm sterile syringe filters. The concentration of the extracts that resulted in 50% of viability (*IC*₅₀) was determined from graph plots of the dose response curve.

RESULTS AND DISCUSSION

RESULTS OF ISOLATION AND GC-FID/MS ANALYSIS OF ESSENTIAL OIL

The aerial parts of *P. dysenterica* contained 0.3% (v/w) of yellow, liquid, fragrant essential oil (EO), and the identified 51 components, accounting for 81.09% of the oil, are listed in Table 1. The EO of aerial parts of *P. dysenterica* was characterized by the presence of a high concentration of oxygenated sesquiterpenes (51.83%). The main compounds were caryophyllene oxide (14.89%), (*E*)-nerolidol (9.18%), *epi*- α -cadinol (3.58%). Nerol (5.92%) was the main representative of the oxygenated monoterpenes, which made up 15.57% of the oil. Sesquiterpene hydrocarbons made up 9.32% of the oil with the dominant compound being β -caryophyllene (4.75%). Non-terpene (other) compounds represented 4.37% of the EO.

Table1. Chemical composition of essential oil from *P. dysenterica* aerial parts

No.	Ret. time ^a	Compound	RIE ^b	EO ^c (%m/m)
1	16.674	α -terpineol	1192.5	0.28
2	18.236	nerol	1229.3	5.92
3	22.814	presilphiperfol-7-en	1337.1	0.33
4	25.599	italicen	1402.6	0.35
5	26.383	β -caryophyllene	1422.6	4.75
6	27.165	(<i>Z</i>)- β -farnesene	1442.0	0.85
7	27.639	geranyl acetone	1454.0	0.50
8	27.71	α -humulene	1456.0	0.29
9	28.63	γ -curcumen	1478.6	0.37

10	28.772	<i>ar</i> -curcumen	1482.3	1.65
11	29.019	(<i>E</i>)- β -jonone	1488.8	0.87
12	29.153	neryl isobutanoate	1492.1	3.60
13	29.484	3-(1,1-dimethylethyl)-4-methoxy-phenol	1500.5	0.16
14	29.484	α -muurolene	1500.5	0.15
15	29.919	β -curcumene	1511.6	0.58
16	30.07	10- <i>epi</i> -italicene ether	1515.8	0.33
17	30.809	silphiperfol-5-en-3-ol B	1534.6	0.27
18	31.517	italicene epoxide	1552.7	0.37
19	32.053	(<i>E</i>)-nerolidol	1566.4	9.18
20	32.533	neryl isovalerate	1579.1	2.01
21	32.6	<i>ar</i> -tumerol	1580.3	1.58
22	32.945	caryophyllene oxide	1589.7	14.89
23	33.045	helifolen-12-al A (syn-anti-anti)	1592.4	0.50
24	33.226	fokienol	1597.0	0.80
25	33.631	(<i>Z</i>)-sesquilavandulol	1607.7	3.11
26	33.795	humulene epoxide II	1612.4	0.72
27	34.032	helifolen-12-al C (anti-syn-syn-)	1620.3	0.60
28	34.452	eremoligenol	1630.1	0.36
29	34.488	murola-4,10(14)-dien-1- β -ol	1631.2	0.34
30	34.543	1- <i>epi</i> -cubenol	1633.0	0.25
31	34.676	caryophylla-4(12),8(13)-dien-5- α -ol	1636.3	0.26
32	34.882	<i>epi</i> - α -cadinol	1642.0	3.58
33	35.138	himachalol	1649.1	0.50
34	35.329	vulgarone B	1654.7	1.58
35	35.473	α -cadinol	1658.1	1.65
36	35.923	14-hydroxy-(<i>Z</i>)-caryophyllene	1670.5	1.64
37	36.107	14-hydroxy-9- <i>epi</i> -(<i>E</i>)-caryophyllene	1675.6	2.30
38	36.125	β -bisabolol	1675.8	1.46
39	36.443	germacra-4(15),5,10(14)-trien-1- α -ol	1684.5	1.94
40	36.692	8-cedren-13-ol	1690.4	1.76
41	37.953	(<i>Z</i>)-nuciferol	1727.3	0.49
42	38.196	zerumbone	1734.2	0.92
43	38.711	fukinone	1749.1	0.45
44	40.123	(8)-cedren-13-ol acetate	1789.7	0.33
45	41.513	(<i>Z</i>)-nuciferol acetate	1830.8	2.11
46	42.027	hexahydrofarnesyl acetone	1846.5	0.92
47	44.41	(5 <i>E</i> ,9 <i>E</i>)-farnesyl acetone	1919.0	0.36
48	47.374	13- <i>epi</i> -mannol oxide	2013.0	0.79
49	50.427	(<i>E</i>)-phytol	2114.3	0.51
50	60.949	<i>n</i> -pentacosane	2498.9	1.16
51	65.819	<i>n</i> -heptacosane	2698.3	0.42
Total			81.09	
Oxygenated monoterpenes			15.57	
Sesquiterpene hydrocarbons			9.32	
Oxygenated sesquiterpenes			51.83	
Others			4.37	

^aRetention time (min)^bRIE – experimental retention indices^cEO – essential oil

The content of essential oils from the aerial parts of *P. dysenterica* from Greece and Iran, obtained by hydrodistillation, ranged from 0.24% to 0.4%. In the present study the content of EO from the aerial parts of *P. dysenterica* from Bosnia and Herzegovinawas in this range. Dominant compounds in the EO from Greece were sesquiterpene lactones β -caryophyllene, caryophyllene oxide, [(*E*),(*Z*)]-nerolidol [11]. Dominant compounds in the EO from Iran were *ar*-curcumin, *epi*- α -cadinol and (*E*)-coniferyl alcohol [2].

In the EO of *P. dysenterica* aerial parts from Turkey, obtained by the petroleum ether extraction, the main compound was identified as terpinolen [13]. In the present study the dominant compounds were the sesquiterpenes caryophyllene oxide, (*E*)-nerolidol, β -caryophyllene and the monoterpene nerol, which were also the dominant compounds in the essential oil of *P. dysenterica* aerial parts from Greece.

RESULTS OF ANTIMICROBIAL ACTIVITY

The antimicrobial activity of EO was determined *in vitro* using the microdilution method. The results are in the form of minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBCs/MFCs) by the bacterial strains already mentioned. They are presented in the Table 2.

Table 2. Antimicrobial activity of essential oil from *P. dysenterica* aerial parts expressed as MIC and MBC

Strain	ATCC	MIC (mgmL ⁻¹)	MBC/MFC (mgmL ⁻¹)
<i>Staphylococcus aureus</i>	6538	4	7
<i>Pseudomonas aeruginosa</i>	27853	3	4
<i>Escherichia coli</i>	35210	1	7
<i>Candida albicans</i>	10231	20	30

MIC - minimum inhibitory concentration

MBC/MFC - minimum bactericidal concentration/minimum fungicidal concentration

The results obtained showed that EO inhibited the growth of all selected ATCC microorganism strains. The best result was obtained against Gram-negative bacteria *Escherichia coli* ATCC 35210 with MIC value of 1 mgmL⁻¹.

There are no previous data on the antimicrobial activity of *P. dysenterica* EO.

The antimicrobial activity of the dominant compounds in the *P. dysenterica* aerial parts EO has been reported in the literature. Schmidt et al. tested caryophyllene oxide, nerolidol, β -caryophyllene against selected ATCC strains of microorganisms in

the concentration range from 0.001 to 1 mgmL⁻¹. The MICs of caryophyllene oxide against Gram-positive bacteria *S. aureus* and Gram-negative bacteria *E. coli* were 0.06 mgmL⁻¹. The MIC of caryophyllene oxide against the yeast *C. albicans* was below 0.06 mgmL⁻¹, while no inhibition was observed against the Gram-negative bacteria *P. aeruginosa*. The MIC of nerolidol against *S. aureus* was below 0.06 mgmL⁻¹ while inhibition against *E. coli*, *P. aeruginosa* and *C. albicans* was not detected. The MIC of β -caryophyllene against *S. aureus* was 0.06 mgmL⁻¹. MIC of β -caryophyllene against *E. coli* was 0.6 mgmL⁻¹ while inhibition against *C. albicans* was not detected [14].

Moura et al. tested nerolidol against selected ATCC strains of microorganisms in the concentration range from 0.25 to 4 mgmL⁻¹. Nerolidol inhibited the growth of *S. aureus* (MIC = 1 mgmL⁻¹) and *P. aeruginosa* (MIC = 0.5 mgmL⁻¹), whereas it showed no activity against the Gram-negative bacteria *E. coli* ATCC [15].

The results of the antimicrobial activity of the *P. dysenterica* aerial parts EO against *E. coli* and *C.*

albicans are in an accordance with the literature data on the antimicrobial activity of dominant constituents of the essential oil. Individual, dominant compounds showed effective antimicrobial activity against *S. aureus* in contrast to the weak antistaphylococcal activity of *P. dysenterica* aerial parts EO. The antimicrobial activity of an EO is not a simple sum of the activities of its constituents. It is a mixture of all components with a unique activity [16].

RESULTS OF CITOTOXICITY FOR THE ESSENTIAL OIL

The cytotoxic activity of *P. dysenterica* aerial parts EO was evaluated on the human cervical adenocarcinoma cells (HeLa) using the MTT test. According to the results shown in Figure 1, the oil showed moderate cytotoxicity against HeLa cell line using MTT method with an IC_{50} of 188.52 μ gmL⁻¹. Extracts with IC_{50} value in the range of 100 to 500 μ gmL⁻¹ are classified as extracts with moderate cytotoxicity [17].

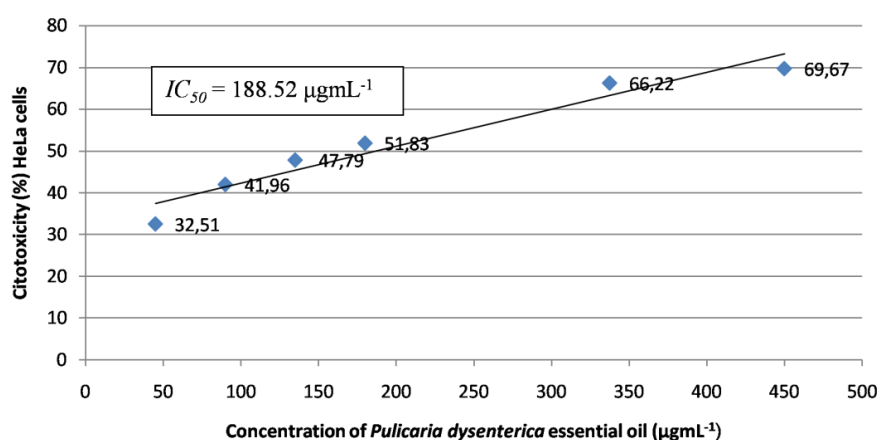


Figure 1. Influence of *Pulicaria dysenterica* essential oil on cell growth in HeLa cell lines

The essential oil induced a dose-dependent inhibition of cell proliferation in tested tumor cell line (Figure 1). There are no published data on the cytotoxicity of *P. dysenterica* aerial parts EO.

The cytotoxicity some of the dominant compounds in the EO has been reported in the literature. Caryophyllene oxide showed potent cytotoxic activity against HeLa with IC_{50} values of 13.55 μ gmL⁻¹. The results showed that caryophyllene oxide exhibited cytotoxicity in both a dose and time-dependent manner [18]. Nerolidol isomers (*trans*-nerolidol, synthetic nerolidol and *cis*-nerolidol) also showed significant cytotoxicity against HeLa cells.

The IC_{50} value of *cis*-nerolidol against HeLa cells was 16.5 μ gmL⁻¹ \pm 6.7 [19], [20]. β -Caryophyllene has also been described as cytotoxic compound against HeLa cells [21].

The results of cytotoxic activity of the *P. dysenterica* aerial parts EO are in accordance with the literature data of cytotoxic activity dominant components in the EO.

CONCLUSION

The chemical composition of *P. dysenterica* aerial parts EO growing wild in Bosnia and Herzegovina was

determined. It is characterized by the presence of a high concentration of oxygenated sesquiterpenes. To the best of our knowledge, this study is the first to describe the antimicrobial and cytotoxic activities of *P. dysenterica* aerial parts EO. All microorganisms tested were sensitive to the EO. The best result was obtained against Gram-negative bacteria *E. coli* ATCC (MIC = 1 mgmL⁻¹). The cytotoxic activity was evaluated on the human cervical adenocarcinoma cells (HeLa) with an IC₅₀ of 188.52 µgmL⁻¹. This study has provided scientific baseline data on the therapeutic properties of *P. dysenterica*.

ACKNOWLEDGMENT

This research is funded by the Federal Ministry of Education and Science (FB&H).

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