

# Original Research Article

## Antioxidant and Cytotoxic Activities of *Pulicaria dysenterica* Methanol Extracts

### ABSTRACT

**Aims:** The aims of the study were to analyse the polyphenols of *P. dysenterica* methanolic extracts from aerial and underground parts, assessment of antioxidant activity and to evaluate their cytotoxicity on HeLa cells of cervical cancer.

**Methodology:** The total phenolic content (TPC) of extracts was determined by the Folin-Ciocalteu spectrophotometric method. The qualitative and quantitative analysis of individual polyphenolic compounds were performed by the RP-HPLC method. The antioxidant capacity was evaluated by DPPH radical assay and FRAP assay while cytotoxicity of the extracts was assessed by MTT assay.

**Results:** TPC of the samples were  $127.62 \pm 2.22$  and  $244.12 \pm 8.84$  mg gallic acid equivalent/g extract. In the extracts chlorogenic acid in amount of  $10.06 \pm 0.96$  and  $11.32 \pm 0.28$  mg/g, flavonoid rutin in amount of  $5.68 \pm 0.13$  mg/g and three caffeic acid derivatives were recorded. Extract from underground parts achieved better antioxidant activity with  $IC_{50}$  value  $52.36 \pm 0.75$   $\mu\text{g/mL}$  and FRAP value  $2411.12 \pm 37.22$   $\mu\text{mol Fe}^{2+}\text{g}^{-1}$  compared to extract from aerial parts. Extract from aerial parts achieved better cytotoxic activity with  $IC_{50}$  value  $0.389 \pm 0.07$  mg/mL, against HeLa cells, compared to extract from underground parts.

**Conclusion:** Analyzed *Pulicaria dysenterica* extracts contained phenolic acids and flavonoids. The extracts showed good antioxidant activity and cytotoxic properties against HeLa cells *in vitro*.

**Keywords:** *Pulicaria dysenterica*, polyphenols, antioxidants, cytotoxicity

### 1. INTRODUCTION

*Pulicaria dysenterica* (L.) Bernh. syn. *Inula dysenterica* L., Asteraceae, is a perennial plant, up to 100 cm high with yellow flowers, growing on damp places. It is found in South, West and Central Europe, Anatolia, Iraq, Iran, Afghanistan, Pakistan and North Africa. The aboveground parts of the *Pulicaria dysenterica* are used in the treatment of diarrhea, dysentery in Iranian traditional medicine. It is also used for the treatment of dysentery in the United Kingdom. The plant has an insecticidal property, as well [1,2]. Previous studies on the composition of aerial parts showed the presence of flavonoids, phenolic acids, sesquiterpene lactones and essential oil [3,4]. A limited number of studies have been carried out concerning the chemical constituents and biological activities of the plant. In favor to its traditional use, previously it was shown that extracts of *Pulicaria dysenterica* aerial parts were active against bacterial strain *Vibrio cholerae*, as well as against parasite *Trichomonas gallinae* in *in vitro* tests [5,6]. Furthermore, study about cytotoxic effects of related *Inula* species against some tumor cell lines showed good results with  $IC_{50}$  values from 17.96  $\mu\text{g/mL}$  [7].

However, data on the composition and activity of aerial and underground parts of this plant still lack. The aims of the study were to analyse the polyphenols of *P. dysenterica* methanolic extracts, assessment of antioxidant activity by different methods and to evaluate their

cytotoxic activities on HeLa cells of cervical cancer, bearing in mind broad set of activities that plant polyphenols might exhibit [8,9].

## 2. MATERIAL AND METHODS

All analyses were performed using analytical grade chemicals and reagents. Folin-Ciocalteu's phenol reagent, sodium carbonate, sodium acetate anhydrous, and ferric (III) chloride were obtained from Merck (Germany). HPLC-grade acetonitrile and formic acid were purchased also from Merck. Methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), ferrous (II) sulfate heptahydrate, hydrochloric acid, glacial acetic acid, HPLC-grade chlorogenic acid were purchased from Sigma-Aldrich (USA). Minimum Essential Medium Eagle, 2 mM L-glutamine, nonessential Amino Acids, heat inactivated fetal bovine serum (HI FBS), penicillin/streptomycin antibiotics and thiazolyl blue tetrazolium bromide (MTT) were purchased also from Sigma-Aldrich. Rutin was obtained from Carl Roth (Germany). Water for HPLC was prepared by Milli-Q Water Purification System. Double-distilled deionized water or culture medium were used for solution preparations and dilutions for MTT assay.

### 2.1 Sampling Plant Material

Aerial and underground parts of *P. dysenterica* were collected at the mountain pass Karaula (N44°10'13.6" E18°39'07.2"), Olovo municipality Bosnia and Herzegovina, during the flowering period in July 2020. The plant material was identified according to the Flora Croatica by authors and the voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Tuzla [10].

### 2.2 Preparation of Methanol Extracts

The air-dried plant material was crushed in a grinder until powder formation. The samples were extracted with 98% methanol (2.5 g plant material with 50 mL of solvent) on a magnetic stirrer under reflux at 50°C for 1 hour. The mixtures were filtered through a filter paper (Whatman No. 1). The solvent was removed by evaporation. The dried extracts were stored in the fridge at 4°C, in glass bottles for further investigations.

### 2.3 Determination of Total Phenolic Content (TPC)

TPC was determined by the Folin-Ciocalteu spectrophotometric method [11]. Stock solutions (2 mg/mL) of extracts in methanol were prepared. One hundred  $\mu$ L of an extract was mixed with 7.9 mL of distilled water. Folin-Ciocalteu reagent (500  $\mu$ L) was added. After 8 min, 1.5 ml of 20%  $\text{Na}_2\text{CO}_3$  was added. After 2 hours of incubation at room temperature ( $20^\circ\text{C} \leq t \leq 25^\circ\text{C}$ ), the absorbance was measured at 765 nm. Quantitative measurements were performed using a standard calibration curve of different concentrations of gallic acid (20, 100, 200, 300, 400 and 500 mg/L), in the same way. The results for TPC are expressed in galic acid equivalents as milligram per gram of dry extract (mg GAE/g).

### 2.4 HPLC analysis

HPLC analyses of extracts (1 mg/mL in methanol) were carried out using an Agilent 1260 Infinity system equipped with an Agilent 1260 Infinity Quaternary Pump, Agilent 1260 Infinity Standard Autosampler, Agilent 1260 Infinity Diode Array Detector, and Agilent 1260 Infinity Thermostatted Column Compartment. The separations were performed on a Merck LiChroCARTR250-4 C18 RP analytical column (250x4.6mm i.d., 5 $\mu$ m). The mobile phase

consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The following gradient was applied: 0-15 min, linear gradient from 10% to 20% B; 15-30 min, linear gradient from 20% to 30% B; 30-35 min, linear gradient from 30% to 40% B; 35-40 min, linear gradient from 40% to 90% B; 40-45 min, then returned to the initial conditions. The injection volume was 10  $\mu$ L; the flow rate was 0.8 mL/min. The detection wavelength was 280 nm, 325 nm, 360 nm and the column thermostat was set at 30°C [12]. Component identification was performed comparing their retention times and UV spectra with those obtained from standards. The calibration curve for chlorogenic acid was obtained by the external standard method in the concentration range of 15.6-500  $\mu$ g/mL ( $R^2 = 0.9996$ ,  $y = 28.93x - 220.2$ ) and the calibration curve for rutin was obtained by the external standard method in the concentration range of 1-20  $\mu$ g/mL.

## 2.5 Determination of Antioxidant Capacity

The *in vitro* antioxidant capacity of *P. dysenterica* extracts was evaluated by DPPH radical scavenging assay and the ferric reducing antioxidant power (FRAP) assay.

### 2.5.1 DPPH Radical Scavenging Assay

Stock solutions (1 mg/mL) of extracts in methanol were prepared. The reaction mixture contained 75  $\mu$ L of extract solution and 75  $\mu$ L of 0.3 mM DPPH solution in methanol. Extracts and DPPH solution were mixed in microtiter plates and incubated in a dark place for 30 min at a room temperature ( $20^\circ\text{C} \leq t \leq 25^\circ\text{C}$ ). A blank was measured for each sample. Instead of DPPH, methanol was added to the blanks. Controls were 98% methanol plus DPPH. Absorbance was measured colorimetrically at 517 nm on microtiter plate reader. The DPPH scavenging activity (SA) in percentage was determined as follows:

$$\text{SA\%} = 100 \times [(\text{Ac} - \text{Ao}) - (\text{As} - \text{Ao})] / (\text{Ac} - \text{Ao})$$
, where Ac is the absorbance of the control, Ao the absorbance of the blank and As the absorbance of the sample. Rutin was used as a reference substance. Results of DPPH assay are expressed as  $IC_{50}$  ( $\mu$ g/mL), defined as the concentration of extract required to remove 50% of free radicals [13].

### 2.5.2 FRAP Assay

Stock solutions (2 mg/ml) of extracts in methanol were prepared. In short, 100  $\mu$ L of extracts diluted with methanol were mixed with 3.0 mL of freshly prepared FRAP reagent consisting of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM  $\text{FeCl}_3$  (10:1:1). Samples were incubated for 30 min and absorbance measured at 593 nm on spectrophotometer. A standard calibration curve has been prepared using different concentrations of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (100-1000  $\mu$ mol/L). The results of FRAP assay are expressed as  $\mu$ mol  $\text{Fe}^{2+}$ /g dry extract [14].

## 2.6 Determination of Cytotoxicity

### 2.6.1 *In vitro* culture of the cell lines

HeLa (cervical cancer) cell line was cultured in Minimum Essential Medium Eagle supplemented with 2 mM glutamine, 1% nonessential Amino Acids, 10% heat inactivated fetal bovine serum (HI FBS) and 1% penicillin/streptomycin antibiotics. Cells were maintained in humidified atmosphere containing 5%  $\text{CO}_2$  at 37°C. For each experiment cells were grown to 80% confluence in cell culture flasks.

### 2.6.2 MTT Cell Proliferation Assay

Cytotoxic effects of *P. dysenterica* extracts were assessed by MTT assay [15]. For each experiment cells were seeded ( $2 \times 10^4$  cells/well) in 96 well plates and incubated overnight. Next day, cells were treated with increasing final concentrations of *P. dysenterica* extracts (40-4000  $\mu\text{g/mL}$ ) and incubated for additional 48h. After incubation the cells, MTT solution 0.5 mg/mL was added in each well, and plates were incubated for another 4 hours at  $37^\circ\text{C}$  in humidified atmosphere containing 5%  $\text{CO}_2$ . Then the medium containing MTT was removed and the remaining MTT-formazan crystals were dissolved by adding 200  $\mu\text{L}$  DMSO to each well with continuous gentle shaking for 15 minutes. Absorbance was read using microplate reader at a wavelength of 570 nm. Experiment was repeated three times and each experiment was performed in triplicate. Untreated cells were used as negative control and positive control were cells treated with 30% DMSO in culture medium. Prepared stock solutions of extracts were sterilized by filtration through 0.2  $\mu\text{m}$  sterile syringe filters. The concentration of the extracts leading to 50% inhibition of viability ( $IC_{50}$ ) was assessed from graph plots of the dose response curve.

### 3. RESULTS AND DISCUSSION

#### 3.1 Yield and polyphenolic composition of extracts

The yields of the methanol extracts of *P. dysenterica* were  $7.28 \pm 0.20$  and  $9.43 \pm 0.71\%$  while the TPC of the samples were  $127.62 \pm 2.22$  and  $244.12 \pm 8.84$  mg GAE/g extract (Table 1).

**Table 1. Yields, content of total phenols,  $IC_{50}$  and FRAP values of *P. dysenterica* methanolic extracts**

Sample	Yields (%) <sup>a</sup>	TPC <sup>b</sup> (mg GAE <sup>c</sup> /g) <sup>a</sup>	DPPH $IC_{50}$ ( $\mu\text{g mL}^{-1}$ ) <sup>a</sup>	FRAP [ $\mu\text{mol Fe}^{2+} \text{g}^{-1}$ ] <sup>a</sup>
PDA	$7.28 \pm 0.20$	$127.62 \pm 2.22$	$157.06 \pm 1.83$	$1296.67 \pm 39.77$
PDU	$9.43 \pm 0.71$	$244.12 \pm 8.84$	$52.36 \pm 0.75$	$2411.12 \pm 37.22$

\*PDA – *Pulicaria dysenterica* aerial parts, PDU – *Pulicaria dysenterica* underground parts,

<sup>a</sup>mean value  $\pm$  standard deviation (n=3), <sup>b</sup>TPC – total phenolic content, <sup>c</sup>GAE – galic acid equivalent

The qualitative and quantitative analysis of individual polyphenolic compounds of the aerial and underground parts extracts of *P. dysenterica* were performed by the RP-HPLC method. In the extract of aerial parts of *P. dysenterica* chlorogenic acid and rutin were identified, as well as one more derivative of caffeic acid. The content of rutin was  $5.68 \pm 0.13$  mg/g. Extract from underground parts contained chlorogenic acid, three derivatives of caffeic acid, but the flavonoid rutin was not detected by the applied method. There was no big difference in chlorogenic acid content in extracts from aerial and underground parts of the plant (Table 2).

**Table 2. Compounds of *Pulicaria dysenterica* extracts**

Retention time (min)	Proposed compound	PDA extract (mg/g) <sup>a</sup>	PDU extract (mg/g) <sup>a</sup>
9.501	chlorogenic acid	$10.06 \pm 0.96$	$11.32 \pm 0.28$
19.329	rutin	$5.68 \pm 0.13$	-
22.449	caffeic acid derivative 1	x	x
35.629	caffeic acid derivative 2	-	x
37.909	caffeic acid derivative 3	-	x

\* PDA – *Pulicaria dysenterica* aerial parts, PDU – *Pulicaria dysenterica* underground parts,

<sup>a</sup>mean value  $\pm$  standard deviation (n=3), x- presence, - not detected

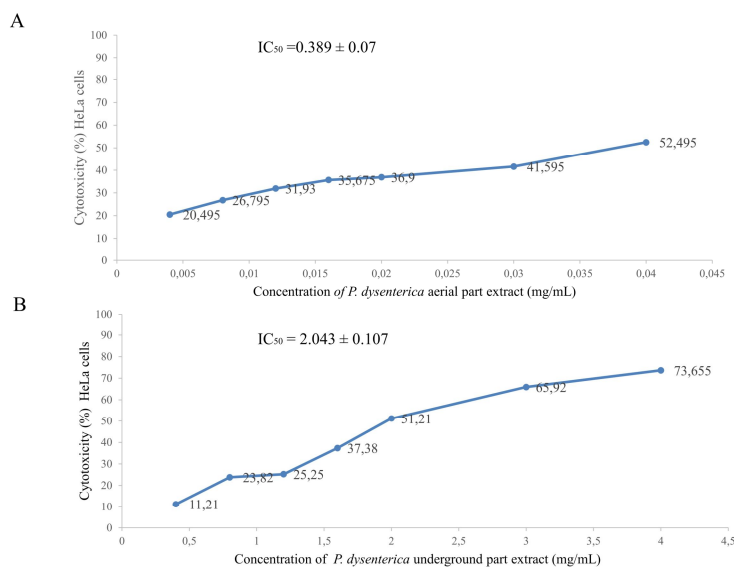
TPC in analysed aerial parts extract is slightly higher compared to published literature data. The phenolic content of *Pulicaria dysenterica* aerial part extracts from Turkey ranged from 80.62±2.87 to 119.40±2.67 mg GAE g<sup>-1</sup>. In those extracts quinic acid, chlorogenic acid and its isomers, dicaffeoylquinic acid and its isomers, luteolin malonyl glucoside, quercetin glucoside were determined [2].

### 3.2 Results of testing antioxidant activity

A number of studies have shown that phenolic compounds have biological activities such as antioxidant, antimicrobial, and antitumor. Thus, phenolic compounds can protect cellular components against oxidative damage and therefore reduce the risk of degenerative disease due to oxidative stress [16]. The results of DPPH and FRAP tests are shown in Table 1. Higher DPPH radical scavenging activity was observed for the extract from *P. dysenterica* underground parts with the  $IC_{50}$  value of 52.36±0.75 µg/mL compared to the extract of aerial parts. Higher antioxidant activity for the extract from *P. dysenterica* underground parts was also observed with the FRAP value of 2411.12±37.22 µmol Fe<sup>2+</sup>g<sup>-1</sup> compared to the extract from aerial parts. The results obtained in this study confirm the well-known positive correlation between the TPC and antioxidant capacity [17].

### 3.3 Results of cytotoxicity testing

The results of the cytotoxic activity suggested that *P. dysenterica* extracts induced a dose-dependent inhibition of HeLa cell proliferation, in accordance to determined  $IC_{50}$ . The measured absorbance values of extracts obtained from aerial and underground plant parts were converted to percent of cell cytotoxicity with respect to negative control (Figure 1).



**Fig. 1. Cytotoxic effects on cancer cell line HeLa, assessed by MTT after 48 hours exposure to increasing concentrations of *P. dysenterica* extract from aerial parts (A),  $IC_{50}=0.389\pm0.07$  mg/mL and *P. dysenterica* extract from underground parts (B),**

**$IC_{50}=2.043\pm 0.107$  mg/mL. Values represent the mean value  $\pm$  standard deviation of three separate experiments**

The  $IC_{50}$  values of methanol extracts from aerial and underground parts of *P. dysenterica* were  $0.389\pm 0.07$  and  $2.043\pm 0.107$  mg/mL respectively, against HeLa cells. For comparison,  $IC_{50}$  for curcumin against HeLa cells was 0.32 mg/mL [18]. In the previous studies methanol extract of *Pulicaria dysenterica* was tested against breast cancer cell lines and showed significant cytotoxic effects. The  $IC_{50}$  values of the extract on MCF-7 cell line was calculated as 27.05  $\mu$ g/mL [19]. To the best of our knowledge, other cytotoxic studies for *P. dysenterica* have not been published.

Previously, MTT assay was used to evaluate the cytotoxic effects of the 19 purified compounds isolated from the *Pulicaria insignis* against four human cancer cell lines, including HeLa. The best results against HeLa cells were obtained with flavonoids and sesquiterpene lactone [20]. *Pulicaria undulata* aerial parts extract was also evaluated for cytotoxicity against breast and hepatoma cancer. One of the flavonoids, among other isolated secondary metabolites, showed the highest activity against tested cell lines [21,22]. In another study on the cytotoxicity one of the isolated pseudoguaianolide sesquiterpene from aerial parts of *P. crispa* (syn. *P. undulata*) showed cytotoxicity with  $IC_{50} = 5.8\pm 0.2$   $\mu$ mol/mL to human bladder carcinoma cell line, EJ-138. [23].

In the extracts analysed in this study, chlorogenic acid and rutin were recorded. A number of studies have shown that treatment with chlorogenic acid has beneficial effects in colon cancer, brain cancer, breast tumours, lung cancer and chronic myelogenous leukaemia [24,25,26,27,28,29]. A study about cytotoxic activity against several cancer cell lines with water soluble green coffee bean extract was conducted. The extract was abundant with chlorogenic acid and its derivatives and showed the highest activity on human colon cancer cells while on HeLa cells the cytotoxicity was low ( $IC_{50} = 1400\mu$ g/mL) [30]. Flavonoid rutin is demonstrated to inhibit the proliferation of breast, colon, lung, and prostate cancers. It affected the cell capture and apoptosis processes, reducing the number of metastatic nodules and cytotoxicity [31,32,33,34,35]. In a study about cervical cancer phytotherapy, rutin showed high cytotoxic activity on HeLa cells with  $IC_{50}$  value 30  $\mu$ g/mL [36]. Considering the fact that currently analysed extract contains approximately 0.5% of rutin, other secondary metabolites also contribute to its cytotoxicity. It was recorded in several studies that effects of flavonoids were linked with their availability and technological formulation. It is found that rutin in prenanoemulsion, which contributes to the improvement of physical and pharmacokinetic properties of this flavonoid, improves its cytotoxicity, as well [33,37].

#### **4. CONCLUSION**

Analyzed *Pulicaria dysenterica* extracts contained chlorogenic acid, caffeic acid derivatives and flavonoid rutin. The extracts showed good antioxidant properties. Stronger antioxidant activity was recorded in extract from underground then from aerial plant parts. Also good cytotoxicity of *P. dysenterica* extracts against HeLa cells *in vitro* was reported. Better result was achieved for extract from aerial compared to extract from underground parts. To our knowledge, this is the first report of *P. dysenterica* showing cytotoxic activity on cervical cancer (HeLa) cell lines. Further research should be directed to isolation and determination the secondary metabolites, flavonoids and sesquiterpene lactones, primarily responsible for the cytotoxicity.

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