Antioxidant and Cytotoxic Activities of *Pulicaria dysenterica* Methanol Extracts

ABSTRACT

Aims: The aims of the study were to analyse the polyphenols of *Pulicaria dysenterica* (L.) Bernh. 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 reverse phase HPLC method. The antioxidant capacity was evaluated by both, 2,2-diphenyl1-picrylhydrazyl radical and FRAP assay, while cytotoxicity of the extracts was assessed by MTT assay.
Results: TPC of the samples were 127.62±2.22 and 244.12±8.84 mg gallic acid equivalent/g extract. In the extracts chlorogenic acid in amount of 10.06±0.96 and 11.32±0.28 mg/g, flavonoid rutin in amount of 5.68±0.13 mg/g and three caffeic acid derivatives were recorded. Extract from underground parts achieved better antioxidant activity with IC_{50} value 55.36±0.75 µg/mL and FRAP value 2411.12±37.22 µmol $Fe^{2+}g^{-1}$ compared to the one from aerial parts. Extract from aerial parts achieved better cytotoxic activity with 50% inhibition of viability (**IC_{50}**) at concentration of 0.389±0.07 mg/mL, against HeLa cells, compared to the 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 height 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 aerial parts of the *Pulicaria dysenterica* are used in the treatment of diarrhea and dysentery in Iranian traditional medicine. They are also used for the treatment of dysentery in the United Kingdom. The plant has an insecticidal property, as well" [1,2]. "Phytochemical analysis of genus *Pulicaria* showed the occurrence of molecules of monoterpenes, diterpenes, sesquiterpenes, triterpenes, flavonoids and steroids. Previous studies on the chemical constituents of aerial parts of *P. dysenterica* included volatile oils, sesquiterpenes, flavonoids and caryophyllenes" [2]. "Flavonoid detected in aerial parts of *P. dysenterica* was quercetin 3-glucuronide. Other chemicals as quercetagetin 3,7-dimethyl ether, 6-hydroxykaempferol 3,4'-dimethyl ether, acetylenes were also recorded" [3,4]. „Studies on the composition of the essential oil of the aerial parts of the *P. dysenterica* from Greece and Iran confirmed well known fact that the chemical composition of the essential oil depends on various parameters such as environmental conditions. Significant difference in the composition and percentage of ingredients was recorded" [1]. 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, it was shown that extracts of *P. dysenterica* aerial parts were active against

bacterial strain *Vibrio cholerae*, as well as against parasite *Trichomonas gallinae* in *in vitro* tests [5,6]. "Cadiz-Gurrea et al. evaluated biological properties of *P. dysenterica* methanolic extracts from aerial parts by *in vitro* inhibitory potential of enzymes (lipase, α-amylase, α-glucosidase, tyrosinase, and cholinesterases). Extracts showed promising results for the management of diabetes type II, Alzheimer's disease and skin hyperpigmentation disorders and obesity. They also examined antioxidant capacity of the extracts" [2]. Furthermore, study about cytotoxic effects of related *Inula species* against some tumor cell lines showed good results with IC_{50} values from 17.96 µg/mL [7]. For some species of genus *Pulicaria* cytotoxic activities have been reported, as well [2].

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 *in vitro* 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). Acetonitrile, high performance liquid chromatography (HPLC) grade and formic acid were purchased also from Merck. Methanol, 2,2-diphenyl1-picrylhydrazyl (DPPH) radical, 2,4,6-tris(2- pyridyl)-s-triazine (TPTZ), ferrous (II) sulfate heptahydrate, hydrochloric acid, glacial acetic acid and HPLC-grade chlorogenic acid were purchased from Sigma-Aldrich (USA). Minimum Essential Medium Eagle (MEM), 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* (PDA and PDU) were collected at the mountain pass Karaula (N44°10'13.6" E18°39'07.2"), municipality Olovo, 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 (No. 20/3.1.I) 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 were prepared in methanol. One hundred µL of an extract was mixed with 7.9 mL of distilled water. Folin-Ciocalteu reagent (500 µL) was added. After 8 min, 1.5

ml of 20% Na_2CO_3 was added. After 2 hours of incubation at room temperature (20°C ≤ t ≤ 25°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 (GAE) as miligram 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 reverse phase (RP) analytical column (250x4.6mm i.d., 5µm). 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 µL; 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 µg/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 µg/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 were prepared in methanol. The reaction mixture contained 75 µL of extract solution and 75 µL 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°C ≤ t ≤ 25°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:

SA% = 100 x [(Ac - Ao) - (As - Ao)] / (Ac - 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} (µg/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 were prepared in methanol. In short, 100 µL 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 $FeCl_3$ (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 $FeSO_4x7H_2O$ from 100 to 1000 µmol/L (R^2=0.9985, y=0.0009x-0.0534). The results of FRAP assay are expressed as µmol 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 MEM supplemented with 2 mM glutamine, 1% nonessential Amino Acids, 10% HI FBS and 1% penicillin/streptomycin antibiotics. Cells were maintained in humidified atmosphere containing 5% 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 ($2x10^4$ cells/well) in 96 well plates and incubated overnight. Next day, cells were treated with increasing final concentrations of *P. dysenterica* extracts (40-4000 µg/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°C in humidified atmosphere containing 5% CO_2. Then the medium containing MTT was removed and the remaining MTT-formazan crystals were dissolved by adding 200 µL 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. Samples were dissolved in 10% DMSO and diluted in culture medium. The final DMSO concentration 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 leading to 50% 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±0.20 and 9.43±0.71% while the TPC of the samples were 127.62±2.22 and 244.12±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 (%)	TPC (mg GAE/g)	DPPH IC_{50} (μgmL^{-1})	FRAP [µmol $Fe^{2+}g^{-1}$]
PDA	7.28±0.20	127.62±2.22	157.06±1.83	1296.67±39.77
PDU	9.43±0,71	244.12±8.84	55.36±0.75	2411.12±37.22

Data presented as mean value ± standard deviation (n=3),

The qualitative and quantitative analysis of individual polyphenolic compounds of the extracts from aerial and underground parts of *P. dysenterica* were performed by the RP-HPLC method. Obtained chromatograms are presented on Fig. 1.

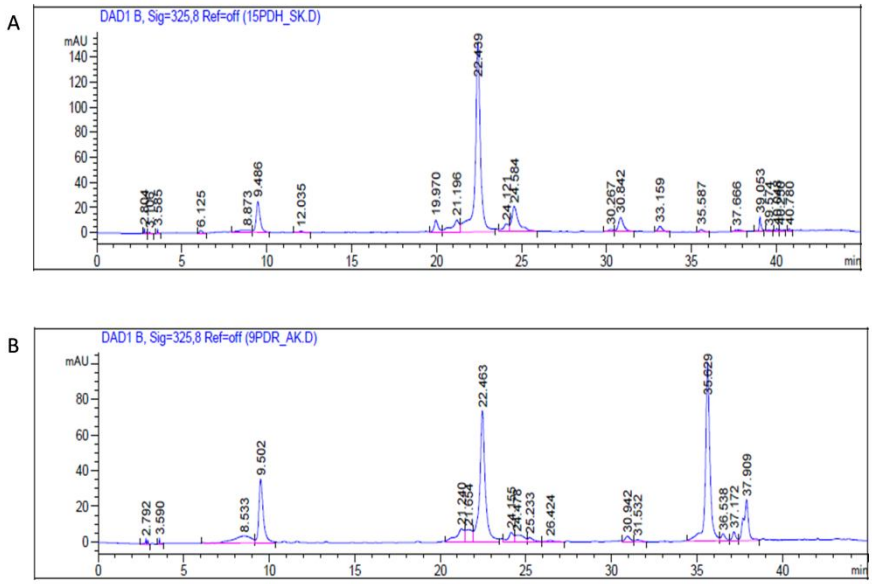

Fig. 1. HPLC chromatograms of *P. dysenterica* extract from aerial parts (A), and underground parts (B) (λ= 325 nm)

In the extract from 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±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)	PDU extract (mg/g)
9.486	chlorogenic acid	10.06±0.96	11.32±0.28
19.970	rutin	5.68±0.13	-
22.463	caffeic acid derivative 1	x	x
35.629	caffeic acid derivative 2	-	x
37.909	caffeic acid derivative 3	-	x

[a]mean value ± standard deviation (n=3), x- presence, - not detected

In methanolic extracts from aerial parts of *P. dysenterica* from Turkey total phenolic, flavonoid and phenolic acid were detected. The phenolic content ranged from 80.62±2.87 to 119.40±2.67 mg GAE g^{-1}. In those extracts quinic acid, chlorogenic acid and its isomers, dicaffeoylquinic acid and its isomers, luteolin malonyl glucoside and quercetin glucoside were determined [2]. TPC in analysed extract from aerial parts is slightly higher compared to published literature data.

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. Graphs about the antioxidant results for DPPH assay are presented on Fig. 2.

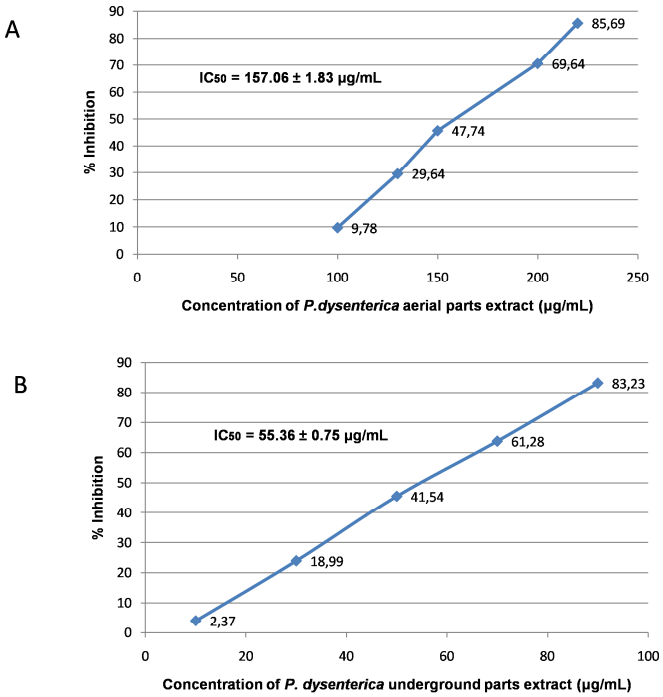

Fig. 2. Antioxidant activity, assessed by DPPH of *P. dysenterica* extract from aerial parts (A), IC_{50}=157.06±1.83 µg/mL and *P. dysenterica* extract from underground parts (B), IC_{50}=55.36±0.75 µg/mL. Values represent the mean value ± standard deviation of three separate experiments

Higher DPPH radical scavenging activity was observed for the extract from *P.dysenterica* underground parts with the IC_{50} value of 55.36±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 $Fe^{2+}g^{-1}$ 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).

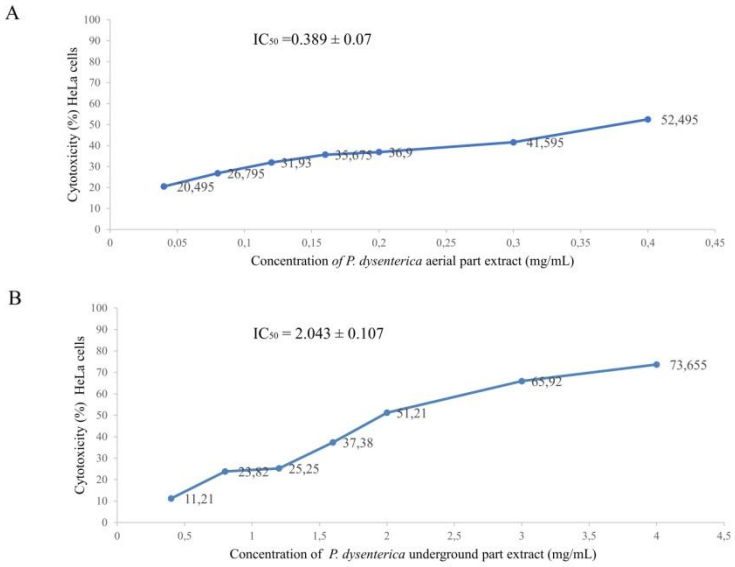

Fig. 3. Cytotoxic effects on cancer cell line HeLa, assessed by MTT after 48 hours exposure to increasing concentrations of *P. dysenterica* extract from aerial parts (A), IC_{50}=0.389±0.07 mg/mL and *P. dysenterica* extract from underground parts (B), IC_{50}=2.043±0.107 mg/mL. Values represent the mean value ± standard deviation of three separate experiments

The IC_{50} values of methanol extracts from aerial and underground parts of *P. dysenterica* were 0.389±0,07 and 2.043±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 µg/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±0.2 µmol/mL to human bladder carcinoma cell line, EJ-138. [23].

Considering the presence of chlorogenic acid and rutin in the analyzed extracts, we investigated previously published data about its cytotoxic and antitumor effects on HeLa and other cancer cell lines. 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 lower with IC_{50} = 1.4 mg/mL [24]. Citotoxic activity of our extract is almost four times stronger compared to the activity of extract abundant with chlorogenic acid and its derivatives. Considering that the content of

chlorogenic acid in our extract is about 1%, its contribution to the cytotoxicity is very small. Chlorogenic acid was tested against breast cancer cell lines. Results were also low with IC_{50} values ranged from 590.5±10.6 µg/mL to 1095±121.6 µg/mL [25]. "On the other side chlorogenic acid isolated from *Piper betel* leaves was found to kill Bcr-Abl-positive chronic myelogenous leukaemia (CML) cells *in vitro* in a dose-dependent manner without any appreciable effects on Bcr-Abl-negative acute T-lymphoblastic leukemia cell line" [26,27]. Chlorogenic acid-induced radical oxigen species production inhibited cell viability in human colon cancer cells [28]. 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 [29,30,31,32,33]. In a study about cervical cancer phytotherapy, rutin showed high cytotoxic activity on HeLa cells with IC_{50} value 30 µg/mL [34]. 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 [31,35].

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. There was also a good cytotoxicity of *P. dysenterica* extracts against HeLa cells *in vitro* 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 of the secondary metabolites, flavonoids and sesquiterpene lactones, primarily responsible for the cytotoxicity.

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Authors ZM and ECK designed the study. ND, LK and LM contributed to samples preparation for total phenolic content (TPC), HPLC analysis and antioxidant assessment. Authors ECK and EH carried out the experiment for total and individual polyphenolic compounds and antioxidant assessment. Authors ED and DH carried out the experiment for cytotoxicity assessment. Authors AS and NS worked out majority of technical details and performed the analytic calculations and the statistical analysis. Author ECK wrote the manuscript with support from author JA, MI and ED. BŠK managed the literature searches and gave critical overview of the manuscript. All authors read and approved the final manuscript.

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