CYTOTOXIC, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF THE METHANOLIC EXTRACT OF SPEEDWELLS (*VERONICA OFFICINALIS L.*)

ORIGINAL SCIENTIFIC ARTICLE

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ABSTRACT:
Speedwells (*Veronica officinalis* L.) is a plant species used in traditional medicine for the preparation of teas, tinctures and other preparations for the treatment of diseases of the skin, respiratory and digestive organs. In this paper, the biological activity of the methanolic extract of the speedwells was examined. To assess the cytotoxic potential, a tetrazolium salt reduction (MTT) viability assay was performed. The experiments were carried out on 3 human cell lines: lung carcinoma (H460), cervical adenocarcinoma (HeLa) and colorectal carcinoma (HCT116). Antimicrobial potential was tested using the diffusion technique on three bacterial strains: *S. aureus*, *E. faecalis* and *S. enterica*. Inhibition of free radicals was tested using the ABTS and DPPH methods, and the reduction potential of the extract of the speedwells was confirmed by the FRAP method. The treatment of HeLa, H460, and HCT116 cell lines with the methanolic extract of speedwells demonstrated a dose-dependent decrease in cell growth. The extract showed a high inhibition of the growth of *S. aureus* but also a complete absence of activity in the case of *E. faecalis*. A high efficiency of inhibition of DPPH and ABTS radicals, as well as reducing ability, was recorded.

KEYWORDS: polyphenols, flavonoids, cytotoxicity, antibacterial activity, antioxidant potential

INTRODUCTION

It is evident that the biologically active components of the plant become an inspiration for the treatment of diseases and the development of medicines in healthcare every day [1]. Interest in the development of natural antioxidants is growing due to their positive impact on health, but also due to the fact that some synthetic antioxidants act as endocrine disruptors and carcinogenic agents [2]. The genus *Veronica L.* is the largest genus of the *Plantaginaceae* family. It includes up to 500 species that are distributed throughout the Northern Hemisphere and in certain parts of the Southern Hemisphere. Their habitats are diverse, from dry steppe habitats to alpine regions [3]. Apart from chemotaxonomic and phytochemical importance genus, *Veronica* species are of particular interest considering their traditional use and biological activities [4]. *Veronica officinalis* is a popular medicinal plant species. It is part of the traditional medicine of many European countries. In Turkish flora 26 of *Veronica* species are endemic. It is important to emphasize that different parts of the plant are often used for different medicinal purposes [5]. Species from the genus *Veronica* represent a valuable source of biologically active compounds.

Extracts show antioxidant, antimicrobial, antifungal, anti-inflammatory and anticarcinogenic effects. The inhibitory potential on acetylcholinesterase, tyrosinase, lipoxygenase and xanthine oxidase has also been proven [6]. *Veronica officinalis L.*, in Balkan traditional medicine is used for the treatment of eczema, liver, wound healing and skin lesions, and also for the treatment of snake bites

[5]. In traditional Chinese medicine, *Veronica* species are used as expectorants, restoratives, tonics, and for the treatment of influenza and other respiratory diseases [7]. *Veronica officinalis L*. has a long history of medicinal use as a diuretic and diaphoretic. In Romanian traditional medicine, it was used for kidney diseases, cough and catarrh, wound healing, and for the treatment of lung diseases and hypercholesterolemia [8,9,10].

Several *Veronica* species are used to treat cancer, influenza, hemoptysis, laryngopharyngitis, hernia, cough and respiratory diseases in different countries [11]. Extracts obtained from the aerial parts of certain species of *Veronica* are used as folk remedies for the treatment of various inflammatory diseases, including rheumatism [12]. In addition, the stems and leaves of some species of *Veronica* are edible, raw or cooked [13]. *Veronica officinalis* extract shows an antimicrobial effect on *Staphylococcus aureus, Listeria monocytogenes and Listeria ivanovii* [3]. There are also reports that extracts of *Veronica officinalis L*. can potentially be used as good natural anti-phytoviral agents [14]. In comparison with V. *peduncularis Bieb., V. baranetzkii Bordz., V. orientalis Miller, V. hederifolia L., Veronica officinalis L*. shows the most significant antioxidant potential due to its rich composition of phenols [12]. Previous phytochemical tests showed that the herb of this plant species contains iridoids (veronicoside, catalpol, aukubin, veproside, musaenoside, landroside): flavonoids (luteolin derivatives): triterpene saponosides, tannins, and phenolic acids (chlorogenic and caffeic acid). It has been proven that the most abundant biologically active ingredient in this species is Acteoside [15].

Of particular importance for medicine is the proven gastroprotective activity of the extract of *Veronica officinalis L*. Namely, the extract of this plant significantly inhibits the formation of stomach ulcers [16]. The molecular docking method proved the potential of Cyclododecane and 2,6-Dimethyl-3-(methoxymethyl)-p-benzoquinone in the treatment of lung cancer. Both biologically active components are present in methanolic extracts of *Veronica officinalis L* [17].

MATERIALS AND METHODS

The aerial part of the dried plant material was purchased in a local market in Tuzla. The dry sample was pulverized in an electric mill and immediately used for extract preparation. Methanol, glacial acetic acid, hydrochloric acid, sodium carbonate were purchased from Merck (Darmstadt, Germany). 2,2`-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), dimethyl sulfoxide (DMSO), gallic acid and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Iron(II) sulphate heptahydrate, vitamin C, iron(III) chloride hexahydrate and potassium persulfate were purchased from Honeywell (Charlotte, North Carolina, USA). Folin & Ciocalteu's reagent was purchased from Semikem (Bosnia and Herzegovina). Spectroscopic measurements were performed on a Perkin Elmer λ25 spectrophotometer.

PREPARATION OF EXTRACT

40 grams of dry crushed plant material was transferred to a flat-bottomed flask and mixed with 160 mL of methanol. The mixture was mixed on a vibromix at 250 rpm/minute. After 24 hours of mixing, the mixture was filtered through filter paper, and the filtrate was immediately evaporated on a rotavapor. The dry extract was collected and further used for analysis. Extract solutions were prepared in DMSO.

DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC)

Total phenolic compounds presented in the extract were quantified spectrophotometrically using the Folin-Ciocalteu test following the protocol [18], with some modifications. 200 μL of extract solution was mixed with 2.54 mL of 10% Folin-Ciocalteu reagent. After 5 min 420 μL of 10% sodium carbonate was added. The absorbance of the resulting blue-coloured solution was measured at 765 nm after incubation at room temperature for 1 hour. Quantitative measurements were performed, based on a standard calibration curve of gallic acid. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrammes per gram of dry extract.

DETERMINATION OF TOTAL FLAVONOID CONTENTS (TFC)

Total flavonoid content in the extract was determined by the previously described method [19], with some modification. 1 mL of extract solution were mixed with 0.3 mL of 5% sodium nitrite. 0.3 mL of 10% aluminium chloride was added after 5 minutes. After 6 minutes incubation at room temperature, 1 mL of 1 M sodium hydroxide was added to the reaction mixture. Immediately the final volume was make up to 10 mL with distilled water. Absorbance of sample was measured against the blank at 510 nm using a spectrophotometer. The results were derived from the calibration curve of quercetin and expressed in quercetin equivalents (QE) per gram of dry extract.

DPPH RADICAL SCAVENGING ACTIVITY

2,2-diphenyl-1-picryl-hydrazyl (DPPH) method was performed according to earlier described method [20]. A series of dilutions of the extract was made, after which 500 uL of 0.5 mM DPPH radical solution was added to each test tube. The samples were incubated for 30 minutes. The absorbance was measured at 517 nm with methanol as a blank sample. 0.5 mL of 0.5 mM DPPH dilution, diluted with 4 mL of methanol, was used as a control sample. The radical scavenging effect (%) or percent inhibition of DPPH radical was calculated according to the equation:

$$[(Ac - As) / Ac] \times 100$$

where As is the absorbance of the solution containing the sample at 517 nm and Ac is the absorbance of the DPPH solution. Results are expressed as IC_{50} value.

ABTS (2,2-AZINO-BIS-3-ETHYLBENZOTHIAZOLINE-6-SULPHONIC ACID) ASSAY

The ABTS scavenging activity was evaluated according to the method of Almeida et al. [21] with some modifications. A stock of ABTS radical cation ($ABTS^{\bullet+}$) was prepared by the reaction of 7 mM ABTS solution (5 µL) with 140 mM potassium persulfate (88 µL), and incubated for 16 h in the dark. The $ABTS^{\bullet+}$ solution was diluted with 95% ethanol to obtain absorbance 1.0 at 734 nm. For the analysis, a series of diluted extract solutions was made and mixed with $ABTS^{+}$ reagent working solution. After 6 minutes of incubation, the absorbance is measured at 734 nm. Results are expressed as IC_{50} value.

FERRIC-REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

The reducing powers of the extracts that reflected their antioxidant activity were determined following the protocol [22]. 3 mL of prepared FRAP reagent is mixed with 100 µL of extract solution. Absorbance at 593 nm is recorded after a 30 min incubation at 37 °C. The FRAP value was calculated from the calibration curve of iron (II) sulfate heptahydrate and expressed in µmol per gram of dry extract.

ANALYSIS OF ANTICANCER POTENTIAL

The experiments were carried out on 3 human cell lines. The following cell lines were used: H460 (lung carcinoma, large cell lung cancer (ATCC®HTB-177™), HeLa (cervical adenocarcinoma, ATCC® CCL-2™), and HCT116 (colorectal carcinoma ATCC® CCL-247™).

Cells were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C.

The panel cell lines were inoculated onto a series of standard 96-well microtiter plates on day 0, at 1.5×10^4 cells/ml. Extract was added at 10 µg/ml, 50 µg/ml, 100 µg/ml, 500 µg/ml and 1000 µg/ml concentration and incubated for a further 72 hours.

After 72 hours of incubation the cell growth rate was evaluated by performing the MTT assay, which detects dehydrogenase activity in viable cells. The MTT Cell Proliferation Assay is a colorimetric assay system, which measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. For this purpose the substance treated medium was discarded and 40 µl of MTT reagent was added to each well at a concentration of 0.5 µg/µl. After four hours of incubation the precipitates were dissolved in 160 µl of DMSO. The absorbance (A) was measured on a microplate reader at 570 nm. The absorbance is directly proportional to the cell viability. The percentage of growth (PG) of the cell lines was calculated according to one or the other of the following two expressions:

$$\text{If } (A_{test} - A_{tzero}) \geq 0 \text{ then:}$$
$$PG = 100 \times (A_{test} - A_{tzero}) / (A_{cont} - A_{tzero})$$
$$\text{If } (A_{test} - A_{tzero}) < 0 \text{ then:}$$
$$PG = 100 \times (A_{test} - A_{tzero}) / A_{tzero})$$

where:
A_{tzero} = the average absorbance before exposure of cells to the test compound,
A_{test} = the average absorbance after the desired period of time (72 h),
A_{cont} = the average absorbance after 72 hours with no exposure of cells to the test compound.

Each test point was performed in quadruplicate in three individual experiments. The results are expressed as concentration-response graphs. A negative percentage indicates cytotoxicity following drug treatment where -100% shows no cells survived the treatment at the specific drug concentration. The results are also expressed as GI_{50}, a concentration necessary for 50% of inhibition.

ANTIBACTERIAL ACTIVITY *IN VITRO*

Antimicrobial activities were investigated by diffusion method for reference bacterial strains *Enterococcus faecalis* (ATCC 51299), *Staphylococcus aureus* (ATCC 25923) and *Salmonella enterica* (ATCC 13076). In the agar sterile

drill-shaped holes were made ("wells") into which 50 and 100 µL of exstract solutions of concentration 100 mg/mL were added. After the plates were left at room temperature for 15 min, the substance was diffused into agar, incubated at 37 °C/24 h. A solution of ciprofloxacin with a concentration of 0.5 mg/mL was used as a control.

RESULTS AND DISCUSSION

CONTENT OF BIOACTIVE COMPONENTS AND ANTIOXIDANT ACTIVITY

Tables 1 and 2 show the results of the analysis of the content of polyphenols and flavonoids, and antioxidant activity of the extract of speedwells. Fig. 1 graphically shows the dependence of free radical inhibition on the concentration of extract and vitamin C.

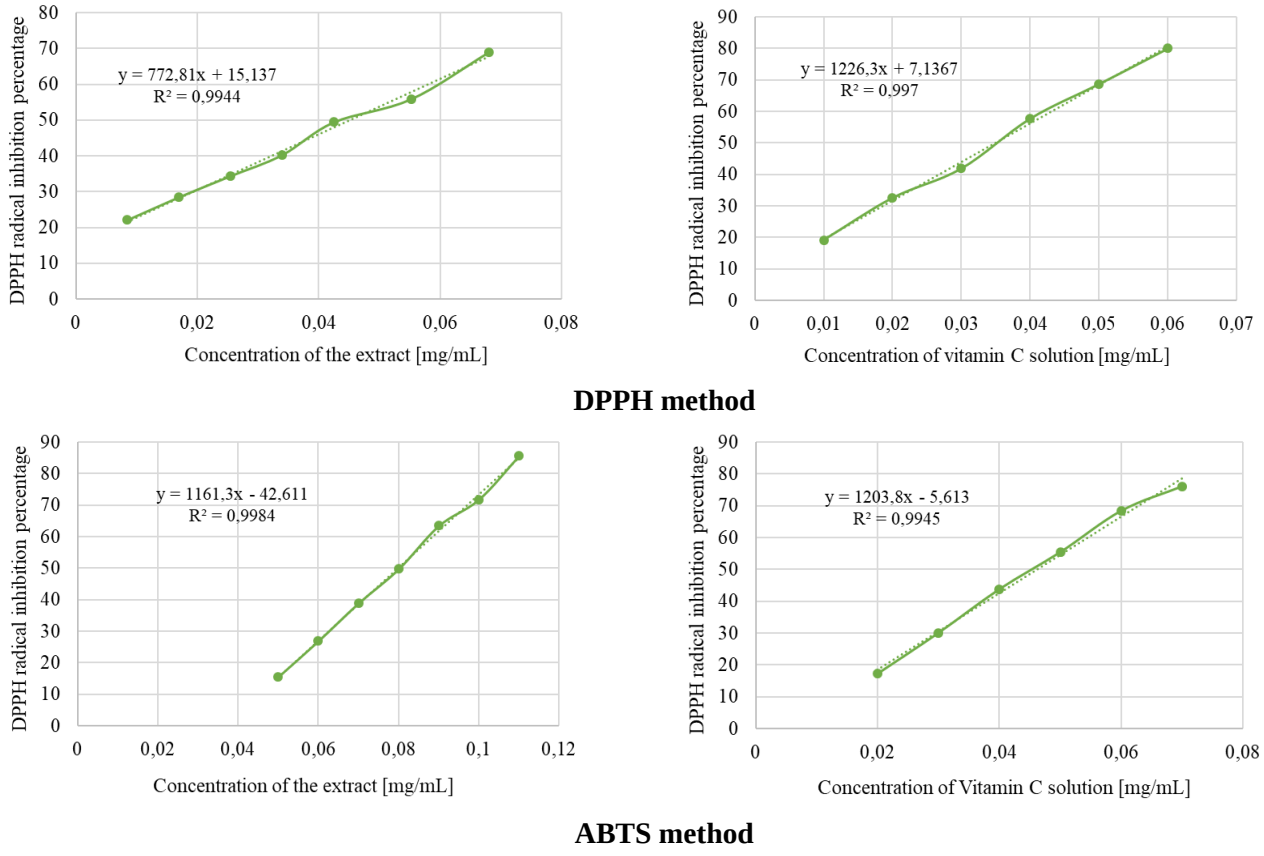

Figure 1. Graphic representation of the dependence of free radical inhibition on the concentration of extract and vitamin C

These graphs were used to calculate IC_{50} values. The content of polyphenols and flavonoids in the methanol extract is high and is correlated with a high ability to inhibit free radicals, and a somewhat weaker reduction potential of the extract. Vitamin C, which was used as a control, showed a higher efficiency of free radical inhibition and a significantly higher reduction potential.

Table 1. Content of bioactive components in the methanolic extract of speedwells

TPC [mg GAE/g]	TFC [mg QE/g]
43,64	2.75

Table 2. Results of the antioxidant capacity of the methanolic extract of speedwells

Sample	DPPH [mg/mL]	ABTS [mg/mL]	FRAP value [µmol/g]
Extract	0.045	0.079	1526.11
Vitamin C	0.035	0.046	14250.05

By looking at the literature data, the presence of numerous polyphenolic components was confirmed by HPLC analysis of the extracts of the speedwells. According to research conducted by Mocan et al. the most abundant are quercitrin, luteolin, ferulic acid, p-coumaric acid and apigenin, while the other components are mostly present in lower

concentrations [23]. This depends on the type of solvent used for extraction, the extraction technique, and the origin and treatment method of the plant material and the resulting extract.

Similar studies of the content of bioactive components and antioxidant capacity were carried out by other scientists for extracts of the mentioned plant species, prepared with different solvents and extraction techniques. Mocan et al. [23] whether the content of bioactive components and the antioxidant capacity of the ethanol extract prepared by ultrasonic extraction at room temperature were examined. The content of polyphenols and flavonoids and their research is lower, and the reason for this can be explained by the use of a different extraction technique and solvent, as well as the different geographical origin of the sample. Žugić et al. [24] examined the content of polyphenolic components and the antioxidant activity of several plant species. For the extract of *V. officinalis*, the polyphenol content was found to be lower than 30 mg GAE/g extract, which is lower compared to the results obtained in our research.

CYTOTOXIC ACTIVITY

The graph below shows the dose-response profiles of a methanolic extract of speedwells on three human cell lines: H460, HeLa and HCT116 (Figure 2). Results were obtained after a 72-hour incubation period with different extract concentrations, as it follows: 10 μg/ml, 50 μg/ml, 100 μg/ml, 500 μg/ml and 1000 μg/ml. The HCT116 and HeLa cell lines exhibited a more complex response, suggesting both stimulatory and inhibitory effects depending on the concentration. At higher doses (above 500 μg/ml), the extract slightly stimulated growth, but the H460 cell line's growth percentage consistently decreased. Altogether, the H460 cells were the most sensitive, showing a significant decrease in PG at lower concentrations compared to HeLa and HCT116 cells. The maximum inhibition of cell growth occurred at a concentration of 500 μg/ml for the HCT116 and HeLa cells, whereas for the H460 cells, it was observed at concentrations higher than 500 μg/ml.

Previous studies correlate with our findings. Several other *Veronica* species have shown an antiproliferative effect on different cell lines (including HeLa and HCT116 cell lines). Authors also suggested potential chemotherapeutic properties [5, 14, 25]. More research is needed to better understand the process and to determine the specific chemicals in the extract that are responsible for these cytotoxic effects.

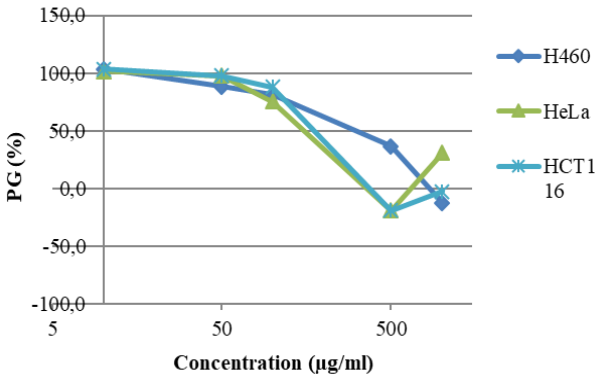

Figure 2 Dose-response profiles for methanolic extract of speedwells on H460, HeLa and HCT116 cell lines

Table 3. GI_{50} values for methanolic extract of speedwells

Cell lines	GI_{50} (μg/mL)
Lung carcinoma (H460)	392±42
Cervical adenocarcinoma (HeLa)	206±99
Colorectal carcinoma (HCT116)	240±15

ANTIBACTERIAL ACTIVITY

The results of the antibacterial activity of the extract of speedwells are shown in table 3. Antibacterial activity was recorded in the case of both tested volumes of the extract solution with a concentration of 100 mg/mL. Greater efficiency of growth inhibition was recorded in the case of *S. aureus*. Ciprofloxacin, which was used as a control, showed a significantly larger zone of growth inhibition on the tested bacterial strains with a zone of inhibition greater than 20 mm.

Table 4. Results of the antibacterial effect of the extract of speedwells

Sample	Inhibition zone [mm]					
	S. aureus		*E. faecalis*		*S. enterica*	
	50 μL	100 μL	50 μL	100 μL	50 μL	100 μL
Extract	14	16	-	-	11	14
Ciprofloxacin	>20	>20	>20	>20	>20	>20

Mocan et al. (2015) investigated the antibacterial activity of ethanolic extract of the speedwells. The research was conducted on a larger number of bacterial strains, which confirmed the antibacterial activity of the ethanolic extract in the case of *S. aureus* and *E. faecalis*, with a minimum inhibitory concentration (MIC) of 7.81 mg/mL.

CONCLUSION

This research has confirmed the biological action of the extract of the speedwells. The content of polyphenols and flavonoids is correlated with the antioxidant capacity of the extract, which was confirmed using three methods. The treatment of HeLa, H460, and HCT116 cell lines with the methanolic extract of speedwells demonstrated a dose-dependent decrease in cell growth. The extract showed high antimicrobial potential in the case of *S. aureus*, and weaker activity against *S. enterica*.

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