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Phenolic profile, antibacterial, antimutagenic and antitumour evaluation of *Veronica urticifolia* Jacq.

Jelena Živković[a*], João C.M. Barreira[b,c,d], Dejan Stojković[e], Tatjana Ćebović[f], Celestino Santos-Buelga[c,*], Zoran Maksimović[g], Slobodan Davidović[h], Isabel C.F.R. Ferreira[b]

[a]Institute for Medicinal Plant Research "Dr. Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia.

[b]Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Ap. 1172, 5301-855 Bragança, Portugal.

[c]Grupo de Investigación en Polifenoles (GIP-USAL), Faculty of Pharmacy, University of Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.

[d]Chemical Sciences Department, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal.

[e]University of Belgrade, Institute for Biological Research "Siniša Stanković", Department of Plant Physiology, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia.

[f]University of Novi Sad, Department of Biochemistry, School of Medicine, Hajduk Veljkova 3, 21102 Novi Sad, Serbia.

[g]University of Belgrade, Department of Pharmacognosy, School of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia.

[h]University of Belgrade, Institute of Molecular Genetics and Genetic Engineering, 11010 Belgrade, Serbia.

*Authors to whom correspondence should be addressed: (Jelena Živković; e-mail: jelenazivkovic1@yahoo.com. Phone: +381 648674921; Fax: +381 113031655; Celestino Santos-Buelga; e-mail: csb@usal.es. Phone: +34 923294537; Fax: +34 923294515).

Abstract

This study was designed to characterize the phenolic profile and evaluate the antibacterial, antimutagenic and antitumor activities of *Veronica urticifolia* Jacq. methanolic extract. HPLC-DAD/ESI-MS analysis revealed the presence of phenolic acids, flavonoids and phenylethanoids, with acteoside as the main component (14.9 mg/g of extract). Antibacterial effect was determined using the microbroth dilution assay and *Staphylococcus aureus* was the most sensitive strain (MIC and MBC = 7.5 mg/mL). Antimutagenic activity was evaluated by Ames mutagenicity assay. At 1 mg/plate, the tested extract afforded high protection against the mutagenicity of nitroquinoline-N-oxide (4NQO) to *Salmonella typhimurium* strain TA100 (inhibition rate 48.3%). Antitumor activity was screened in Ehrlich ascites carcinoma (EAC) model. Pretreatment with 2 mg/kg body weight showed statistically significant decrease in tumor cell viability, while ascites volume and tumor cell count became slightly decreased, but not to a statistically significant extent. Above results indicate that *V. urticifolia* deserves further research into chemoprevention.

Keywords: *Veronica urticifolia*; Phenolic profile; HPLC-DAD/ESI-MS; Antibacterial; Antimutagenicity; Ehrlich carcinoma.

1. Introduction

Cancer, the second leading cause of death worldwide next to cardiovascular diseases, is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion and distant metastases (Madhusudan and Middleton, 2005). According to World Health Organization, more than 10 million new cases of cancer are diagnosed every year, and the statistical trends indicate that this number would double by 2020 (Mignogna et al., 2004). The demand for new anticancer agents is escalating due to the accumulation of carcinogenic and mutagenic agents in the environment. Although many synthetic anticancer agents are used in the treatment of cancer, side effects and emergence of synthetic drug resistant cancer cells among patients limits their purpose (Madhusudan and Middleton, 2005). Recent studies have shown that cancer management by phytochemicals from vegetables, fruits and medicinal plants is one of the most feasible approaches to treat this cascading disease (Dhamija et al., 2013).

In the fight against cancer, the search for antimutagenic agents is essential, since mutagenic and carcinogenic factors are present in the human environment and their total elimination seems to be impossible (Abdillahi et al., 2012). Humans are provided with many defences and their failure could lead to DNA damage and consequently to cancer (El-Sayed and Hussin, 2013). Carcinogenesis is generally a slow process and often takes decades from tumor initiation to diagnosis, offering a considerable time frame for chemopreventive approaches. Chemoprevention is defined as the use of specific natural or synthetic agents to prevent, delay, or slow the carcinogenic process (Martin et al., 2013). The prevention of cancer can be achieved by avoiding exposures to mutagens, by fortifying physiological defence mechanisms, or by supplying the intake of protective factors (De Flora, 1998). Human epidemiology and animal studies have indicated that cancer risk may be modified by changes in dietary habits or by dietary supplements (Miyazawa and Hisama, 2004).

More than 15% of malignancies worldwide are established to have an infectious cause (Mager, 2006). Therefore, application of methods to prevent and/or treat infection, such as the use of antimicrobial treatments, could have an effect on future burden of cancer worldwide. Although antibiotics decreased the spread and severity of a wide variety of infectious diseases, bacteria and fungi have developed numerous mechanisms to escape old and new antimicrobial agents due to their uncontrolled use. In this concern, the proper treatment of cancer and microbial infections would have a great impact on the population's health (Assaf et al., 2013).

The identification of dietary components as potential cancer chemopreventive agents in the form of functional foods or as nutraceuticals has become an essential subject of many studies. One of the best approaches in search for anticancer agents from plant resources is the selection of plant species based on ethnomedical leads and testing the selected species efficacy in light of modern science.

In traditional medicine, several *Veronica* species are used to treat cancer (Harput et al., 2002a). As currently circumscribed, *Veronica* (Plantaginaceae) is a genus of 450 species found in temperate regions of both hemispheres. Regardless of widespread use, mostly as diuretics, for their wound-healing properties and in medical treatment of respiratory diseases, there is a scarcity of physiological evidence to support any claim of therapeutic values for *Veronica* species. Only few studies confirm that certain *Veronica* genus reveal considerable bioactivity such as antibacterial (Stojković et al., 2013; Gusev et al., 2012), antioxidant (Živković et al., 2012), anti-inflammatory (Harput et al., 2002a) and cytotoxic (Harput et al., 2002a; Saracoglu and Harput, 2012) activities.

On the other hand, the phytochemistry of the genus has been extensively studied with many species surveyed for their iridoid (Jensen et al., 2005) and phenylethanoid derivatives

(Taskova et al., 2006) and flavonoid glycosides (Albach et al., 2003, 2005; Saracoglu et al., 2004; Taskova et al., 2008) with most of these being considered beneficial for human health. Previously we reported *in vitro* and *in vivo* antioxidant activity of *Veronica urticifolia* Jacq. (Živković et al., 2012). Nevertheless, to the best of our knowledge, there are no reports on other biological activities of the selected species. Phenolic profile of the species *V. urticifoila* is presented for the first time. The aim of this study was profiling the phenolic compounds of *V. urticifolia* and evaluating the antibacterial, antimutagenic and antitumor effects of its methanolic extract.

2. Materials and methods

2.1. Plant samples

The aerial parts of *Veronica urticifolia* were collected during their flowering period in June 2008 from Mountain Goč in central Serbia. Plant material was taxonomically determined and deposited in the Herbarium collection of the Institute of Botany, School of Pharmacy, Belgrade (voucher specimen numbers were VR 165).

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Dimethylsulfoxide (DMSO) (Merck KGaA, Germany) was used as a solvent in antimicrobial assays. Formic acid was purchased from Prolabo (VWR International, France). N-acetyl-L-cysteine and Trypan blue solution were obtained from Sigma-Aldrich (Steinheim, Germany). Nitroquinoline-N-oxide (4NQO) was purchased from Sigma, USA. The phenolic compound standards were from Extrasynthese (Genay, France). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Extraction procedures

The powdered plant sample (~1 g) was extracted by stirring with 30 mL of methanol:water 80:20 (*v/v*), at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman no. 4 paper. The residue was then re-extracted twice with additional portions (30 mL) of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove methanol. The aqueous phase was lyophilized and re-dissolved in 20% aqueous methanol at 5 mg/mL and filtered through a 0.22-μm disposable LC filter disk for high performance liquid chromatography (HPLC-DAD-MS) analysis.

For biological activity evaluations, the air dried plant material was reduced to a fine powder and extracted by maceration with methanol in a solid/solvent ratio of 1:20 at room temperature for 48 h. The obtained extract was evaporated under reduced pressure and further kept in a vacuum desiccator to remove traces of solvents.

2.3. Elucidation of phenolic compounds structures

The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min; the injection volume was 100 μL. Double online detection was carried out in the DAD using 280

nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500 V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to record full scan spectra to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1000. Analysis in EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) detected in the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25 V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the samples were characterized according to their UV and mass spectra and retention times compared with commercial standards when available. For the quantitative analysis of phenolic compounds, a calibration curve was obtained (using the UV detector with λ = 280 nm for phenolic acids and λ = 370 nm for flavonoids) by injection of known concentrations (1-100 μg/mL) of different standards compounds: luteolin-7-*O*-glucoside (y = 80,829$\varkappa$ - 21,291; R^2 = 0.999); apigenin-7-*O*-glucoside (y = 159.62$\varkappa$ + 7.5025; R^2 = 0.999); 5-*O*-Caffeoylquinic acid (y = 313.03$\varkappa$ - 58.2; R^2 = 0.999); caffeic acid (y = 611.9$\varkappa$ -4.5733; R^2 = 0.999); *p*-coumaric acid (y = 884,6$\varkappa$ + 184,49; R^2 = 0.999).

2.4. Antibacterial activity

2.4.1. Microorganisms and culture conditions.

For the bioassays seven bacterial strains were used, four Gram-positive: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (human isolate), *Micrococcus flavus* (ATCC 10240) and *Listeria monocytogenes* (NCTC 7973), and three Gram-negative: *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (human isolate) and *Escherichia coli* (ATCC 35210).

All of the tested microorganisms were obtained from the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, University of Belgrade, Serbia. The bacteria were cultured on Mueller-Hinton agar (MH) and cultures were stored at +4 °C and subcultured once a month.

2.4.2. Microdilution method.

The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the modified microdilution method (Espinel-Ingroff, 2001). Briefly, fresh overnight culture of bacteria was adjusted to a concentration of 1×10^5 CFU/mL by counting the spores in suspension. The inocula were prepared daily and stored at +4 ºC until use. Dilutions of inocula were cultured on solid medium to verify the absence of contamination and check the validity of the inoculum. Different solvent dilutions of methanolic extract were carried out over the wells containing 100 μL of Tryptic Soy Broth (TSB) and afterwards, 10 μL of inoculum was added to all the wells. The microplates were incubated for 24 h1 at 37 ºC. The MIC of the samples was detected following the addition of 40 μL of iodonitrotetrazolium chloride (INT) (0.2 mg/mL) and incubation at 37 °C for 30 min. MBC was determined by serial sub-cultivation of 10 μL into microplates containing 100 μL of TSB. The lowest concentration that shows no growth after this sub-culturing was read as the MBC.

Streptomycin (Sigma P 7794) (0.05-3 mg/mL) was used as positive control for bacterial inhibition. A 5% solution of DMSO in water was used as negative control.

2.5. Antimutagenicity

A variation of the Ames test was used to screen for antimutagenic activity of *Veronica urticifolia* methanolic extract. For this activity, *Salmonella typhimurium* TA100 (Molecular Toxicology, Inc) without S9 metabolic activation were used due to cost implications. It is also known that these two strains are capable of identifying up to 90% of the mutagens (Mortelmans and Zeiger, 2000). Here, 500 µL of 0.1 M phosphate buffer was added to 50 µL of test sample in a test-tube. Fifty microlitres of the nitroquinoline-*N*-oxide (4NQO) in DMSO (20 µg/mL) was added to the mixture and then pre-incubated for 3 min before the addition of 100 µL of overnight TA100 strains of bacterial culture. After incubation for 48 h at 37 °C, the number of viable cells (cultured on MH) and revertant colonies were determined (on minimal glucose agar) and percentage of inhibition (Index of antimutagenicity) calculated using the formula shown below. The extract was tested in triplicate and it was repeated twice. The percentage inhibition was calculated by the following formula: Percent of inhibition (%) = (1 - T/M) × 100, where T is the number of revertants per plate in the presence of the mutagen 4NQO, and M is the number of revertants per plate in the positive control (mutagen).

The mutagenic index (MI) was also calculated for each concentration tested, this being the average number of revertants per plate with the tested extract divided by the average number of revertants with the negative (solvent) control. A tested extract was considered mutagenic when a dose-response relationship was detected and a two-fold increase in the number of mutants (MI≥2) was observed for at least one concentration (Resende et al., 2012).

Cell viability was also determined to assess the potential bactericidal effect of the mutagens. A substance was considered bactericidal when the bacterial survival was less than 60% of that observed in the negative control (Resende et al., 2012).

2.6. Cytotoxic activity against Ehrlich's tumor cells in mice

2.6.1. Animals and experimental procedure.

Dry extract was dissolved under sonication in water to make 5% (*w/v*) solution, and filtered through a 0.45 μm membrane filter. The resulting solutions were kept in the refrigerator (4-8 °C) until applied.

Animal care and all experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animal Resources* edited by the Commission of Life Sciences, National Research Council and according to approval from ethical committee for animals care at University of Novi Sad. Male and female Hannover National Medical Institute (Hann:NMRI) mice were obtained from the Biochemical Laboratory, Clinical Centre Novi Sad (Novi Sad, Serbia). Animals were fed standard mouse chow (LM2, Veterinarski zavod, Subotica, Serbia) with free access to tap water, in a temperature (25 °C) and humidity-controlled (30-50%) animal house under 12 h night/day cycles. NMRI mice of both sexes (6-8 weeks old) weighing 25±2.5 g were used in the experiments. Animals were divided into three groups of six mice under the following conditions and treatments: I, EAC group (mice with implanted EAC cells), (n =6); II, mice pre-treated with the *Veronica urticifolia* extract, 2 mg/kg b.w. per day, i.p., starting 7 days before the EAC implantation (n = 6), III, NALC group, mice pre-treated with the *N*-acetyl-L-cysteine (5 mM), 2 mL/kg b.w. per day, i.p., starting 7 days before the EAC implantation (n = 6). Fourteen days after EAC implantation, all mice were killed, and the ascites of the carcinoma were collected for further experiments.

2.6.2. Determination of tumour cell number and cell viability.

Ascites from the abdomen was transferred to Krebs-Ringer phosphate buffer solution (0 °C, pH 7.4), then subjected to subsequential centrifuging at 4500 rpm (MSE high speed centrifuge at 4 °C) and 12 000 rpm (Eppendorf 3200 centrifuge, 2.5 min) to obtain a dense cell suspension (1:1). The cell weight and cell number expressed as number of cells/mm^3 (counted in a Neubauer's compartment) were determined from the suspension. Cell viability was determined by the Trypan blue exclusion method: Trypan blue (0.4% solution in Krebs-Ringer phosphate buffer) stained only the damaged cells. These data were expressed as percentage of damaged cells.

2.7. Statistical analysis

All data are presented as mean ± standard deviations. One way analysis of variance (ANOVA) was used to detect any significant differences among different means as well as interactions between the variables used in biochemical analyses. Statistically significant effects were further analyzed and means were compared using Bonferroni test. A level of $p<0.05$ was taken as statistically significant.

3. Results and Discussion

3.1. Phenolic compounds characterization

The characterization of the phenolic compounds was performed by HPLC-DAD-ESI/MS analysis, and data of the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification and concentration of phenolic acid derivatives and flavonoids are presented in **Table 1**. The HPLC phenolic profile recorded at 280 nm can be observed in **Figure 1A**.

UV and mass spectra obtained by HPLC-DAD-ESI/MS analysis showed that the phenolic composition of *V. urticifolia* was characterized by the presence of flavonoids and hydroxycinnamoyl derivatives. The analysis of the MS^2 fragments revealed *O*-glycosides from two flavones (luteolin and apigenin) and hydroxycinnamoyl (caffeic acid) derivatives. Sugar substituents consisted of hexoside, deoxyhexosides, disaccharides and glucuronides, as deduced from the losses of 162 Da, 146 Da, 308 Da and 176 Da, respectively.

3.1.1. Hydroxycinnamoyl derivatives.

These compounds correspond to caffeic (peaks 1-4, 6-8 and 10 in **Table 1**) or *p*-coumaric acid (peak 11) derivatives. Peak 1 was identified as 3-*O*-caffeoylquinic acid due to the yielded deprotonated quinic acid (*m/z* at 191) as base peak and another ion at *m/z* 179 [caffeic acid-H]$^-$, with an intensity >50% of the base peak, a fragmentation pattern characteristic of 3-acylchlorogenic acids (Clifford et al., 2005). Peaks 2 and 3 presented the same pseudomolecular ion [M-H]$^-$ at *m*/*z* 487 and similar fragmentation pattern with the loss of 308 mu (hexosyl + deoxyhexosyl moieties) yielding a base peak at *m*/*z* 179 mu ([caffeic acid-H]$^-$) and other two fragments at *m/z* 161 mu ([caffeic acid-H-H_2O]$^-$) and 135 mu ([caffeic acid-H-CO_2]$^-$). The fact that no alternative losses of hexosyl (-162 mu) deoxyhexosyl (-146 mu) residues were observed suggested that the two sugars were constituting a disaccharide (*e.g.*, rutinose or neohesperidose), although the actual nature of the sugar residues could not be established from the MS analysis. Thus, the compounds were tentatively assigned as as caffeoyl deoxyhexosyl-hexosides I and II.

Peak 4 corresponds to caffeic acid as identified by comparison of its UV and mass characteristics and retention time with those of the commercial standard. Peaks 6 and 8, with pseudomolecular ions [M-H]$^-$ at *m*/*z* 477 and *m*/*z* 453, respectively, were attributed to caffeic

acid derivatives, according to the observation of the characteristic peaks at *m*/*z* 179 mu, 161 mu and 135 mu, as mentioned above.

The UV spectrum of compound 7 and 10 showed the characteristic of phenylethanoid glycosides with maximum absorption peaks at 234 and 330 nm (**Figure 1B**). The HPLC-DAD/ESI-MS of compound 7 in the negative mode gave *m*/*z* 623 as the deprotonated molecular ion $[M-H]^-$, which could be assigned to acteoside (also known as verbascoside), identity that was also supported by its UV spectrum and MS^2 fragmentation pattern as reported by Li et al. (2005). Thus, MS^2 of the *m*/*z* 623 ion ($[M-H]^-$) produced a main fragment ion at *m*/*z* 461 considered to be from the loss of the caffeoyl moiety ($[M-H-161]^-$); the weak ion at *m*/*z* 315 was consistent with the further loss of a rhamnose unit, and ions at *m*/*z* 161 mu and 135 mu revealed the presence of the caffeoyl residue. Similar characteristics were observed for peak 10, suggesting that both compounds were isomers; they were tentatively assigned as acteoside and isoacteoside, respectively, based on their relative elution order according to Li et al. (2005). Peak 7 corresponds to the majority (14.9 mg/g extract) phenolic compound in *V. urticifolia* extract. In fact, acteoside is a phenylethanoid glycoside, a group of phenolic compounds that is widely distributed in plants and was previously reported in *Veronica* species (Harput et al., 2002b; Johansen et al., 2007; Crişan et al., 2007), being inclusively considered as an important chemotaxonomic marker. The detected amounts of acteoside highlight *V. urticifolia* as a potential natural source of this bioactive compound, already reported as having antimicrobial (Avila et al., 1999), antigenotoxic (Zaabat et al., 2011) and antitumor (Ohno et al., 2002) properties.

Finally, peak 11 had a molecular ion $[M-H]^-$ at *m*/*z* 437 that produced an MS^2 base peak at *m/z* 163 ([coumaric acid-H]$^-$), which linked to its UV spectrum (λ_{max} at 312 nm) allowed to assign it to an unidentified coumaric acid derivative.

3.1.2. Flavones.

Flavones were the only detected flavonoids in *V. urticifolia* extracts (**Table 1**). Peaks 5 and 12 were identified as apigenin derivatives, while peaks 9 and 13 were identified as luteolin derivatives, according to their UV and mass spectra characteristics. Peaks 5 and 12 presented pseudomolecular ions $[M-H]^-$ at *m/z* 621 and 445 both losing an MS^2 fragment ion at *m/z* 269 ($[M-176-176]^-$ and $[M-176]^-$, corresponding to the loss of two and one glucuronyl moieties, respectively), being tentatively identified as apigenin-*O*-diglucuronide (peak 5) and apigenin-*O*-glucuronide (peak 12) . The observation of the ion at *m/z* 445 in peak 5 suggested that each glucuronide residue was located on different positions of the apigenin aglycone.

Peak 9 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 461 releasing an MS^2 fragment ion at *m/z* 285 ($[M-H-176]^-$, corresponding to the loss of one glucuronyl moiety) so that it could be identified as a luteolin-*O*-glucuronide. Peak 13 showed the same pseudomolecular ion $[M-H]^-$ at *m/z* 461 but it released a main fragment ion at *m/z* 299 ($[M-H-162]^-$, loss of a hexose) together with another ion at *m/z* 285 that might be due to the further loss of a methyl residue (-14 mu). These characteristics suggested that it could be a methyl derivative of luteolin, *e.g.*, as chrysoeriol (3'-methyl-luteolin) or diosmetin (4'-methyl-luteolin), although its actual identity could not be established. Thus, the peak was tentatively assigned as a methyl-luteolin hexoside. Its elution order after peak 9 is also coherent with the loss of the polarity induced by the additional methyl group.

3.2. Antibacterial activity

The results of antibacterial activity of *V. urticifolia* methanolic extract and standard antibiotic streptomycin, tested by microdilution assay, are presented in **Table 2**. *Staphylococcus aureus* was the most sensitive species with equal values of MIC and MBC of 7.5 mg/mL. The antibacterial activity of acteoside, the main phenolic compound, has already been

demonstrated in various studies. According to Avila et al. (1999) acteoside induced lethal effect on *S. aureus* by affecting protein synthesis and inhibiting leucine incorporation. As a result of these findings, activity of *V. urticifolia* methanolic extract against *S. aureus* could be attributed at least in part to acteoside. Nevertheless, other constituents of the extract should be taken into consideration for the global activity, *e.g.*, the high amounts of apigenin and especially luteolin glycosides.

An overwhelming body of evidence has determined the association among certain bacteria and cancer (Mager, 2006). Bacteria have been linked to cancer by two mechanisms: induction of chronic inflammation and production of carcinogenesis. The staphylococcal α-toxin has been shown to activate both several cytokines which have been linked to all steps in tumor development and nuclear factor κB, known tumor promoter in the development of hepatocellular carcinoma (Kullander et al., 2009).

Micrococcus flavus, *Listeria monocytogenes* and *Enterococcus faecalis* were similarly susceptible to the inhibitory (MIC = 7.5 mg/mL) and bactericidal (MBC = 15 mg/mL) effects of the extract. The most resistant species were *Bacillus cereus*, *Pseudomonas aeruginosa* and *Escherichia coli* with MIC = MBC = 15 mg/mL. As shown in **Table 2**, streptomycin has stronger activity than tested extract. The obtained results for antibacterial activity are comparable to those published previously for *V. montana* water extract (Stojkovic et al., 2013).

3.3. Antimutagenicity testing

As mutations are known to be the most important cause of the initiation of many diseases, phytochemicals exhibiting antimutagenic potential could be of immense significance (Kumar et al., 2013). It has been suggested that the use of antimutagen in daily life will be the most effective procedure for preventing human cancer and genetic disease (Bhagavathy et al.,

2011). Antimutagenic compounds (*e.g.* carotenoids) interfere with mutagen metabolism, they may act as mutagen scavengers, or they may also inhibit either the initiation or promotion phase of the carcinogenic process (Bhagavathy et al., 2011). Although an antimutagenic effect found in a plant extract does not necessarily mean that it is an anticarcinogen, it is however an indication of the possibility of acting as one (Abdillahi et al., 2012). The Ames *Salmonella* test is widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. The test uses *Salmonella* strains with pre-existing mutations that leave the bacteria unable to synthesize the required amino acid, histidine, and therefore unable to grow and form colonies in its absence. New mutations at the site of these pre-existing mutations, or nearby in the genes, can restore the gene's function and allow the cells to synthesize histidine. These newly mutated cells can grow in the absence of histidine and form colonies (Mortelmans and Zeiger, 2000).

Lesions to DNA resulting from attack by reactive oxygen species (ROS) can be a major cause of mutagenesis and carcinogenesis (Mimica-Dukić et al., 2010). Therefore, antioxidants may act as universal antimutagenic agents (Ananthi et al., 2010). In a previous research, we demonstrated the protective role of methanolic extract of *V. urticifolia* in oxidative stress helping to keep normal levels of enzymatic and non-enzymatic antioxidants and lipid peroxidation (Živković et al., 2012). Based on this, we extent our evaluation using the Ames *Salmonella* test.

In this study, the mutagenicity of *V. urticifolia* methanolic extract against *S. typhimurium* TA 100 was measured by comparing the ratio of induced revertants and spontaneous revertants in plates. According to **Table 3**, the mutagenicity index showed that tested extract in the range 0.2-1 mg/plate, did not induce increase in the number of revertant colonies relative to the negative control, indicating the absence of any mutagenic activity.

As reported in **Table 3**, tested extract displayed protective effects against 4-NQO induced mutagenicity. In determining the antimutagenic potential of a sample, a value smaller than 25% inhibition of the mutagen activity indicates a weak or non-antimutagenic effect, a moderate effect when the value is between 25 and 40% and strong antimutagenicity when the value is greater than 40% (Negi et al., 2003). In the present study induced inhibition was dose dependent: 0.2 mg/plate and 0.5 mg/plate induced moderate antimutagenic activity reducing histidine-prototrophic revertant colonies by 25.8% and 36.2%, respectively. At 1 mg/plate the extract showed strong inhibitory effect against direct-acting mutagen 4NQO (inhibition rate was 48.3%). Since the extract plus bacterial strain treated plate did not produce any change in the background growth of bacteria with respect to the plate used to detect the spontaneous revertant, the antimutagenic activity of extract cannot be correlated to its toxicity to bacterial strains.

The exhibited antimutagenic activity of the extract might be ascribed to its constituents, in particular to the major phenolic compounds (acteoside and luteolin-*O*-glucuronide) polyphenols. In fact, some phenylpropanoids act as potent antimutagenic and anticarcinogenic agents (Miyazawa and Hisama, 2003). According to Zaabat et al. (2011) acteoside showed pronounced antigenotoxic effect. Therefore, taking into consideration the concentration of acteoside in the extract, it can be suggested that acteoside could be an essential compound for the antimutagenic activity of *V. urticifolia*.

Keeping in prospect our previous publication regarding the antioxidant activity of tested extract, the exhibited antimutagenic activity might also be partially ascribed to its antioxidant activity.

3.4. Cytotoxic activity against Ehrlich's tumor cells in mice

Plants belonging to the genus *Veronica* and several of their constituents have shown potent cytotoxic activity on different cell lines (Harput et al., 2002a; Teng et al., 2008; Saracoglu and Harput, 2012). In the present study the *V. urticifolia* methanolic extract was evaluated for its *in vivo* antitumor properties.

The Ehrlich tumor was initially described as a spontaneous murine mammary adenocarcinoma. It is a rapidly growing carcinoma with very aggressive behaviour. After intraperitoneal inoculation of Ehrlich tumor cells, the ascitic volume and cells number increase drastically. This has been associated to an increase in peritoneal vascular permeability (De Matos Gomes et al., 2008). In clinical situations, formation of ascites is often observed in patients with advanced cancer.

The involvement of free radicals in tumors is well documented. Previous investigations showed that EAC-bearing mice are under higher oxidative stress than control animals indicated by elevated lipid and protein oxidation and reduced endogenous antioxidants in the liver (Al Abdan, 2012). If oxidative stress is involved in the origin of EAC-induced oxidative injury, then a successful antioxidant treatment should protect against that injury. To test the hypothesis that tumor cell growth is based on the change in antioxidant status in EAC cells *N*-acetyl-L-cysteine, a thiol antioxidant precursor of glutathione (NALC) is used as a positive control in investigation.

Antitumor properties of *V. urticifolia* methanolic extract were assessed by parameters such as ascites volume, tumor cell count and cell viability; the results are shown in **Table 4.** A single dose of tested extract was chosen since the preliminary results using different doses (0.2; 0.5; 1.0; 1.5 and 2.0 mL/kg b.w.) indicated that 2.0 mg/kg b.w. was the most effective dose. For the purpose of preliminary elucidation i.p. administration of the extract prior to oral application was chosen, in order to avoid possible chemical changes upon oral consumption of the extract.

The Ehrlich ascitic tumor implantation induces *per se* a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration and progressive ascitic fluid formation (Agrawal et al., 2011). The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells (De Matos Gomes et al., 2008). Our data showed that administration of NALC significantly reduced the ascitic fluid volume. On the other hand, after application of *V. urticifolia* methanolic extract the ascites volume became slightly decreased, but not to a statistically significant extent.

Ascites fluid accumulation occurs in parallel with the proliferation of tumor cells (Ozaslan et al., 2011). Cytotoxic activity can be determined as a decreased-cell number because of cell death, while cell-growth inhibitory or cytostatic activity can be determined as a suppression of the increase of cell number without causing cell death. Administration of NALC or investigated extract during the course of this experiment led to slight, but insignificant reduction of number of tumor cells in peritoneal cavity as compared to control group, pointing to their oncostatic activity.

Prolongation of survival is directly related to decrease in viable cell count (Agrawal et al., 2011). In the present study a significantly decreased EAC cell viability after administration of the tested extract in comparison to the control group was observed. Administration of the NALC showed no effect on tumor cell viability.

In conclusion, the observed antibacterial, antimutagenic and oncostatic effects of *V. urticifolia* methanol extract indicates that this species may play a role as a cancer-preventing agent. The effect can be partially ascribed to the phenolic compounds identified in the extract. Nevertheless, further studies are needed to explain the role of phytochemicals in the bioactivities of *V. urticifolia*.

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FIGURE CAPTION

Figure 1. HPLC chromatogram (the period from 5.5 to 6.1 minutes was zoomed to elucidate peaks 1, 2 and 3) of the phenolic compounds of *Veronica urticifolia* recorded at 280 nm (**A**). The UV spectrum of the dominant compound (acteoside) is also shown (**B**).