NETTLE (*Urtica dioica* L.) SEEDS AS A SOURCE OF FREE AND BOUND PHENOLICS: THE ANTIOXIDANT, ANTIMICROBIAL ACTIVITY AND THE COMPOSITION

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In this study, the composition and the antioxidant and antimicrobial activity of free and bound phenolics from *Urtica dioica* L. seeds were studied. The composition of phenolics was determined by isocratic and gradient HPLC methods, and the antioxidant activity by determining DPPH scavenging capacity, reducing the power and FRAP value. The antimicrobial activity was tested by using the disc diffusion method. The content of free phenolics was higher (6000.71 µg gallic acid equivalent per g dry seeds mass) than the content of bound phenolics (2801.16 µg g⁻¹). The extract of free phenolics had a higher FRAP value than the bound, while the bound phenolics had a better DPPH scavenging capacity and the reducing power. Eighteen phenolics have been detected in the free phenolic extract, among which kaempferol 3-O-rutinoside-7-O-rhamnoside was the most abundant (428.55 µg g⁻¹), while ten phenolics have been detected in the bound phenolic extract, among which monocaffeoyl-meso-tartaric acid (406.83 µg g⁻¹) was the most abundant. Both of the extracts have shown a good inhibitory effect on *Bacillus cereus* ATCC 11778 and *Klebsiella pneumoniae* ATCC 700603. The study showed that nettle seeds are a good source of natural antioxidants and antimicrobials for potential use in the food and pharmaceutical industry.

Keywords: phenolics, nettle seeds, antioxidant activity, antimicrobial activity

Introduction

Urtica dioica L., also known as stinging nettle, is a perennial flowering herb that belongs to the *Urticaceae* family. It is known as a cosmopolitan plant, widespread throughout moderate and tropical areas [1]. This plant is a good source of secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids, but it is also rich in minerals, especially iron, vitamin C and pro-vitamin A [2, 3]. Since ancient times, nettle has been used in traditional medicine due to its therapeutic potential and a wide spectrum of activities such as antioxidant, antimicrobial, antiulcer, analgesic, diuretic, antidiabetic, anti-inflammatory and antirheumatic [4].

The antioxidant activity is mainly expressed as the ability to scavenge free radicals [5] which are continuously formed in biological systems as a result of normal metabolism or a disease. Free radicals are unstable molecules or highly reactive ions that start chemical reactions with almost all classes of biomolecules, and tend to damage a number of cellular systems changing their functions. In the literature, there are many studies regarding the evaluation of the antioxidant activity of various parts of plant extracts, mainly leaves [6]. The investigations of Singh et al. [7] showed that the antioxidant activity of seeds is generally increased during the germination, and this was confirmed by Lin and Lai [8] for mature and germinated seeds of selected legume

varieties including soybeans, azuki and mung beans. The increase in the antioxidant activity, measured as the inhibition of peroxidation was observed by Hudec et al. [9] after the root, stem, and leaf of the nettle plant treatment by O-phosphoethanolamine and carboxymethyl chitin glucan.

Phenolics are believed to be the effective plant antioxidants for maintaining the cellular redox status, and they are one of the most numerous and structurally diverse plant phytochemicals which are synthesized during the normal development of a plant or as a response to stress (infection, temperature, UV-irradiation) [10]. They can be classified in diversified categories such as flavonoids, phenolic acids, phenolic polymers (hydrolyzable and condensed tannins), stilbenes and lignans [11]. In radical-scavenging reactions, the phenolics take part as donors of electrons and form stable radicals [12] protecting biomolecules (such as proteins, nucleic acids, polyunsaturated lipids, and sugars) from oxidation. Many earlier studies have been dealing with the antioxidants of free phenolics. However, in order to consider the total antioxidant activity, it is important to study the bound phenolics too.

In our available literature, there are many studies concerning the content, antioxidative and antimicrobial activities of phenolics from various parts of *Urtica dioica* L.,

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especially leaves, but the appropriate data about these compounds, especially bound phenolics from seeds are missing. Therefore, the aim of the present research is to estimate the content and composition of both free and bound phenolics, their antioxidative and antimicrobial activities, as well as to assess nettle seeds as a source of antioxidants and antimicrobials for the potential usage in food and pharmaceutical industry.

Materials and methods

Plant material

A sample of *Urtica dioica* L. seeds was purchased from "Jeligor", Svrlijig in Serbia. The nettle seeds were ground in an electric grinder (Bosh, MKM 600, Germany) and sieved through a 0.40 mm riddle.

Extraction of free phenolics

A free phenolics extract was prepared by 30 g of the sample and 150 mL of 70% methanol. The extraction was carried out at room temperature for 24 h, with occasional stirring.

The extract was separated by filtration and the seeds remnant left from the filtration was used as a material for a re-extraction process. Methanol (20 mL) was added to the remnant and the extraction was carried out at room temperature for 30 minutes. The re-extract was separated by filtration, the filtrates were combined and a final volume of 150 mL was made by methanol in order to obtain the extract of free phenolics for further analysis. To obtain different concentrations of the extract, the appropriate dilutions ranging from 1:1 to 1:10 (v/v) were made. In order to carry out HPLC analysis of the free phenolics composition, the extract was filtered through a 0.45 µm Millipore filter (Millipore Corporation, Billerica, MA, USA). The extraction procedure was performed in triplicate, and the seeds from which free phenolics had been extracted were preserved and used for the extraction of bound phenolics.

Extraction of bound phenolics

The bound phenolics were liberated by alkaline hydrolysis, as described by Verma et al. [13]: 30 g of the seeds from which free phenolics had been removed, 900 mL of distilled water and 150 mL of 6 M NaOH were mixed and left at room temperature for 24 h. The pH of mixture was adjusted to 2 with 5 M HCl, and 800 mL of diethyl ether and the ethyl acetate mixture in the ratio of 1:1 (v/v) was added. The mixture was shaken for every 30 minutes during 3 h and left overnight. The upper layer was then removed and evaporated by rotary evaporation (IKA®-WERKE, RV06-ML, Germany) until it was dry. The dry residue was dissolved in 200 mL of 70% methanol. To obtain different concentrations of the extract, dilutions in the same range as in the case of the free phenolics extract were made. Before HPLC analysis, the extract was filtered through a 0.45 µm filter as in the case of free phenolics.

Determination of the total phenolics content

The total phenolic content of the extracts was assayed as described by Singleton and Rossi [14] by using the Folin-Ciocalteu colorimetric method with final reaction measurements at 765 nm. The extract of free or bound phenolics (0.5 mL) was mixed with 0.5 mL of Folin-Ciocalteu reagent and 4.5 mL of deionized water, and 7.5% aqueous Na₂CO₃ (5 mL, w/v) was added after 5 min. After 90 minutes of incubation, the absorbance was measured by using UV 2100 Spectrophotometer (Cole Parmer Instrument Company, Vernon Hills, Illinois, USA). The blank was prepared by using the same procedure, whereby the extract was replaced with an equal volume of deionized water. The phenolics content was calculated by using the equation ($C = 175.44 \times A_b - 5.30$, $R^2 = 0.9959$, µg mL⁻¹) of the calibration curve obtained with gallic acid in the concentration range 0 to 300 µg mL⁻¹. The phenolics content was calculated to µg of gallic acid equivalent (GAE) per g of the dry plant material.

DPPH radical scavenging capacity (DPPH SC)

The SC of the extracts of free and bound phenolics was determined by the method described by Mensor et al. [15]. The absorbance at 517 nm was measured and converted into the percentage of radical SC (%):

$$\text{DPPH SC (\%)} = 100 - \left[\frac{(A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \right] \times 100 \quad (1)$$

where A_{sample} is the absorbance at 517 nm of the extract in methanol (2.5 mL) treated by the DPPH radical solution, A_{blank} is the absorbance at 517 nm of the extract in methanol (1 mL of methanol added to 2.5 mL of the extract), and A_{control} is the absorbance at 517 nm of methanol of the DPPH radical (1 mL of a 0.3 mM added to 2.5 mL of methanol). The IC₅₀ value was determined by using Microsoft Excel ed50plus v1.0 software (INER/México) and expressed as mg of the dried residue per mL. Butylated hydroxyanisole (BHA) was used as a standard.

Reducing power

The reducing power of the extracts was determined by measuring the absorbance at 700 nm as described by Oyaizu [16]. Various concentrations of the dry residue of the free and bound phenolics extract (0.5 to 8.5 mg mL⁻¹) were mixed with 2.5 mL of the 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. Then, 2.5 mL of trichloroacetic acid (10%) was added and the mixture was centrifuged at 3000 rpm for 10 min. Supernatant (2.5 mL) was mixed with redistilled water (2.5 mL) and 0.5 mL of the ferric chloride solution (0.1%), while the absorbance was measured at 700 nm. Using Microsoft Excel ed50plus v1.0 software, the IC₅₀ value was determined (INER/México) and expressed as mg of the dried residue per mL, while BHA was used as a standard.

Ferric reducing antioxidant power (FRAP)

The FRAP of methanol extracts was measured according to the method of Benzie and Strain [17] with some modifications: the extracts (0.2 mL) were mixed with 6 mL of the FRAP reagent in test tubes and subjected to Vortex-induced vibrations. After 30 min of incubation at 37 °C in the water bath, the absorbance was measured at 593 nm. A blank sample was prepared with methanol (0.2 mL) and the FRAP reagent (6 mL).

To determine a FRAP value, the amount of the standard solution (0.2 mL) of $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ in the concentration range 0-350 $\mu\text{g mL}^{-1}$ was mixed with 6 mL FRAP reagent. The calibration curve was designed based on absorbance values and the investigated concentrations of $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ solutions ($C_{\text{FeSO}_4 \times 7\text{H}_2\text{O}} = 1.6\text{Ab} + 14.6 \times 10^{-3}$, $\text{mmol Fe}^{2+} \text{ L}^{-1}$). The FRAP value is expressed as $\mu\text{mol Fe}^{2+} \text{ g}^{-1}$ of the dry plant material. Analogous tests were carried out with BHA as a standard.

HPLC analysis

The isocratic HPLC method [18] was used to determine the phenolic acids composition. The content of phenolic acid was calculated based on the calibration curve obtained for each phenolic acid separately [19]. In order to test the extracts to hydroxycinnamic acid, caffeoylshikimic and monocaffeoyl-*meso*-tartaric acid and kaempferol and quercetin derivatives, the HPLC method by the gradient elution program [20] was applied. Due to poor reproducibility, the relative retention indices were calculated as the ratio between the retention time of the compound and naringenin as the internal standard. The peak identity was checked by comparison of their relative retention indices with the previous ones and with reference compounds (hydroxycinnamic acid, 5-*O*-caffeoylshikimic acid, and quercetin 3-*O*-rhamnoside purchased from Sigma Chemical, St. Louis, Missouri, USA). The content of these compounds was calculated based on the HPLC peak area and the corresponding calibration curve, and the mass of other compounds was quantified based on the calibration curve of the most similar compound by retention time.

Antimicrobial activity determination

The antimicrobial activity was analysed using nine bacterial strains: *Proteus vulgaris* ATCC 8427, *Bacillus cereus* ATCC 11778, *Bacillus luteus*, *Bacillus subtilis*, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 15313, *Klebsiella pneumoniae* ATCC 700603 and one fungal strain *Candida albicans* ATCC 10259. Microorganisms were cultivated on Nutrient agar (Torlak, Belgrade, Serbia) for bacterial, and Sabouraud maltose agar (Torlak, Belgrade, Serbia) for the fungi growth. The mediums were sterilized for 15 minutes in the autoclave at 121 °C and under the pressure of 101 kPa, and the antimicrobial activity was determined by using the agar discdiffusion method [21]. In order to achieve the cell number of 1×10^8 CFU

mL^{-1} , the inoculums of the strains were prepared by suspending the culture in the sterile 0.8% NaCl solution and adjusting the turbidity value to 0.5 McFarland standard. Afterward, the suspension was swabbed on the surface of solidified agar plates. Paper disks (diameter 6 mm) were impregnated with 30 μL of the extracts and placed on the inoculated agar surface. The incubation was performed at 37 °C for 24 h for bacterial, and at 28 °C for 48 h for fungi strains. The antimicrobial activity was determined by measuring the diameter of the inhibition zone.

Statistical analysis

The results were presented as the mean of three determinations $\pm$ standard deviation. In order to compare the differences in mean values of the content of free and bound phenolics in the obtained extracts, a statistical analysis by t-test at the 5% level was done.

Results and discussion

Phenolics content

The content of free and bound phenolics in the nettle seeds is shown in Table 1.

Table 1. The content of phenolics, IC_{50} value for the DPPH and the reducing power and FRAP value for the extracts of free and bound phenolics from nettle seeds

	Extract of free phenolics	Extract of bound phenolics
Extract dry matter [*]	13.46 $\pm$ 0.98 ^a	8.45 $\pm$ 1.01 ^b
Phenolics content ^{**}	6000.71 $\pm$ 28.81 ^a	2801.16 $\pm$ 49.31 ^b
Total^{***}	8801.87$\pm$57.11	
IC_{50} , DPPH SC ^{***}	0.73 $\pm$ 0.14 ^a	0.38 $\pm$ 0.09 ^b
1/ IC_{50}	1.37	2.63
Phenolics content in dry matter of IC_{50} value of DPPH SC ^{***}	65.09 $\pm$ 12.48 ^a	18.89 $\pm$ 8.95 ^b
IC_{50} , Reducing power ^{***}	5.68 $\pm$ 0.55 ^a	1.43 $\pm$ 0.2 ^b
1/ IC_{50} , Reducing power	0.18	0.70
Phenolics content in dry matter of IC_{50} value of reducing power ^{***}	506.44 $\pm$ 49.04 ^a	71.10 $\pm$ 19.89 ^b
FRAP value ^{****}	37.85 $\pm$ 1.75 ^a	27.16 $\pm$ 2.32 ^b
BHA equivalent for FRAP value^{***}	157.35 $\pm$ 35.96 ^a	563.14 $\pm$ 47.79 ^b

Within rows, the values assigned to different letters (a, b) are significantly different at $P < 0.05$

^{*}mg dried residue per mL

^{**} μg gallic acid equivalent per g of the dry plant material

^{***} μg of dry residue per mL

^{****} $\mu\text{mol Fe}^{2+}$ per g of the dry plant material

The results show that the content of phenolics in the extract of free phenolics (6000.71 $\mu\text{g g}^{-1}$) was significantly ($p < 0.05$) higher than in the extract of bound phenolics (2801.16 $\mu\text{g g}^{-1}$).

Most of the previously published results consider the content of phenolics extracted from other parts of nettle plants, especially leaves, and point out the dependence of the phenolics content on the used solvent, the extraction technique and the geographical origin of the plant [22-27]. Our results are similar to the results of the total phenolics content in roots, stalks, and leaves of nettle of 7.82, 9.91, 7.62 mg GAE per g dry mass, respectively, reported by Hudec et al. [9]. In some papers, the content of the phenolics is expressed in μg per dry residue of the extract, so it is not possible to properly compare the

results with the results obtained in this research. Thus, the phenolics content of 208.37 mg GAE per g dry matter extract was found in the nettle leaves extracts obtained by using 80% ethanol (v/v) and ultrasonic extraction at 30 Hz [27]. The extracts obtained by Soxhlet extraction showed that the phenolics content was in the range 1.27-24.22 mg GAE per g dry mass of the solvent fraction, depending on the solvent (hexane, benzene, chloroform, diethyl ether, ethyl acetate, acetone, ethanol, methanol, and water) and solvent ratios [25].

Antioxidant activity

In Table 1, IC_{50} values of DPPH SC and the reducing power, as well as FRAP values are shown. Since the lower IC_{50} values show higher DPPH RSC and the reducing power, the reciprocal IC_{50} values are calculated to obtain the proper dependence.

The extract of bound phenolics showed IC_{50} of 0.38 mg mL⁻¹ and the extract of free phenolics, 0.73 mg mL⁻¹ for DPPH SC, indicating that the bound phenolics have better DPPH SC, and the difference is statistically significant ($p < 0.05$). In literature, there are data of IC_{50} values for free phenolics extracted by 50% aqueous-methanolic extracts from nettle leaves in the range of 0.105-132 mg mL⁻¹ [28] and for free phenolics extracted by using the 5% aqueous-ethanolic extract IC_{50} was 1.2 mg mL⁻¹ [29]. Similar results were achieved for the extract obtained by using 54% aqueous-methanol by maceration and ultrasound-assisted extraction [30] when IC_{50} was 0.293-0.337 mg mL⁻¹ and 0.226-0.240 mg mL⁻¹, respectively. A higher IC_{50} value of 88.33 µg mL⁻¹ for DPPH SC was reported by Khare et al. [31] for phenolics of the aerial part of the nettle extracted by two steps of the Soxhlet extraction first by 98% petroleum and then by 50% hydro-alcohol, as well as by Zeković et al. [26] when IC_{50} values of 29.77, 21.47 and 16.93 µg mL⁻¹ were obtained for the extracts from nettle leaves by using ultrasound-assisted, microwave-assisted and subcritical water extraction, respectively. The higher antioxidant activity probably exists due to a different composition of the extracted phenolics. They are extracted by using different solvents, techniques and plant parts.

The presented results in Table 1 show that besides DPPH SC, the extract of bound phenolics has a statistically higher reducing power (IC_{50} was 1.43 mg mL⁻¹) compared to the extract of free phenolics (5.68 mg mL⁻¹, respectively) ($p < 0.05$). In available literature, there are data concerning the reducing power of methanol extracts obtained by Soxhlet extraction of phenolics from nettle leaves [32], when IC_{50} was 125 mg mL⁻¹, and the water extract from aerial parts of the nettle achieved by Gülçin et al. [5] when the IC_{50} value was 110 mg mL⁻¹. Compared to our results, it is evident that phenolics from the seeds have a stronger reducing power than phenolics from the aerial parts of nettle. On the other hand, the extracts of nettle leaves obtained by novel techniques of the extraction such as ultrasound, microwave, and a subcritical water extraction showed a stronger reducing power: IC_{50} values were 43.93, 35.60

and 30.07 µg mL⁻¹, respectively [26].

The results of FRAP values show that the extract of free phenolics had a higher value (37.85 µmol Fe²⁺ per g of dry plant material, i.e. 7.57 mM Fe²⁺/L) than the extract of bound phenolics (27.16 µmol g⁻¹, i.e. 3.87 mM Fe²⁺/L). The BHA equivalent for free and bound phenolics was 157.35 and 563.14 µg mL⁻¹, respectively and the difference is statistically significant ($p < 0.05$). The results are in accordance with Cvitanović et al. [33] investigations who reported the FRAP values in the range of 2.38-13.47 mM Fe²⁺/L for the extracts from nettle leaves obtained by infusion, maceration, and decoction.

Differences in the antioxidant activity between the extracts of free and bound phenolics could be explained by the content and the composition of phenolics (different in the chemical structure and the content of individual compounds) which show a different antioxidant activity depending on the applied method for investigation [34].

In order to compare the DPPH SC and reducing the power of the investigated extracts to BHA, the content of phenolics contained in the dry residues of IC_{50} values were calculated and also presented in Table 1. For the DPPH SC test, standard BHA had the IC_{50} value of 2.27 µg mL⁻¹ and for the reducing power test it was 16.34 µg mL⁻¹. These results indicate that the extract of free phenolics has 28.7 times weaker DPPH SC and 30.4 times weaker reducing power. The extract of bound phenolics has 8.3 times weaker DPPH SC and the 2.3 times weaker reducing power than BHA.

Comparing the extract of free phenolics to the extract of a bond, the bound phenolics had better DPPH SC and the reducing power. The reason for that may be a higher content of caffeic acid in the extract of bound than in the extract of free phenolics, as well as tannic acid, moncaffeoyl-*meso*-tartaric acid, NID 1 and NID2 that are present only in the extract of bound phenolics (Table 2). The investigation of Chen and Ho [35] supported this fact by showing that the caffeic acid had a better DPPH SC than chlorogenic and ferulic acids. The investigation of Masek et al. [36] also shows that in comparison to *p*-coumaric acid, the caffeic acid exhibited better antioxidant properties investigated by DPPH, FRAP and CUPRAC methods.

Composition of phenolics

The results of the composition of free and bound phenolic acids, rutin and myricetin in the extracts, determined by HPLC isocratic elution, are presented in Table 2. In the extract of free phenolics, nine phenolics had been detected, while in the extract of bound phenolics, seven. Among these phenolics, the chlorogenic acid was the most abundant in the extract of free phenolics (323.72 µg g⁻¹), and the caffeic acid in the extract of bound phenolics (165.49 µg g⁻¹). In the extract of free phenolics, the content of gallic, chlorogenic and trans-ferulic acid and rutin was 17.5, 15.3, 4.9 and 49.4 times higher, respectively, compared to the extract of bound phenolics. On the other hand, in the extract of bound

phenolics, the content of caffeic and coumaric acid was 7.3 and 6.9 times higher, respectively, than in the extract of free phenolics. The obtained results by Pinelli et al. [37] showed that the leaves of cultivated and wild nettle contain large amounts of chlorogenic and 2-O-caffeoyl-malic acid, which represented over 70% of total phenolics, respectively. By using LC/MS and HPLC analysis, Vajić et al. [30] detected the chlorogenic acid, rutin, and 2-O-caffeoyl malic acid as the most abundant phenolics in the extracts obtained by maceration and ultrasound-assisted extraction of phenolics from stinging nettle leaves. Furthermore, the investigations of Otles and Yalcin [22] showed that different plant parts of nettle (root, stalk, and leaves) collected from different regions of Turkey have a different composition and content of phenolics, where the content of syringic acid was the most abundant in fresh leaves of one sample of nettle from Aegean region. Based on the presented data, it might be concluded that the nettle has different quantitative and qualitative profiles of phenolics depending on the part of a plant, growing region, extraction techniques, solvents used for the extraction etc. Therefore, it is necessary to examine each part of the plant separately.

Table 2. The content of phenolic compounds in the extracts of free and bound phenolics from nettle seeds identified by an isocratic HPLC method

Content of compound ($\mu\text{g g}^{-1}$)	Extract of free phenolics	Extract of bound phenolics	Total free and bound
Galllic acid	149.27 $\pm$ 13.43 ^a	8.51 $\pm$ 1.18 ^b	157.78 $\pm$ 13.48
Chlorogenic acid	323.72 $\pm$ 27.50 ^a	21.19 $\pm$ 5.69 ^b	344.91 $\pm$ 28.08
Caffeic acid	22.51 $\pm$ 2.03 ^a	165.49 $\pm$ 12.4 ^b	188.00 $\pm$ 12.57
Tannic acid	-	52.47 $\pm$ 3.67	52.47 $\pm$ 3.67
Syringic acid	11.46 $\pm$ 0.92	-	11.46 $\pm$ 0.92
Ellagic acid	94.75 $\pm$ 8.05	-	94.75 $\pm$ 8.05
Coumaric acid	1.66 $\pm$ 0.15 ^a	11.47 $\pm$ 0.80 ^b	13.13 $\pm$ 0.15
Trans-ferulic acid	49.74 $\pm$ 3.48 ^a	10.22 $\pm$ 0.92 ^b	59.96 $\pm$ 3.59
Rutin	216.04 $\pm$ 17.28 ^a	4.37 $\pm$ 0.39 ^b	220.41 $\pm$ 17.28
Myricetin	34.47 $\pm$ 2.75	-	34.47 $\pm$ 2.75
Total	903.62$\pm$36.39	273.72$\pm$14.24	1177.34$\pm$39.07

Within rows, the values assigned to different letters (a,b) are significantly different at $P < 0.05$
^a μg phenolic compound per g of the dry plant material

Table 3. The content of phenolic compounds in the extracts of free and bound phenolics from nettle seeds identified by a gradient HPLC method

Phenolic compound content ($\mu\text{g g}^{-1}$)	Extract of free phenolics	Extract of bound phenolics	Total free and bound
Hydroxycinnamic acid	48.13 $\pm$ 4.09	-	48.13 $\pm$ 4.09
5-O-caffeoylshikimic acid	8.03 $\pm$ 0.76	-	8.03 $\pm$ 0.76
Kaempferol 3-O-7-O-diglucoside	185.07 $\pm$ 15.73 ^a	13.36 $\pm$ 1.23 ^b	198.43 $\pm$ 14.57
Phenolic 'P5'	12.78 $\pm$ 1.21	-	12.78 $\pm$ 1.21
Protogenkwanin-4-O-glucoside	27.03 $\pm$ 2.29	-	27.03 $\pm$ 2.29
Quercetin 3-O-glucoside-7-O-rhamnoside	33.44 $\pm$ 2.17	-	33.44 $\pm$ 2.17
Kaempferol 3-O-rutinoside-7-O-rhamnoside	428.55$\pm$23.57	-	428.55 $\pm$ 23.57
Quercetin 3-O-rhamnoside	46.34 $\pm$ 3.47	-	46.34 $\pm$ 3.47
Kaempferol 3-O-rutinoside	9.58 $\pm$ 0.58	-	9.58 $\pm$ 0.58
Monocaffeoyl-meso-tartaric acid	-	406.83$\pm$36.61	406.83 $\pm$ 36.61
Kaempferol 3-O-rutinoside-7-O-glucoside	-	50.65 $\pm$ 4.05	50.65 $\pm$ 4.05
NID 1	-	217.75 $\pm$ 1.82	217.75 $\pm$ 1.82
NID 2	-	1450.66$\pm$2.63	1450.66 $\pm$ 2.63
Total	798.95$\pm$29.05	2139.26$\pm$36.99	2938.21$\pm$28.44

Within a row, the values assigned to different letters (a,b) are significantly different at $P < 0.05$

^a μg phenolic compound per g of the dry plant material

^aNID – non-identified compound

By the gradient HPLC method, nine phenolic compounds were identified in the extract of free, and five in the extract of bound phenolics (Table 3). Among identified compounds in the extract of free phenolics, kaempferol 3-O-rutinoside-7-O-rhamnoside (428.55 $\mu\text{g g}^{-1}$) was the most abundant, and the total kaempferol derivatives content was 623.20 $\mu\text{g g}^{-1}$. Among the phenolics in the extract of bound phenolics, a compound with the retention time of 67.73 min, NID 2, was the most abundant (1450.66 $\mu\text{g g}^{-1}$).

Based on the total phenolics content and the total amount of compounds observed by using HPLC methods, it is evident that only 33% of phenolics were detected. This percentage is probably higher since the Folin-Ciocalteu reagent reacts with phenolics, but also with a variety of non-phenolic reducing compounds (tertiary aliphatic amines, tryptophan, hydroxylamine, hydrazine, certain purines and organic and inorganic reducing agents) [38]. A statistical analysis showed that the investigated extracts of free and bound phenolics are different in the content of the identified compounds.

Antimicrobial activity

The antimicrobial activity of the investigated extracts is shown in Table 4.

Table 4. Antimicrobial activity of the extracts of free and bound phenolics from nettle seeds

Test microorganism	Diameter of inhibition zone (mm)	
	Extract of free phenolics	Extract of bound phenolics
<i>Proteus vulgaris</i> ATCC 8427	-	-
<i>Bacillus cereus</i> ATCC 11778	12.66 $\pm$ 1.07	17.33 $\pm$ 1.64
<i>Bacillus luteus</i>	-	13.00 $\pm$ 0.91
<i>Bacillus subtilis</i>	-	15.66 $\pm$ 1.17
<i>Staphylococcus aureus</i> ATCC 25923	-	10.33 $\pm$ 1.01
<i>Escherichia coli</i> ATCC 25922	-	-
<i>Pseudomonas aeruginosa</i> ATCC 27853	-	-
<i>Listeria monocytogenes</i> ATCC 15313	-	-
<i>Klebsiella pneumoniae</i> ATCC 700603	11.66 $\pm$ 1.05	19.66 $\pm$ 1.57
<i>Candida albicans</i> ATCC 10259	-	-

The extract of free phenolics had an effect on two, while the extract of bound phenolics on five of ten tested microbial species. Both of the extracts had the best inhibitory effect on the growth of the same microbial species, *Bacillus cereus* ATCC 11778 and *Klebsiella pneumoniae* ATCC 700603: the extract of free phenolics had a diameter of the inhibition zone on these bacteria of 12.66 and 11.66 mm, while with the extract of bound phenolics it was 19.66 and 17.33 mm, respectively. The extracts had no effect either on the growth of other investigated bacteria (*Proteus vulgaris* ATCC 8427, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 15313) or on the fungi *Candida albicans* ATCC 10259. In literature, there are data about the antimicrobial activity of phenolics extracts from aerial parts of nettle to different bacteria and pathogenic fungi [5, 39, 40]. The Körpe et al. [41] studied

the antibacterial activity of methanolic and aqueous extracts of *Urtica dioica* L. and *Urtica pilulifera* L. seeds on some pathogenic bacteria. These extracts had no effect on *Klebsiella pneumoniae* and *Escherichia coli* but showed a high-antibacterial activity on *Pseudomonas aeruginosa* and *Listeria monocytogenes*.

Conclusion

The investigated *Urtica dioica* L. seeds had a higher content of free than bound phenolics, and the extract of free phenolics had a better FRAP value than the extract of bound phenolics. By using isocratic and gradient HPLC methods, a 33% of phenolics were identified: eighteen phenolics in the extract of free phenolics with kaempferol 3-O-rutinoside-7-O-rhamnoside as the most abundant, and ten in the extracts of bound phenolics among which the monocateoyl-meso-tartaric acid had the highest content. The extract of bound phenolics showed a better inhibitory effect on ten tested microbial species than the extract of free phenolics, and the best among them was a negative bacterium *Klebsiella pneumoniae* ATCC 700603 used per gram. The results indicate that nettle seed is a good source of natural antioxidants and antimicrobials that might find a potential use in food and pharmaceutical industry.

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Izvod

SEME KOPRIVE (*Urtica dioica* L.) KAO IZVOR SLOBODNIH I VEZANIH FENOLA: ANTIOKSIDATIVNA, ANTIMIKROBNA AKTIVNOST I SASTAV

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U radu je ispitan sastav, antioksidativna i antimikrobna aktivnost ekstrakata slobodnih i vezanih fenola iz semena koprive. Sastav fenola određen je HPLC metodom, antioksidativna aktivnost određivanjem kapaciteta neutralisanja DPPH radikala, redukcione snage i FRAP vrednosti, a antimikrobna aktivnost ispitana je diskdifuzionom metodom. Sadržaj slobodnih fenola bio je veći (6000,71 µg ekvivalenta galne kiseline po g suvog semena) u odnosu na sadržaj vezanih (2801,16 µg g⁻¹). Ekstrakt slobodnih fenola imao je veću FRAP vrednost od ekstrakta vezanih fenola, dok je ekstrakt vezanih fenola imao bolji kapacitet neutralisanja DPPH radikala i redukcione snagu. U ekstraktu slobodnih fenola detektovano je osamnaest fenolnih jedinjenja među kojima je kaempferol 3-O-rutinozid-7-O-ramnozid (428,55 µg g⁻¹) bio najzastupljeniji, dok je deset fenolnih jedinjenja detektovano u ekstraktu vezanih fenola, među kojima je monokafeoil-mezo-vinska kiselina (406,83 µg g⁻¹) bila najzastupljenija. Oba ekstrakta pokazala su najbolji inhibični efekat na rast *Bacillus cereus* ATCC 11778 i *Klebsiella pneumoniae* ATCC 700603. Rezultati istraživanja pokazuju da je seme koprive dobar izvor prirodnih antioksidanasa i antimikrobnih jedinjenja za potencijalnu upotrebu u prehrambenoj i farmaceutskoj industriji.

Ključne reči: fenoli, seme koprive, antioksidativna aktivnost, antimikrobna aktivnost