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CHARACTERIZATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF NETTLE LEAVES (*Urtica dioica* L.)

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Samples of stinging nettle or common nettle (Urtica dioica L.*) were collected from the area of Banja Luka. To measure and evaluate the content of chlorophyll (a and b), carotenoids, and soluble proteins, as well as peroxidase activity (POD, EC 1.11.1.7.), fresh nettle leaves of different developmental stages were used. Dried nettle leaves were used to obtain ethanol extract. The dry residue of ethanol extract was dissolved in methanol and the obtained solution was used to determine the content of total phenols, flavonoids, flavonols, as well as non-enzymatic antioxidant activity and antimicrobial activity. The non-enzymatic antioxidant activity was determined by different methods: FRAP, DPPH, and ABTS. The results were compared to those of standard substances like vitamin C, BHT, and BHA. Antimicrobial activity was screened by using macrodilution method.*

The obtained results showed insignificantly higher content of chlorophyll, carotenoids, and proteins in young nettle leaves as well as an increase in the soluble peroxidase activities. Native electrophoresis of the soluble fraction showed the presence of two peroxidase isophorms in the soluble protein fraction of nettle leaves. The total phenolic content in nettle extracts amounted to 208.37 mg GAE/g_{dw},, the content of total flavonoids was 20.29 mg QE/$g_{dw,}$ and the content of total flavonols was 22.83 mg QE/g_{dw}. The antioxidant activity determined by FRAP method was 7.50 mM Fe(II)/g_{dw}, whereas the antioxidant activity measured by using DPPH and ABTS methods, with IC_{50} values, were 31.38 and 23.55 μg mL^{-1}, respectively. These results showed the weak and moderate antioxidant capacity of stinging nettle.

Extract of Urtica dioica L. was tested for antibacterial acivity against various Gram-positive and Gram-negative bacteria: Bacillus subtilis IP 5832, Lactobacillus plantarum 299v (Lp299v), Pseudomonas aeruginosa, and Escherichia coli isolated from food and Escherichia coli isolated from urine samples. Ampicillin, erythromycin, ciprofloxacin, and gentamicin were used as positive control. The results showed that minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract ranged from 9.05 to more than 149.93 mg mL^{-1}.

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INTRODUCTION

Common nettle (*Urtica dioica L*), a herbaceous perennial flowering plant, is a member of the *Urticaceae* family. Traditional herbal medicine in the Balkan countries uses stinging nettle leaves in the form of an herbal infusion as a remedy for the treatment of diarrhea, vaginal discharge, internal/external bleeding (1). Being rich in chlorophyll, nettle leaves are used for the treatment of anemia as well as general well-being, and more recently as natural food colorant. The nettle extract is a common ingredient in cosmetics, e.g. in shampoos and hair growth lotions.

The stinging nettle leaf contains chlorophyll, vitamin C, vitamin K, panthotene acid, carotenoids, B group vitamins (B1 and B2), tannins, essential oil, proteins, and minerals (Fe, Cu, Mn and Ni) (2). Stinging nettle hairs contain acethylcholin and histamine (1), while the stem and root contain flavonoids (3,4).

Animal studies proved that nettle leaf extract may inhibit blood clotting (platelet aggregation), can decrease total cholesterol levels as well as enhance the overall liver function (5,6,7). Water extract of stinging nettle makes significant inhibition of adenosine deaminase activity in prostate tissue in the patients with prostate cancer (8). Adding dried powder of nettle into laying hens diets significantly increases egg production, proves the modulating effects of the immune parameters (9), and lowers the total cholesterol and triglycerides concentration (10). It has also been reported that the stinging nettle extract exibits antioxidant, antimicrobial, antiulcer, and analgesic activities. (11)

The aim of the present study is to evaluate the total, non-enzymatic and enzymatic, antioxidant capacities of ethanol nettle extract as well as its antimicrobial activity.

EXPERIMENTAL

Materials and instruments

All reagents used in this research were of pro analysis grade. Folin-Ciocalteu; DPPH; TPTZ (Sigma Chemical Co., St. Luis, USA); Gallic acid; BHT; BHA; $AlCl_3$; Quercetin hydrate (Acros, New Jersey, USA); Vitamin C; $K_2S_2O_8$ (Merck, Darmstadt, Germany); $NaHCO_3$; $FeCl_3$ x 6 H_2O; $FeSO_4$ x 7H_2O (Lach-Ner, s.r.o., Czech Republic); Sodium acetate (Zorka Pharma a.d., Šabac, Serbia); Pyrogallol; TRIS (Carlo Erba Reagent, Italy); 4-chloro-α-naphthol; Ammonium persulfate; PMSF (Sigma, Germany); Riboflavin; TEMED (Semikem, B&H); Glicine; NaOH (Lach-Ner, Czech Repulic); NaH_2PO_4 (Centrohem, Serbia); H_2O_2 (Sineks Laboratory, B&H); Acrylamide and bis-acrylamide (Carl Roth GmbH, Germany). Antibiotics: Ampyciline; Erytromicine; Ciprofloxacine (HemofarminfectoLogica, Vršac, Serbia); Gentamicin (Alkaloid Skopje, FJR Macedonia); culture media: Mueller Hinton broth (MHB) i Mueller Hinton agar plate (MHA) (Liofilchem, Italy). The bacterial cultures used in this study: *Bacillus subtilis* IP 5832 („Diastop", Alkaloid, Skopje, FJR Macedonia); *Lactobacillus plantarum* 299v (Lp299v) („Flo-

bion", Abela Pharm in cooperation with Probi AB and Insitut Rosell, Italy); *Pseudomonas aeruginosa* and *Escherichia coli* isolated from food (Veterinary Institute "Vaso Butozan", Banja Luka, Republic of Srpska, B&H) and *Escherichia coli* isolated from urine samples (Institute for Health Protection of Republic Srpska, B&H).

Measurements were performed on the following instruments: 6305 UV-VIS spectrophotometer equipped with a thermal cell (Jenway, England), UV-VIS Spectrophotometer (Shimadzu, Japan), Electrophoresis (BioRad, Germany), and pH meter (Hanna, USA).

Preparation of plant material

Top fresh nettle (*Urtica diocia* L.) leaves were collected for the purposes of this study from the Laktaši forest community in June of 2010. The plant material was authenticated by Dr Ljiljana Topalić-Trivunović of the Faculty of Technology, University of Banja Luka. The preparation of plant material was performed according to the following analysis:

a) for the determination of protein solubility and peroxidase activity
Top nettle leaves (L1) and the first pair of leaves below the top (L2) were used in the experiment (Figure 1). To obtain soluble proteins, the nettle leaves were air-dried, chopped in small pieces and ground to powder with liquid nitrogen and homogenized in 0.1 M sodium-phosphate buffer, pH 6.4 containing 1 mM phenylmethyl-sulfonyl fluoride (PMSF). The homogenate was centrifuged at 4000 x g for 15 min at 4 ºC. After centrifugation, the supernatant was separated and marked as 'soluble protein fraction'.

b) for determination of chlorophyll and carotenoids
Chlorophyll a and b and carotenoids were extracted from the fresh nettle leaves in acetone (0.5 g of the plant material in 5 mL acetone). After the centrifugation at 3000 rpm for 15 min, the supernatant was used and the absorbance of extract was measured at 662 nm, 644 nm and 440 nm.

c) for determination of total phenols, flavonoids, and flavonols, non-enzymatic antioxidant and antimicrobial activity
The collected nettle leaves were dried at room temperature at a draft, protected from direct light, for a few days. Then they were chopped up and stored until used in a glass jar. The air-dried and ground material (20 g) was extracted (three times) with 100 mL of 80% ethanol (v/v) at 25 ^{o}C, in ultrasonic bath (30 Hz) for 5 minutes. After that, the solution was continuously stirred for 30 minutes at the same temperature and then filtered. The combined extract was evaporated to dryness under reduced pressure at 40 ^{o}C. The obtained resin-like dry extract was dissolved in methanol and the contents of total phenols, flavonoids, and flavonols were determined as well as antioxidant and antimicrobial activity.

Determination of soluble proteins, chlorophyll and carotenoids

Protein content was determined according to Lowry (12). The concentrations of chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoids were calculated according to Holm (13) and Van Wattstein (14).

Determination of total phenolic content

Total phenols were determined by a modified Folin-Ciocalteu method (15). The measurement was conducted by mixing 1.5 mL of working Folin-Ciocalteu solution (stock Folin-Ciocalteu solution dissolved with water in 1:10 ratio), 1.5 mL of 7.5% $NaHCO_3$ and 200 µL of the methanol extract solution (100 µg mL^{-1}). The absorbance was measured after 30 minutes at 765 nm, along with the blank.

The standard gallic acid diagram was prepared by adding 200 µL of gallic acid of different concentration (50-250 µg mL^{-1}) instead of 200 µL of sample. The total phenolic content was calculated as phenols equivalent to gallic acid (mg GAE/$g_{\text{dry extract}}$) using the following equation based on the calibration curve: $y = 0.0016x + 0.0234$; $R^2 = 1$, where y is the absorbance and x the gallic acid concentration (µg mL^{-1}).

Determination of total flavonoids

Total flavonols in the plant extracts were estimated by using the method of Kumaran and Karunakaran (17). To 1 mL of sample solution (1 mg mL^{-1}), 1 mL of 2% $AlCl_3$ ethanol and 1.5 mL (50 g L^{-1}) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 h at 20°C. The standard quercetin diagram was prepared by adding 1 mL of quercetin of different concentration (10-80 µg mL^{-1}) instead of 1 mL of sample. The total flavonoid content was calculated as quercetin (mg QE/$g_{\text{dry extract}}$) using the following equation based on the calibration curve: $y = 0.0214\ x + 0.004$; $R^2 = 0.9993$, where y is the absorbance and x is the quercetin concentration (µg mL^{-1}).

Determination of total flavonols

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Determination of antioxidant activity

a) enzymatic antioxidant activity

For the determination of peroxidase activity, pyrogallol (A_{430}; $\varepsilon = 12$ mM^{-1} cm^{-1}) was used as hydrogen donor and the absorbance increase at 430 nm was measured. The reaction mixture consisted of 20 mM pyrogallol, 3.3 mM H_2O_2 in 100 mM sodium - phosphate buffer (pH 6.4) and an aliquot of the extract.

Peroxidase (POD) isoforms were separated by native electrophoresis on a 10% polyacrylamide gel at 100V for 120 min. For the visualization of POD isoforms, the gel was

incubated in a staining solution consisting of (5 mg 4-chloro-α-naphthol, 5 mL methanol in 45 mL of 100 mM potassium-phosphate buffer (pH 6.5) and 0.03% H_2O_2.

b) non-enzymatic antioxidant activity

FRAP method (Ferric Reducing Antioxidant Power) is based on the reduction of Fe^{3+} ions to Fe^{2+} ions in the presence of an antioxidant (18). The obtained Fe^{2+} ions in the presence of TPTZ reagent [2,4,6 three(2-pyridyl)-S-triazine] make colored complex, with the absorbance maximum at 593 nm. The reaction takes place in acid medium.

The reagents were: 10 mM TPTZ solution, 40 mM HCl, 20 mM $FeCl_3$ x $6H_2O$ solution, 300 mM Na-acetate buffer, pH 3.6 and 20 mM $FeSO_4$ x 7 H_2O solution.

The working solution was prepared by mixing 25 mL of acetate buffer solution, 2.5 mL of TPTZ reagent and 2.5 mL of $FeCl_3$ x $6H_2O$ solution. The FRAP working reagent must always be freshly prepared and kept until used in a water bath at 37 °C.

The measurement was performed by mixing 200 µL of *Urtica dioica* L. extract (500 µg mL^{-1}) with 1.8 mL of FRAP working reagent, incubating for 10 minutes at 37 ºC and finally measuring the absorbency at 593 nm, with a blank (1.8 mL FRAP working reagent + 200 µL distilled water).

The standard diagram for $FeSO_4$ solution was prepared by adding 200 µL of $FeSO_4$, concentration 0.1-1.0 mM (the dependence is linear in the concentration range from 0.2 to 1.0 mM $_{FeSO4}$) instead of 200 µL of extract.

The results were presented as mM Fe(II)/g of dried extract and compared with the standard antioxidant compounds: BHA, BHT and vitamin C.

DPPH method. The DPPH method is based on the ability of stable free radical 2,2-diphenyl-picrylhydrasyl (DPPH) to react with hydrogen donors, including phenol compounds. DPPH shows an intensive absorption in the visible part of the spectrum and is easily determined spectrophotometrically (19).

The solution of 0.135 mM DPPH in methanol was used.

The measurement was done by mixing 2 mL of DPPH solution with 2 mL of extract or standard compounds. The ranges of concentration of the compounds used were: gallic acid 0.25-2.5 µg mL^{-1}; vitamin C 2-10 µg mL^{-1}; BHA 1.5-12 µg mL^{-1}; nettle extract 20-80 µg mL^{-1}. The reaction mixture was kept in the dark at room temperature and the absorbance was measured after 30 minutes at 517 nm, along with a blank.

The antiradical activity (AA%) was calculated from the following relation:

$$AA\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad [1]$$

where:

$A_{control}$ - DPPH working solution absorbency + methanol,

A_{sample} - DPPH working solution absorbency + sample (or the standard solution).

Based on the diagram representing the antiradical activity vs. different sample concentrations or the reference compound, the value of IC_{50} was determined. This value represented the sample (or the reference compound) concentration needed for inhibiting 50% of DPPH radicals.

The results were also presented as antioxidant activity index (AAI) (20):

$$\text{AAI} = \frac{c_{DPPH}^{final}\left(\mu gmL^{-1}\right)}{IC_{50}\left(\mu gmL^{-1}\right)} \quad [2]$$

AAI was compared with reference values, vitamin C, BHA and BHT.

$ABTS^{+}$ radical scavenging assay. For the ABTS assay, the method of Re et al. (21) was adopted. The stock solutions included 7 mM $ABTS^{\cdot+}$ solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal volumes and allowed to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL $ABTS^{\cdot+}$ solution with 60 mL methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. $ABTS^{\cdot+}$ solution was freshly prepared for each assay. Plant extracts (1 mL) were allowed to react with 1 mL of the $ABTS^{\cdot+}$ solution and the absorbance was taken at 734 nm after 7 min, using the spectrophotometer. The radical scavenging capacity was calculated by equation 3 as percentage of inhibition (I%) of $ABTS^{\cdot+}$ radicals..

$$I\% = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad [3]$$

where $A_{control}$ is the absorbance of $ABTS^{\cdot+}$ radical + methanol; A_{sample} is the absorbance of $ABTS^{\cdot+}$ radical + sample extract/standard.

The value of the IC_{50} was determined based on the diagram representing the percentage of inhibition vs. the concentration of the sample or the reference compound. This value represented the sample (or the reference compound) concentration needed for inhibiting 50% of $ABTS^{\cdot+}$ radicals.

The IC_{50} was compared with the reference values, BHT, BHA and vitamin C..

Determination of antimicrobial activity

The antimicrobial activity of ethanol nettle extract was screened by using macrodilution method with slight modifications. All analyzed cultures were incubated until log-phase when the density of suspension was adjusted to 1.5 x 10^8 cfu mL^{-1} equal to that of the 0.5 McFarland standard (22). Two rows of eight tubes were diluted with MHB medium and then a two-fold serial dilution of the nettle extract concentration was made. The last test tubes contained 1.13 mg mL^{-1} of the extract. One row of test tubes was filled with the bacterial suspension. The density of each bacterial culture in the tubes was 5 x 10^5 cfu mL^{-1}. The transport time was within 15 minutes of standardization, and the tubes were incubated at 37 ^{o}C for 24 hours.

After the incubation this row of test tubes was compared with the row of test tubes containing the same MHB and extract concentrations, but without the culture. The first test tubes (the tubes were ordered from the lowest to the highest concentration) in which the absence of visible bacterial growth occurred, represented the MIC. The MBC was determined by subcultivation with a loop from all test tubes without visible growth of microorganisms on the Petri dish with MHA. After the 24-hour incubation at 37 ^{o}C, the lowest extract concentration contained in the test tube from which solid plates were

cultured without bacterial colony growth, was recorded as MBC. In the same way, MIC and MBC were determined for the appropriate antibiotics, with the antibiotics solutions being prepared using an appropriate procedure (23). All experiments were carried out in triplicates.

RESULTS AND DISCUSSION

Among the leaves of different ages, there are differences in the chlorophyll content, chlorophyll a/b ratio, carotenoids content, as well as the intensity of photosynthesis. It has been shown that the level of chlorophyll increased in young expanding leaves and decreased during senescence (24). Our result showed that the content of total chlorophyll as well as the carotenoid content were higher in younger nettle leaves (L1) (Table 1).

Table 1. Content of chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll Chl(a+b), carotenoids and soluble proteins in nettle leaves of different age.

Samples	Chla (mg/g_{FW})	Chlb (mg/g_{FW})	Chl(a+b) (mg/g_{FW})	Carotenoids (mg/g_{FW})	Protein content (mg/g_{FW})
L1	0.882±0.002*	0.285±0.01*	1.174±0.006*	0.323±0.006*	17.505±0.765*
L2	0.698±0.053*	0.320±0.03*	1.02±0.026*	0.216±0.05*	14.365±0.1976*

* mean values of three measurements ± SD

In the photosynthetic tissues, carotenoids are synthesized in the chloroplasts, where they accumulate primarily in association with the light-harvesting complex and reaction centres (25). Higher carotenoid content in L1 nettle leaves may be associated with their photo-protective role. As the leaves of different ages represent different physiological states, this may contribute to significant changes in the quantity and quality of the leaf proteins. The obtained results showed higher protein content in younger nettle leaves (Table 1). Yeoh and Paul (26) showed that young apical cassava leaves had a high protein content.

Table 2. Content of total phenols, flavonoids and flavonols in extract of ***Urtica dioica*** **L**.

Sample	Total phenols mg GAE/g_{DW**}	Total flavonoids mg QE/g_{DW}	Total flavonols mg QE/g_{DW}
***U. dioica* L**	208.37 ± 4.39*	20.29± 0.48*	22.83 ± 0.30*

* mean value of three measurements ± SD

** DW- dry extract

Phenolic components are found in the natural world, especially in the plant kingdom, and their diverse biological functions have been proven, including the antioxidant (27, 28) and antimicrobial activities (29). Many studies of phenolic components have reported that the environmental, climatic, or geographic factors as well as extraction techniques may significantly influence the quality and the quantity of phenolic components present

in nettle (30,31,32). As shown in Table 2, the total phenolic content in ethanol extract of nettle leavess is high (208.37 mg GAE/g_{DW}), whereas the content of total flavonoids and flavonols is relatively low (20.29 and 22.83 mg QE/g_{DW}, respectively).

When the values of total phenolic components in ethanol extract of nettle leaves are compared (Table 2), it is evident that they are considerably higher than the ones in methanol extract of nettle collected from the Nothern provinces of Iran (24.1mg GAE/g_{DW}) (31). On the other hand, the values of total phenolic components in ethanol nettle extracts were lower than the values found by Soxhlet extraction with methanol from nettle leaves collected from Turkey (332 mg GAE/g_{DW}) (30). Accordingly, the content of total flavonoids in methanol nettle extract is significantly higher than that in ethanol extract (43.3 and 33.94 mg QE/g_{DW}, respectively) (30,31) (Table 2)

Determination of antioxidant activity

Enzymatic antioxidant activity. Peroxidases (POD, EC 1.11.1.7.) are members of a large group of heme-containing glycoproteins that catalyze oxidoreduction between H_2O_2 and various reductants. They are widely distributed in higher plant parts and are involved in several physiological functions such as organogenesis (33), auxin catabolism (34), lignification (35), suberization (36), cross-linking of cell wall structural proteins (37), auxin catabolism (38), self-defense against pathogens (39), salt tolerance (40), and senescence (41).

The activity of soluble peroxidase with pyrogallol as electron donor was higher in younger nettle leaves, sample L1 (Table 3). Using native electrophoresis, two peroxidase isoforms, labeled as POD1 and POD2, were detected in both samples (Figure 1, b).

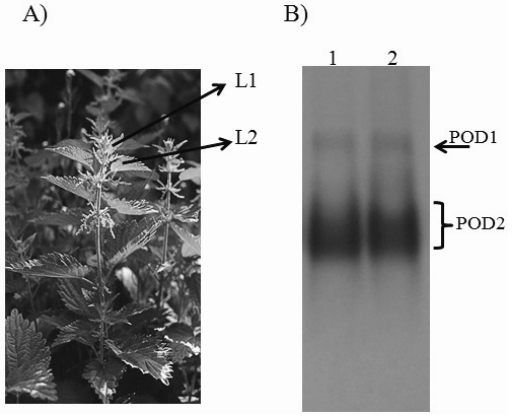

Figure 1. A) Nettle plant with marked leaves used for the experiment: L1- Top young nettle leaves (L1) and the first pair of leaves below the top (L2) were used in the experiment (Figure 1). B) Peroxidase pattern in L1 and L2 sample obtained on native 10% polyakryamide gel. Arrows indicate POD isomorfs

Table 3. Peroxidase activity in nettle leaves of different age

Samples	Peroxidases activity ($\mu mol/mg_{prot}$ /min)
L1	1.174±0.145 *
L2	0.675±0.171

* mean value of three measurements ± SD

Peroxidases (POD, EC 1.11.1.7.) are members of a large group of heme-containing glycoproteins that catalyze oxidoreduction between H_2O_2 and various reductants. They are widely distributed in higher plant parts and are involved in several physiological functions such as organogenesis (33), auxin catabolism (34), lignification (35), suberization (36), cross-linking of cell wall structural proteins (37), auxin catabolism (38), self-defense against pathogens (39), salt tolerance (40), and senescence (41).

The activity of soluble peroxidase with pyrogallol as electron donor was higher in younger nettle leaves, sample L1 (Table 3). Using native electrophoresis, two peroxidase isoforms, labeled as POD1 and POD2, were detected in both samples (Figure 1, B). Peroxidase isoforms differ in electrophoretic mobility (Rf_{POD1} = 0.82, Rf_{POD2} = 0.67). Most higher plants possess several isozymes and their number and relative concentration in different tissues vary according to the stage of plant development or to environmental factors. Peroxidases are considered as ubiquitous enzymes in the vacuoles, which are also the target compartment for the accumulation of secondary metabolites (42). It has been shown that secondary metabolites, including phenols, may act as substrates for class III peroxidases (POD) (43,44,45) and they consume excess of H_2O_2. Furthermore, it is shown that phenols may form a co-operative regenerating cycle with POD and ascorbic acid, enabling to scavenge high doses of H_2O_2 (42). Acting together, POD and phenols are important part of the antioxidant metabolism in response to different types of biotic and abiotic stresses.

Non-enzymatic antioxidant activity. To evaluate the antioxidant activity accurately, only one method is not sufficient since many factors can affect the evaluation. It is required to take more than one measurement and also to take into consideration different mechanisms of antioxidant activity.

The total antioxidant capacity of ethanol extract of nettle was determined by FRAP method and the results showed a weak antioxidant activity compared to the control antioxidants such as vitamin C and BHA (20 times more powerful than the nettle extract), whereas the BHT showed to have two times higher antioxidant activity than ethanol extract of stinging nettle. (Table 4).

The stable free radical DPPH is often used to evaluate the antioxidant properties of natural products. The stable free radical DPPH scavenging effect is otherwise expressed as an IC_{50} value, i.e. the extract concentration required to inhibit 50% DPPH radicals. It was shown, however, that the observed values of IC_{50} may vary depending on the initial DPPH concentration, so that the AAI has been used instead and antioxidants are classified as weak, when AAI < 0.5, moderate, when AAI between 0.5-1.0, strong, when AAI between 1.0-2.0, and very strong, when AAI >2.0 (20).

Table 4. Antioxidant activity of *U.dioica* L. leaf determined by FRAP, DPPH and ABTS methods

Samples	FRAP	DPPH		ABTS
	mM(Fe II)/g $_{DW}$	AAI	IC_{50} (μg/g $_{DW}$)	IC_{50} (μg/g_{DW})
Vitamin C	143.09 ± 11.29**	4.97 ± 0.01**	5.36 ± 0.01**	1.37 ± 0.01**
BHA	147.28 ± 13.87	3.96 ± 0.17	6.58 ± 0.28	1.72 ± 0.08
BHT	16.64 ± 0.30	1.15 ± 0.04	23.16 ± 0.84	6.27 ± 0.16
Extract ***U.*** *dioica* L.	7.50 ± 0.43	0.85 ± 0.003	31.38 ± 0.102	23.55 ± 0.64

* DW- dry extract

** mean value of three measurements ± SD

It can be seen from Table 4 that the AAI values for standard antioxidants, vitamin C (4.97) and BHA (3.96), correspond to the antioxidants classified as showing "very strong antioxidant activity", whereas BHT (1.15) is classified as an antioxidant with "strong antioxidant activity". The nettle extract has the AAI value of 0.82, and it is classified as an antioxidant with "moderate antioxidant activity".

When the values of total antioxidant activity of nettle leaves were compared by using stable free radical DPPH (values were expressed as IC_{50}), it was shown that ethanol extract of nettle leaves had significantly higher antioxidant activity (31.38 μg mL^{-1}) than some of the methanol extracts ranging from 1.45 mg mL^{-1} (32), and 105.16 μg mL^{-1} (46), to 175 μg mL^{-1} (47).

ABTS method is also a common method for determination of antioxidant activity of herbal extracts. The nettle leaves have the ability to inhibit ABTS radical and this method is used to measure that. The results, (Table 4) show that ethanol extract of nettle leaves had significantly lower antioxidant activity measured according to ABTS than the other compared to the standard control antioxidants such as vitamin C, BHA and BHT.

The nettle extract IC_{50} value was 23.55 μg mL^{-1}, vitamin C and BHA had 1.37 and 1.72 μg mL^{-1}, respectively, and BHT 6.27 μg mL^{-1}. These values show that nettle extract has 17.2 times lower antioxidant activity than vitamin C, 13.7 times lower than BHA, and 3.8 times lower than BHT. Statistical discrepancy was observed between these results and those obtained for methanol extracts of nettle leaves from Turkey (40.59 mMTE/g of dry extract) (30) and Poland (17.3 μM TE/g of dry extract) (48) (TE-trolox equivalent), which have been found to have significant antioxidant potential.

Antimicrobial activity

The ethanol extract of nettle leaves diluted with methanol showed a weak antibacterial activity. The nettle extract exibited best antibacterial activity against the cultures *B. subitilis* IP 5832 and *E. coli* which were isolated from food, with the lowest MIC values recorded (Table 5). All MIC values were above the highest tested concentration, except for the culture *P. aeruginosa* (144.86 mg mL^{-1}). *P. aeruginosa* is a naturally resistant Gram-negative bacterium that causes various infections in humans, and is rather insusceptible to herbal extracts. Water extract of nettle (*U. dioica L.* WEN*),* at the doses of 250 μg per disk had no effect on the growth of *P. aeruginosa* ATCC 9027 (11), whereas *P.*

aeruginosa (clinical isolate) was less susceptible to the combined fractions of hexane extract of *U. dioica* in comparison to the other Gram-positive and Gram-negative bacteria tested (49). The plant extract Ankaferd Blood Stopper®, which contains nettle extract, also showed the lowest inhibitory activity against *P. aeruginosa* (50).

E. coli is a Gram-negative bacterium which is commonly found in the intestinal tract of humans and animals as normal flora but which can cause urinary tract infections and foodborne disease. The ethanol extract of nettle leaves diluted with methanol showed a MIC value that was lower against *E. coli* isolated from food samples in comparison to the bacterial strain isolated from urine samples. The values of MIC against both *E. coli* strains were higher than the highest tested concentrations. The ethanol extract of nettle leaves did not inhibit the growth of *E. coli* ATCC 9837 (51), unlike the water extract, which exhibited considerable antibacterial activity (11).

B. subtillis, an endospore-forming Gram-positive bacterium, showed a higer susceptibility to the ethanol extract of nettle leaves diluted with methanol in comparison to the other tested bacteria, but no MIC values against this bacterium were determined. *L. plantarum* 299v is a probiotic Gram-positive bacterium found in many fermented food products and in the human intestinal tract. This bacterium has many beneficial effects on human health (52), and the MIC value of the ethanol extract of nettle leaves diluted with methanol showed a potent antibacterial activity against this bacterium, which is of importance since nettle leaves have been consumed as food for centuries.

Table 5. Antibacterial activity of ethanol extract of *Urtica dioica* L. leaves diluted with methanol

Samples	Extract (mg/mL)		E[a]		AMP[a]		CIP[a]		G[a]	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. subtilis	36.21	>144.86	5	20	0.078	0.156	0.3125	1.25	0.325	5
[b]*E. coli*	72.43	>144.86	0.625	1,25	8	16	<0,078	0.078	1.25	2.5
[c]*E. coli*	36.21	>144.86	20	40	64	256	0.156	0.625	1.25	5
P. aeruginosa	72.43	144.86	20	40	-	-	0.3125	0.625	1.25	1.25
L. plantarum	72.43	>149.93	0.3125	1,25	0.078	1.25	2.5	-	0.156	-

[a]Antibiotics (μmL^{-1}): Erythromycin (E); Ampicillin (AMP); Ciprofloxacin (CIP); Gentamicin (G)
[b]*Escherichia coli* isolated from urine samples; [c]*Escherichia coli* isolated from food

CONCLUSION

The obtained results showed insignificantly higher content of chlorophyll, carotenoids, and proteins in young nettle leaves as well as an increase in the soluble POD activities. Native electrophoresis of the soluble fraction showed that the presence of two peroxidase isophorms were detected in the soluble protein fraction of nettle leaves. The total phenolic content in ethanol extract of nettle leaves is high (208.37 mg GAE/g_{DW}), whereas the content of total flavonoids and flavonols is relatively low (20.29 and 22.83 mg QE/g_{DW}, respectively). Ethanol extract of *U. dioica* leaves has significant enzymatic and moderate non-enzymatic antioxidative action compared to the control antioxidants

(vitamin C, BHA and BHT). Acting together, POD and phenols are important part of the antioxidant metabolism in response to different types of biotic and abiotic stresses.

Ethanol extract of nettle leaves diluted with methanol showed a weak antibacterial activity against *B. subtilis* IP 5832 and *E. coli* isolated from food. The other tested bacteria strains, of *E. coli* isolated from urine, *P. aeruginosa,* and *L. plantarum* did not exhibit any antibacterial activity at test concentration of ethanol extract of *U. dioica*. A weak antimicrobial activity of the tested extract could be of importance since nettle leaves have a wide range of uses, for food, medicinal purposes, fibers, and may have other positive effects on human health.

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КАРАКТЕРИЗАЦИЈА АНТИОКСИДАТИВНЕ И АНТИМИКРОБНЕ АКТИВНОСТИ ЛИСТА КОПРИВЕ (*Urtica dioica* L.)

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Коприва *(Urtica dioica* L.) за потребе овог рада прикупљена је у региону Бања Луке. У свјежим листовима различите старости одређен је садржај хлорофила а, хлорофила б, каротеноида и солубилних протеина као и активност пероксидаза (POD, EC 1.11.1.7.). Суви листови коприве су коришћени за добијање етанолног екстракта. Суви остатак етанолног екстракта је растворен у метанолу и у добијеном раствору је одређен садржај укупних фенола, флавоноида, флавонола, неензимска антиоксидативна и антимикробна активност. Неензимска антиоксидативна активност одређена је FRAP, DPPH и ABTS методом. Резултати су поређени са антиоксидативном активношћу стандардних једињења (витамин Ц, BHT и BHA). Антимикробна активност рађена је методом макроразређења.

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Добијени резултати су показали незнатно повећање садржаја укупног хлорофила, каротеноида и протеина у млађим листовима. Такође је у млађим листовима измерена већа активност солубилних пероксидаза. Нативном електрофорезом детектоване су две пероксидазне изоформе у солубилној протеинској фракцији листа коприве. Садржај укупних фенола у коприви је износио 208,37 мг GEA/г сувог екстракта (dw), укупних флавоноида 20,29 мг QE/$г_{dw}$,а укупних флавонола 22,83 мг QE/$г_{dw}$.

Антиоксидативна активност добијена FRAP методом износила је 7,50 mM Fe(II)/g_{dw}, док је за DPPH и ABTS методу, изражена као IC_{50} била 31,38 и 23,55 μg mL^{-1}, респективно. Резултати указују на слабу и умерену антиоксидативну активност коприве.

Екстракт коприве је тестиран на различите Грам-позитивне и Грам-негативне бактерије *(Bacillus subitlis IP 5832, Lactobacillus plantarum 299v (Lp299v), Pseudomonas aeruginosa* и *Escherichia coli* која је изолована из узорака хране и *Escherichia coli* која је изолована из узорака урина). Комерцијални антибиотици (ампицилин, еритромицин, ципрофлоксацин и гентамицин) су коришћени као позитивна контрола. Резултати показују да је минимална инхибиторна концентрација (MIC) и минимална бактерицидна концентрација MBC екстракта листа коприве у распону од 9,05 до више од 149,93 mg mL^{-1}.

Кључне речи: коприва, садржај хлорофила, каротеноида и солубилних протеина, укупни феноли, флавоноиди и флавоноли, антиоксидативна и антимикробна активност.

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