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TOTAL PHENOLIC CONTENT, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *Equisetum arvense* L.

The antioxidant and antimicrobial activity of Equisetum arvense L. harvested in a polluted urban area were investigated. The total phenolic content determined by a modified Folin-Ciocalteu method was 355.8 mg of GEA/g of the dried ethanol extract. Antioxidant activity determined by FRAP method was 28.7 mM Fe(II)/g of extract, whereas antioxidant activity index for stable DPPH free radicals was 3.86. The results were compared with control antioxidants (gallic acid, vitamin C, BHA and BHT). Antimicrobial activity was tested by monitoring the impact of different extract concentrations through measuring the change in optical density of bacterial suspension over time and by determining MIC and MBC using macrodilution method. E. arvense extract has been effective on the tested microorganisms depending on the concentration and exposure time. The extract had the strongest antibacterial activity on Staphylococcus aureus, with MIC and MBC of 11.14 and 22.28 mg/mL respectively, and the weakest effect on Bacillus cereus, with MIC of 89.10 mg/mL.

Keywords: Equisetum arvense L., antioxidant activity, antimicrobial activity.

Equisetum arvense L. (field horsetail) is a fern from the *Equisetaceae* family, widely spread across the northern hemisphere as a weed in fields and uncultivated land. Multiple healthfulness properties of field horsetail have been known since ancient times and it has been used in the treatment of pulmonary tuberculosis and haemorrhage, anaemia, peptic and other types of ulcers, fistulas and colon polyps, inflammation, bleeding, kidney and bladder tuberculosis [1].

A large number of papers verify various biological effects of the *E. arvense* extracts, such as sedative and anticonvulsive, hepatoprotective, antioxidant, antibacterial and antifungal activity [2-6]. *E. arvense* is well-known for its high content of bioactive components, such as: phenolic compounds, saponins, aconite, oxalic and malic acid, resins, tannins, pectin, flavonic compounds, vitamin C, carotenoids and mineral substances [7-9].

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Studies have shown that there are differences in the content of the bioactive substances in *E. arvense* plants collected in different geographical areas [10].

This study investigated the antioxidant and antimicrobial activity of field horsetail collected in ruderal habitats of the urban area of Banja Luka, which were exposed to various types of pollution, as a part of research on medicinal plants.

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 (Acros, NJ, USA); vitamin C (Merck, Darmstadt, Germany); Cephalexin (Panfarma, Beograd, Srbija); Doxicyclin (Hemofarm, Vršac, Srbija). Nutrient broth and nutrient agar were acquired at The Institute of Virology, Vaccines and Sera "Torlak", Belgrade. *E. coli*, *S. aureus* and *B. cereus* were isolated from foodstuffs at the Veterinary Institute "Vaso Butozan", Banja Luka. All spectrophotometric measurements were performed on a Jenway 6305 spectrophotometer, equipped with a thermal cell.

Herb material extraction

In this work, sterile stems of *E. arvense*, collected in the Banja Luka city in June 2010 were used. The herbs were determined and a voucher specimen of the plant was deposited at the Herbarium of the Institute of Botany and Botanical garden Jevremovac, University of Belgrade (BEOU, no. 16502). The air dried and ground material (20 g) was extracted (strongly stirred) three times with 200 mL of 50 vol.% ethanol in water at 25 °C for 30 min [11]. After filtering, the combined extract was evaporated to dryness under reduced pressure at 40 °C. The obtained resin-like dry extract was dissolved in methanol and the total phenolic content was determined, as well as antioxidant and antimicrobial activity.

Determination of total phenolic content

Total phenolic contents were determined by a modified Folin-Ciocalteu method [12]. The measurement was conducted by mixing 1.5 mL of working Folin-Ciocalteu solution (stock Folin-Ciocalteu solution diluted by 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 min at 765 nm, along with a 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 μg of sample. The results were expressed as the total phenolic equivalent to gallic acid: mg GAE/g of dried ethanol extract.

Determination of antioxidant activity

FRAP working solution was prepared by mixing 25 mL of 300 mM Na-acetate buffer solution (pH 3.6), 2.5 mL of 10 mM TPTZ reagent in HCl (40 mM) and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ solution [13]. The determination was performed by mixing 200 μL of *E. arvense* extract (0.125 mg mL^{-1}) with 1.8 mL of FRAP working reagent, incubating for 10 min at 37 °C and measuring the absorbance at 593 nm, along with a blank.

The standard $FeSO_4$ diagram was prepared by adding 200 μL of $FeSO_4$ (concentration range 0.1-1.0 mM) instead of 200 μL of extract. The results were presented as mM Fe(II)/g of dried extract.

The measurement using DPPH method was performed by mixing 2 mL of 0.135 mM DPPH methanol solution with 2 mL of extract or standard compounds [14]. Concentration ranges of standard compounds were as follows: gallic acid 0.25-2.5 μg mL^{-1}; vitamin C 2-10 μg mL^{-1}; BHA 1.5-12 μg mL^{-1}; *E. arvense* extract 10-30 μg mL^{-1}.

The reaction mixture was kept in the dark at room temperature and the absorbance was measured after 30 min at 517 nm, along with a blank.

Antiradical activity (AA%) was calculated from Eq. (1):

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

where $A_{control}$ is the absorbance of DPPH radical + methanol and A_{sample} the absorbance of DPPH radical + sample extract or standard.

Based on a diagram representing antiradical activity in relation to various sample concentrations or the reference compound (Figure 1), the value of $IC50$ was determined.

The results were also presented as antioxidant activity index (AAI), Eq. (2), and compared with reference values for gallic acid, vitamin C and BHA [15].

$$AAI = \frac{C_{DPPH}^{final}\left(\mu g\ mL^{-1}\right)}{IC50\left(\mu g\ mL^{-1}\right)} \quad (2)$$

where C_{DPPH}^{final} is the final concentration of DPPH in the reaction mixture.

Determining antimicrobial activity

Bacterial cultures (*E. coli*, *S. aureus* and *B. cereus*) were kept on NA until use. A two-step subcultivation was done with NB and the working inoculums' density was approximately 1×10^6 mL^{-1}.

Determining antimicrobial activity of different concentrations of dried ethanol extract of *E. arvense* in methanol was performed by hourly monitoring of the test microorganisms growth in liquid medium for the total of eight hours. The bacterial population growth was determined spectrophotometrically, measuring the optical density of bacterial suspension at 600 nm which was afterwards correlated with the number of colonies on the agar plate (cfu mL^{-1}). The components (sterile NB, methanol solution of the extract, test microorganisms' culture) were dosed into sterile test tubes and the final extract concentrations in the test tubes were: 0.21, 0.42, 0.83 and 1.67 mg mL^{-1}. The antimicrobial activity of different concentrations of *E. arvense* was expressed in two different ways [16]:

a) Through the influence of the extract on the bacterial growth rate (L), Eq. (3):

$$L(\%) = \left(1 - T_{1/2} / T_{1/2E}\right) \times 100 \quad (3)$$

where $T_{1/2}$ is the mean generation time of bacterial culture growth without the extract and $T_{1/2E}$ the mean

generation time of bacterial culture growth with the extract.

b) Through the influence of the extract on the total number of bacteria (M), Eq. (4):

$$M(\%) = (1 - a/b) \times 100 \quad (4)$$

where:

$a = \log N_{\text{stationary phase}} - \log N_{\text{lag phase}}$

with the extract,

$b = \log N_{\text{stationary phase}} - \log N_{\text{lag phase}}$

without the extract.

In order to determine MIC and MBC, macrodilution method was applied, with slight modifications [17]. For each bacterial culture seven test tubes were used and the extract was successively diluted in a way that the seventh test tube contained the extract methanol solution of 1.39 mg mL^{-1} concentration. Consequently, in each test tube 100 µL of appropriate culture was added and the test tubes were incubated at 37 °C for 24 h. After the incubation this row of test tubes was compared with the row of test tubes containing the same NB and extract concentrations, but without the culture. The first test tube (the tubes were ordered from the lowest to the highest concentration) in which the absence of visible bacterial growth occurred represented MIC. MBC was determined by subcultivation with a loop from all test tubes without visible growth of microorganisms on the Petri dish with NA. After the 24 h incubation at 37 °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 appropriate antibiotics, with antibiotics solutions being prepared using an adequate procedure [18].

RESULTS AND DISCUSSION

Phenols are largely present in plants and it is proven that they have numerous biological functions including antioxidant [19,20] and antimicrobial activity [21]. The total phenolic content in the field horsetail extract was high, 355.80±17.8 mg GAE/g of the dried extract. Quantitative and qualitative variations in the content of some phenolic compounds present in *E. arvense* depending on environmental and geographical factors have been reported [22]. Horsetail collected in the region of mountain Zlatibor, Serbia, was reported to have total phenolic compounds content in the *n*-butanol, ethyl-acetate and water extract: 96.4, 26.4 and 15.4 mg GAE/g of the dried extract, respectively [5]. Data regarding total phenolic content and antioxidant properties of sterile stems of *E. arvense* collected in the polluted urban area are still scarce.

The total antioxidant potential of *E. arvense* extract determined by FRAP method and compared to standard compounds indicated a moderate total antioxidant activity (Table 1).

Table 1. Total antioxidant activity determined by FRAP method; results represent a mean value of five measurements ± standard deviation

Sample	mM (FeII)/g of dried extract
E. arvense extract	28.7±0.1
Vitamin C	71.5±0.1
BHA	73.6±0.1
BHT	8.3±0.4

A moderate reductive activity of field horsetail extract points to the fact that the extract is capable of donating electrons to free radicals which can be formed in biological systems, thus making free radicals stable and non-reactive.

Antioxidant activity of the extract largely depends on the extract composition and the method applied to determine it. It can depend on a number of factors and cannot be entirely assessed by a single method. It is necessary to perform more than one type of test and take into account different mechanisms of the antioxidant effect. DPPH is the stable radical and is often used for assessing the antioxidant activity of natural products [23].

Increasing concentration of *E. arvense* extract and reference compounds evidently led to an increase of their antiradical activity (*AA*%) based on the stable DPPH radical (Figure 1).

The scavenging effect of the *E. arvense* extract on DPPH radicals sharply increased from 10 µg mL^{-1} (35.6%) to 30 µg mL^{-1} (91.3%), with good correlation (r^2 = 0.9645), indicating the extract's hydrogen donating capability.

The capability of scavenging free radicals with *E. arvense* extract is represented with antioxidant activity index and with the sample concentration necessary for inhibition of 50% DPPH radicals (IC50 value, Table 2).

Although *AAI* of *E. arvense* ethanol extract was 2, 2.5 and 9 times lower than *AAI* values for BHA and gallic acid reference compounds, respectively, based on the stable DPPH radical and according to the classification by R. Scherer and H.T. Godoy, this extract belongs to a group of very strong antioxidants [15].

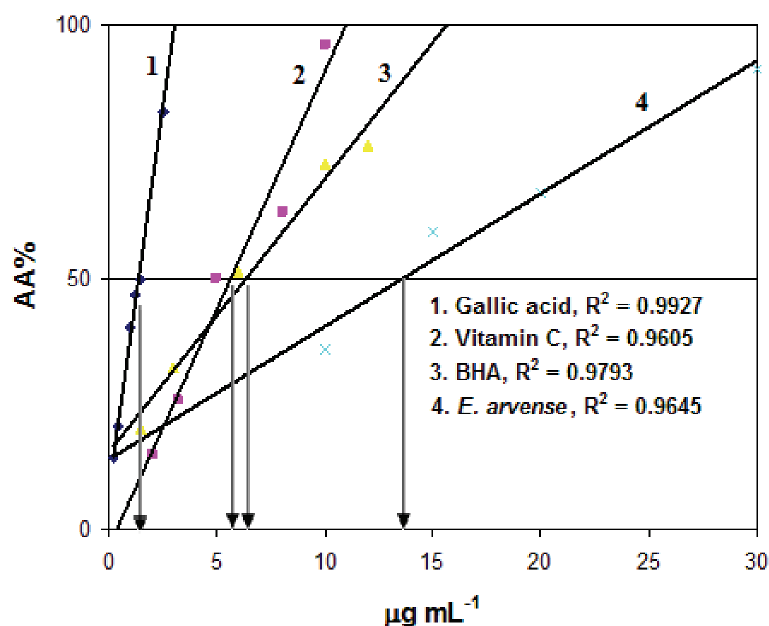

Figure 1. The antiradical activity of tested samples versus concentration.

Comparing the *IC*50 values of horsetail ethanol extract with the *IC*50 values of some other herbal extracts, we can notice the following: the lowest IC50 value has the extract of *Acacia confusa* (5 µg mL^{-1}) [24], then *Reynoutria japonica* (13.68 µg mL^{-1}) [16], *Uncaria tomentosa* (18 µg mL^{-1}) [25], *Anthriscus cerefolium* (45 µg mL^{-1}) [26] and *Polygonum aviculare* (50 µg mL^{-1}) [27].

These results point to the following order of antioxidant capacity of herbal extracts: *Acacia confusa* > *Equisetum arvense* ≈ *Reynoutria japonica* > *Uncaria tomentosa* > *Anthriscus cerefolium* > *Polygonum aviculare*.

The influence of *E. arvense* extract on all tested bacterial cultures depends on the concentration and exposure time. An increase in extract concentration slows down the growth of tested bacterial cultures and decreases the total number of bacteria (Figure 2 and Tables 3 and 4).

Table 2. IC50 and antioxidant activity index (AAI); results represent a mean value of three measurements ± standard deviation

Sample	*IC*50 / µg mL^{-1}	*AAI*	Classification
E. arvense extract	13.5±0.6	3.9±0.2	Very strong antioxidant
BHA	6.5±0.3	8.1±0.4	Very strong antioxidant
Vitamin C	5.4±0.2	9.6±0.4	Very strong antioxidant
Gallic acid	1.5±0.0	35.1±0.7	Very strong antioxidant

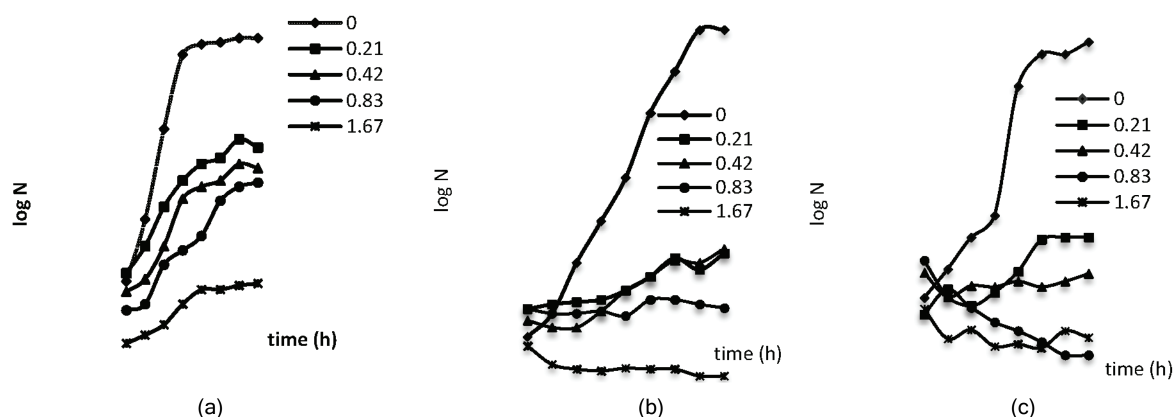

Figure 2. The influence of methanol solution of E. arvense extract on bacterial cell number: a) E. coli, b) S. aureus and c) B. cereus.

Table 3. The influence of methanol solution of E. arvense extract on bacterial cultures growth

Extract concentration, mg mL^{-1}	*E. coli*			*S. aureus*	*B. cereus*
	$T_{1/2}$ / h	*L* / %	*M* / %	*M* / %	*M* / %
0	0.33	-	-	-	-
0.21	0.98	66.67	48.30	81.98	74.32
0.42	0.67	51.13	49.17	74.46	95.54
0.83	1.05	68.76	47.44	98.14	136.89
1.67	1.75	81.26	75.43	103.72	104.57

Table 4. MIC and MBC (both in mg mL^{-1}) for E. coli, S. aureus and B. cereus; Results represent a mean value of two measurements

Bacterium	Extract		Antibiotic[a]	
	MIC	*MBC*	*MIC*	*MBC*
E. coli	44.50	89.10	0.13	0.26
S. aureus	11.14	22.28	0.01	0.02
B. cereus	89.10	-	0.03	0.06

[a]Antibiotic cephalexin was used for *E. coli* and *S. aureus* and doxicyclin for *B. cereus*

The extract had a more pronounced effect on G^+ bacteria *S. aureus* and *B. cereus* than on G^- bacteria *E. coli*. G^+ bacteria are generally more sensitive to herbal extracts compared to G^- bacteria [28,29].

An increase in extract concentration extended the mean generation time ($T_{1/2}$) and decreased the growth rate (L) of *E. coli*. Decrease in the total number of microorganisms (M) was the most significant at the highest concentration of the extract added, so that *MIC* and *MBC* were of expectedly high values. The extract had the strongest antibacterial activity on *S. aureus*, with *MIC* and *MBC* of 11.14 and 22.28 mg mL^{-1} respectively, and the weakest effect on *B. cereus*, with *MIC* of 89.10 mg mL^{-1}.

All extract concentrations influenced *S. aureus* and *B. cereus* cultures and consequently no exponential growth of the populations took place. Therefore, the influence of *E. arvense* extract could not be quantified by calculating the change in bacterial growth rate (L%). After the initial reduction in cell numbers of *B. cereus*, a certain part of bacterial population survived, probably due to ability to form endospores, and thus *MBC* could not be determined.

The ethanol extract of *E. arvense* tested in our experiments had a stronger inhibitory effect (lower MIC values) on *E. coli*, *S. aureus* and *B. cereus* than ethyl acetate, n-butanol and aqueous extracts [5], but weaker than methanol extract against *S. aureus* and *B. cereus* [30]. The ethanol extract also showed a stronger inhibitory effect against various bacteria causing urinary tract infections, observed as a greater inhibition zone [31]. Contrary to our results, *S. aureus* had the lowest sensitivity to the extract, whereas *E. coli* was the most sensitive among the tested bacteria. Similar results with higher sensitivity of *E. coli* compared to the Gram-positive bacteria *B. subtilis* [32] and *S. aureus* [33] were obtained in testing the ethanol extract of *E. arvense* collected in India and Serbia.

Regardless of strain of bacterial culture, it is obvious that *E. arvense* extract exhibited a significant antibacterial and antifungal [6,34] activity. It is especially important to emphasize that much lower concentrations than *MIC* have a certain inhibitory effect on the growth of G^+ bacteria, which is particularly significant since both types of bacteria are known to cause food poisoning.

CONCLUSION

E. arvense ethanol extract exhibits a significant antioxidant and antimicrobial activity. The total phenolic content detected in herbal material amounted to 355.80±17.8 mg GAE/g of the dried extract. Since total phenolic content is often related to extinguishing free radicals and antibacterial activity, it can be assumed that high antioxidant and significant antimicrobial activity of *E. arvense* ethanol extract is directly related to high total phenolic content.

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Nomenclature

GAE	Gallic acid equivalents
FRAP	Ferric reducing antioxidant power
DPPH	2,2-Diphenyl-1-picrylhydrazyl
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene

MIC Minimum inhibitory concentration
MBC Minimum bactericidal concentration
TPTZ 2,4,6-Tri(2-pyridyl)-S-triazine
NA Nutrient agar
NB Nutrient broth
cfu Colony forming units.

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SADRŽAJ UKUPNIH FENOLA, ANTIOKSIDATIVNA I ANTIMIKROBNA AKTIVNOST EKSTRAKTA POLJSKE PRESLICE (*Equisetum arvense* L.)

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NAUČNI RAD

Pripremljen je etanolni ekstrakt osušene biljke preslice, ubrane na području banjalučke regije. Suvi ostatak etanolnog ekstrakta je rastvoren u metanolu i u njemu je određen sadržaj ukupnih fenola, antioksidativna i antimikrobna aktivnost. Sadržaj ukupnih fenola je određen modifikovanom metodom po Folin-Ciocalteu, a antioksidativna aktivnost DPPH i FRAP metodom. Sadržaj ukupnih fenola u ekstraktu preslice iznosi 355,8 mg GEA/g ekstrakta. Antioksidativna aktivnost po FRAP metodi je 28,7 mM Fe(II)/g ekstrakta, dok je ta aktivnost izražena kao indeks antioksidativne aktivnosti (AAI) za stabilne DPPH slobodne radikale iznosila 3,86. Rezultati su upoređeni sa rezultatima dobijenim za kontrolne antioksidanse (galna kiselina, vitamin C, BHA i BHT). Antimikrobna aktivnost je ispitivana na dva načina: praćenjem uticaja različitih koncentracija ekstrakta mjerenjem promjene optičke gustoće bakterijske suspenzije u funkciji vremena i određivanjem MIC i MBC metodom makrorazrjeđivanja u tečnom mediju. Rezultati pokazuju da ekstrakt poljske preslice na testirane mikroorganizme djeluje zavisno od koncentracije i vremena izloženosti. Posmatrajući MIC i MBC, ekstrakt ima najizraženije antibakterijsko djelovanje na Staphylococcus aureus sa MIC i MBC od 11,14 i 22,28 mg/mL, redom, a najslabije na Bacillus cereus sa MIC od 89,10 mg/mL.

Ključne reči: Equisetum arvense L., sadržaj fenola, antioksidativno i antimikrobna aktivnost.