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Anti-oxidative and antimicrobial activities of *Hieracium pilosella* L. extracts

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Abstract: The anti-oxidative and antimicrobial activities of different extracts from *Hieracium pilosella* L. (Asteraceae) whole plant were investigated. The total dry extracts were determined for all the investigated solvents: methanol, dichloromethane, ethyl acetate and dichloromethane:methanol (9:1). It was found that the highest yield was obtained by extraction with methanol (12.9 g/100 g of dry plant material). Qualitative and quantitative analysis were performed by the HPLC method, using external standards. Chlorogenic acid, apigenin-7-*O*--glucoside and umbelliferone were detected in the highest quantity in the extracts. The qualitative and quantitative composition of the extracts depends on the solvent used. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect of the extracts was determined spectrophotometrically. The highest radical scavenging effect was observed in the methanolic extract, both with and without incubation, EC_{50} = 0.012 and EC_{50} = 0.015 mg ml^{-1}, respectively. The antimicrobial activities of the extracts towards the bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella enteritidis* and *Klebsiella pneumoniae*) and the fungi (*Aspergillus niger* and *Candida albicans*) were determined by the disc diffusion method. The minimal inhibitory concentrations were determined for all the investigated extracts against all the mentioned microorganisms.

Keywords: *Hieracium pilosella* L.; anti-oxidant activity; antimicrobial activity; HPLC determination.

INTRODUCTION

Hieracium pilosella L. (family Asteraceae) is a perennial herbaceous plant. It is widely spread in mountains and foothill pastures, in the areas of oak woods and undergrowth. It is mainly used as a traditional medicine for bronchitis, bronchial asthma, edema and as an ointment for wound healing. It is especially recommended for intensifying urination and eliminating slime, sand and small stones from the urinary tract and the kidneys.[1]

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Hieracium pilosella is used in traditional European medicine for its diuretic and anti-inflammatory effects.[2] Extracts obtained from *Hieracium pilosella* leaves have an antibacterial effect on grown cultures of veterinary pathogens: *Brucella abortis* and *Brucella melitensis*.[3] The identification of flavonoids and phenolic acids in this plant species was mostly carried out on the material harvested in areas of New Zealand.[4] The phenolic components most frequently represented in methanol extracts from all *Hieracium* species are: chlorogenic acid (3-{[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid), caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid), and umbelliferone (7-hydroxy-2*H*-1-benzopyran-2-one), and of these, umbelliferone is the most active one.[4–6] The phenolic acids and flavonoids present in the plants are natural antioxidants.[7,8] They also have anti-mutagenic and anti-cancerogenic properties,[9] cardio-protective[10] and antimicrobial activity.[11] Chlorogenic, caffeic and dihydrocaffeic acid, which are present in many plant species, are cinnamic acid derivates. These acids have anti-inflammatory, anti-mutagenic and anti-cancerogenic properties.[12] Antioxidants are important species which have the ability to protect the organism from the damage caused by a free radical-induced oxidative stress.[13] Phenolic acids are mainly antioxidant active due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators. Their antioxidant activity is generally based on the number and the location of hydroxyl groups present, as well as on the presence of a 2–3 double bond and 4-oxo function.[14,15]

Free radicals contribute to more than one hundred disorders in humans, including atherosclerosis, arthritis, ischemia and repercussion injuries of many tissues, central nervous system injury, gastritis and cancer.[16–19] Due to environmental pollutants, radiation, chemicals, toxins, deep-fried and spicy foods, as well as physical stress, free radicals cause depletion of the antioxidants of the immune system, changes in gene expression and induce abnormal proteins. Oxidation processes are one of the most important routes for producing free radicals in food, drugs, and even in living systems.[16,20,21]

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value.[22,23] Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led the authors to investigate the antimicrobial activity of medicinal plants.[22,24,25] Moreover, the increasing use of plant extracts in the food, cosmetic and pharmaceutical industries suggests that, in order to determine the active compounds, a systematic study of medicinal plants is very important.[13,22]

The anti-oxidative and antimicrobial activity of different extracts from dried and ground *Hieracium pilosella* L. (Asteraceae) whole plant material from south-east Serbia were studied. The qualitative and quantitative compositions of the extracts obtained were determined by HPLC analyses.

EXPERIMENTAL

Plant material

Hieracium pilosella L. whole plant was harvested in June 2005 in the area of southeast Serbia. The plant material was dried in the shade in an airy place and then stored in paperbags and kept at room temperature.

Standards

Chlorogenic acid was obtained from Sigma-Aldrich (Steinheim, Germany), and apigenin-7-*O*-glucoside and umbelliferone were purchased from Extrasynthese (Genay, France).

Solvents and reagents

Methanol and acetonitrile were of HPLC grade from Merck (Darmstadt, Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemicals Co., St. Louis, MO, USA. All other chemicals were of analytical-reagent grade (Sigma).

Extraction method

Dried, ground plant material (10 g) was extracted for 48 h at room temperature (plant material to solvent ratio: 1:20, m/v) employing the following solvents: methanol, dichloromethane:methanol (9:1), dichloromethane and ethyl acetate. All the extracts were filtered through No. 1 Whatman filter paper.

Determination of a total yield of extracted substance in Hieracium pilosella *L. plant extracts*

10 ml of extract was added into the vessel of a Scaltec SMO 1 apparatus (Scaltec Instruments, Germany). After drying at 105 °C, the contents of the dry residue mass were read on the apparatus display.

Free radical scavenger activity

The capacity of a compound to scavenge free 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals is determined by the use of the so-called DPPH test.[26-29] The extracts obtained using the various solvents (10 ml) were evaporated on a rotary evaporator at 40 °C until dry, then dissolved in methanol and various concentrations of the methanolic extract solutions were prepared. A 1.0 ml of methanolic solution of DPPH radicals (3×10^{-4} mol dm^{-3}) was added to 2.5 ml sample. The capacity of the scavenging free radicals was calculated using Eq. (1):

$$\text{DPPH radicals scavenging capacity} = 100 - \frac{100(A_S - A_B)}{A_C} \quad (1)$$

where A_S is the sample absorbance at 517 nm of the sample of a methanolic solution of the extract treated with the DPPH radical solution, A_B is the blank absorbance at 517 nm of the blank methanol solution of the extract not treated with the DPPH radical solution and A_C is the control absorbance at 517 nm of the control solution of a pure, non-irradiated methanolic solution of DPPH radical (1.0 ml of DPPH radical of 3×10^{-4} mol dm^{-3} concentration + 2.5 ml of methanol).

The absorbance of the samples was measured on a VARIAN UV–Vis Cary-100 Conc. spectrophotometer. The EC_{50} value (mg ml^{-1}) was determined for all the extracts. The antioxidant capacities of the samples were compared with those of butylated hydroxytoluene (BHT). A decrease by 50 % of the initial DPPH concentration was defined as the EC_{50}.

HPLC analysis

For the quantification of phenolic substances, the extracts of dry homogenized leaves and roots from individual plants were analyzed by HPLC under the following conditions. Appa-

ratus: Agilent 110 Series, Waldborn, Germany; column: Zorbax-Eclipse XDB-CN, 4.6×250 mm, 5 μm; eluent: acetonitrile:water = 30:70 v/v; flow rate: 1.0 ml min^{-1}; task volume: 20 μl; temperature: 25 °C; detection: UV detector at 205 nm.

The quantities of chlorogenic acid, apigenin-7-*O*-glucoside and umbelliferone were determined from calibration curves of the compounds, using external standards.

Microbiological tests

Microorganisms and substrates. The following microorganisms were used for the antimicrobial test. Bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Salmonella enteritidis* (ATCC 13076) and *Klebsiella pneumoniae* (ATCC 13883); fungi: *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231). Substrates used for microorganism growth: antibiotic agar no. 1 for microbiology for bacteria and tryptic soy agar for fungi (Merck, Darmstadt, Germany).

Disc diffusion test. The extracts evaporated on a rotary evaporator (40 °C) were dissolved in dimethyl sulfoxide (DMSO, BDH, Milan, Italy). The substrates were sterilized for 15 min in an autoclave at 121 °C. 0.50 ml of microorganism was added to 50 ml of agar and a 10 ml sample was poured into a petri dish. Filter paper discs (12.7 mm, Schleicher & Schuell) were placed on the inoculated substrate and impregnated with 70 μl of the sample. The plates are incubated for 18 h at 37 °C for the bacteria and 48 h at 25 °C for the fungi. All tests were performed in duplicate and the antibacterial activity is expressed as the average value of the inhibition zones (mm) realized by the plant extracts. The minimum inhibitory concentration (MIC) of the extracts is defined as the smallest extract concentration causing a visible inhibition of the microorganisms.

RESULTS AND DISCUSSION

The DPPH test is based on the exchange of hydrogen atoms between the antioxidant and the stable DPPH free radical. Practically, the reaction brings about the reduction of DPPH radicals to the corresponding hydrazine, which is manifested by a color change from violet to yellow, which is monitored spectrophotometrically. The results for methanol, dichloromethane:methanol, ethyl acetate and dichloromethane extracts, are shown in Fig. 1.

All the extracts show a higher DPPH radicals scavenging capacity after incubation (20 min) with a free radical solution.

The DPPH scavenging capacity of the studied extracts at a concentration of 0.18 mg ml^{-1} decreased in the following sequence: MeOH > EtOAc > CH_2Cl_2: :MeOH >> CH_2Cl_2, the values being 95.53, 94.54, 87.62, and 19.83 % (20 min incubation time) and 95.33, 67.7, 51.75, and 12.7 % (without incubation), respectively. The methanol extract showed the highest anti-oxidative activity. The EC_{50} values for all extracts and BHT are given in Table I.

Unlike the examined extracts, the DPPH test performed without incubation showed that the standard BHT antioxidant did not attain the EC_{50} value at a concentration of 0.18 mg ml^{-1}. In the case of DPPH test performed with a 20-min incubation, the BHT concentration necessary for reaching EC_{50} was 0.021 mg ml^{-1}. The concentrations of the dichloromethane:methanol, ethyl acetate and dichloromethane extracts required to attain EC_{50} were higher than that of BHT. In

producing this effect, the methanol extract concentration was lower than that of BHT. The obtained data show that the methanol extract was a better antioxidant than the BHT standard of the same concentration.

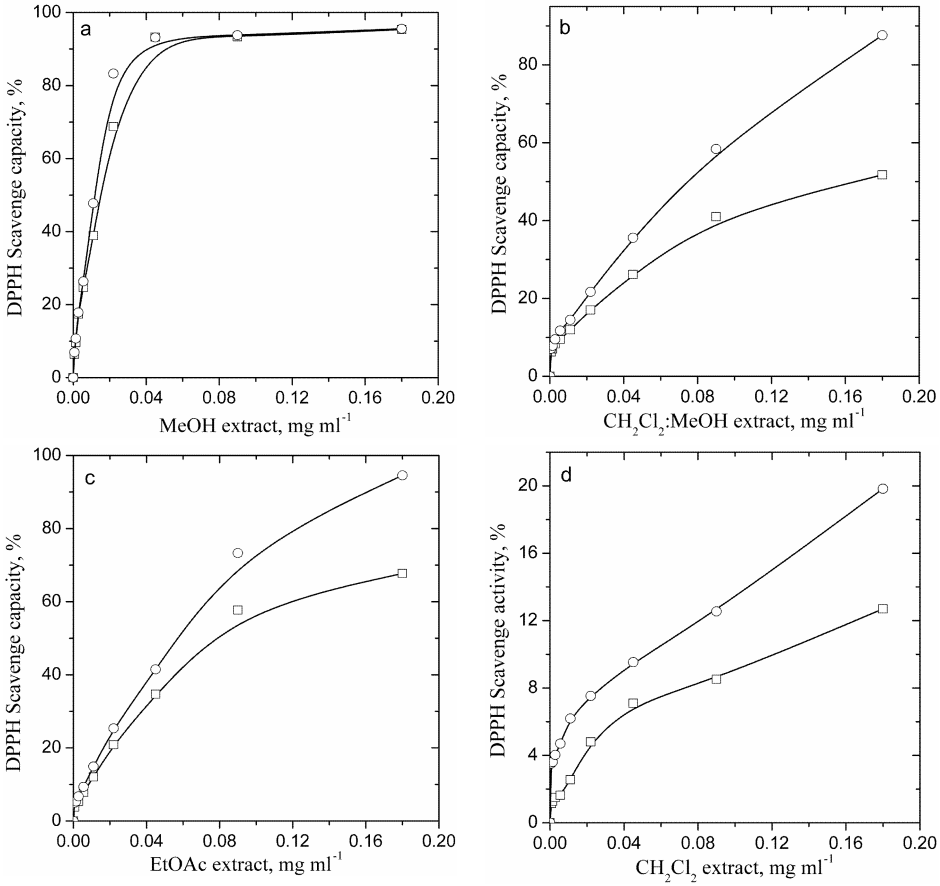

Fig. 1. DPPH scavenging capacity of the MeOH (a), CH_2Cl_2:MeOH (b), EtOAc (c) and CH_2Cl_2 (d) extracts of *Hieracium pilosella* L.; (–□–) without incubation; (–○–) 20 min of incubation.

The carriers of the anti-oxidative activity are phenolic acids and flavonoids extracted from the plant material.[7,8] In the DPPH test, different extracts showed different anti-oxidative activities. This is the consequence of the different polarities of the employed solvents, which extract different components from the plant material and, therefore, the composition of the extracts differed.

Total quantities of substances extracted using the different solvents per 100 g of dry plant material are given in Table II, while the retention times and calibration curves of the estimated compounds in the *Hieracium pilosella* L. extracts are given in Table III.

TABLE I. EC_{50} values (mg ml^{-1}) of the different extracts

	Extract				
	MeOH	CH_2Cl_2:MeOH (9:1)	EtOAc	CH_2Cl_2[a]	BHT
Without incubation	0.015	0.167	0.079	> 0.18	EC_{50} not achieved
20 min incubation	0.012	0.075	0.058	> 0.18	0.021

TABLE II. Total quantity of dry substance extracted by the different solvents

Solvent	Total extract, g per 100 g of d.p.m.[a]
MeOH	12.9
CH_2Cl_2:MeOH (9:1)	5.2
CH_2Cl_2	3.7
EtOAc	4.0

[a]Dry plant material

TABLE III. Calibration curves and retention times of the estimated compounds in the *Hieracium pilosella* L. extracts

Compound		Chlorogenic acid	Apigenin-7-*O*-glucoside	Umbelliferone
Retention time		2.01	4.81	5.67
min				
Concentration range		1–500	0.15–15	4–670
μg ml^{-1}				
Calibration curve[a]	q	75.84	60.08	235.61
$P = q + rc$	r	30891.11	79938.97	153295.95
Correlation coefficient		0.9998	0.9997	0.9998

[a]P, mAU: peak area; c, mg ml^{-1}: concentration of the standard sample; q and r: constants

Based on HPLC analysis and the calibration curves of the standard samples, the contents of the bioactive components were determined in all the extracts (Table IV).

TABLE IV. The content of bioactive components in g per 100 g of the total dry extracts or the dry plant material

Extract	Chlorogenic acid		Umbelliferone		Apigenin-7-*O*-glucoside	
	Dry extract	Dry plant material	Dry extract	Dry plant material	Dry extract	Dry plant material
MeOH	35.45	4.58	2.54	0.32	1.20	0.150
CH_2Cl_2:MeOH (9:1)	16.60	0.86	11.66	0.61	1.80	0.094
CH_2Cl_2	0.58	0.02	6.35	0.23	0.03	0.01
EtOAc	2.65	0.12	5.15	0.20	0.97	0.039

The highest quantities of chlorogenic acid and apigenin-7-*O*-glucoside were extracted using methanol: 4.58 and 0.15 g/100 g of d.p.m., respectively, and the highest yield of umbelliferone was obtained using dichloromethane:methanol, 0.61 g/100 g d.p.m. Chlorogenic acid is a natural antioxidant.[7,8,23] The methanolic extract contained the highest quantity of chlorogenic acid and showed the highest antioxidant activity. Such a composition results in the extracts having dif-

ferent anti-oxidative and antimicrobial activities. Since phenolic acids are well--known natural antioxidants[7,8] and have antimicrobial properties,[11] such an extract composition only confirms the good anti-oxidative properties, especially of the extracts obtained with methanol.

The results of the microbiological tests and MIC determinations are given in Fig. 2.

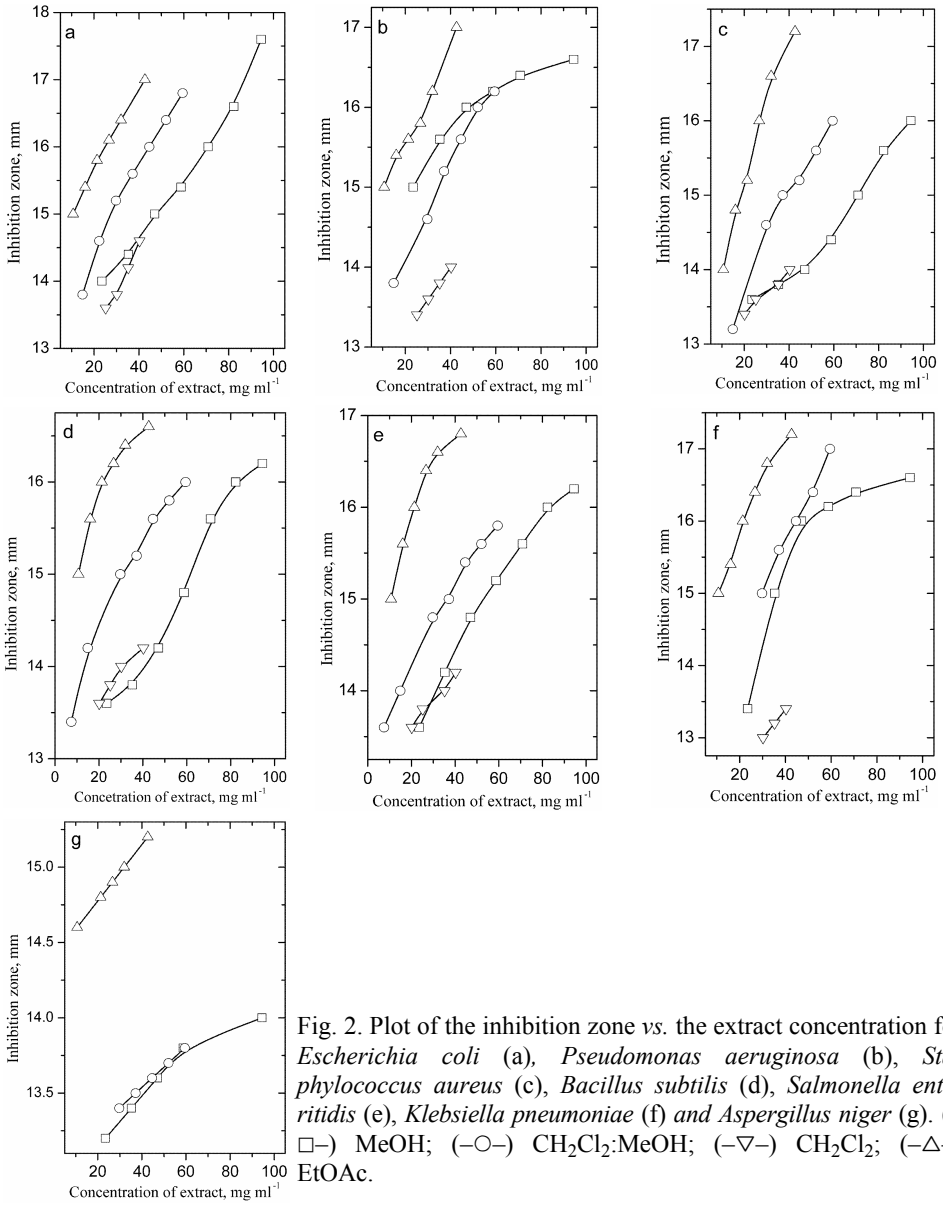

Fig. 2. Plot of the inhibition zone *vs.* the extract concentration for *Escherichia coli* (a)*, Pseudomonas aeruginosa* (b), *Staphylococcus aureus* (c), *Bacillus subtilis* (d), *Salmonella enteritidis* (e), *Klebsiella pneumoniae* (f) *and Aspergillus niger* (g). (–□–) MeOH; (–○–) CH_2Cl_2:MeOH; (–▽–) CH_2Cl_2; (–△–) EtOAc.

All the tested extracts (Fig. 2) demonstrated some antimicrobial activity. All the extracts concentrations less than 30 mg ml^{-1} had an antimicrobial effect on the bacteria *Staphylococcus aureus* and *Bacillus subtilis*. All the extracts showed antimicrobial activity against *Escherichia coli*, whereby the ethyl acetate extract was active at the lowest concentration. In comparison to the other extracts, the ethyl acetate extract was active against the bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* at the lowest concentration (Figs. 2b and 2f). The dichloromethane:methanol extract was active at the lowest concentration (7.44 mg ml^{-1}) against *Salmonella enteritidis* (Fig. 2e). The ethyl acetate extract had the best effect on the fungus *Aspergillus niger*, while dichloromethane extract had no effect at all (Fig. 2g). All the extracts studied were inactive against *Candida albicans*.

The *MIC* values of the different extracts are given in Table V.

TABLE V. *MIC* values (mg ml^{-1}) of the different extracts

Microorganism	Extract			
	MeOH	CH_2Cl_2:MeOH (9:1)	CH_2Cl_2	EtOAc
Escherichia coli	11.75	14.87	25.16	10.66
Pseudomonas aeruginosa	23.5	14.87	25.16	10.66
Staphylococcus aureus	23.5	14.87	20.12	10.66
Bacillus subtilis	23.5	7.44	20.12	10.66
Salmonella enteritidis	23.5	7.44	20.12	10.66
Klebsiella pneumoniae	23.5	29.75	30.19	10.66
Aspergillus niger	23.5	29.75	No effect	10.66

The lowest *MIC* values for all microorganisms was shown by the ethyl acetate extract, while dichloromethane:methanol extract has a wide range of *MIC* values, the lowest *MIC* value (7.44 mg ml^{-1}) being for *B. subtilis* and *S. enteritisdis*. The activity of the obtained extracts was more pronounced against bacterial than against fungal organisms. Some authors attribute the antimicrobial activity to the phenolic components.[30] Investigations have demonstrated the presence of flavonoids and triterpenes in the species of the genus *Hieracium*, which are carriers of antimicrobial activity.[22] The antifungal compounds of the plants are not well known. However, the presence of flavonoids and terpenes and a certain degree of lipophilicity might determine the toxicity by interactions with the membrane constituents and their arrangement.[22] However, it is difficult to compare the data with the literature because several variables influence the results, such as the environmental and climatic conditions of the plant, and the choice of the extraction method and the antimicrobial test. Moreover, standard criteria for the evaluation of the plant activity are lacking and therefore the results obtained by different authors differ widely.[22]

CONCLUSIONS

The highest yield was obtained by extraction with methanol (12.9 g/100 g of dry plant material). Chlorogenic acid, apigenin-7-*O*-glucoside and umbelliferone

were detected in the highest quantities in the extracts. The qualitative and quantitative composition of the extracts depended on the solvent used. The highest radical scavenging effect was observed in a methanolic extract with and without incubation, EC_{50} = 0.012 and 0.015 mg ml^{-1}, respectively. The lowest *MIC* values for all microorganisms was shown by the ethyl acetate extract, while the dichloromethane:methanol extract had a wide range of *MIC* values, the lowest *MIC* value (7.44 mg ml^{-1}) being for *Bacillus subtilis* and *Salmonella enteritidis*.

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И З В О Д

АНТИОКСИДАТИВНА И АНТИМИКРОБНА АКТИВНОСТ ЕКСТРАКАТА *Hieracium pilosella* L.

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Испитивана је антиоксидативна и антимикробна активност различитих екстраката добијених из целе биљке *Hieracium pilosella* L. (Asteraceae). Укупни суви екстракти одређени су за све екстракте, метанолни, дихлорметански, етилацетатни и дихлорметан:метанолни (9:1) и показано је да је највећи принос постигнут са метанолом (12.9 g/100 g сувог биљног материјала). Квалитативни и квантитативни садржај је анализиран помоћу HPLC методе коришћењем екстерних стандарада. Идентификоване су три најзаступљеније компоненте у екстрактима: хлорогенска киселина, апигенин-7-*O*-глукозид и умбелиферон. Установљено је да квалитативни и квантитативни састав екстраката зависи од врсте растварача. Способност неутралисања 1,1-дифенил-2-пикрил-хидразил (DPPH) радикала екстрактима одређена је спектрофотометријски. Највећа способност неутралисања радикала остварује се метанолним екстрактом са инкубацијом и без ње (EC_{50} = 0.012, односно 0.015 mg ml^{-1}). Антимикробна активност на бактерије (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Salmonella enteritidis* и *Klebsiella pneumoniae*) и гљиве (*Aspergillus niger* и *Candida albicans*) одређена је применом диск дифузионе методе. Одређене су минималне инхибиторне концентрације (*MIC*) за све испитиване екстракте за наведене микроорганизме.

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