International Journal of Food Properties

ISSN: 1094-2912 (Print) 1532-2386 (Online) Journal homepage: https://www.tandfonline.com/loi/ljfp20

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To cite this article: Sanja Vlaisavljević, Biljana Kaurinović, Mira Popović & Sanja Vasiljević (2017) Profile of phenolic compounds in *Trifolium pratense* L. extracts at different growth stages and their biological activities, International Journal of Food Properties, 20:12, 3090-3101, DOI: 10.1080/10942912.2016.1273235

To link to this article: https://doi.org/10.1080/10942912.2016.1273235

INTERNATIONAL JOURNAL OF FOOD PROPERTIES
2017, VOL. 20, NO. 12, 3090–3101
https://doi.org/10.1080/10942912.2016.1273235

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Profile of phenolic compounds in *Trifolium pratense* L. extracts at different growth stages and their biological activities

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ABSTRACT

Although *Trifolium pratense* (Red Clover) is considered to be one of the leading crops for livestock grazing, it could also be used as a potential source of bioactive compounds in phytopharmacy. The aim of this study was to investigate the phenolic content and its biological activity at the growth phases (30 cm, 50 cm, and bud) of this plant. The phenolic compounds in methanolic extracts of *T. pratense* leaves at three growth stages, obtained by Microwave Assisted Extraction, were quantified using the HPLC-ESI-MS/MS technique, and their antioxidant and antimicrobial activity were assessed. Isoflavonoids, genistein, and daidzein, as well as other phenols, p-hydroxybenzoic and caffeic acids, kaempferol 3-O-glucoside, quercetin 3-O-glucoside, and hyperoside were found in all the extracts, but the content of these compounds was the highest in the extract of the plant at the lowest growth stage (30 cm, vegetative). Therefore, this extract showed the best antioxidant potential and it was most effective against bacterial strains such as *Escherichia coli* and *Enterobacter aerogenes*. These results indicated that red clover has potential health benefits, and that growth phase contributes to its biological activity. The extract of red clover at the growth stage of 30 cm is a great source of bioactive compounds and could be used in phytotherapy and nutrition.

ARTICLE HISTORY
Received 29 August 2016
Accepted 13 December 2016

KEYWORDS
Antibacterial activity; Antioxidant activity; HPLC-ESI-MS/MS technique; Phenols; *Trifolium pratense* L

Introduction

Trifolium pratense L. (Fabaceae) is widely spread forage crop in temperate and subtropical regions of both hemispheres. In Serbia, red clover is the second most important perennial forage legume.[1] Institute of Field and Vegetable Crops in Novi Sad began a concentrated effort on the collection of red clover in order to obtain as much genetic variability as possible for the subsequent breeding of this crop. Research of the current Institute's red clover collection has been started on the characterization and evaluation of the most important morphological, biological, phytochemical, and agronomic traits in the field conditions. This species has both agronomic importance and many health benefits. The bioactive compounds have been identified in this plant exhibit health benefits for humans, including antioxidative and anti-inflammatory properties.[2,3] These bioactive compounds also occur in a normal diet, in a transformed form, via meat of livestock that feed on clover as well as milk and dairy products.[4] There are several reports on polyphenols with emphasis on isoflavonoid contents. [5–7] The isoflavonoids belong to the group of phytoestrogens that are used in hormone replacement therapy.[8] It is well known that they are wide spread in many plants.[9]The most studies which are related to investigation of their activity and content were focused on extracts *Glycine max* (Soya)[10] and *Cimicifuga racemosa*. The aim of some other studies was to evaluate the biological activity of *T. pratense* as a potential source of dietary supplements and herbal remedies administered as an alternative to the conventional hormonal replacement therapy.-[11–13]. The activity and benefits of phenolic compounds in red clover, used in human nutrition, have

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received little attention. **Only certain classes of phenolic compounds, presented in red clover**, have been described and mentioned in previous works.[14–17]. Hence, it encouraged us to extend them through analyzing the phenol profile and study some of *in vitro* biological activities of methanolic extracts. Investigations were carried out on the dry plant at three stages of growth, starting from the assumption that the growth may have a significant influence on the content of phenolic compounds and therefore on the antioxidant and antimicrobial properties. The obtained results could represent a novel opportunity for human food science and health promotion.

Phytochemical investigation of methanolic extracts included qualitative and quantitative determination of 44 selected phenolic compounds using the LC/MS-MS technique. Also, the total phenolics and flavonoids were quantified. The antioxidant activity of the MeOH extracts of *T. pratense* was determined *in vitro* via neutralization of the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical, hydroxyl ($OH^{\bullet}$) radical, superoxide anion ($O_2^{\bullet -}$) radical, nitric oxide ($NO^{\bullet}$), and hydrogen peroxide (H_2O_2), as well as measuring total reducing potential of the examined samples and the inhibition of lipid peroxidation (LPx) in the Fe^{2+}/ascorbate induction system. Furthermore, a collection of six test organisms, including both Gram-positive and Gram-negative bacterial strains, was used for the evaluation of antibacterial activity.

Materials and methods

Standards and reagents

Deionized water was produced using the Elga Lab Water Purelab Option-Q water purification system. HPLC gradient grade methanol and p.a. methanol were purchased from J.T. Baker (Deventer, The Netherlands) (purity> 98% by HPLC), and p.a. formic acid (purity> 98% by HPLC) and DMSO from Merck (Darmstadt, Germany). Reference standards of the compounds used in this study were obtained from Sigma–Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland), and from Chromadex (Santa Ana, USA).

Plant material

The leaves of cultivated *Trifolium pratense* L. (*Una* variety) were collected from planting at Institute of Field and Vegetable Crops, BačkoGradište – Rimski Šanac in the Vojvodina Province, Republic of Serbia. Samples: No 1: *Trifolium pratense* L. – 30 cm heights of growth, vegetative, stage of forming leaf rosette; No 2: *Trifolium pratense* L. – 50 cm heights of growth, vegetative, the beginning stage of forming stems and branching; No 3: *Trifolium pratense* L. – bud phase. Red clover herbage was dried at 60°C for about 48 h. Particular attention was paid to making sure that the samples were homogenized and ground to a particle size of Æ = 0.8 mm

Extracts preparation

The extracts were prepared using the Microwave Assisted Extraction. The method was used for the first time in this study. Experiment was performed with a single-mode Discover BenchMate microwave reactor from CEM Corporation (Matthews NC USA) with a maximum output power of 300 W and with an IR temperature sensor positioned at the bottom of the cavity below the vessel. The microwave reactor covers a variety of reaction conditions in open- (up to 125 mL) and closed-vessel systems (up to 50 mL filling volume). Extraction in the present study was performed in the open-vessel system. Since the experiments were carried out in temperature control mode, the microwave magnetron power constantly regulates itself from a maximum value at the beginning of heating (thus reaching the desired temperature faster) to a lower one after the set temperature of 70°C is reached and also during the experiment to keep the sample of water heated at 70°C. Previously chopped dried plant material (5 g) was placed in the flask (100 mL) and coated with 50 mL of methanol (MeOH). The extraction lasted 20 min. After the filtration

the solvent evaporated *in vacuo* at 45°C. Dried extracts were dissolved in 70% MeOH (v/v) to obtain 100 mg/mL (stock solution).

LC-MS/MS quantification of the selected phenols and flavonoids

The analyses were performed on the Agilent Technologies 1200 series HPLC with Agilent Technologies 6410A series electrospray ionization triple-quadrupole MS/MS with electrospray ionization (ESI). The injection volume for all samples was 5 µL. The separation was achieved using a Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm × 1.8 µm (Agilent Technologies)), and thermostated at 50°C, with a flow rate of 1 mL/min of mobile phase whose composition is: A = 0.05% aqueous formic acid, B = methanol. Gradient elution was performed using the following solvent gradient: starting with 30% B, reaching 70% B in 6 min, and holding until 12 min, with post-time of 3 min. Detection of eluted components, ionized by ESI was performed in dynamic Selected Reactions Monitoring mode (SRM), in following ion source: negative ion polarity, nebulizer gas (N_2), flow 9 L/min, pressure 40 psi, temperature 350° C, capillary voltage 4 kV. Peak areas in the chromatograms were read using the Agilent Mass Hunter Workstation Software-Qualitative Analysis (Ver. B.03.01). The calibration curves were constructed from peak areas of different concentrations of the reference standard (from 1.53 ng/mL to $25.0.10^3$ ng/mL) using the equation for linear regression obtained from the calibration curves (R^2>0.995). LC-MS/MS data for standard compounds are presented in Table 1.

Total phenolic content

A modification of the method of Fukumoto and Mazz[18] was performed.[19] Extracts were used in concentration of 0.125, 0.25 and 0.5 mg ml^{-1}. Gallic acid (0.625 to 100 µg ml^{-1}), was used as a standard. Thirty microliters of each extract or standard solution, except in a blank probe when only the solvent was used, was added to 150 µL of 0.1 $molL^{-1}$ FC reagent and mixed with 120 µL of sodium carbonate (7.5%) after 10 min. The mixture was incubated in the dark at room temperature (2 h). The absorbance of the resulting solution was measured at 760 nm. The phenolics concentration was determined by comparison with the standard calibration curve of gallic acid, and results are presented as a mean value of triplicate tests. The total phenol value was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (dw) of extract, calculated according to the standard calibration curve.

Total flavonoid content

The aluminum chloride colorimetric assay described by Jia et al.[19] adapted for 96-well microplates, was used to determine the total content of flavonoids. Test samples were prepared in concentrations 1.0, 2.0, and 4.0 mg ml^{-1}, whereas quercetin solutions were prepared ranging from 0.625 to 80 µg ml^{-1} and used as a standard. Thirty microliters of extract or standard solution was diluted with 90 µL of methanol, and 6 µL of 10% aluminum chloride (substituted with distilled water in blank probe), 6 µL of 1 mol/L potassium acetate, and 170 µL of distilled water were added. Absorbance at 415 nm was determined after 30 min. All samples were made in triplicate, and mean values of flavonoid content are expressed as milligrams of quercetin equivalents (QE) per gram of dry weight (dw) of extract, calculated according to the standard calibration curve.

DPPH Assay

Plant extracts were tested for the scavenging effect on the DPPH radical according to Sanchez-Moreno et al.[20] Synthetic antioxidants, BHT and BHA, were used as a positive control. Ten microliters of the examined extract solutions, in series of different concentrations (2.50–125.00 µg ml^{-1}), was added to 100 µL of 90 µ$molL^{-1}$ DPPH solution in methanol and the mixture was diluted with 190 µL of methanol. In control, the exact amount of the extract was substituted with solvent,

Table 1. LC-MS/MS data for standard compounds.

	LC-MS/MS conditions for quantification of standard compounds				
Compound	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Fragmentor voltage (V)
Hinic acid	0.52	191	85	20	150
Gallic acid	0.61	169	125	10	90
Catechin	0.75	289	245	10	150
Protocatechuic acid	0.80	153	109	9	105
Epigallocatechin gallate	0.80	457	169	16	165
Chlorogenic acid	0.84	353	191	10	100
Epicatechin	1.01	289	245	10	150
2,5-Dihydroxybenzoic acid	1.05	153	109	9	100
p-Hydroxybenzoic acid	1.11	137	93	10	80
Aesculetin	1.12	177	133	15	105
Caffeic acid	1.17	179	135	10	100
Vanillic acid	1.26	167	108	15	100
Syringic acid	1.32	197	182	7	90
p-Coumaric acid	1.68	163	119	9	90
Scopoletin	1.73	191	176	8	80
Umbelliferone	1.76	161	133	19	120
Ferulic acid	1.89	193	134	11	90
Sinapic acid	1.89	223	193	17	100
Vitexin	1.91	431	311	22	200
Luteolin 7-O-glucoside	2.15	447	285	30	230
Hyperoside	2.18	463	300	30	200
Rutin	2.21	609	300	42	135
Quercetin 3-O-glucoside	2.25	463	300	30	210
Apiin	2.61	563	269	36	250
o-Coumaric acid	2.63	163	119	5	100
Myricetin	2.70	317	179	20	150
Apigenin 7-O-glucoside	2.71	447	284	30	190
Quercitrin	2.78	447	300	27	190
Kaempferol 3-O-glucoside	2.85	431	268	41	135
Secoisolariciresinol	2.87	361	165	26	130
3,4-Dimethoxycinnamic acid	3.00	207	103	7	110
Baicalin	3.36	445	269	22	140
Daidzein	3.43	253	208	31	145
Matairesinol	3.66	357	122	24	130
Quercetin	3.68	301	151	15	130
Naringenin	3.86	271	151	16	130
Cinnamic acid	3.91	147	103	5	100
Luteolin	3.96	285	133	25	135
Genistein	4.13	269	133	32	145
Kaempferol	4.52	285	285	0	130
Apigenin	4.64	269	117	25	130
Isorhamnetin	4.79	315	300	21	160
Chrysoeriol	4.81	299	284	20	125
Baicalein	5.15	269	269	0	165
Amentoflavone	5.81	537	375	35	220

and in the blank probe, only methanol (290 μL) and extract (10 μL) were mixed. After 1 h, measurements of absorbance were done at 515 nm.

Hydroxyl-radical (HO˙) scavenger capacity assay

Scavenging capacity was determined by monitoring the chemical degradation of 2-deoxy-D-ribose.-[21] A 50 $\mu molL^{-1}$ solution of 2-deoxy-D-ribose in phosphate buffer pH 7.4 (0.1 mL) was mixed with 20 μL of extract (concentration ranging from 2.5 to 100 μgml^{-1}) or solvent in control, 0.1 mL H_2O_2 (0.015%), 0.1 mL $FeSO_4$ (10 $mmolL^{-1}$) and diluted with 2.7 mL of phosphate buffer, pH 7.4. Three milliliters of phosphate buffer and 20 μL of extract were added in the blank probe. After incubation at 37°C for 1 h, a 0.1 mol/L EDTA solution (0.2 mL) was added to all the samples. Thiobarbituric

acid-reactivity was developed by adding 2 mL of aqueous mixture containing TBA (3.75 $mgml^{-1}$), $HClO_4$ (1.3%), and trichloroacetic acid (0.15 g mL^{-1}), followed by heating at 100°C for 10 min. The absorbance of the cooled mixtures was measured at 532 nm.

Superoxide anion scavenger capacity ($O_2^{\bullet-}$) assay

The capability of extracts to neutralize superoxide anion formed by the reduction of nitroblue tetrazolium (NBT) with NADH mediated by phenazinemethosulfate (PMS) under aerobic conditions was conducted according to Cos et al. (1998).[22] The mixture of 0.2 mL NBT (144 $\mu molL^{-1}$), 10 µL of extract (concentration ranging from 2.50 to 125.0 μgmL^{-1}, substituted with solvent in control), 0.1 mL of NADH (0.68 $mmolL^{-1}$), and freshly prepared PMS (60 $\mu molL^{-1}$) was diluted with 1.1 mL of phosphate buffer, pH 8.3. The blank probe was prepared by mixing 1.5 mL of buffer and 10 µL of extract. Absorbance was measured at 560 nm after 5 min.

NO scavenger capacity assay

Test of nitric oxide radical($NO^{\bullet}$) scavenging capacity was based on method of Green et al. (1982), adapted for 96-well microplates.[23] The reaction mixture containing sodium nitroprusside (10 $mmolL^{-1}$, 75 µL), phosphate buffer, pH 7.4 (75 µL) and extract (10 µL, concentration ranging from 2.50 to 125.0 μgmL^{-1}) or standard solution (BHT, BHA) was incubated at 25° C for 90 min. Ten microliters of extract and 150 µL of buffer were added in the blank probe. After incubation, 150 µL of solution prepared by mixing equal amounts of sulfanilamide (2% in 4% phosphoric acid) and *N*-(1-naphthyl) ethylenediaminedihydrochloride (0.2%) was added to the reaction mixture and allowed to stand for 3 min. The absorbance of these solutions was measured at 546 nm against appropriate blanks.

Reduction of the H_2O_2

Scavenging activity on H_2O_2 was carried out according to the method of Ruch et al. (1989).[24] A solution of H_2O_2 (40 mM) was freshly prepared in 0.05 M KH_2PO_4–K_2HPO_4 phosphate buffer (pH 7.4). The samples (from 2.50 to 50.00 μgmL^{-1}) were mixed with phosphate buffer (3.4 mL) and 40 mM H_2O_2 (0.6 mL).

Inhibition of lipid peroxidation

The inhibition of lipid peroxidation was modified from the methods of Buege and Aust[25] and Fukuzawa et al.[26] The inhibition of lipid peroxidationwas determined by measuring the intense color of adduct produced in the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde (MDA), as an oxidation product in the peroxidation of membrane lipids. The commercial preparation of liposomes 'PRO-LIPO S' (Lucas-Meyer) pH = 5–7 was used as a model system of biological membranes. The liposomes, 225–250 nm in diameter, were obtained by dissolving the commercial preparation in demineralized water (1:10), in an ultrasonic bath. The content of the MDA (TBARS) was determined by measuring the absorbance of adduct at 532 nm. In a test tube, a suspension of liposomes (50 µL) was incubated with 0.01 M $FeSO_4$ (20 µL), 0.01 M ascorbic acid (20 µL), and extract (10 µL; concentration ranging from 0.5 to 100 μgmL^{-1}, substituted with solvent in control) in 0.05 M KH_2PO_4–K_2HPO_4 buffer (2.90 mL, pH 7.4, 3 mL final solution). Samples were incubated at 37°C for 1 h. LP was terminated using the reaction with 1.5 mL of TBA reagent and 0.2 or 0.1 mL of EDTA, heated at 100°C for 20 min. After precipitated proteins were cooled and centrifuged (4000 rpm for r 10 min), the content of the MDA (TBARS) was determined by measuring the absorbance of adduct at 532 nm.

Reducing power – FRAP assay

To evaluate the reducing power of the extracts, the ferric ion reducing antioxidant power (FRAP) assay,[27] modified for 96-well microplates, was undertaken. Extracts were prepared in concentration of 0.5, 1.0, and 2.0 mg mL^{-1}, in which ascorbic acid ranging from 1.25 to 140 μgmL^{-1} was used to create a standard curve. FRAP reagent was prepared by mixing 10 mmolL^{-1} 2,4,6-tripyridyl-*s*-triazine (TPTZ) in 40 mmolL^{-1}HCl, 0.02 mol/L $FeCl_3$, and acetate buffer, pH 3.6, in ratio of 1:1:10, respectively. Following the addition of extract or ascorbic acid (10 μL) to 290 μL of FRAP reagent (substituted with distilled water in the blank probe), absorbance at 593 nm was determined after 6 min. Three replicates were performed for each sample and mean values of reducing power were expressed as milligrams of ascorbic acid equivalents (AAE) per gram of dry weight of extract, calculated according to the standard calibration curve.

Antimicrobial activity

Bacterial cultures

The antimicrobial activity of the extract tested against the bacterial strans: *Pseudomonas aeruginosa* (ATCC 35554), *Escherichia coli* (ATCC 11775), *Enterococcus faecalis* (ATCC 19433), *Enterobacter aerogenes* (ATCC 13048), *Staphylococcus aureus* (ATCC 25923), and *Bacillus cereus* (ATCC 11778). Microorganism strains were employed for the determination of antimicrobial activity. The cultures of the test bacteria were grown 20–24 h in Müller-Hinton agar (Torlak, Belgrade, Serbia) at 37°C.

Disc diffusion assay

Evaluation of the antibacterial activity of the samples was performed according to the Kirby–Bauerdisk-susceptibility standard method. Agar disc diffusion method was used for the evaluation of the antibacterial activity of the samples. The strains were grown on Mueller–Hinton agar slants at 37°C for 24 h and checked for purity. After the incubation, the cells were washed off the surface of agar and suspended in sterile physiological solution. The number of cells in 1 mL of suspension for inoculation measured by McFarland nefelometer was 5×10^7 CFU mL^{-1}.[28] One mL of this suspension was homogenized with 9 mL of melted (45°C) Mueller–Hinton agar and poured into Petri dishes. On the surface of the agar, 6 mm diameter paper discs (Bioanalyse Ltd., Ankara, Turkey) were applied and impregnated with 15 μL of samples. The plates were incubated 24 h at 37°C and the diameters of the resulting zones of inhibition (ZI) were measured and expressed in mm.[28] The diameters of the resulting zones of inhibition (ZI) were measured and expressed in mm.

Microdilution assay

This method was performed in order to determine the minimum inhibitory concentration (MIC).[29] Minimal concentration of the extracts which prevents the visible growth of bacteria represents the minimal inhibitory concentration (MIC). Microtiter plates (Spektar, Čačak, Serbia) were filled with nutrient broth, double dilution series of essential oils (from 0.35 to 45.4 % v/v) and suspension of bacteria in the ratio 10:1:0.1. Tests were performed in triplicates at each concentration. Microtiter plates were left at 37°C over night and the growth was compared with control well without oils. Determination of MBC was done when 100 μl from the well with no turbidity was plated in Petri plates with nutrient agar and left for 24 h. Two fold dilutions of essential oils in 0.5% v/v TWEEN 80 due to their lipophility were analyzed in the concentration range from pure oil to 1:500. Control wells without tested compounds or tested bacteria were also included for each plate. Minimal concentration of the extracts which prevents the visible growth of bacteria represents the minimal inhibitory concentration (MIC).

Statistical analysis

All measurements were performed in triplicate. The percentage of inhibition *I (%)* (obtained by different concentration) for each radical species was calculated using the following equation: $I\ (\%) = 100 \times (A_{blank} - A_{sample})/A_{blank}$, where A_{blank} was the absorbance of the control reaction and A_{sample} was the absorbance of the examined samples, corrected for the value of the blank probe. From the obtained *I (%)* values, the IC_{50} values (which represented the concentrations of the examined extracts that caused 50% neutralization) were determined by linear regression analysis, using Origin software, version 8.0. All of the results were expressed as mean ± SD of three different trials. A comparison of the group means and the significance between the groups were verified by one-way ANOVA. Statistical significance was set at $p<0.05$.

Results and discussion

LC-MS/MS analysis of the selected phenols and flavonoids

Evaluation of phytochemical composition of extracts of samples No1 (30 cm growth), No2 (50 cm growth), and No3 (bud phase) included qualitative and quantitative analysis of 43 selected phenolic components (15 phenolic acids, 23 flavonoids, 3 coumarins, and 2 lignans; Table 1) using a specific and highly selective LC-MS/MS technique, whilst the MRM mode was applied as the preferred acquisition method. Targeted MS analysis using MRM allows monitoring of the specific ions of targeted analytes, which enables more accurate quantification and increases the lower detection limit. Data concerning the content of examined phenolics are presented in Table 2.

From the literature data it can be concluded that isoflavones are the most common compounds in *T. pratense* extracts. Plant isoflavones are mostly present in the glycoside form in plant. They can be transformed into their corresponding aglycones by intestinal microflora, high temperature or by addition of acid.[5,7,14]

However, tested extracts in this work contained many other phenols. Their identification is also important for a better understanding of the biochemical mechanisms and interactions which occur between them. This the first study done on the Una variety of red clover phenolic production related to the growth stage under the environmental conditions typical of Vojvodina, Serbia (45°39′58.02″N 19°04′51.16″E).

Daidzein and genistein were detected in all three methanolic extracts of samples No1 (30 cm growth), No2 (50 cm growth), and No3 (bud). The highest content of these compounds was determined in the sample No1 (1.527 μg g^{-1} of daidzein and 0.588 μg g^{-1} of genistein). This result (Table 2) can be explained by the fact that the difference in daidzein and genistein content among the growth stages reflects biochemical changes occurring during growth, as well as differences in reactions to biotic and abiotic stresses at different growth stages.[15]

Fifteen phenolic acids were examined in this study. The largest amount of caffeic acid was detected in the sample of *T. pratense* No 1 (30 cm growth) (1.041 μg g^{-1}), while protocatechuic acid, 2,5-dihydroxybenzoic acid and p-coumaric acid were found only in the extracts of No 1 (30 cm growth) and No 2 (50 cm growth). In addition, very low amounts of apigenin and luteolin were quantified in all three samples. Evidently, investigated *T. pratense* extracts were rich in glycosides, kaempferol 3-O-glucoside, quercetin 3-O-glucoside (8.544 μg g^{-1}; 24.243 μg g^{-1} in extract No1-30 cm growth) while their corresponding aglycones were found in the traces (kaempferol, quercetin). The most dominant flavonol glycosides identified in all three samples of *T. pratense* was hyperoside that possess cytoprotective properties, but the largest amount of this compound was found in sample No 1 (30 cm growth) (45.259 μg g^{-1}). Furthermore, attempts were made to detect selected compounds from other classes of flavonoids, such as flavanones or flavonols, but only naringenin was detected in No1 (30 cm growth) and No2 (50 cm growth) extracts. Interestingly, the derivate of coumarin, aesculetin, was detected in all of three samples of *T. pratense*, whereas it was the most common in the sample No1 (30 cm growth). The most dominant, health-benefiting phenols were found in extract of the *T. pratense* in the lowest growth

Table 2. Quantification of selected phenolics in examined *T. pratense* MeOH extracts.

	Content of selected phenolics in *T. pratense* extract (μg g^{-1} of dw)		
Compound	No 1	No 2	No 3
Phenolic acids			
Hinic acid	nd[b]	Nd	nd
Galic acid	nd	Nd	nd
Protocatechuic acid	0.197 ± 0.004[a]	0.079 ± 0.003[b]	nd
Chlorogenic acid	nd	Nd	nd
2,5-Dihydroxybenzoic acid	0.088 ± 0.001[a]	0.087 ± 0.001[a]	nd
p-Hydroxybenzoic acid	0.713 ± 0.034[a]	0.180 ± 0.021[b]	0.042 ± 0.004[c]
Caffeic acid	1.041 ± 0.052[a]	[b]0.922 ± 0.043	0.086 ± 0.011[c]
Vanillic acid	nd	Nd	nd
Syringic acid	nd	Nd	nd
p-Coumaric acid	0.246 ± 0.027[a]	0.080 ± 0.013[b]	nd
Ferulic acid	nd	Nd	nd
Sinapic acid	nd	Nd	nd
o-Coumaric acid	nd	Nd	nd
3,4-Dimethoxycinnamic acid	nd	Nd	nd
Cinnamic acid	nd	Nd	nd
Flavonoids			
Apigenin	0.320 ± 0.037[a]	0.144 ± 0.041[b]	0.012 ± 0.001[c]
Apigenin 7-O-glucoside	0.288 ± 0.052[a]	0.034 ± 0.001[b]	0.014 ± 0.002[c]
Apigein	nd	Nd	nd
Vitexin	0.246 ± 0.001[a]	0.080 ± 0.001[b]	Nd
Amentoflavone	0.345 ± 0.001[a]	0.018 ± 0.002 [b]	Nd
Baicalein	nd	Nd	Nd
Baicalin	nd	Nd	Nd
Daidzein	1.527 ± 0.077[a]	0.878 ± 0.064[b]	0.121 ± 0.031[c]
Genistein	0.588 ± 0.056[a]	0.561 ± 0.038[a]	0.031 ± 0.004
Isorhamnetin	nd	Nd	Nd
Kaempferol	0.062 ± 0.001	0.015 ± 0.001	Nd
Kaempferol 3-O-glucoside	8.544 ± 0.095	1.344 ± 0.081	0.277 ± 0.049
Chrysoeriol	nd	Nd	Nd
Luteolin	0.229 ± 0.032[a]	0.028 ± 0.003[b]	0.011 ± 0.001[c]
Luteolin 7-O-glucoside	nd	0.032 ± 0.004	Nd
Quercetin	0.128 ± 0.042[a]	0.018 ± 0.002[b]	0.017 ± 0.002[b]
Quercetin 3-O-glucoside	24.243 ± 0.371[a]	3.137 ± 0.109[b]	0.817 ± 0.016[c]
Quercitrin	nd	Nd	Nd
Hyperoside	45.259 ± 0.628[a]	5.624 ± 0.212[b]	1.540 ± 0.059[c]
Rutin	0.157 ± 0.027[a]	0.020 ± 0.001[b]	Nd
Myricetin	nd	Nd	Nd
Naringenin	0.062 ± 0.005	0.014 ± 0.002	Nd
Epicatechin	nd	Nd	Nd
Catechin	nd	Nd	Nd
Epigallocatechin gallate	nd	Nd	Nd
Coumarins			
Umbelliferon	nd	Nd	Nd
Aesculetin	0.606 ± 0.033[a]	0.293 ± 0.028[b]	0.076 ± 0.017[c]
Scopoletin	nd	Nd	Nd
Lignans			
Matairesinol	nd	Nd	Nd
Secoisolariciresinol	nd	Nd	Nd

[b]Nd – not detected Data are means ±SD of three measurements. In each different letters mean significant differences ($p<0.05$)

stage. In view of the quantitative content of main phenolic compounds, there are some implications for potential biological activity of this sample. The quantified polyphenols play an important role in prevention of cancers, cardiovascular diseases, expressing strong antioxidant effects, increasing the activities of antioxidant enzymes and related to that and also decreasing the oxidative damage to tissues.[12,16] Therefore, further investigation should be directed toward wider quantification and identification of other bioactive compounds presented in the plant at the lowest growth stage (30 cm growth, vegetative) as well as investigation of *in vivo* antioxidant activity.

Total phenolic content, total flavonoid content, and in vitro antioxidant activity

The data presented in Table 3 indicated that the highest total phenol as well as flavonoid content of *T. pratense* was observed in the extract No 1 (30 cm growth). There are no reports of studies on antioxidant activity of *T. pratense* in different growth stage. The antioxidant activity of *T. pratense* extracts has been evaluated in a series of *in vitro* assays. Methanolic extracts of samples No1 (30 cm growth), No2 (50 cm growth), and No3 (bud phase) were evaluated for their scavenging capacity of DPPH$^{\bullet}$, OH$^{\bullet}$, $O_2^{\bullet-}$, NO$^{\bullet}$ radicals, neutralization of H_2O_2 and inhibition of LP and their activity compared to synthetic antioxidants BHA and BHT. Obtained results were given in Table 3. According to the results obtained for the values of investigated system, it can be concluded that all of the assessed extracts were able to reduce the stable radical DPPH, but the extract of the sample No1 (30 cm growth) was exhibited by shown the best scavenging capacity (22.98±0.14 μg mL^{-1}). The greatest ability to neutralize $O_2^{\bullet-}$ has extract of *T. pratense* No1 (30 cm growth) (25.73±0.36) and this ability is a bit weaker compared to the one exhibited by the synthetic antioxidants. Similar results were obtained examining the neutralization of NO radical where the extract No1 (30 cm growth) exhibited the strongest inhibitory effect (11.23±0.45 μg mL^{-1}). Inhibition of NO radicals is very significant, having in mind the ability to neutralize the superoxide anion radicals as well. The extract No1 (30 cm growth) is especially suited in this process since it neutralizes both superoxide anion radical and NO radical. Scavenging capacity of all extracts to OH$^{\bullet}$ is significantly higher than those of BHT and BHA. Hence, *T. pratense* extracts can be used to minimize the adverse effects from the hydroxyl radicals. Hydrogen peroxide itself is not very reactive, but it could be toxic to cells because of its ability to penetrate biological membrane. Based on the results of the spectrophotometric measurement of the H_2O_2, all the examined *T. pratense* extracts showed the ability to remove H_2O_2. However, this ability is a bit weaker compared to the one exhibited by the synthetic antioxidants, BHT and BHA. All tested extracts exhibited much weaker protective effect of the process of lipid peroxidation than synthetic antioxidants. Among them, the strongest inhibitory effect was shown by MeOH extract No1 (30 cm growth) (53.07±1.72 μg mL^{-1}). The extracts No1 showed the strongest antioxidant potential which could be explained by higher content of phenolic compounds presented in it.

Antibacterial activity

The antibacterial activity of different isoflavones has been described in many studies.[30] The antibacterial activity of *T. pratense* extracts against human pathogenic bacteria was not previously

Table 3. Total phenolics and flavonoid content, antioxidant potential of extracts of *T. pratense* extracts and standard compounds.

		T. pretense			Standards	
		No1	No3	No2	BHA	BHT
Content	*Total phenolics* (mg gallic acid equivalents g^{-1} of dw)	41.96 ± 0.93$^{\S a}$	36.00 ± 0.55^{b}	30.99 ± 1.02^{c}	-	-
	Total flavonoids (mg quercetin equivalents g^{-1} of dw)	7.32 ± 0.59^{a}	5.78 ± 0.30^{b}	3.87 ± 0.23^{c}	-	-
Antioxidant activity	*IC_{50} values* (μg/mL for extracts)					
	DPPH$^{\bullet}$	22.98 ± 0.14^{c}	35.09 ± 0.51^{d}	44.85 ± 1.32^{e}	11.07 ± 0.07^{a}	14.32 ± 024^{b}
	HO$^{\bullet}$	188.42 ± 1.65^{c}	204.30 ± 1.84^{d}	213.06 ± 0.28^{e}	119.88 ± 0.2^{b}	108.73 ± 1.3^{a}
	$O_2^{\bullet-}$	25.73 ± 0.36^{c}	27.40 ± 6.25^{c}	30.77 ± 1.20^{d}	8.53 ± 0.49^{a}	10.12 ± 0.34^{b}
	NO$^{\bullet}$	11.23 ± 0.45^{c}	33.34 ± 0.63^{d}	42.89 ± 1.05^{e}	62.33 ± 0.10^{a}	87.90 ± 0.18^{b}
	H_2O_2	32.43 ± 0.39^{c}	44.10 ± 1.61^{d}	55.08 ± 1.05^{e}	17.20 ± 0.60^{a}	20.67 ± 0.63^{b}
	LP	53.07 ± 1.72^{c}	68.30 ± 1.12^{d}	74.90 ± 1.86^{e}	34.76 ± 1.14^{b}	26.08 ± 0.98^{a}
	Reducing power (mg of ascorbic acid equivalents (AAE)/g of dw)					
	FRAP	428 ± 2.60^{c}	411.40 ± 0.98^{d}	376.90 ± 2.35^{e}	587.70 ± 1.80 ^{a}	54.20 ± 1.79^{b}

§Values are means ± SD of three measurements; a,b,c,d,e means in the same row not sharing the same superscript are significantly different (p<0.01).

Table 4. Evaluated antibacterial activity of *T. pratense* extracts.

	Disc diffusion assay (Inhibition zone measured in mm)					
	Test microorganisms					
Tested sample	Pseudomonas aeruginosa	Escherichia coli	Enterobacter aerogenes	Enterococcus faecalis	Staphylococcus aureus	Bacillus cereus
T. pratense No1	abs*	13.4 ± 0.25[§b]	12.5 ± 0.68[b]	8.6 ± 1.38[b]	7.4 ± 0.64[b]	9.30 ± 0.74[b]
T. pratense No2	abs	5 ± 0.09[c]	3.9 ± 1.04[c]	2.3 ± 1.74[c]	1.2 ± 0.46[c]	2.59 ± 0.77[c]
Streptomycin	13.2 ± 0.11	29 ± 0.55[a]	18 ± 0.55[a]	14 ± 0.00[a]	31.2 ± 0.44[a]	19.30 ± 2.20[a]
Agar dilution assay (MIC; $\mu g\ ml^{-1}$)						
T. pratense No1	nd[¥]	25	25[b]	50	50[b]	100
T. pratense No2	Nd	nd	50[c]	nd	100[c]	nd
Streptomycin	16	4	8[a]	16	2[a]	8

[§]Values are means ± SD of three measurements; [a,b,c] means in the same column not sharing the same superscript are significantly different ($p<0.01$)

*abs – absence of inhibition zone; [¥]nt – not determined.

reported (Table 4). Only one report has confirmed that some other species from *Trifolium* genius, *Trifolium alexandrum*, shows antimicrobial activity.[31] Antimicrobial activity of the tasted extracts is the consequence of the fact that phenolic content found in them are partially hydrophobic and are considered to interact with the bacterial cell wall and lipopolysaccharide interfaces by decreasing membrane stability.[32] The antibacterial activity of tested MeOH extract of *T. pratense* No 1 (30 cm growth) and *T. pratense* No 2 (50 cm growth) against Gram-positive and Gram-negative bacteria was shown in Table 4. The results obtained from extract of *T. pratense* No 3 (bud phase), were not presented, because the MICs determined were greater than the highest concentration tested. These extracts were more effective in growth inhibition of both *Escherichia coli* and *Enterobacter aerogenes* bacterial strains, where the extract, No1 (30 cm growth), has shown better antibacterial activity than the extract No 2 (30 cm growth). These results could be explained by the fact that presence of greater content of antimicrobial compounds in this extract contributes to its antibacterial properties.

Conclusion

Variation in phenolic content may have value in determining the best source of a functional food. The present study indicates that the extract of the *T. pratense* at the lowest growth is a valuable source of natural antioxidants which could indicate their use as possible agents in prevention of oxidative-stress and thereby reduce the development of many diseases. This is due to the presence of a higher amount of phenolic compounds that has been identified in this extracts such as genistein, daidzein, p-hydroxybenzoic and caffeic acid, kaempferol 3-O-glucoside, quercetin 3-O-glucoside, hyperoside, aesculetin, as well as glycosides kaempferol 3-O-glucoside, quercetin 3-O-glucoside and apigenin 7-O-glucoside. Due to the presence of the highest content of phenolic compounds in the extract No1, this extract expressed the best antioxidant and antimicrobial activity. These data indicate that growth stage of the plant is one of the important factors that should be taken into account when using this plant in phytomedicine and also in human nutrition.

Declaration of interest

The authors declare that they have no conflict of interest.

Funding

This work was supported by the Ministry of Science and Environmental Protection of the Republic of Serbia (Project No. 172058).

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