ORIGINAL ARTICLE

POLYPHENOLIC PROFILE AND ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES FROM TWO *TRIFOLIUM* SPECIES

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Abstract

In the present study, flowers and leaves of *Trifolium pratense* and *T. repens* were screened for evaluating the polyphenolic contents and the antioxidant and antibacterial activities. A characterization of the main phenolic compounds was carried out using HPLC-MS method. The total polyphenolic and flavonoid content was spectrophotometrically determined. Antioxidant activity was evaluated by using several methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, TEAC (Trolox equivalent antioxidant capacity), HAPX (haemoglobin ascorbate peroxidase activity inhibition) and EPR (electron paramagnetic resonance) method. The polyphenolic profile revealed the presence of common components as *p*-coumaric acid, ferulic acid, hyperoside, quercetin, luteolin, and other that make the differences (miricetol, quercitrin, isoquercitrin). *T. pratense* showed better antioxidant and antibacterial activity than *T. repens*. The quantitative and qualitative differences between the two species of *Trifolium* could be used as a potential taxonomic marker in order to distinguish the species.

Rezumat

În acest studiu, florile şi frunzele de *Trifolium pratense* şi *T. repens* au fost analizate în vederea evaluării conţinutului de polifenoli şi activităţile antioxidante şi antimicrobiene. Caracterizarea compuşilor fenolici a fost realizată utilizând metoda HPLC-MS. Conţinutul total de polifenoli şi flavonoide a fost determinat spectrofotometric. Activitatea antioxidantă a fost evaluată prin utilizarea mai multor metode: DPPH (2,2-difenil-1-picrilhidrazil), TEAC (*Trolox equivalent antioxidant capacity*), HAPX (*haemoglobin ascorbate peroxidase activity inhibition*) şi EPR (rezonanţă electonică paramagnetică). Profilul polifenolic a relevat prezenţa unor componente comune: acizii *p*-cumaric, ferulic, hiperozida, quercetol, luteolina, precum şi altele care fac diferenţele între cele două specii: miricetol, quercitrina, izoquercitrina. *T. pratense* a prezentat activitate antioxidantă şi antibacteriană mai puternică decât *T. repens*. Diferenţele cantitative şi calitative între cele două specii de *Trifolium* ar putea fi folosite ca un marker taxonomic, în scopul identificării speciilor vegetale.

Keywords: *Trifolium pratense*, *Trifolium repens*, DPPH, TEAC, HAPX, EPR

Introduction

The Romanian flora comprises around 40 species and more than 20 subspecies of the *Trifolium* genus. *Trifolium pratense* L. and *Trifolium repens* L. are two spontaneous medicinal species that are also cultivated as fodder plants. These species contain isoflavonoids and other phenolic compounds. Biological activities include antioxidant, estrogenic, cytotoxic, tyrosine kinase inhibitory activities [1-7]. The aim of this work was to analyse the chemical composition of *T. pratense* and *T. repens* extracts and to investigate their antioxidant and antimicrobial properties, for a better characterization and exploitation of these natural products.

Materials and Methods

Plant materials. The flowers and leaves of *T. pratense* (Voucher No. 959) and *T. repens* (Voucher No. 960) were harvested from Valea Drăganului, Cluj, Romania. Voucher specimens were deposited in the Herbarium of the Department of Pharmacognosy of the Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. The plant material was extracted with 70% ethanol [8-10].

HPLC–MS analysis was performed on an Agilent 1100 HPLC Series system using the chromatographic conditions previously described [8-10]. Quantitative

determinations were performed using an external standard method. Calibration curves in the 0.5 - 50 mg/mL range with good linearity ($R^2 = 0.999$) for a five points plot were used in orders to determine the concentration of polyphenols.

Determination of total polyphenolic (TPC) and flavonoidic contents. This analysis was made using spectrophotometric methods [9-13].

Antioxidant activity tests. The extracts were screened for their antioxidant activities using four *in vitro* assay models: DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, TEAC (Trolox equivalent antioxidant capacity), HAPX (haemoglobin ascorbate peroxidase activity inhibition) and EPR (electron paramagnetic resonance) methods [10, 12, 14-19]. The positive controls were those using the standard solution of quercetin, BHT (butylated hydroxytoluene) and Trolox. The HAPX assay measures the capability of the extract components to quench the damage inflicted by hydrogen peroxide upon haemoglobin. EPR measurements were performed on a Bruker Elexsys E500 spectrometer.

Antibacterial activity test. The antimicrobial properties were demonstrated using a disk-diffusion method on the following strains: *Staphylococcus aureus* (ATCC 6538P), *Listeria monocytogenes* (ATCC 13932), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 13076) [10].

Statistical analysis. All the samples were analysed in triplicate; the average and the relative SD were calculated using the Excel software package.

Results and Discussion

The concentrations of the identified polyphenolic compounds in all analysed samples are shown in Table I. Gentisic, caffeic, chlorogenic, *p*-coumaric, ferulic acids were identified in the extract of leaves and flowers of *T. pratense* and *T. repens*. Sinapic acid was detected only in *T. pratense* leaves. Hyperoside, isoquercitrin, rutin, quercitrin, myricetin, quercetin, luteolin, kaempferol and apigenin were identified and quantified. Iso-quercitrin, rutin and kaempferol were detected only in the extracts of *T. pratense*. Myricetin was found in *T. repens*. This could serve as an important chemo-taxonomic marker that could avoid adulterations among these species.

Table I

Polyphenolic compounds content in *T. pratense* and *T. repens* extracts (mg/100 g)

Polyphenolic compounds	**t_R ± SD (min)**	***T. pratense* leaves**	***T. repens* leaves**	***T. pratense* flowers**	***T. repens* flowers**
Caftaric acid	3.54 ± 0.05	NF	NF	< 0.2	NF
Gentisic acid	3.52 ± 0.04	< 0.2	NF	< 0.2	< 0.2
Caffeic acid	5.60 ± 0.04	< 0.2	NF	< 0.2	< 0.2
Chlorogenic acid	5.62 ± 0.05	< 0.2	NF	< 0.2	< 0.2
p-Coumaric acid	9.48 ± 0.08	0.85 ± 0.12	1.12 ± 0.04	3.04 ± 0.09	< 0.2
Ferulic acid	12.8 ± 0.10	1.85 ± 0.08	1.09 ± 0.03	22.55 ± 0.22	< 0.2
Sinapic acid	15.00 ± 0.10	1.85 ± 0.04	NF	NF	NF
Isoquercitrin	19.60 ± 0.10	49.91 ± 0.85	NF	64.50 ± 2.1	NF
Hyperoside	18.60 ± 0.12	42.91 ± 0.72	11.19 ± 0.07	68.84 ± 1.63	NF
Rutin	20.20 ± 0.15	NF	NF	5.27 ± 0.05	NF
Myricetin	21.13 ± 0.12	NF	0.91 ± 0.02	NF	NF
Quercitrin	23.64 ± 0.13	NF	2.99 ± 0.1	25.87 ± 1.2	NF
Quercetin	26.80 ± 0.15	2.98 ± 0.13	0.5 ± 0.03	9.48 ± 0.07	1.33 ± 0.2
Luteolin	29.10 ± 0.19	NF	1.34 ± 0.4	2.87 ± 0.05	2.87 ± 0.09
Kaempferol	32.48 ± 0,17	NF	NF	6.27 ± 0.04	NF
Apigenin	33.10 ± 0.15	NF	0.30 ± 0.01	1.15 ± 0.07	0.57 ± 0.04

Note: NF - not found, below limit of detection.

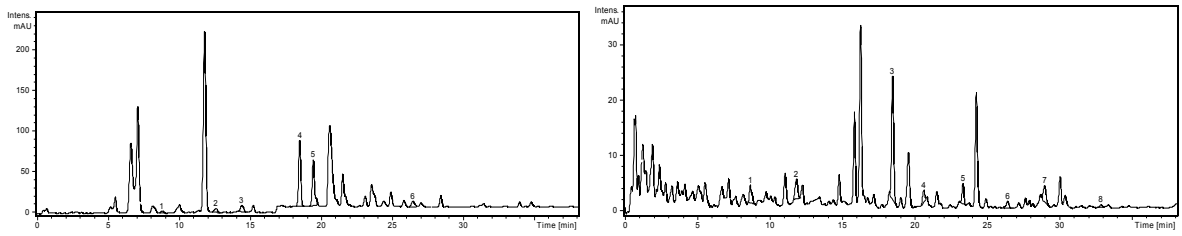

Figure 1.

HPLC chromatograms of *T. pratense* (left) and *T. repens* (right) leaves

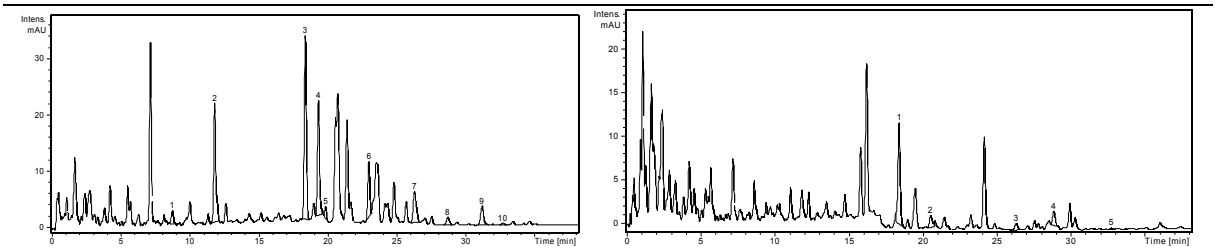

Figure 2.
HPLC chromatogram of *T. pratense* (left) and *T. repens* (right) flowers

TPC and flavonoid values are presented in Table II. The highest amount of total polyphenols was determined for *T. pratense* leaves (76.16 mg/g) followed by *T. repens* leaves (62.06 mg/g). Concerning the content of flavonoids, the extract of *T. pratense* flowers (14.1 mg/g) was richer in flavonoids, than the extract of *T. repens* flowers (8.2 mg/g).

Table II
The content of total polyphenols and flavonoids and antioxidant activity

Samples	TPC (mg GAE/g)	Flavonoids (mg RE/g)	IC_{50} (µg/mL)	TEAC (µmol Trolox/mg)	HAPX (%)
T. pratense flowers	61.80 ± 1.20	14.10 ± 0.40	104.45 ± 0.75	50.86 ± 1.21	21.68 ± 1.51
T. pratense leaves	76.16 ± 0.84	7.67 ± 0.23	135.55 ± 0.65	-	-
T. repens flowers	49.80 ± 2.20	8.20 ± 1.30	180.91 ± 0.29	39.50 ± 1.34	30.22 ± 0.95
T. repens leaves	62.06 ± 1.94	2.048 ± 0.59	361.78 ± 0.62	-	-
Quercetin	-	-	5.40 ± 0.32	-	-
BHT	-	-	15.6 ± 0.44	-	-

The highest radical scavenging activity was showed by the extract of *T. pratense* flowers, followed by the extract of *T. pratense* leaves. This is in agreement with the TPC values listed in Table II. According to TEAC, the extract of *T. pratense* flowers was significantly higher regarding antioxidant capacity than the extract of *T. repens* flowers, in agreement with the DPPH results and the TPC values. In the TEAC assay, the stable radical is dissolved in an aqueous solution, thus expecting a different mechanism of interaction between the antioxidantive molecules and the radical since TEAC assesses the more hydrophilic components while DPPH describes all components. This can be observed by the final TEAC values, even though the antioxidant power was significantly higher in *T. pratense* flowers extract. The difference in absolute value was not as great as in the case of DPPH method. *T. pratense* contains specific phenolic compounds which are much more antioxidant than many others, or other electron rich compounds responsible for the anti-oxidant activity (isoflavonoids). Also, it can be assumed that the extract of *T. repens* flowers contains antioxidant compounds which better act in aqueous solution than in organic solvent (ethanol). By contrast, the HAPX results revealed that *T. repens* flowers possess a higher antioxidant potential than *T. pratense* flowers. This HAPX assay measures the capability of the extract components to quench the damage inflicted by hydrogen peroxide upon haemoglobin. For EPR spectroscopy study, a common encountered nitrone spin probe, nitroxidic radical TEMPO has been used. The rate of reaction between the antioxidant compounds and TEMPO radical was monitored by using normalized double integrated residual EPR signal, which is correlated with the number of paramagnetic species in time (Figure 3).

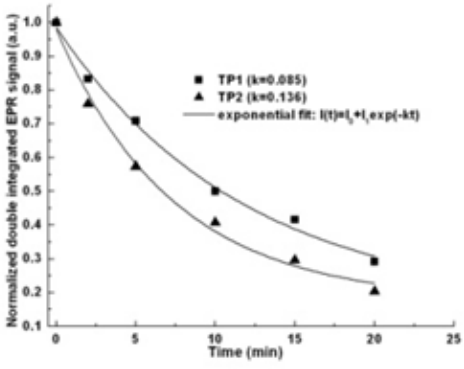

Figure 3.
The rate of reaction between antioxidant compounds and TEMPO radical

The best fit has been obtained using the first order exponential decay: $I(t) = I_0 + I_1e^{-kt}$, where I_0 and I_1 are the fit constants representing the double integral EPR signal intensity immediately after adding free radicals and after time t, respectively; k is the kinetic constant of the reaction corresponding to each type of extracts. The k constant is specific to each type of sample and processing way. It

represents the oxido-reduction rate of the Tempo radical in time and it is a fingerprint of the quality of the antioxidant compound. Comparing the calculated kinetic rates of the both samples, one can observe that *T. pratense* (TP2) extract has a higher antioxidant capacity (k_{TP2} = 0.136) than *T. repens* (TP1) extract (k_{TP1} = 0.085).

Table III

Antibacterial activity of *T. pratense* and *T. repens* extracts

Samples	Inhibition zone in diameter (mm)			
	Staphylococcus aureus (ATCC 6538P)	*Listeria monocytogenes* (ATCC 13932)	*Escherichia coli* (ATCC 25922)	*Salmonella typhimurium* (ATCC 13076)
T. pratense flowers	12.0	11.0	10.0	16.0
T. pratense leaves	17.0	10.0	16.0	17.0
T. repens flowers	10.0	10.0	14.0	8.0
T. repens leaves	10.0	10.0	11.0	12.0
Gentamicin	19.0	18.0	22.0	18.0

The results of antibacterial activity are shown in the Table III. All the extracts were found to be active against the tested bacterial strains, these samples showing a moderate antibacterial activity.

Conclusions

In order to supply new information on *T. pratense* and *T. repens*, their polyphenolic composition and the antioxidant and antimicrobial activities were evaluated. The phytochemical study showed qualitative and quantitative differences between the two *Trifolium* species and these were related to the respective flavonoid and flavonol profiles. The antioxidant activity evaluated using several methods indicated that *T. pratense* is superior compared to *T. repens*, related with the total polyphenolic and flavonoidic content. The results suggested the great value of flowers and leaves of *Trifolium* species for their use in phytotherapy, due to their content of polyphenols responsible for the antioxidant activities and other bioactive properties.

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