Red Clover Characterization: *Trifolium patulum* Tausch

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Although clovers (Trifolium ssp, Fabaceae) are considered one of the leading crops for cattle grazing, they could also be used as a potential source of bioactive compounds. in fact, they have a long history of use in popular medicine. The Trifolium pratense (Red Clover) is certainly the most common in this family, but endemic or sub endemic italian species are interesting for the investigation of their bioactive compounds content and their potential application. The aim of the study was to investigate the tirfolium pratensis versus Trifolium patulum Tausch, an endemic species of southern appennine. The analysis focused on GC / MS determinations of volatile content, and HPLC determinations of polar compounds. In addition, antioxidant activity tests were performed.

Keywords: Trifolium patulum; poliphenols content; ginestein; daidzein

Trifolium pratense L. (Fabaceae) is widely spread forage crop in temperate and subtropical regions of both emispheres. 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 antiinflammatory properties [1,2]. Secondary metabolites are synthesized in the plant to facilitate adaptation to the vegetative habitats that host them. Moreover, such adaptation is favored by genetic mutations, and they are also capable of influencing the content in active principles. It is for this reason that many endemic species, derived from species of wide distribution, have scientific interest, especially for the content of specific active principles. There are several reports on polyphenols with emphasis on isoflavonoid contents. [3-5] The isoflavonoids belong to the group of phytoestrogens that are used in hormone replacement therapy [6]. Red clover is known to have high concentrations of isoflavones, mainly formononetin and biochanin A [7- 8]. In red clover-grass silage and pasture the isoflavones daidzein, genistein, prunetin, and glycitein, the flavone chrysin and the flavanone naringenin have also been detected [9 -10]. no studies on the phytochemical composition of *Trifolium patulum* have been reported in the literature. *Trifolium patulum* is a perennial herb which occurs in rocky woodland and scrub. It has been reported from very steep mountainsides and in association with *Abies, Acer pseudoplatanus, Carpinus betulus, Coronilla, Corylus avellana, Cotinus cogygria, Fraxinus ornus* and *Juniperus oxycedrus*. Due to its occurrence in protected areas, wide distribution range and apparently stable population, *Trifolium patulum* is listed as Least Concern. However, the current assessment has been carried out with information from old specimens. Further surveys are needed to determine the current status of the species on its native range. for this reason we have harvested T. *patulum* on the mountains of Sila (calabria), and we have studied it in comparison with T. *pratense*, collected in the same localities. Phytochemical investigation of methanolic extract, but also portions of this soluble in increasing polarity solvents (Esano, ChCl2, AcOEt), included qualitative and quantitative determination of both volatile compounds and polar metabolites (polyphenols and flavonoids) using the GC/MS and LC/MS-MS technique. Also, the total phenolics and flavonoids were quantified. The antioxidant activity of of T. *pratense* and T. *patulum* was determined in vitro via neutralization of the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical.

Experimental part

Materials and methods

Chemicals

Chlorogenic acid, quercetin, Folin–Ciocalteu reagent, aluminium chloride, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene and ethanol were purchased from VWR International s.r.l. (Milan, Italy). Propyl gallate linoleic acid and Tween 20 were purchased from Sigma-Aldrich S.p.A. (Milan, Italy). All other reagents, of analytical grade, were Carlo Erba products (Milan, Italy).

Plant materials

Trifolium patulum Tausch and *Trifolium pratense* L. are spontaneous plant, has been widely described in regard of agricultural relevance (forage) both pharmacological action (estrogenic effects, in particular). Aerial parts samples were collected in different sites located in the Sila Massif, Cecita lake (39.397913, 16.511862; 39.400395, 16.502876) chosen as representative of ecogeopedological characteristics.

Extraction procedure

Dried plant material was extracted by maceration with EtOH at room temperature. The procedure was repeated three times for 48 h. Solvent was evaporated using a Rotavapor® R-220 SE (BUCHI Labortechnik AG, Flawil, Switzerland).

Fractionation extract

The extract we obtained was suspended in a 9: 1 water-ethanol mixture and extracted with n-hexane. The hydroalcoholic fraction, after removal of ethanol, was extracted firts with chloroform ($CHCl_3$) and then with ethyl acetate (AcOEt). The fractions thus obtained were dried under vacuum with rotavapor at a temperature below 40 ° C and then stored at -20 ° C.

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Total phenols content

Total phenol content of the total extracts was determined using Folin-Ciocalteu reagent and chlorogenic acid used as standard, modified according to Casacchia et al., 2017 [11].

A concentration 2 mg/mL in a solution composed by cloridric acid: water, 5:95 of each sample was heated at 60 °C for 1h. Then, 1.0 mL of Folin-Ciocalteu reagent and, after 3 min, 1.0 mL of Na_2CO_3 (7.5%) were added. The tubes were vortexed and heated at 40ºC (water bath) for 30 min. The absorbance produced by the blue colour was measured at 726 nm with a Perkin Elmer Lambda 40 UV/Vis spectrophotometer (Milano, Italy). Analysis were made in triplicate. The calibration curve was determined with seven standards with concentrations ranging from 50 to 1200 μg/mL. Values were calculated from a calibration curve of chlorogenic acid, and the total phenol content expressed as mg of chlorogenic acid equivalents (CAE) per g of dry material (DW).

Total flavonoid content

The total flavonoid content of crude extract was determined by the $AlCl_3$ colorimetric method on the same extracts used for total phenols determination [12].

A solution 2 mg/mL in EtOH 80% of each sample (1 mL) was mixed with 2% $AlCl_3$ in EtOH (1 mL). Absorbance was measured at 430 nm after 15 min. Experiments were performed in triplicate. Total flavonoid content was expressed as mg of quercetin equivalent (QE) per g of dry plant material (DW).

HPLC Analysis

The HPLC analyses were realized on a VWR-Hitachi, a liquid chromatograph fitted with a pulse-free pump and a suitable detection device (model L-2455). A C18 endcapped Lichrospher column (250 x 4.6 mm I.D.; 5 μm particle size) was employed (Merck, Darmstadt, Germany), at 25 °C. The mobile phase was composed of 0.1% acetic acid and ethanol (56:44, v/v), at a flow rate of 0.8 mL/min. The injection volume was 20μL and UV-detection was performed at 254 nm.

Data were collected at 261 nm for genistein and at 248 nm for daidzein. Each extract and five solutions of each standard were injected three times.

Our standard solutions contained 800 μg/mL daidzein and 1000 μg/mL genistein in HPLC grade ethanol. Calibration ranges were 0.100–200 μg/mL for daidzein and 0.050–150 μg/mL for genistein. Triplicates of 10 μL injections were made for each standard solution.

GC-MS

Analysis of volatile compounds was performed using a Hewlett-Packard gas-chromatograph, model 5890, equipped with a mass spectrometer, model 5972 series II, and controlled by HP software with capillary column 30 m 9 0.25 mm, static phase SE30, using programmed temperatures from 60 to 280 °C (rate 16°/min); detector and injector were set at 280 and 250 °C respectively (split vent flow 1 mL/min). Compound identification was verified according to relative retention time and mass spectra with those of Wiley 138 library data of the GC-MS system (Hewlett-Packard Co., Milan, Italy). Extracts were analysed, also using the Shimadzu GC17A gas chromatograph system (Columbia, MD, USA). An SE- 30 capillary column (30 m, internal diameter 0.25 mm and film thickness 0.25 μm) was used with nitrogen as the carrier gas.

GC oven temperature and conditions were as described above. Percentages utilized for composition of samples analysed were computed by the normalization method from GC peak areas related to GC peak area of external standards, injected into the GC equipment in isothermal conditions, at 180 °C. Percentage of their total area was obtained by their addition. All determinations were performed in triplicate and averaged.

Free radical scavenging assay

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was adapted according to Marrelli et al., 2014 [13]. Different concentrations of each sample (5–1000μg/mL) were tested. Test sample solutions (200 μL) were added to a 10^{-4} M ethanol solution of DPPH (800 μL). Mixtures were kept in the dark for 30 min and absorbance was measured at 517 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer (Milano, Italy). Ascorbic acid was used as positive control and analyses were run in triplicate.

This activity was given as % DPPH radical scavenging, calculated by the following equation: % DPPH radical scavenging= (absorbance control " absorbance sample) / absorbance control × 100

Antilipoperoxidation activity

The antilipoperoxidation activity was measured using the â-carotene bleaching (BCB) test method [13]. The test solutions were realized adding 0.04 mL of linoleic acid and 0.4 mL of Tween 20 to 2 mL of a β-carotene solution in chloroform (0.5 mg/mL). The solvent was then evaporated under reduced pressure at 40°C for 10 min through a rotary evaporator and the dry residue immediately diluted with 150 mL of distilled water. Water was slowly added to the mixture and vigorously stirred to form an emulsion. Then, 0.2 mL of sample solutions at different concentrations (120, 60, 30, 15, 8, 4, 2, 0,5 mg/mL) were added to 5 mL of the emulsion. Ascorbic acid was used as positive control. The tubes were stirred slowly and kept at 45°C in a water bath. The absorbance was read at λ=470 nm at the starting incubation time (t_0) and at t=30 min. The antioxidant activity (AA) was calculated using the following equation: AA=[1–(A0–At)/(A0°–At°)]×100, where A0 and A0° are the absorbance values measured at t=0 min for samples/standard and control, respectively, and At and At° are the absorbance values measured in the samples/standard and control at t=30 min, respectively.

Results and discussions

Extraction yield

The hydroalcoholic extracts of two different species of *Trifolium* were obtained by maceration. The aerial parts have been collected in July 2015 in a grazing area, in Sila, near Cecita lake, chosen as representatives of the ecogeopedological conditions of the area of interest. Plants were dried in the dark at room temperature.

The extraction yields were 14.52% for T. *patulum* Tausch and 14.05%. for T. *pratense* L.

Total content of phenols and flavonoids

One of the targets of the study was to evaluate the difference between the content of polyphenols and total flavonoids in the two different *Trifolium* species comparing.

The total phenolic content of T. *patulum* and T. *pratense* extracts expressed in chlorogenic acid equivalent in mg / g of dry matter was evaluated in the different biofractionated portions and are reported in table 1.

The two different species of *Trifolium* showed a different phenolic content. T. *pratense* extract showed the highest

Species	Total phenolic content (mg CAE / g FW)	Total flavonoid content (mg QE / g FW)
T. *pratense* L. tot	150.67 ± 3.24 [a]	13.31 ± 3.01 [a]
T. pretense L. AcOEt	233.07± 2.58 [b]	17.21 ± 1.10 [b]
T. pretense L. $CHCl_3$	63.132 ± 2.78 [c]	2.28 ± 2.86 [a]
T. *patulum* T. tot	121.67 ± 4.20 [a]	9.2 ± 0.91 [a]
T. *patulum* T. AcOEt	188.98 ± 3.55 [b]	11.76 ± 2.4 [b]
T. *patulum* T. $CHCl_3$	57.14 ± 4.75 [c]	2.22 ± 1.4 [c]

Table 1
TOTAL PHENOLIC AND FLAVONOID CONTENT IN TOTAL AND FRACTIONATED EXTRACT OF *TRIFOLIUM PRATENSE* L. AND *TRIFOLIUM PATULUM* TAUSCH

Total phenolics are expressed as chlorogenic acid equivalents (CAE) per g of fresh material (FW). Total flavonoids are expressed as quercetin equivalents (QE) per g of fresh material (FW). Means (n = 5) ± SD with different letters within the same straight line are significantly different at p < 0.05

content, with a value of 150.67 mg of polyphenols per g of extract.

In T. *patulum* sample was observed a content of 121.67 mg/g.

Also as regards the concentration of total flavonoids in the two different clover species, we notice that this is higher in the T. *pratense* species (13.3 mg / g), perfectly in line with the total polyphenols content.

The total extract was solubilized with water and partitioned with increasing polarity solvents to obtain the following fractions: n-hexane, chloroform and ethyl acetate.

The portions in Ethyl Acetate (AcOEt) are characterized by a higher content of polyphenols:

233.07 ± 2.58 mg/g in T. *pratense* versus 188.98 ± 3.55 mg/g of T. *patulum* while content of flavonoids is 17.21 ± 1.10 mg/g versus 11,76 ± 2.4 mg/g, T. *pratense* and T. *patulum*, respectively. All results are shown in table 1.

The total flavonoid content was compared with the results verified by other authors [14].

Antioxidant activity

The antioxidant activity was determined by the DPPH test and the beta-carotene bleaching test. The 1,1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl, DPPH) molecule is a stable free radical that is capable of relocating the electron. Delocalisation also generates deep purple color, characterized by an absorption band, in methanol solution, at about 520 nm. When a DPPH solution is mixed with substances that can donate a hydrogen atom, we obtained a reduced form of the radical free and the loss of this purple color (although one would expect a pale yellow colour due to the picryl group still present). In DPPH assay, we studied the ability of the investigated extracts to act as hydrogen atoms or electrons donor in the DPPH transformation in its reduced form DPPH-H.

In our work, we evaluated how the fraction of T. *pratense* in ethyl acetate, which is characterized by a higher content of polyphenols and total flavonoids, has the best radical scavenging activity with an IC50 value of 17.49 ± 0.21 μg/mL and the best antioxidant activity evaluated with the beta-carotene bleaching test (IC50 of 7.53 ± 1.02 after 30 min of incubation). Less active is the ethyl acetate T. *patulum* portion in which the radical scavenging activity has an IC 50 value of 46.66 ± 0.18 μg/mL and an antioxidant activity, estimated by IC50, of 58.35 ± 1.54 μg/mL. The lowest radical scavenging activity as reported in table 2, is present in AcOEt fraction of T. *pratense*, followed by that of T. *patulum*.

Since quercetin glycosides and flavonoids (kaempferol-3-O-Glc, quercetin-3-O-Glc, luteolin-7-O-Glc and apigenin-7-O-Glc) were detected in EtOAc extract, and g that flavonoid-rich plants could be itself good sources of antioxidants that would help improve the overall antioxidant capacity of an organism and protect against lipid peroxidation [15]. Also Erkan et al. [16] reported a close correlation between the radical scavenging activity and the total phenolic content of extract of various natural sources.

HPLC

The method used was validated by determining the detection limit (LOD), the quantification limit (LOQ), and the consequent linearity. The linearity of the calibration curve was evaluated using standard solutions of Daidzein and Genistein prepared at different concentrations, between 0.25 and 100μg/mL.

Calibration curves were determined with 3 different injections of 1ì of each standard solution and the standard

Species	IC_{50} (μg/ml)		
	DPPH	βcarotene 30'	βcarotene 60'
T. *pratense* L. tot	48.44 ± 0.41	8.78 ± 0.69	36.41 ± 2.57
T. pretense L. AcOEt	17.49 ± 0.21	7.53 ± 1.02	22.03± 1.97
T. pretense L. $CHCl_3$	327.9 ± 4.73	> 100	> 100
T. *patulum* T. tot	83.84 ± 2.62	> 100	> 100
T. *patulum* T. AcOEt	46.66 ± 0.18	58.35 ± 1.54	90.16 ± 0.45
T. *patulum* T. $CHCl_3$	400.3 ± 7.40	95.84 ± 3.03	> 100

Positive reference DPPH: ascorbic acid (IC_{50} = 0,89 ± 0.82 μg/ml); positive reference βcarotene bleaching test: propyl gallate (IC_{50} = 1 ± 0.02 μg/ml)

Table 2
ANTIOXIDANT ACTIVITY IN TOTAL AND FRACTIONATED EXTRACT OF *TRIFOLIUM PRATENSE* L. AND *TRIFOLIUM PATULUM* TAUSCH

Table 3
GENISTEIN AND DAIDZEIN CONTENT IN TOTAL AND FRACTIONATED EXTRACT OF *TRIFOLIUM PRATENSE* L. AND *TRIFOLIUM PATULUM* TAUSCH

Species	genistein (µg/g)	daidzein (µg/g)
T. *pratense* L. tot	7.09 ± 2.34 [a]	8.66 ± 2.50 [a]
T. pretense L. AcOEt	6.01± 2.11 [b]	8.01 ± 1.00 [a]
T. pretense L. $CHCl_3$	0.98 ± 3.71 [c]	0.12 ± 2.46 [b]
T. *patulum* T. tot	8.99 ± 1.89 [a]	6.90 ± 3.01 [a]
T. *patulum* T. AcOEt	7.78 ± 3.05 [b]	6.05 ± 3.06 [a]
T. *patulum* T. $CHCl_3$	0.74 ± 2.72 [c]	0,22 ± 1.45 [b]

Results were expressed aa mean ±SD

deviation was calculated to evaluate the reproducibility of the data.

The regression equation and the correlation coefficient determined for Genistein and Daidzein are, respectively: y = 1.478x - 0.6392 R^2 = 0.9983 and y = y = 1.491x - 1.78 R^2 = 0. (9991).

Samples and standards have been solubilized in ethanol because it's considered one of the best organic solvents for the separation of isoflavones [17].

The results are shown in table 3.

GC – MS

The results of the GC-MS analyses of the n-exane extract of T. pratensis e T. *patulum* parts are presented in tables 4 and 5.

Various compounds were identified on the basis of their retention time and intensity of the peak.

A total of 41 compounds were identified from the extract of P. *pratense* and 27 compounds were identified in T. *patulum*.

It is important to note that many compounds are common to both clover species. In some cases we find percentages of the total, of the same compounds, which is the double or triple in the T. *pratense* relative to T.*patulum*. These include Thiosulfuric acid (an inorganic acid) and Hexadecanoic acid (saturated fatty acid), Nonacosane (straight chain hydrocarbon), Aristolone (sesquiterpene). On the other hand, T.*patulum* has a greater amount of compounds such as 2-decenal (2-Decenal is a component of essential coriander oil, belongs to the family of medium chain aldehydes) or Stigmast-5-en3-ol (a phytosterol widespread in the vegetable kingdom) compared to T-*pratense*. What matters is that both species have many sterols, terpenes, fatty acids, and flavonoids essential to determine their activiy.

Statistical analysis

All the data were expressed as the mean values ± SD and were obtained from experiments repeated three times.

Compounds	*tR / min*	*Corr. % max*	*% of total*
Octanoic acid	11,118	0,84	0,104
2-Decenal	11,947	0,67	0,083
2,4- Decadienal	12,324	0,75	0,093
Hexadecane	14,090	1,47	0,183
Nonanoic acid	14,547	2,33	0,290
Tetradecene	16,033	5,16	0,641
Cycloundecane	16,559	1,28	0,159
Tetradecanoic acid	16,902	2,93	0,364
p-Menth-8(10)-en-9-ol	16,954	4,78	0,587
Tetradecanoic acid	17,085	1,44	0,179
1-Docosanol	17,296	0,52	0,065
Thiosulfuric acid	17,453	14,57	1,810
9-Octadecenoic acid	18,171	56,27	6,990
Hexadecanoic acid	18,685	62,89	7,811
Octadecanoic acid	19,394	57,70	7,167
9,12,15-Octadecatrienoic acid	19,954	51,49	6,395
7- Heptadecene	20,622	12,49	1,522
1-Octadecanethiol	22,028	21,22	2,636
1,2-Benzenedicarboxylic acid	22,451	23,85	2,963
Octadecanoic acid	22,759	7,28	0,904
Heptacosane	23,463	16,54	2,054
Octadecanoic acid	24,315	6,09	0,756
Nonacosane	25,418	28,85	3,584
3α,5-cyclo-ergosta-7,22-dien-6-one	28,281	19,62	2,437
Vit E	29,104	8,75	1,087

Table 4
VOLATILE COMPOUNDS IN n - HEXANE EXTRACT OF *TRIFOLIUM PRATENSE* L.

24,26-Dimethylergosta-5-24-dien-3-beta-ol	29,676	3,19	0,396
Ergost-5-en-3β-ol	29,756	2,32	0,289
5-Cholesten-3β,16β,26-triol	30,064	10,57	1,313
Stigmast-5,22-dien-3-ol	31,162	10,06	1,250
24-Methyl-26,26-dimethyl-27-horcholesta-5,22	31,893	3,45	0,428
Stigmasta-5,23dien-3β-ol	31,957	16,83	2,090
Cyclotriacontane	32,968	8,89	1,105
Stigmast-5-en-3-ol	33,642	61,88	7,686
Aristolone	34,459	40,32	5,008
3-Keto-urs-12-ene	34,974	2,54	0,315
Viminalol	35,123	21,49	2,670
4-Stigmasten-3-one	37,357	9,40	1,167
Rotenalone	38,294	4,74	0,588
A-Friedooleanan-3-one	39,906	5,64	0,700
Neophytadiene	41,495	2,29	0,285
1-Naphthacenecarboxylic acid	43,581	5,40	0,671

Continuated table 4

Table 5
VOLATILE COMPOUNDS IN N –HEXANE EXTRACT OF *TRIFOLIUM PATULUM* TAUSCH

Compounds	*tR / min*	*Corr. % max*	*% of total*
2-Heptenal	7,083	0,93	0,114
Benzoic acid	9,695	1,01	0,124
2-Decenal	11,941	2,10	0,259
Benzene	14,198	5,04	0,622
2(4H)-Benzofuranone	14,976	3,20	0,394
Phenol	15,541	2,42	0,298
Methyl ester of 2-(cyclohex-1enyl)4-hydroxybutan	16,079	3,39	0,418
Hexadecanal	16,444	10,28	1,268
Tetradecanoic acid	16,822	2,54	0,314
Pentadecanoic acid	17,336	0,88	0,108
Neophytadiene	17,468	23,79	2,935
Hexadecanoic acid	18,140	25,31	3,123
Tetradecanoic acid	18,473	41,23	5,087
Heptadecanoic acid	18,896	6,87	0,847
9,12-Octadecadienoic acid	19,468	52,10	6,428
Thiosulfuric acid	19,914	9,71	1,198
Tricosane	20,788	15,48	1,910
Eicosanoic acid	20,971	46,52	5,741
Hexadecanoic acid	22,194	30,94	3,818
8-Acetyl-3,3-epoxymethano-6,6,7-trimetylbiciclo	22,611	15,42	1,903
Nonacosane	25,383	17,91	2,210
3α-5-cyclo-ergosta-7,22-dien-6-one	28,224	15,10	1,864
24-Methyl-26,26-dimethyl-27-horcholesta-5,22	31,824	13,63	1,682
Cicloexane	32,693	7,29	0,900
Stigmasten-5-en-3-ol	33,373	100,00	12,339
Aristolone	35,436	10,55	1,302

The percentage of radical scavenging activity (RSC) was calculated using the following equation:

RSC (%) = 100 × (Ablank - Asample / Ablank)

IC5O is the calculated value that highlights the neutralization of 50% of the radicals and was determined by linear regression analysis. The differences between the extracts were analyzed using t-test Student (significant difference at $p \leq 0.05$ confidence level).

Our aim was to characterize the metabolomic profile of *Trifolium patulum* Tausch, considered a Calabrian endemic

species, and then compare it with another red clover, T. *pratense* L., widely studied in literature.

Since it has been fully recognized by the scientific community that secondary metabolites are heavily dependent on climatic conditions, biotic and abiotic stresses, geophysical characteristics and altitude, both samples were collected in the same area for the purpose of being equated.

In fact, any constituent derived from secondary plant metabolism, such as phenolic derivatives, flavonoids, terpenes, steroids, anthraquinones, alkaloids, are often characterized by a species or a certain taxonomic group, thus allowing characterization of the species investigated.

Total polyphenols and total flavonoids were evaluated in total extract and in polar solvents of the two species of *Trifolium*, *pratense* and *patulum*, as well as their antioxidant activity.

The ethanol extracts in CHCl3 and AcOEt showed different amounts of metabolites with antioxidant activity, apparently depending on their extractive capacity. The polar portion, AcOEt, has extracted a higher content of polyphenols and flavonoids, while chloroform has mainly extracted polyphenols, aglycols.

This kind of analysis confirm how the two species of clover are qualitatively similar, and so can be used both for their purpose.

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