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Original article

DETERMINATION OF ISOFLAVONE CONTENT BY HPLC-UV METHOD AND *IN VITRO* ANTIOXIDANT ACTIVITY OF RED CLOVER (*TRIFOLIUM PRATENSE* L.)

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Abstract

In this study, the methanol and acid hydrolyzed methanol extracts prepared from the aerial parts of two varieties of Trifolium pratense (red clover) collected from different parts of Turkey, namely T. pratense L. var. pratense and T. pratense L. var. sativum Schreb., have been analyzed to determine the concentrations of four isoflavones, daidzein (D), genistein (G), formononetin (F) and biochanin A (B). A RP-HPLC-UV method for the determination of these isoflavones in red clover was developed. Chromatographic separation was carried out by using a gradient system consisting of 0.3 % acetic acid and methanol on a reversed phase column, Supelcosil™ LC-18 (15 cm x 4.6 mm id, 5µm). The highest content of total D, G, F, and B were found as 0.0447, 0.0115, 0.0251 and 0.0039 % in T. pratense var. pratense, respectively. Antioxidant capacity of the methanol extracts of aerial parts of plant samples was also studied. Studied extracts were found to possess some antioxidant activities, as determined by the DPPH free radical scavenging test, thiobarbituric acid and trolox-equivalent antioxidant capacity assays. The phenolic contents of the samples were determined using Folin-Ciocalteu's reagent.

Key words: *Trifolium pratense, Fabaceae, Isoflavones, HPLC, Antioxidant activity.*

Kırmızı yonca (*Trifolium pratense* L.)'nın İzoflavon İçeriğinin YPSK-UV Metodu ile Tayini ve *In vitro* Antioksidan Aktivitesi

Bu çalışmada, Türkiye'nin farklı yerlerinden toplanan Trifolium pratense'nin (kırmızı yonca) iki varyetesinin, T. pratense L. var. pratense and T. pratense L. var. sativum Schreb., toprak üstü kısımlarından hazırlanan metanol ve asitle hidrolize edilmiş metanol ekstrelerinde dört izoflavonun, daidzein (D), genistein (G), formononetin (F) ve biokanin A (B), konsantrasyonları tayin edilmiştir. Kırmızı yonca içerisindeki bu izoflavonların tayini için bir ters faz YPSK-UV metodu geliştirildi. Kromatografik ayırım % 0.3 asetik asit ve metanol içeren bir gradient sistemin kullanıldığı ters faz bir kolonda, Supelcosil™ LC-18 (15 cm x 4.6 mm id, 5µm) gerçekleştirilmiştir. En yüksek total D, G, F ve B miktarları T. pratense var. pratense içerisinde sırasıyla % 0.0447, % 0.0115, % 0.0251 ve % 0.0039 olarak bulunmuştur. Bitki örneklerinin toprak üstü kısımlarının metanol ekstreleri antioksidan aktiviteleri bakımından da incelenmiştir. İncelenen ekstreler DPPH serbest radikal süpürücü, tiyobarbitürik asit ve troloks'a eşdeğer antioksidan kapasite tayinlerinde bazı antioksidan aktivitelere sahip bulunmuştur. Örneklerin fenolik madde içerikleri Folin-Ciocalteu reajanı kullanılarak belirlenmiştir.

Anahtar kelimeler: *Trifolium pratense, Fabaceae, İzoflavonlar, YPSK, Antioksidan aktivite.*

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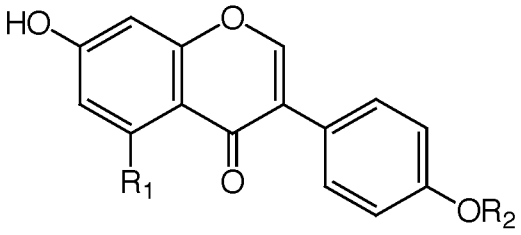
INTRODUCTION

Trifolium pratense L. (Leguminosae), also known as red clover, is a perennial plant with trifoliate leaves, obovate to broadly elliptic, inflorescence globose to ovoid, and corolla reddish-purple to pink, rarely whitish. *T. pratense* is a very variable species. It has three varieties in Turkey, namely *Trifolium pratense* L. var. *pratense*, *Trifolium pratense* L. var. *sativum* Schreb., and *Trifolium pratense* L. var. *americanum* Harz. All varieties are widely cultivated as a fodder crop throughout the North temperate region (1). Red clover is traditionally used in the treatment of chronic skin diseases such eczema and psoriasis and whooping cough (2,3). Recently, it has been used for many human health benefits due to phytoestrogen isoflavones (4).

Red clover contains a great number of flavone derivative compounds, principally isoflavones. Many flavonoids have been identified as glycoside and glycoside malonate derivatives of flavonoids in red clover. The main bioactive isoflavones are formononetin (F) and biochanin A (B), with smaller concentrations of daidzein (D) and genistein (G) (4). Therefore, it has received considerable interest as a rich source of estrogenic isoflavones. Phytoestrogens reported to have protective effects on estrogen-related conditions such as menopausal symptoms and estrogen-related diseases, such as several cancers, prostate, breast, etc., osteoporosis and cardiovascular diseases (4-6). Therefore, red clover preparations have recently suggested for alternative hormone replacement therapy (HRT) as selective estrogen receptor modulators and in the management of menopause symptoms (5). According to the substituents in the main bioactive isoflavone structure in red clover, the molecular structures of D, G, F and B are presented in Figure 1.



No	Isoflavones	Substituents	
		R_1	R_2
1	Daidzein	H	H
2	Formononetin	H	CH_3
3	Genistein	OH	H
4	Biochanin A	OH	CH_3

Figure 1. Chemical structures of major isoflavones in *Trifolium pratense* L.

The Genus *Trifolium* L. comprises 128 taxa, in which 11 of them are endemic to Turkey (1,7-10). A number of analytical methods have been reported for the detection and the quantification of the isoflavones in red clover (11-20). In our previous study, we applied new HPLC-chemometric approaches for the simultaneous chromatographic quantification of daidzein, genistein, formononetin, and biochanin A in the samples consisting of the aerial parts of *Trifolium lucanicum* Gasp. In addition, conventional HPLC was used for the determination of each compound in the extracts of *T. lucanicum* (21). As a part of our ongoing research on *Trifolium* species growing in Turkey, we investigated isoflavone contents of the aerial parts of two *Trifolium pratense* varieties. Additionaly, we determined total polyphenol contents and *in vitro* antioxidant potentials of methanolic extracts of these samples.

In the present study, our aim is (i) to quantify principally bioactive isoflavones (daidzein, genistein, formononetin and biochanin A) in the extracted samples of aerial parts of red clover;

(ii) to determine the content of total polyphenols in methanol extracts of the aerial parts of red clover; (iii) to evaluate the antioxidant activity and free radical scavenging activity of methanol extract prepared from the aerial parts of *T. pratense* varieties growing in Turkey.

EXPERIMENTAL

Chemicals

In our study, methanol used in the HPLC analysis was of chromatographic grade (Merck, Darmstadt, Germany). Acetic acid (Merck, Darmstadt, Germany) was also used to prepare the mobile phase. In the extraction procedure, methanol and trifluoroacetic acid (TFA) were of analytical grade (Merck, Darmstadt, Germany). Isoflavone standards; daidzein (D), genistein (G), formononetin (F) and biochanin A (B) were purchased from Fluka (Buchs, Switzerland). Dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) was used by dissolving the isoflavone standards. All other chemicals were analytical reagent grade.

Plant materials

Plant materials were collected in flowering periods from different localities in Turkey. The plant samples were collected and identified by *N. Adıgüzel* and *B. Bani*. Authenticated voucher specimens were deposited in the Herbarium of GAZI, Ankara, Turkey. Their collecting locations are given below:

T. pratense var. *pratense:* B6 Adana: Tufanbeyli, Yamanlı village 1550 m, 20.05.2006 (B.B. 2774).

T. pratense var. *sativum*: C9 Van: Çatak 1500 m, 16.6.2006 (B.B. 1836).

HPLC Analysis of Isoflavones

Sample preparation

Two-hundred miligrams powdered materials were extracted with 80 % methanol for 15 min under reflux at 85 °C. This procedure was repeated two times. After cooling, the solution was filtered and the residue washed with 5 mL of 80 % methanol. The combined extract was evaporated under reduced pressure. The extract was dissolved in methanol and then aliquots of 3 mL were applied onto pre-conditioned SPE cartridges (Sep-Pak C18, Waters). Isoflavones were eluted with 80 % methanol and elution solvent was distilled in *vacuo*. This extracts (E_1) were analysed for free isoflavones.

Two-hundred miligrams powdered materials were extracted by using 20 mL of 80 % methanol (acidified to pH 3 with TFA) for 15 min under reflux at 85 °C. This procedure was repeated to times as a sequence treatment. After filtering the collected extract samples, 1 mL filtrate was diluted with 9 mL water and loaded on to a Sep-Pak C18 cartridge (Waters). Isoflavones were retained on the Sep-Pak C18 cartridge which was then washed with 10 mL of water twice and eluted with 80 % methanol. This extracts (E_2) were analyzed for total isoflavones.

Four isoflavones, D, G, F and B in the both final samples were determined by using the proposed conventional HPLC method. All samples were filtered through a 0.45 µm PTFE membrane filter prior to HPLC separations. Each sample was analyzed by triplicate injections.

Chromatographic conditions

HPLC analysis was performed on an Agilent Technologies 1200 model Liquid chromatograph using a UV detector. Chromatographic separation was carried out by using the HPLC column, a Supelcosil^{TM} LC-18 (4.6 x 150 mm id, 5 µm). Column temperature was 25 °C. The mobile phase consisting of (A) 0.3 % acetic acid (v/v) and (B) methanol was prepared daily, filtered through a 0.45 µm membrane filter and degassed before use. Gradient elution program is applied for chromatographic analysis (Table 1). Flow rate was maintained at 1mL/min and the injection volume was 5 µL. The wavelength of detection was 270 nm. The

quantitative determination of isoflavones was carried out by the external standard method based on the peak areas.

Table 1. Gradient elution program for reversed-phase HPLC-UV method

Time (min)	Mobile Phase (%)		Flow rate (mL/min)
	A	B	
0	50	50	0.6
14	50	50	0.6
15	50	50	0.8
20	35	65	0.8
25	30	70	0.8
27	50	50	0.8

Solvent A: 0.3 % acetic acid (v/v); Solvent B: methanol

Preparation of standard solutions

The stock solutions of 5 mg/mL daidzein, genistein, formononetin and biochanin A were prepared in DMSO and stored in the dark at 4 °C. The standard solutions of four isoflavones were prepared daily from the stock solutions by dilution with methanol. The calibration concentrations of standards were prepared in the range between 2.17-17.5 µg/mL of D, 1.08-8.75 µg/mL of G, 1.08-8.75 µg/mL of F, and 0.156-12.5 µg/mL of B. Six different concentrations of isoflavones were evaluated. All solutions were filtered through a 0.45 µm PTFE membrane filter prior to HPLC analysis. The regression equations calculated for each standard are shown in Table 2.

Table 2. Calibration curves of daidzein, genistein, formononetin and biochanin A.

Standards	Regression equtaions	Correlation coefficient, r^2
Daidzein	y=26.5x-7.5	0.9990
Genistein	y=26.6x-1.8	0.9963
Formononetin	y=20.1x-1.6	0.9997
Biochanin A	y=10.2x+0.5	0.9934

y: unit; x: concentration in µg/mL

Antioxidant Activity

Sample preparation

Each 5 g powdered aerial parts of plant materials were extracted with methanol (2 × 50 mL) for 2 h under reflux at 85 °C. After cooling, the extracts were filtered and concentrated under reduced pressure at 40 °C in *vacuo*.

Determination total phenols

The total phenol content of methanol extracts of the aerial parts of *T. pratense* var. *pratense* and *T. pratense* var. *sativum* was determined using the Folin-Ciocalteu technique (22). Briefly, 50 µL sample were added to 250 µL Folin-Ciocalteu reagent. After 1 min, 750 µL of 20 % (w/v) aqueous Na_2CO_3 were added, and the volume was made up to 5.0 mL with H_2O. After 2 h incubation at 25 °C, the absorbance was measured at 760 nm and to comparison was made via gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg gallic acid/g extract), and the values are presented as means of triplicate analyses.

DPPH free radical-scavenging activity

The DPPH free radical scavenging activities of the plant extracts were assessed by their ability to bleach the stable radical DPPH (23). A 0.75 mL of the aqueous extract at different concentrations was mixed with 1.5 mL of a DPPH methanolic solution (20 mg/L). The controls

contained all the reaction reagents except the extract or positive control substance. After 20 min incubation in darkness and at ambient temperature, the absorbance was recorded at 517 nm.

The percentage of DPPH decolorization of the sample was calculated according to the equation % decolorization = [1 - (ABS_{sample} / $ABS_{control}$)] x 100. The decolorization was plotted against the sample extract concentration, and a linear regression curve was established in order to calculate the IC_{50} (mg/mL) which is the amount of sample necessary to decrease by 50 % the absorbance of DPPH. Butylated hydroxytoluen (BHT), quercetin and ascorbic acid were used as positive controls. All the analyses were carried out in triplicate.

Anti-lipid peroxidation activity

The thiobarbituric acid (TBA) test was used to assess the efficacy extracts in the inhibition of the lipid peroxidation of the liposomes (24,25). The extracts were re-dissolved in methanol and tested at seven different concentrations (0.016-1 mg/mL). Propyl gallate was the reference compound prepared at seven different concentrations (0.000064-1 mg/mL). The assays were carried out in four replicates.

Percentage inhibition of lipid peroxidation was assessed by comparing the absorbance of the reaction mixture containing no inhibitor with that of the extract test reaction mixtures where the substance to be assessed was included. The absorbance readings of the extract alone and the liposomes alone were also taken in account as follows:

$$\% \text{ inhibition} = 100x \frac{(FRM-B)-(ET-B-EA)}{(FRM-B)}$$

Where FRM is the absorbance of the full reaction mixture (liposomes and iron source plus solvent water without the test substance), B is the absorbance of the blank mixture (liposomes only), ET is absorbance of the extract test mixture (full reaction mixture plus test substance), EA is the absorbance due to the extract alone. The half-maximal inhibitory concentration (IC_{50}) of the extract was calculated by linear regression analysis.

Trolox-equivalent antioxidant capacity (TEAC)

The TEAC assay was carried out according to the procedure of Re et al. (26). Briefly, $ABTS^{+}$ radical cation was generated by a reaction of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate. The reaction mixture was allowed to stand in the dark for 16 h at room temperature and used within 2 days. The $ABTS^{+}$ solution was diluted with methanol to an absorbance of 0.700±0.020 at 734 nm. All samples were diluted appropriately to provide 20-80 % inhibition of the blank absorbance. Then, 10 µL of the diluted sample were mixed with 1 mL of diluted $ABTS^{+}$ solution. The assay with the mixture was carried out in triplicate, the mixture was allowed to stand for 6 min at room temperature and the absorbance was immediately recorded at 734 nm. Trolox solution (final concentration 0.50-2.25 mmol/L) was used as a reference standard. The results were expressed as mmol/L Trolox/g dry weight of plant material.

RESULTS AND DISCUSSION

As a part of our continuing research on *Trifolium* species growing in Turkey, we have investigated the content of isoflavones of *Trifolium pratense*. For this purpose, the methanol and acid hydrolyzed methanol extracts was obtained from the aerial parts of two varieties of *Trifolium pretense*, namely *T. pratense* var. *pratense* and *T. pratense* var. *sativum*. The extracts have been investigated to determine the concentrations of four main isoflavones, daidzein, genistein, formononetin and biochanin A, by the use of reversed-phase HPLC.

In our chromatographic application, new approaches were applied for the simultaneous chromatographic quantification of D, G, F and B in the samples consisting of *T. pratense var. pratense* and *T. pratense var. sativum*. After the optimization of chromatographic separation,

elution of isoflavones was performed using a gradient system, and the mobile phase consisting of % 0.3 acetic acid (v/v) and methanol. A good chromatographic separation was observed between D, G, F and B at 1 mL/min of flow rate. In our study, standard series of analyzed isoflavones were prepared between 2.17-17.5 µg/mL for D, 1.08-8.75 µg/mL for G, 1.08-8.75 µg/mL for F, and 0.156-12.5 µg/mL for B. A good linearity was obtained for all standards. Chromatogram of the standard mixture of D, G, F and B was shown in Figure 2.

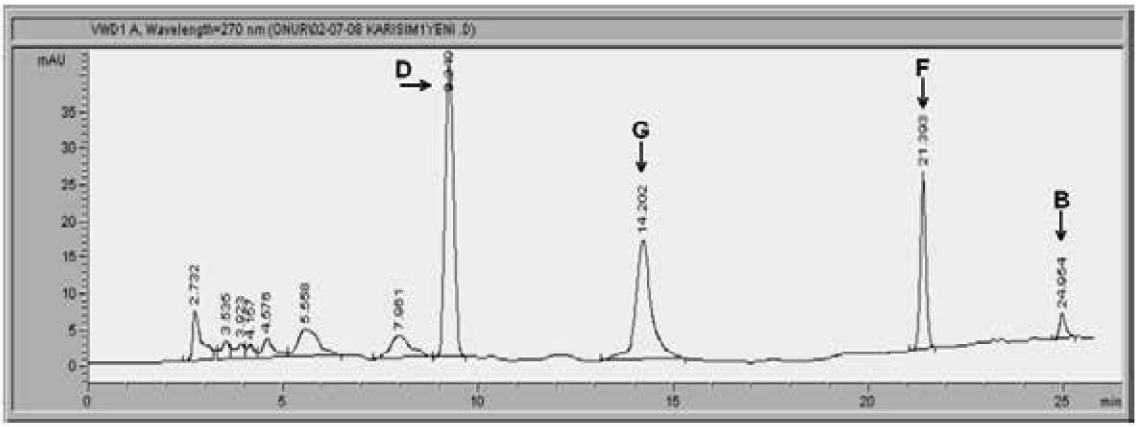


Figure 2. Chromatographic separation of standard mixture of daidzein (D), genistein (G), formononetin (F) and biochanin A (B).

As described in the context of this study, developed HPLC method were subjected to the quantitative analysis of D, G, D and B in two different extracts, the methanol and acid hydrolyzed methanol extracts, obtained from plant samples. The content of D, G, F and B in the extracted samples of *T. pratense* is given in Table 3. While the methanol extracts contain only free isoflavones in red clover, the acid hydrolyzed methanol extracts consist of total isoflavones in plant. In our results, the highest content of total D, G, F and B were found as 0.0447, 0.0115, 0.0251 and 0.0039 % in the acid hydrolyzed methanol extracts of *T. pratense* var. *pratense*, respectively. HPLC chromatograms of D, G, F and B in the extracted samples of *T. pratense* are given in Figures 3 and 4.

Table 3. Content of daidzein, genistein, formononetin and biochanin A in *Trifolium pratense* var. *pratense* and *Trifolium pratense* var. *sativum.*

Samples	Daidzein (%)	Genistein (%)	Formononetin (%)	Biochanin A (%)
T. pratense var. *pratense* (E_1)	0.0398±0.0022	0.0025±0.0014	0.0175±0.0015	0.0023±0.0002
T. pratense var. *pratense* (E_2)	0.0447±0.0007	0.0115±0.0006	0.0251±0.0008	0.0039±0.0001
T. pratense var. *sativum* (E_1)	0.0204±0.0007	0.0035±0.0026	0.0059±0.0002	0.0022±0.0001
T. pratense var. *sativum* (E_2)	0.0257±0.0028	0.0086±0.0002	0.0079±0.0001	0.0035±0.0003

Data are presented as mean ± standard deviation.

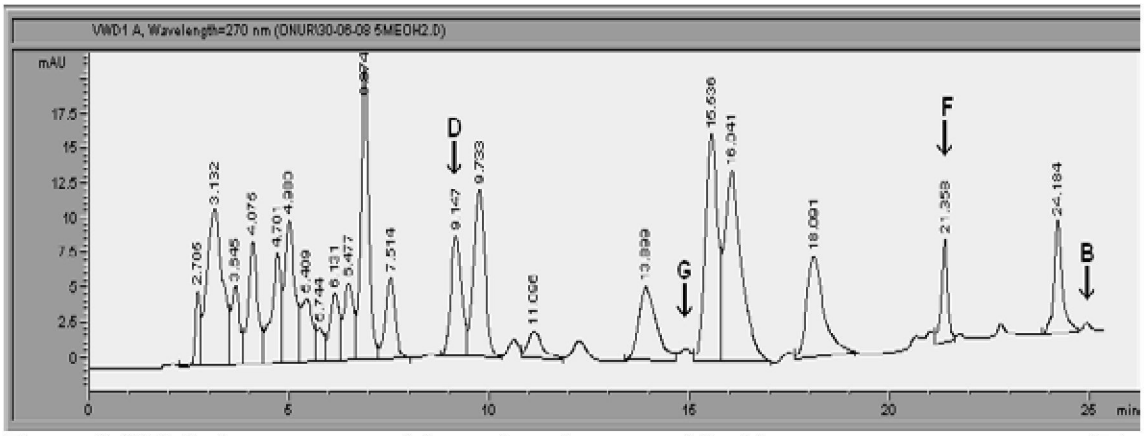


Figure 3. HPLC chromatogram of the methanol extract of *Trifolium pratense* var. *pratense* (E_1).

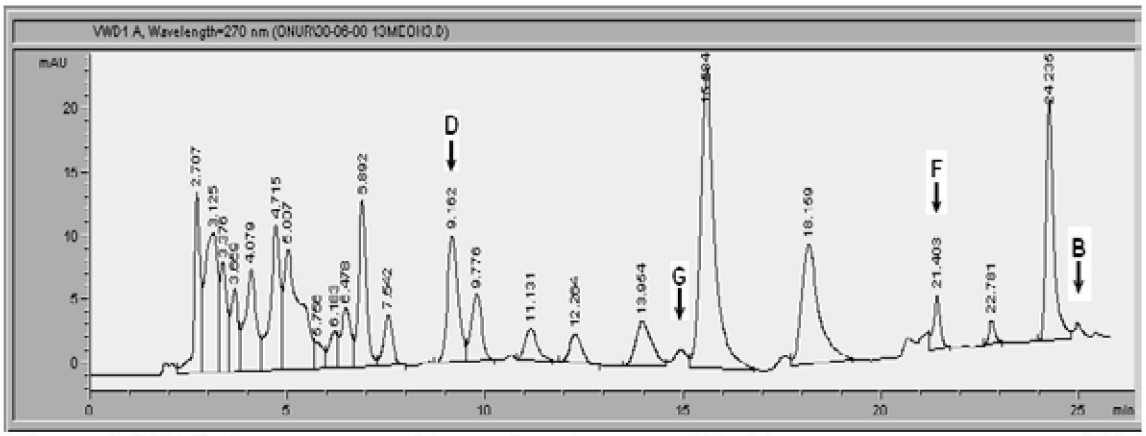


Figure 4. HPLC chromatogram of the methanol extract of *Trifolium pratense* var. *sativum* (E_1).

In this study, we prepared two different extracts from plant materials, methanol and acid hydrolyzed methanol extracts, for the determination of isoflavones by the use of HPLC-UV method. The chromatographic conditions described enable the quantification of daidzein, genistein, formononetin and biochanin A in red clover after hydrolysis of the respective glycosides. Our results showed that developed RP-HPLC-UV method for the determination of major isoflavones in red clover was also rapid and simple to perform.

There are a number of records in the literature related the detection and quantification of isoflavones in *Trifolium pratense* (11-20). In a previous study, Saviranta et al. reported that the content of isoflavones, found in the leaves, stems, roots, and different coloured flowers of red clover, is affected by some factors such as growing period, genetic factors, cultivar, temperature, light, nutrition, etc. (12). Ramos et al.'s work stated that the content of D, G, F and B of five populations of red clover, from a greenhouse in Brazil, ranged from 7.87-91.31, 51.60-131.30, 6568.33-23461.82, to 2499.55-10337.33 µg/g of dried material, respectively (14). In a previous study, the formononetin and biochanin A content in fresh leaves of red clover collected from Netherlands was found 0.06 % and 0.033 %, respectively. It was revealed that genistein and daidzein was not detected in the leaves of red clover (15). In Delmonte et al.'s study, the D, G, F and B content in the herb of *T. pratense* grown in Austria was found 0.011 %, 0.010 %, 0.289 % and 0.204 %, respectively (11). In other study, the D, G, F and B were quantified after hydrolytic extraction in red clover cultivars by an RP-HPLC method, and F and B were found as the main compounds in all studied samples (varied from 0.025 to 0.3 %), while only small amounts of D and G were detected (13). These results showed that the content of isoflavones in red clover samples were varied depending on the cultivar, origin, vegetative stage, genetic factors, etc. (12,13,20).

In addition, the antioxidant activity of methanol extracts from the aerial parts of *T. pratense* var. *pratense* and *T. pratense* var. *sativum* were investigated by three different *in vitro* antioxidant test systems, DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation scavenging and thiobarbituric acid (TBA) assays. The extracts yields (w/w) are given as follows: methanol extract of *T. pratense* var. *pratense* (10.70 %) and methanol extract of *T. pratense* var. *sativum* (7.41 %). The results obtained with methanol extracts of two varieties of *T. pratense* and the references in the antioxidant activity tests are given in Table 4. Both extracts of red clover displayed the highest activity in DPPH radical scavenging and trolox-equivalent antioxidant tests, except thiobarbituric acid test. The inhibition of lipid peroxidation of both extracts was found as low values compared with propyl gallate. The methanol extract of *T. pratense* var. *sativum* proved to be much weaker inhibitors of lipid peroxidation than the other extract (Table 4).

Table 4. Antioxidant activities and total phenol contents of the extracts of *Trifolium pratense* var. *pratense* and *Trifolium pratense* var. *sativum.*

Samples	Total phenols[a]	DPPH[b]	TBA[c]	TAC[d]
T. pratense var. *pratense*	52.30 ± 1.20	1.84 ± 0.13	1209.16 ± 1.01	0.98 ± 0.06
T. pratense var. *sativum*	48.60 ± 0.86	1.87 ± 0.09	2500> ± 4.48	1.02 ± 0.22
BHT	-	0.53 ± 0.01	-	-
Quercetin	-	0.06 ± 0.01	-	-
Ascorbic acid	-	0.09 ± 0.01	-	-
Propyl gallate	-	-	2.38 ± 0.01	-

Data are presented as mean ± standard error
BHT, quercetin, propyl gallate and ascorbic acid were used as positive controls.
[a] Values expressed as mg gallic acid/g extract
[b] Values expressed as IC_{50} (mg/mL)
[c] Values expressed as IC_{50} (µg/mL)
[d] Values expressed as mmol/L Trolox/g

The content of total polyphenols was determined in the methanol extract of red clover by the Folin-Ciocalteu spectrophotometric method. The total phenolic contents expressed as gallic acid equivalents (mg gallic acid / g extract) is shown in Table 4. The amounts of total phenolics were found to be similar levels as 52.30±1.20 mg/g in the extract of *T. pratense* var. *pratense* and 48.60±0.86 mg/g in the extract of *T. pratense* var. *sativum*.

During the literature survey, several reports were indicated on the antioxidant activity of isoflavones, especially genistein, and red clover extracts in several models (27-31). Kroyer studied the *in vitro* DPPH free radical scavenging activity and total polyphenols of the ethanol extract of red clover leaves, and the results were reported as EC_{50}: 0.32 mg/ml for radical scavenging activity and 153 mg/g for total polyphenols (30). Kaurinovic et al. measured the antioxidant properties of five different extracts (ether, chloroform, ethyl acetate, *n*-buthanol and water) of *T. pratense* leaves by using various assays; free radical scavenging capacity tests (DPPH, hydroxyl, superoxide anion and nitric oxide), lipid peroxidation assay, also calculated the total phenolic and flavonoid contents. They found that the water and EtOAc extracts showed a potent antioxidant effect compared well-known synthetic antioxidants (31).

In conclusion, the results of this research represent that *T. pratense* samples collected from Turkey is a good source of phytoestrogenic isoflavones, daidzein, genistein, formononetin and biochanin A, as natural antioxidant compounds.

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