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Original article

Quantitative Determination of Isoflavones by HPLC-UV Method and Antioxidant Activity of *Trifolium longidentatum*

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In this study, aerial parts of *Trifolium longidentatum* Nábělek (Fabaceae) were quantitatively analyzed for their content of four isoflavones, daidzein (D), genistein (G), formononetin (F) and biochanin A (B), by the use of developed RP-HPLC-UV method. The chromatographic separation was performed using a gradient system with a mobile phase of methanol, acetate buffer (pH=4.75) and acetonitrile with on the reversed phase column, Agilent Eclipse XDB-18 (15 cm x 4.6 mm i.d.). The contents of D, G, F and B in the aerial parts of *T. longidentatum* were determined as 0.0095, 0.0056, 0.0021 and 0.0073 %, respectively. Antioxidant potential of the methanol extract prepared from the aerial parts of the plant was also investigated with different *in vitro* methods, namely DPPH radical scavenging activity, lipid peroxidation inhibition and trolox equivalent antioxidant capacity assays. As a consequence, it was observed that the methanol extract of *T. longidentatum* had *in vitro* antioxidant activities in these methods. The phenolic content of the extract was also measured as 53.98 ± 0.95 mg gallic acid/g extract by the Folin-Ciocalteu's reagent.

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

Trifolium longidentatum'un İzoflavonlarının YPSK-UV Metodu ile Kantitatif Tayini ve Antioksidan Aktivitesi

Bu çalışmada, *Trifolium longidentatum* Nábělek (Fabaceae) bitkisinin toprak üstü kısımlarındaki daidzein (D), genistein (G), formononetin (F) and biokanin A (B) olmak üzere dört izoflavonun içeriği Ters faz YPSK-UV metotu kullanılarak kantitatif olarak analiz edilmiştir. Kromatografik ayırım, metanol, asetat tamponu (pH=4.75) ve asetonitril mobil fazının Agilent Eclipse XDB-18 (15 cm x 4.6 mm i.d.) ters faz kolon üzerinde uygulandığı gradient bir sistem kullanılarak gerçekleştirilmiştir. *T. longidentatum*'un toprak üstü kısımlarındaki D, G, F ve B içerikleri sırasıyla % 0.0095, 0.0056, 0.0021 ve 0.0073 olarak tayin edilmiştir. Ayrıca bitkinin toprak üstü kısımlarından hazırlanan metanol ekstresinin antioksidan potansiyali DPPH radikal süpürücü aktivite, lipid peroksidasyon inhibisyon ve troloks'a eşdeğer antioksidan kapasite gibi çeşitli *in vitro* metotlarla incelenmiştir. Sonuç olarak, *T. longidentatum*'un metanol ekstresi incelenen metotlarda antioksidan aktivitelere sahip bulunmuştur. Ayrıca ekstrenin fenolik madde içeriği Folin-Ciocalteu reaktifi kullanılarak 53.98 ± 0.95 mg gallik asit/g ekstre olarak ölçülmüştür.

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

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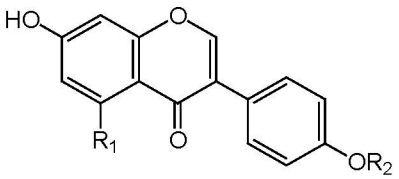
INTRODUCTION

Genus *Trifolium* L. (Fabaceae) is represented by 130 taxons, in which 13 are endemic in flora of Turkey (1-5). *Trifolium longidentatum* Nábělek is an endemic plant with narrow distribution in southeastern of Turkey. It is also an Irano-Turanian element. *T. longidentatum* is an erect or ascending perennial plant with oblong-elliptic leaflets, inflorescence ovoid, elongating in fruit, and corolla pinkish (1).

In traditional medicine, *Trifolium* species are used in the treatment of several disorders; chronic skin diseases such as eczema and psoriasis, whooping cough, asthma, bronchitis and rheumatism (6-8). The blossoms of some *Trifolium* species, *T. pratense* L. and *T. ambiguum* M. Bieb., are used as vegetables in the vicinity of Erzurum and Kars in Turkey (6). In addition, lots of *Trifolium* species are important feeding plants for farm animals in the Mediterranean region (9).

T. pratense, also known as red clover, is a prominent natural source of phytoestrogenic isoflavonoids. Several researches have shown that red clover extracts contain significant high amounts of four major estrogenic isoflavones; genistein (G), daidzein (D), and their methyl ether derivatives biochanin A (B) and formononetin (F) (Figure 1). Therefore, red clover extracts indicate the potential application of red clover preparations for alternative hormone replacement therapy as selective estrogen receptor modulators and in the management of menapouse symptoms (10).



Isoflavones	Substituents	
	R_1	R_2
Daidzein	H	H
Formononetin	H	CH_3
Genistein	OH	H
Biochanin A	OH	CH_3

Figure 1. Chemical structures of isoflavones

A number of analytical methods have been reported for detection and quantification of the isoflavones in red clover as well as other *Trifolium* species and several biological samples by chromatographic methods (11-19). As a part of our ongoing research on *Trifolium* species growing in Turkey, we have investigated the content of isoflavones and antioxidant activities of some *Trifolium* species. In our previous study, the methanol extracts of two varieties of *Trifolium pratense*, namely var. *pratense* and var. *sativum*, were analyzed to determine the amounts of four main isoflavones, D, G, F and B, by the use of developed HPLC method, as well as evaluating for their antioxidant activities (11). In another our previous study, we proposed new HPLC-chemometric approaches for the simultaneous chromatographic quantification of D, G, F, and B in the aerial parts of *T. lucanicum* Gasp. In addition, conventional HPLC was used for the determination of each compound in the extracts of *T. lucanicum* (12). Up to date, any analytical and biological activity studies have not been performed on *T. longidentatum*. As a part of our ongoing research on *Trifolium* species growing in Turkey, we firstly analyzed the amount of main isoflavones, D, G, B and F, of the aerial parts of *T. longidentatum*. Moreover, the content of total polyphenols and *in vitro* antioxidant potentials of the plant extracts were measured in this study.

The aims of the present study focuses on the quantify of biologically active isoflavones, D, G, B, and F, in the methanol extracts of the aerial parts of *T. longidentatum* and investigation of their total phenolic contents and antioxidant activities. The antioxidant properties of the extracts were investigated by three complimentary tests; namely, lipid peroxidation inhibition activity, DPPH radical scavenging activity and ABTS cation radical scavenging activity assays.

EXPERIMENTAL

Chemicals

In our study, methanol was of analytical grade (Merck, Darmstadt, Germany) in the extraction procedure. HPLC grade methanol and acetonitrile (Merck) were used in the

HPLC analysis. The standard isoflavones; daidzein (D), genistein (G), formononetin (F) and biochanin A (B) were purchased from Fluka (Buchs, Switzerland). Dimethyl sulfoxide (DMSO) (Merck) was used by dissolving the isoflavone standards. All other chemicals were analytical reagent grade.

Plant material

Trifolium longidentatum Nábělek was collected from B9 Van: Çatak, Kıyıcak village, Kato mountain, north slopes of Lisek hill, steppe, 2200 m, 13.7.2003 during flowering periods. The plant was identified by Prof. N. Adıgüzel and Assoc. Prof. B. Bani from the Department of Biology. Voucher specimen (BB 2267) was deposited in the Herbarium of GAZI, Ankara, Turkey.

Sample preparation

The extraction procedure was carried out as previously, described (11). Briefly, about 5 g of powdered aerial parts of the plant material was extracted with 80% methanol (2 x 50 mL) for 30 min under reflux at 85 °C. After cooling, the solution was filtered and the residue washed with 5 mL of 80 % methanol. The combined extract was evaporated under reduced pressure to afford the methanolic extract. This extract was used for its isoflavone analysis by the use of HPLC and for evaluating its antioxidant activities.

HPLC analysis of isoflavones

Instrumentation

The HPLC analysis was performed on an Agilent Technologies (Santa Clara, CA) 1200 series Liquid chromatograph with a G1311A pump, G1322A degasser, G1316A column heater, G1328B manual injector, and G1314B variable wavelength UV-detector. An Agilent Eclipse XDB-18 (15 cm x 4.6 mm ID, 5µm) column was used as the stationary phase.

Chromatographic conditions

For chromatographic separation, the mobile phase consisting of (A) methanol, (B) acetate buffer (pH:4.75, 0.1 M) and (C) acetonitrile was prepared daily, filtered through a 0.45 µm nylon-membrane filter (Millipore, Barcelona, Spain) and degassed before use. Gradient elution program is applied for chromatographic analysis. A two-step solvent gradient was used, starting from 21% methanol, 58% acetate buffer, 21% acetonitrile at flow rate 1.0 mL/min and conditions were stabilized until 13 min and installing a gradient to obtain 41% methanol, 38% acetate buffer, 21% acetonitrile at flow rate 1.5 mL/min at 15 min, then obtained new conditions were stabilized until end of the analysis (until 30 min). The injection volume was set at 10 µL. The wavelength of detection was adjusted 270 nm with a UV-VIS detector. The column temperature was set to 25 °C. The quantitative determination of isoflavones was carried out by the external standard method based on peak areas. 6-Methoxy flavone was used as internal standard (IS).

Preparation of stock and standard solutions

Individual stock solutions of the isoflavones were prepared in DMSO and methanol. 2 mg of daidzein, genistein, formononetin and biochanin A standards were weighed and the standards were dissolved with 0.5 mL DMSO and diluted with methanol to prepare 20 mg/L stock solutions. The standard solutions of four isoflavones were prepared daily from the stock solutions by dilution with methanol and stored in the dark at 4 °C during the study. The concentrations of standard solutions were adjusted to 0.25, 0.5, 1.0, 2.5 ve 5.0 µg/mL.

All solutions were filtered through a 0.45 µm PTFE membrane filter prior to HPLC analysis. The internal standard (6-methoxy flavone) was added into all of the standard solutions to a final concentration of 5 µg/mL. These standard solutions were directly injected into the HPLC system.

Sample preparation for HPLC analysis

The methanolic extract of the aerial parts of *T. longidentatum* was analyzed by using the proposed HPLC method. For HPLC analysis, 20 mg from the extract sample was dissolved 100 µL DMSO and diluted to 2 mL with methanol, respectively. The internal standard (6-methoxy flavone) was added into the prepared sample to a final concentration of 5 µg/mL. The extract was filtered through a 0.45 µm PTFE membrane filter and 10 µL were analyzed by HPLC. The sample was analyzed by triplicate injections.

Method validation

The developed method was validated by determination of the linear range, limit of detection (LOD), and limit of quantification (LOQ).

The linearity of the calibration curve was assessed by the analysis of reference compounds. In this study for the system linearity, standard solutions of daidzein, genistein, formononetin and biochanin A at five concentration levels were prepared: the range of 0.25-5.0 µg/mL for four isoflavones. A calibration curve was obtained with the experimental results, and the corresponding statistical study was carried out. Calibration curves were determined on three separate injections for all of the standards and the standard deviation was calculated to evaluate reproducibility.

The LOD and LOQ values were calculated from the calibration curves that defined linearity. LOD, which is expressed in units of concentration, describes the lowest concentration level that can be distinguished from the blank. The slope of the calibration curves (b) and the standard error of the independent term of the regression (Sb) were used to calculate the LOD and LOQ with reference to International Union of Pure and Applied Chemistry definitions.

Antioxidant Activity

Determination of total phenolic content

Total phenolic content of the extract was determined using the Folin-Ciocalteu method (20). According to the method, 250 µL of Folin-Ciocalteu reagent and 750 µL of 20 % (w/v) aqueous Na_2CO_3 solution were added to 50 µL extract. The volume was made up to 5.0 mL with distilled H_2O. After 2 h of staying at 25 °C, the absorbance was measured at 760 nm on spectrophotometer versus blank sample. Total phenolic content was determined gallic acid equivalents (mg gallic acid/g extract). The total phenols are presented as means of triplicate analyses.

DPPH free radical scavenging activity

The ability of the extracts to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals were estimated to the method of Brand-Williams et al. (21). Briefly, 0.75 mL of the methanolic 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 percent 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. BHT and ascorbic acid were used as positive controls. All the analyses were carried out in triplicate.

Lipid peroxidation inhibition activity

The thiobarbituric acid (TBA) test was used to determine the potential of the methanol extract on the inhibition of the lipid peroxidation of the liposomes (22). The extract was tested at seven different concentrations (0.016-1 mg/mL). Propyl gallate was used the reference compound at (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:

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

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 IC_{50} concentration of the extract was calculated by linear regression analysis.

Trolox equivalent antioxidant capacity (TEAC)

TEAC was determined as described by Re et al. (23). 2,2'-Azinobis-(3-ethyl-benzothiazoline-6-sulfonate) (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. 10 µL of the diluted sample were mixed with 1 ml of diluted $ABTS^{+}$ solution. The mixture was allowed to stand for 6 min at room temperature and then 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. The assays were carried out in triplicate.

RESULTS AND DISCUSSION

In this study, as a part of our ongoing research on *Trifolium* species growing in Turkey, we have investigated the content of main isoflavone compounds D, G, F, and B of the methanol extract prepared from the aerial parts of *T. longidentatum* using by RP-HPLC for the first time. In addition, antioxidant activity of the plant was firstly evaluated by the use of three *in vitro* tests, namely, DPPH radical scavenging activity, lipid peroxidation inhibition activity and ABTS cation radical scavenging activity assays. We also calculated the total phenolic content of the methanol extract of the plant.

In our chromatographic analysis, new applications were carried out for the simultaneous chromatographic quantification of D, G, F, and B in the samples obtained from *T. longidentatum*. For this purpose, the elution solvent composed by methanol, acetate buffer (adjusted to pH 4.75, 0.1 M) and acetonitrile was chosen as mobil phase. Different gradient systems were tested for the separation of *T. longidentatum* extract by using HPLC-UV method. The best separation was achieved using a two-step solvent gradient as shown in Table 1. 6-Methoxy flavone was chosen as an internal standard. After the optimization of chromatographic separation, analysis of *T. longidentatum* extract was performed using a gradient system under determined conditions.

Table 1. Gradient elution program for RP-HPLC analysis.

Time (min)	Mobile phase (%)			Flow rate (mL/min)
	A	B	C	
0	21	58	21	1.0
13	21	58	21	1.0
15	41	38	21	1.5
30	41	38	21	1.5

A: Methanol; B: Buffer; C: Acetonitrile.

For the validation of the developed HPLC-UV method, external calibration curves were obtained for linear regression analysis. A linear response was obtained for investigated standards. The linear response function parameters were summarized in Table 2. The calibration curves were obtained in the range of 0.25-5.0 µg/mL for four isoflavones. The r^2 values for isoflavones and the experimentally determined values of LOD and LOQ were given in Table 2. The LOD values were found to be 0.11 µg/mL for daidzein, 0.16 µg/mL for genistein, 0.08 µg/mL for formononetin, and 0.08 µg/mL for biochanin A. The LOQ values were found to be 0.33 µg/mL for daidzein, 0.48 µg/mL for genistein, 0.24 µg/mL for formononetin, and 0.24 µg/mL for biochanin A. These results showed that the developed RP-HPLC-UV method is sensitive and has a good linearity for quantification of

isoflavones present at low concentrations in the extract of *T. longidentatum*.

The chromatogram of the standard mixture of D, G, F and B was shown in Figure 2 at a concentration of 5 µg/mL. The retention times of daidzein, genistein, formononetin, biochanin A and IS were monitored at 3.92 min, 7.84 min, 15.51 min, 19.51 min and 21.64 min, respectively.

Table 2. Regression equations for HPLC-UV determination of four isoflavones.

Compounds	Regression equation	r^2	Linear range (µg/mL)
Daidzein	y = 2.4795x + 0,127	0.9994	0.33-5.2
Genistein	y = 2.5711x - 0,195	0.9988	0.48-5.8
Formononetin	y = 2.3682x + 0,017	0.9995	0.24-5.4
Biochanin A	y = 1.7751x -0.020	0.9993	0.24-4.8

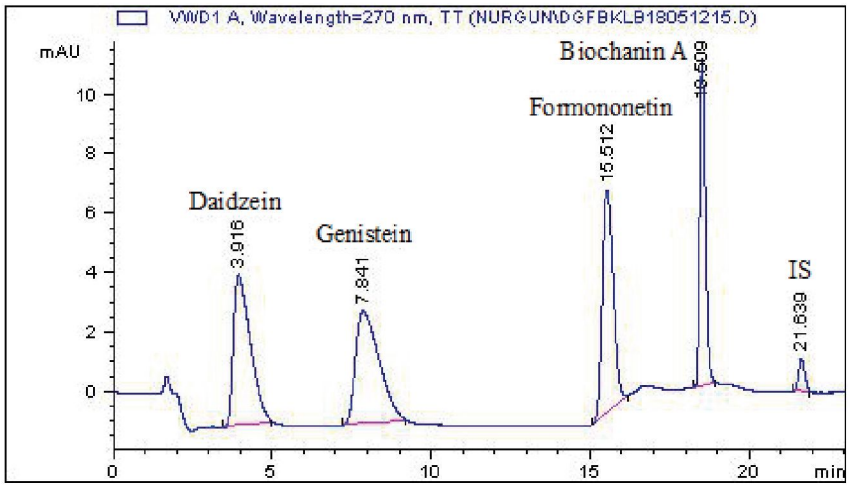


Figure 2. Chromatographic separation of standard mixture of D, G, F, B and IS 5 µg/mL of each.

The developed HPLC-UV method was applied to the quantification of four isoflavones in the methanol extract of *T. longidentatum*. HPLC chromatogram of D, G, F, and B in the extracted samples of *T. longidentatum* are given in Figure 3. The retention times of daidzein, genistein, formononetin, biochanin A and IS in the extract of *T. longidentatum* were monitored at 5.52 min, 9.99 min, 16.14 min, 18.84 min and 21.99 min, respectively. In present study, the highest content of total daidzein, genistein, formononetin, biochanin A were found as 0.0095, 0.0056, 0.0021 and 0.0073 % in the methanol extract of *T. longidentatum*, respectively (Table 3). The developed RP-HPLC-UV method allows plant samples to determine isoflavones. This method is rapid and simple to perform the quantification of the isoflavones.

Table 3. Content of D, G, F and B in the methanol extract of *T. longidentatum*.

Sample	Daidzein	Genistein	Formononetin	Biochanin A
T. longidentatum	0.0095±0.30	0.0056±0.24	0.0021±0.80	0.0073±0.13

Results were expressed as mean ±S.D.

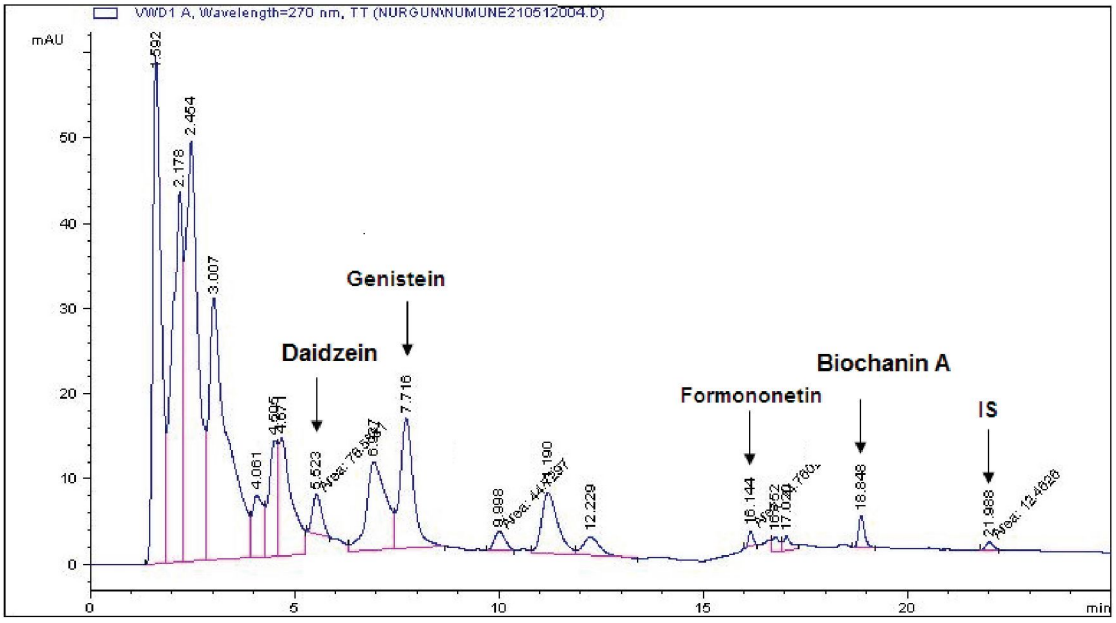


Figure 3. The HPLC-UV chromatogram of the methanol extract of *T. longidentatum*.

Many records for the quantification of isoflavones especially in *T. pratense* and different *Trifolium* species were reported in the literature (11-19). In previous studies on the content of isoflavones of *T. pratense* showed that the content of isoflavones in red clover samples were varied depending on the cultivar, origin, vegetative stage, genetic factors, temperature, light, growing period, etc. (14,15,19). In a previous study, the content of D, G, F and B of five populations of red clover from 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 (16). In another study, the percentage of formononetin and biochanin A in fresh leaves of red clover from Netherlands was determined as 0.06 and 0.033 %, respectively. Otherwise, genistein and daidzein were not detected in the samples of red clover (17). In Delmonte et al.'s study, the content of D, G, F and B in the herb of *T. pratense* from Austria was quantified as 0.011, 0.010, 0.289 and 0.204 %, respectively (13). Krenn et al. reported that the content of D, G, F and B were determined after hydrolytic extraction in red clover samples by the use of RP-HPLC method, and formononetin and biochanin B were found as the major compounds in all investigated samples (varied from 0.025 to 0.3 %), while only small amounts of D and G were determined (15). In our previous study, we developed an HPLC-UV method for the determination of these isoflavones in red clover and 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 (11). In other our previous study, new HPLC-chemometric approaches were proposed for the simultaneous chromatographic quantification of D, G, F, and B in the methanol extracts of the aerial parts of *T. lucanicum* Gasp. collected from Turkey. In addition, we used conventional HPLC system and DAD detections at four different wavelengths for the determination of isoflavones in the extracts of *T. lucanicum* (12). Renda et al. reported that the content of D, G, F, and B of the aqueous methanolic extracts (80 %) of 13 *Trifolium* species collected from different regions of Turkey was determined by the use of validated HPLC method. Genistein (10 species) and formononetin (nine species) were the most common isoflavones in studied *Trifolium* samples, while biochanin A and daidzein were present only in seven and four species, respectively. *T. canescens* was found the richest species containing four isoflavones, totally 1835.73 µg/g, with the highest

amounts of genistein and biochanin A among the all analyzed *Trifolium* samples, and following *T. pratense* var. *pratense* was determined as the second species contains four isoflavones (24).

In this study, the antioxidant activity of methanol extract from the aerial parts of *T. longidentatum* was investigated by three different *in vitro* antioxidant test systems, DPPH radical scavenging, ABTS radical cation scavenging and TBA assays. The methanol extract of the plant is yielded as 10.41 % (w/w). The results of the antioxidant activity and total phenolic content of the methanol extract are given in Table 4. The extract displayed the moderate activity in DPPH radical scavenging and trolox equivalent antioxidant tests, with 1.71 ± 0.09 mg/mL and 1.07 ± 0.12 mmol/L Trolox/g of IC_{50} values, respectively. The inhibitory activity of the extract against lipid peroxidation was determined thiobarbituric acid method. The inhibition of lipid peroxidation of the extract was found as low value (441.58 ± 4.33 µg/mL) compared reference compound propyl gallate. The total phenolic content was determined in the methanol extract of the plant by the spectrophotometric method with Folin-Ciocalteu reagent. The total phenolic content expressed as gallic acid equivalent (mg gallic acid/ g extract) is shown in Table 4. The amount of total phenolics was found as 53.98 ± 0.95 mg/g in the extract of *T. longidentatum*.

Table 4. Antioxidant activities and total phenolic content of the extracts of *T. longidentatum*.

Samples	Total phenolic content[a]	DPPH[b]	TBA[c]	TAC[d]
T. longidentatum	53.98 ± 0.95	1.71 ± 0.09	441.58 ± 4.33	1.07 ± 0.12
BHT	-	0.016 ± 1.00	-	-
Ascorbic acid	-	0.006 ± 1.10	-	-
Propyl gallate	-	-	2.38 ± 0.01	-

Data are presented as mean ± standard error
BHT, 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

Natural antioxidants have been proved to play prevention roles against harmful effects of oxygen radicals and other reactive oxygen species. A lot of studies for the finding more effective antioxidants of natural origin have been studied. Phenolic compounds are known as responsible for the potential antioxidant and radical scavenging activities. Isoflavones, a specific group of phenolic compounds, especially genistein and red clover extract, have been reported to possess significant antioxidant and radical scavenging potentials in different assays (11, 25-30). In these studies, the various extracts of red clover showed potent antioxidant effects by using different assays, etc. free radical scavenging capacity tests (DPPH, hydroxyl, superoxide anion and nitric oxide), lipid peroxidation assay, compared reference substances (28,30).

In conclusion, the results of this research revealed that *T. longidentatum* contain all investigated isoflavones, genistein, daidzein, formononetin and biochanin A, which are known as natural antioxidant compounds. The developed RP-HPLC-UV method successfully might be applies for the determination of isoflavones in *Trifolium* species and other plant materials as well as products containing *T. pratense* extracts.

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