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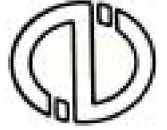

ARAŞTIRMA MAKALESİ /**RESEARCH ARTICLE**

ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF *LINUM OLYMPICUM* EXTRACTS

Nilgün Öztürk[1*], Berrin Bozan[2], Belma Konuklugil[3]

ABSTRACT

The antioxidant activities of extracts *Linum olympicum* leaves were evaluated using a DPPH assay, a β-caroten/linoleic acid assay and the Rancimat method. Leaves were extracted with 70% methanol and fractionated with ethyl acetate. Total phenolic content varied from 303 to 493 mg gallic acid equivalent/g extract. Only methanol extract of the leaves showed free radical scavenging activity and ethyl acetate fraction exhibited the strongest neutralizing activity. Phenolic groups (i.e. phenolic acids and flavonoid) in the methanolic extract were identified previsionaly by HPLC-DAD method.

Keywords: *Linum olympicum* leaves, Antioxidant activity, Total phenolic content, HPLC-DAD.

LINUM OLYMPICUM EKSTRELERİNİN ANTİOKSİDAN VE SERBEST RADİKAL SÜPÜRÜCÜ ETKİLERİ

ÖZ

Linum olympicum yaprak ekstrelerinin antioksidan aktivitesi 3 farklı yöntem kullanılarak değerlendirilmiştir; DPPH üzerinden serbest radikal süpürücü etki, β-karoten/ linoleik asit tayini ve Ransimat metodu. % 70 metanol ile ekstre edilen yapraklar, etilasetat ile fraksiyonlanmıştır. Toplam fenolik madde içeriği gr ekstrede 303-493 mg gallik asite eşdeğer olarak bulunmuştur. Yaprakların metanol ekstresi serbest radikal süpürücü etki gösterirken, eti-lasetat fraksiyonu serbest radikalleri güçlü nötralleştirme etkisi göstermiştir. Metanol ekstresinin fenolik grupları (fenolik asit ve flavonoidler gibi) YBSK-DAD metoduyla tayin edilmiştir.

Anahtar Kelimeler : *Linum olympicum*, Antioksidan aktivite, Toplam fenol miktarı, YBSK-DAD

[1] Anadolu University, Faculty of Pharmacy, Department of Pharmacognosy, Tepebaşı, 26470 Eskişehir, Turkey.
e-mail: nozturk@anadolu.edu.tr
[2] Anadolu University, Faculty of Enginering and Architecture, Department of Chemical Enginering , 26470 Eskişehir, Turkey
[3]Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, Tandoğan, 06100 Ankara, Turkey
*To whom all correspondance should be addressed

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1. INTRODUCTION

Phenolic compounds, secondary metabolites in plant materials, are known to be responsible for antioxidant effect. Recent epidemiological studies have strongly suggested that consumption of food plants may reduce risk of chronic diseases related to oxidative stress on account of their antioxidant activity and promote general health benefits (Halliwell, 1997). On the other hand, in the food industry, antioxidants are used to retard the oxidative degradation of fats by inhibiting the formation of free radicals. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propylgallate (PG) are widely used, however the use of synthetic antioxidants in food products is being questioned (Branen, 1975; Takajashi and Hiraga, 1978). Consumers have also become more cautious about the nutritional quality and safety of food additives. In response to the growing consumer demand, investigations on antioxidants from natural sources have gained interest (Pokorny, 1991). Fruits and vegetables are main sources of phenolic compounds in human diet. Other sources, grains, herbs and spices, also have received particular attention as sources of antioxidants (Hannum, 2004; Nakatani, 2000).

The genus *Linum* contains about 230 species mainly annual or perennial herbs with some small shrubs, and they are distributed all over the world in a very wide variety of habitats (Heywood, 1979). The genus *Linum* is represented by 39 species (51 taxa) in the Flora of Turkey and East Aegean Islands. Twenty-four taxa of these, including *Linum olympicum* Boiss., are endemicplants for Turkey (Davis, 1967; Davis, et al. 2002). There is only one study concerning chemistry of *Linum olympiacum* leaves (Konuklugil, 2004), and no study found on the antioxidant properties of this plant. Therefore, the objective of this study was to evaluate antioxidant activity of polar extract of *L. olympiacum* leaves. Priliminary identification of phenolic compound groups in the extracts were also investigated.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Linum olympicum Boiss., (syn. Linum kotschyanum Hayek) (LINACEAE) was collected from Bursa-Uludağ, 2245 m in Turkey in September 2002 and identified by Prof. Hayri Duman from The Department of Biology (University of Gazi). A voucher specimen (AEF No: 22951) has been deposited in the herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy (University of Ankara). BHT and Folin Ciocalteu’s phenol reagent, b-caroten, linoleic acid and Tween 80 were purchased from Sigma Chemical Co. (St. Louis, MO), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH.) was from Aldrich Chemical Co. (Milwaukee, WI). Crude sunflower oil was kindly provided by Demircanlar Co., Eskişehir, Turkey.

2.2 Preparation of the Extracts

Dried and powdered leaves were extracted with petroleum ether to remove fats. Fat-free air dried material was extracted with aqueous methanol (%70) at 40°C water bath 30 min (x 4). MeOH was removed under reduced pressure by a rotary evaporator and the remaining aqueous solution was lyophilizied (LMF). This extraction process was repeated and aqueous methanol extraction was partitioned with ethylacetate, in order to obtain compounds in different polarity. After concentration under reduced pressure a dark green ethyl acetate fraction was obtained (LEF). The remaining water fraction was freeze dried (LWF). All fractions (LMF, LEF and LWF) obtained were weighed to determine the yields of soluble constituents, total phenolic content and their antioxidant activity.

2.3 Total Phenolics Determination

The total phenolics were determined by the Folin-Ciocaltaeu colorimetric method (Hoff and Singleton, 1977) as follows: Samples (0.5 ml) were introduced into test tubes; 2.5 ml of Folin Ciocalteu (10% in water) reagent solution and 7.5 ml of Na_2CO_3 (20% in water) solution were added. The tubes were mixed and allowed to stand at room temperature in the dark for 2 h. Absorption at 750 nm was measured. Total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g dry material. The results are expressed as average of three measurements..

2.4 Antioxidant Activity Evaluation

2.4.1 Rancimat method

Antioxidant activities of fractions were evaluated by measuring the oxidation induction time with the use of A743 Rancimat apparatus (Metrohm AG, Switzerland). A flow of air (20L/h) was bubbled through the oil heated at 100°C, and the volatile compounds were collected in cold water, increasing the water conductivity. Each sample was dispersed in 3 g of sunflower oil rich in linoleic acid (65% of fatty acids) at the concentration of 1%. Sunflower oil without added antioxidant as the control were run similarly. The test was run in triplicate. Induction index was calculated by following equation:

$$\text{Induction index (II)} = \frac{\text{Induction time of sample}}{\text{Induction time of control}}$$

2.4.2 Free radical scavenging activity on DPPH

Free radical scavenging effects of the fractions on DPPH were estimated according to the method of Sanchez-Moreno (1998) with some modification. An aliquot of methanol (0.4 ml), solution containing different sample concentrations (4mg/ml)) was added to 3 ml of 0.05 mM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH.) in methanol prepared daily. The mixture

was shaken vigorously and left standing at room temperature for 30 min; absorbance of the resulting solution was then measured spectrophotometrically at 517 nm. The radical scavenging activity of the tested samples, expressed as % Inhibition against DPPH., was calculated as follows:

$$\text{Inhibition (\%)} = \left[\frac{A_{Control} - A_{Sample}}{A_{Control}}\right] x100$$

Free radical scavenging activity determination was repeated four times for each sample and the means are reported. EC_{50} values were obtained from the inhibition curve. Free radical scavenging activity determination was repeated four times for each sample and the means are reported.

2.4.3 β-Carotene-linoleic acid assay system

The antioxidative activity of extracts was evaluated using a β-carotene-linoleic acid model system (Maure et al., 2001). Briefly, 10 mg β-carotene was dissolved in 3 ml of chloroform. The solution was added to 40 mg linoleic acid and 400 mg Tween 80. After removing the chloroform using a rotary evaporator at 40°C, oxygenated distilled water (100 ml) was added to the β-carotene-linoleic acid emulsion with vigorous shaking. 3 ml of this solution were mixed with 200 ml extract prepared at 0.2 mg/ml concentration. After incubation at 50°C for 180 min, absorbance of each sample at 470 nm was monitored at time intervals of 15 min during 180 min. Antioxidant activities of extracts were compared with those of BHT at the same concentration and blank consisting of 0.2 ml of methanol. The antioxidant activity (AA) was calculated from the following equation:

$$AA = \frac{A_s - A_c}{A_0 - A_c} x100$$

where, A_s and A_c are absorbances of sample and control, respectively at 180 min; A_0 is absorbance at 470 nm of sample at the start of test..

2.4.4 HPLC-DAD analysis of phenolic compounds

Analysis of phenolic compounds was determined by high performance liquid chromatography (HPLC). The HPLC system used consists of a Shimadzu SCL 10V equipped with Diode Array Detector (SPD-M 10AVP). Phenolic acids were separated on a reversed phase column (C18, 25 cm x 4.6 mm I.D., 5 µm particle size, Beckman) with the mobile phase flow rate at 1 mL/min. Separation was achieved by elution gradient using the mobile phase A: Methanol:Water:Acetic acid (10:88:2)(v/v) and B: Methanol:Water:Acetic acid (90:8:2) (v/v). The linear gradient program used is as follow: The composition of B was increased from 0% to 15% in 15 min, increased to 50% in 10 min, and finally to 70% in another 9 min. The data were integrated and analyzed using Shimadzu Class-VP Chromatography Laboratory Automated Software system. Reference phenolic acids and flavonoids were obtained from Sigma. Gallic, protocatechuic, *p*-OH-benzoic, vanillic, isovanillic, syringic acids were used as the reference compounds represents the benzoic acids. For cinnamic acids, six commercially available acids as caffeic, *p*-coumaric, ferulic, sinapinic, *o*-coumaric acids were selected as reference compounds. In order to identify flavonoids, quercetin, luteolin, kaempherol, apigenin, which were available commercially in their agycone form, were used.

Table 1. Yields of fractions and total phenolics contents obtained from *Linum olympicum* leaves

Fractions	Extraction Yield (%)[1]	Total phenolics content [2,3]
L. olympicum - methanol (LMF)	19.44	356.60 ± 8.15
L. olympicum - ethylacetate (LEF)	5.42	492.59 ± 7.20
L. olympicum - water (LWF)	14.01	302.86 ± 3.45

[1] %, w/w on dry weight basis, [2] mg GAE*/g extract,
[3] Results are represented as means±standard deviation (n=3).

3. RESULTS and DISCUSSION

3.1 Total phenolics content

Table 1 shows the fraction yields expressed as percentage of dry matter of fractions from *L. olypicum* leaves and the total phenolics content was determined with Folin Ciocalteu reagent, using gallic acid as standard for calibration curve. Results of triplicate analyses are expressed as milligram of gallic acid equivalents (GAE) per gram of extract. Total extractable material with methanol:water (70:30) (LMF) from defatted Linum olympicum leaves was found to be 19.4%. 27.88% of crude extract was obtained by ethyl acetate (LEF) and 72.12 % of the extract remained in water phase (LWF). The phenolic content in the crude aqueous methanolic extract was 356.60 mg GAE/g extract, it was 492.59 mg GAE/g extract. and 302,86 mg GAE/g extract in LEF and LWF, respectively.

3.2 Evaluation of antioxidant activity

3.2.1 Rancimat method

The antioxidant activity, as determined by the Rancimat method, of the fractions on the peroxidation of sunflower oil is shown in Table 2. The data obtained from three replications given as Induction Index (induction time of sunflower oil + sample / induction time of sunflower oil). Higher induction index indicates higher antioxidant activity. None of the extract tested in this study showed antioxidant activity measured by Rancimat method at the concentraction of 1%.

3.2.2 Free radical scavenging activity on DPPH

The DPPH free radical method determines the antiradical power of antioxidants. DPPH is a stable free radical that accepts an electron or hydrogen radi-

cal to became a stable diamagnetic molecule (Sanchez-Moreno et al. 1998). Free radical scavenging activities of the fractions of *Linum olympicum* leaves are given in Table 3. The concentration of antioxidant needed to decrease by 50% the initial sample concentration (EC_{50}) is a parameter widely used to measure the antioxidant power. The lower the EC_{50} the higher the scavenging activity. Only methanol extract of the leaves showed free radical scavenging activity. However, its EC_{50} value was almost 50% higher than that of BHT.

Table 2. Antioxidant activity of *L. olympicum* fractions and BHT at the 1% of concentration

Treatment	Induction Index [1,2]
Sunflower oil +LMF	1.02 ± 0.03
Sunflower oil +LEF	1.00 ± 0.03
Sunflower oil +LWF	1.00 ± 0.02
Sunflower oil +BHT	1.15 ± 0.03

[1] Induction Index = Induction time of sunflower oil+sample / Induction time of sunflower oil,
[2] Results are represented as means±standard deviation (n=2).

Table 3. Free radical scavenging effects of fractions obtained from *Linum olympicum* on DPPH at concentration of 1.6 µg/ml

Samples	Inhibition (%)	EC_{50}
LMF	15.69±0.82	5.10±0.27
LEF	3.10±0.55	26.21±4.60
LWF	4.65±0.27	17.22±1.02
BHT	31.97±0.84	2.50±0.08

3.2.3 β-Carotene-linoleic acid assay system

The β-carotene bleaching method is based on the competitive bleaching β-carotene during the autoxidantion of linoleic acid in aqueous emulsion monitored as decay of absorbance in the visible region (Roginsky and Lissi, 2005). Figure 1 shows the antioxidant activity of the extracts in β-carotene-linoleic acid system. The absorbance of emulsions decreased with time.

It can be seen that *Linum olympicum* fractions exhibited varying degrees of antioxidant activity. For easier comparison the antioxidant activities of the extracts, the data calculated at 180 min are additionally presented in the form of values collected in Table 4. In this work, the highest antioxidant activity found was from the synthetic antioxidant (i.e. BHT).

The antioxidant activity of extracts ranged from 3.28% to 14.01%. As can be seen from results in Table 4, ethylacetate fraction exhibited the strongest neutralizing activity (14.01%) of free radicals. However activity of fractions was not comparable with the activity of BHT

Table 4. Percentage of inhibition estimated by means of β-carotene/linoleic acid system for extracts from *L. olympicum* fractions at 180 min

Extracts	Pericarp
LMF	3.28±0.60
LEF	14.10±0.30
LWF	4.72±00.6
BHT	85.44±0.60

Results are represented as means±standard deviation (n=3).

3.2.4 HPLC analysis of methanol extract of Linum olympicum

Although mass spectrometry coupled with suitable separation techniques is necessary for complete identification, HPLC-DAD emerged as the suitable tool in the preliminary identification of the compounds (Escarpa et al., 2002). Therefore the preliminary identification of the compounds present in the *L. olympicum* fractions was made by High Pressure Liquid Chromatography-Diode Array Detection technique.

Identification of commercially available compounds in the fractions was made by comparing their t_R values and UV spectra with those regarded as standards and previously stored in a data bank. Phenolic classification of commercially unavailable phenolic compounds was made on the basis of their characteristics spectra.

Retention times and the maximum wavelenghts of the reference compounds representing phenolic acids as well as flavonoids separated by the HPLC at the conditions given in the experimental section is shown in Table 5. HPLC chromatogram of metanol extract obtained *L. olympicum* leaves is shown in Figure 2.

None of the retention times and spectral values of the peaks in the methanol extract was identical with those of commercial standards used in this study. Since no literature data of phenolic compounds of *L. olympicum* and no match found with the commercial standarts, only previsional phenolic group identification based on the major peaks (>%5 of total peak areas) was made by comparing UV spectra with those regarded as standards and results showed in Table 6.

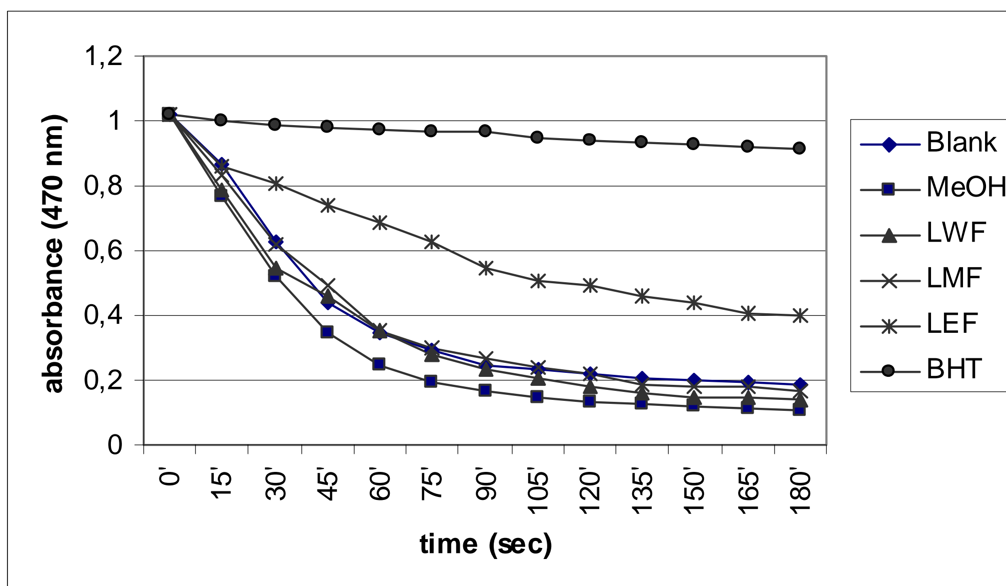

Figure 1. Antioxidant activity of extracts obtained from *Linum olympicum* measured by β-carotene-linoleic acid system over 180 min

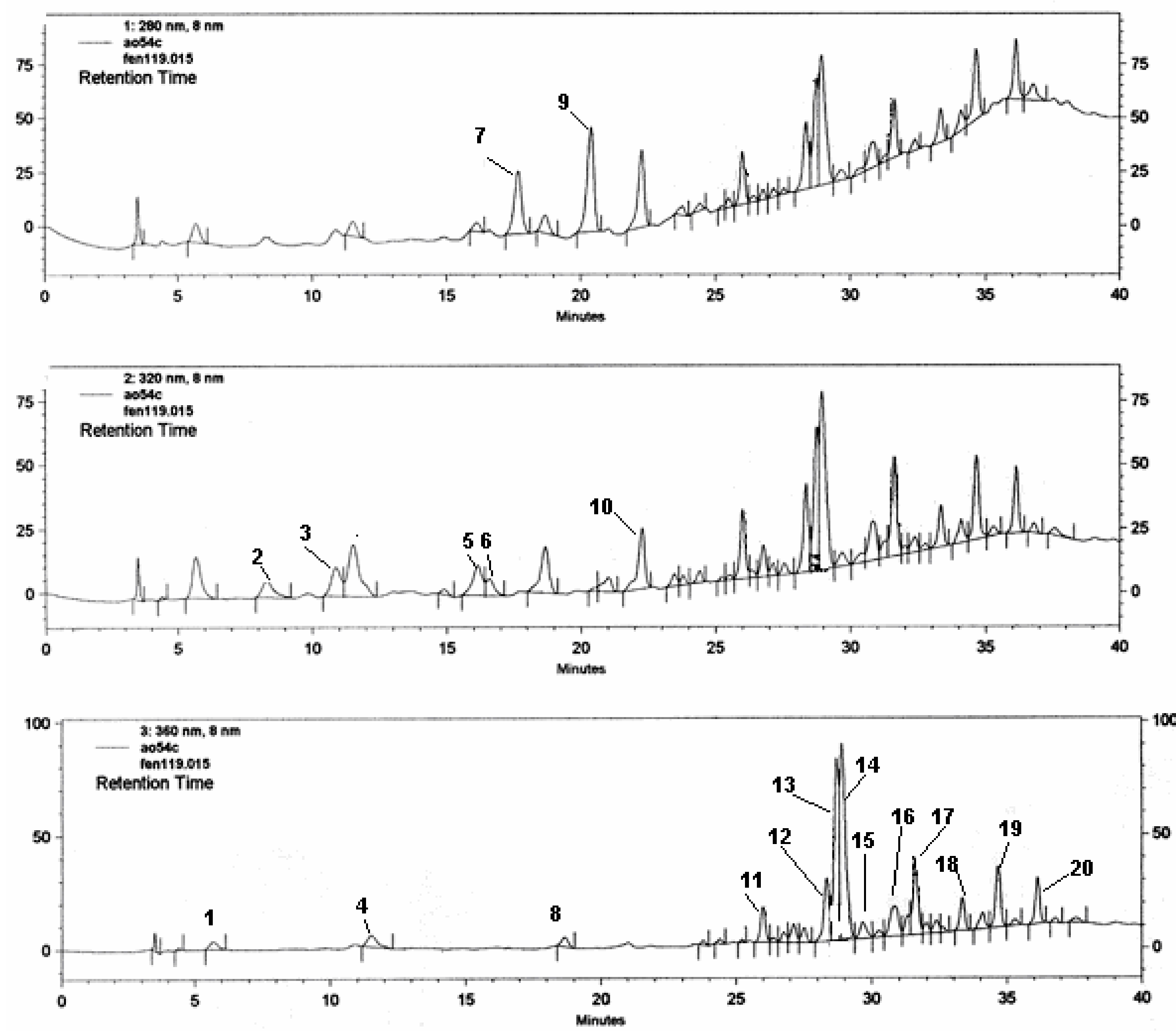

Figure 2. HPLC-DAD chromatogramme of the *Linum olympicum* methanolic extract.

Table 5. Retention times and maximum wavelenghts based on the spectral values obtained by Diode Array Detector of phenolic acids and flavonoid reference compouds

Compounds	RT (min)	Maximum wavelenghts	Classification
Gallic acid	3.8	260, 230, 280	Hydroxy benzoic acid
Protocatechuic acid	6.0	254, 230, 290	Hydroxy benzoic acid
p-OH-benzoic acid	9.5	246, 250, 280	Hydroxy benzoic acid
Vanillic acid	12.8	254, 230, 280	Hydroxy benzoic acid
Caffeic acid	13.3	320, 290, 234	Hydroxy cinnamic acid
Isovanillic acid	14.3	254, 230, 290	Hydroxy benzoic acid
Syringic acid	15.5	270, 230	Hydroxy benzoic acid
p-coumaric acid	19.9	320, 290, 234	Hydroxy cinnamic acid
Ferulic acid	22.9	320, 310, 290	Hydroxy cinnamic acid
o-coumaric acid	26.2	320, 270, 234	Hydroxy cinnamic acid
Myrcetin	29.1	370, 254, 234	Flavonoid
Quercetin	31.6	370, 254, 234	Flavonoid
Luteolin	32.9	350, 234, 254	Flavonoid
Kaempherol	33.8	360, 234, 260	Flavonoid
Apigenin	34.7	340, 234, 260	Flavonoid

Table 6. Previsional group assignments in the chromatogram obtained from fractions of *Linum olympicum*

Peak	RT(sec)	Maximum wavelenghts	Previsional group
1	5.6	320, 280, 360	Flavonoid
2	8.3	320, 280	Hydroxy cinnamic acid
3	10.8	320, 280	Hydroxy cinnamic acid
4	11.5	320, 280, 360	Flavonoid
5	16.1	320, 280	Hydroxy cinnamic acid
6	16.5	320, 280	Hydroxy cinnamic acid
7	17.6	280	Hydroxy benzoic acid
8	18.7	320, 280, 360	Flavonoid
9	20.3	280	Hydroxy benzoic acid
10	22.2	320, 280	Hydroxy cinnamic acid
11	25.9	320, 280, 360	Flavonoid
12	28.3	360, 320, 280	Flavonoid
13	28.7	360, 320, 280	Flavonoid
14	28.9	360, 320, 280	Flavonoid
15	29.6	360, 320, 280	Flavonoid
16	30.8	360, 320, 280	Flavonoid
17	31.6	360, 320, 280	Flavonoid
18	33.3	360, 320, 280	Flavonoid
19	34.6	360, 320, 280	Flavonoid
20	36.1	360, 320, 280	Flavonoid

Spectral data showed that peaks at the retention times of 8.3, 10.8, 16.1, 16.5, 22.2 were hydroxcinnamic acid derivative. Only a peak at 17.66 was assigned as hydroxybenzoic acid derivative. Rests of the major peaks in the chromatogram were assigned as flavonoid derivative since they produced absorbance at 360 nm.

4. CONCLUSION

Although total phenolic content of the extracts from *L. olympicum* leaves ranged from 303 to 493 mg GAE/g extract, only methanol extract of the leaves showed free radical scavenging activity and ethyl acetate fraction exhibited the strongest neutralizing activity. However antioxidant activity of the fractions tested was not comparable with the activity of BHT.

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Nilgün ÖZTÜRK, born in Kocaeli, had completed primary, secondary and high school education in Eskişehir. After graduation from Faculty of Pharmacy, Anadolu Univercity, Eskişehir, Turkey, in 1987, she began working as a research assistant at the same institution in 1988. She has completed her M.Sc. study in 1990 and received Ph.D. degree from the Department of Pharmacognosy Faculty of Pharmacy, Anadolu University in 1997. She has appointed as an assistant professor in the same year. Her professional specialization area covers medicinal plants, natural antioxidants and chromatographic techniques. She is married with a child.

Berrin BOZAN, was graduated from Department of Chemical Engineering, Faculty of Engineering and Architecture, Anadolu University. She has received her MSc and PhD degrees in 1988 and 1994, respectively. She has been working at the same department since 2001

Belma KONUKLUGİL, was graduated from the Faculty of Pharmacy, Ankara University, Ankara, Turkey. She has reached M.Sc. degree at the Department of Pharmacognosy, Faculty of Pharmacy, Ankara University and completed her Ph.D. degree at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Nottingham University, U.K.She is currently working as professor at the Department of Pharmacognosy, Faculty of Pharmacy, Ankara University.