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DETERMINATION OF THE FREE RADICAL SCAVENGING ACTIVITY OF *Lycium* EXTRACTS

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Lycium *species growing in Turkey have not so far been studied sufficiently. For this reason, non-polar and polar extracts obtained from the fruits of* Lycium barbarum *L. and* L. ruthenicum *Murray (Solanaceae) were assessed both in vitro for their potential as free radical scavenger crude extracts and their phenolic composition. Fruits of* Lycium *species were sequentially extracted with petroleum ether, ethyl acetate, methanol,* n*-butanol, and water in a Soxhlet extractor. All the extracts were assessed for the scavenging of the nitrogen-centered free radical 1,1'-diphenyl-2-picrylhydrazyl (DPPH˙) by in vitro method. Furthermore, the composition of each extract was investigated both in terms of its Folin-Ciocalteau reactive components and its qualitative content. The phenolic compounds within the extracts were determined as benzoic acid and hydroxycinnamic acid derivatives, flavonoids, and anthocyanins according to their retention time and UV spectral data by HPLC-DAD system.*

Key words: *Lycium,* Solanaceae, free radical scavenging activity, DPPH˙, HPLC.

Lipid peroxidation is the principle cause of the organoleptic deterioration of foodstuffs during processing, distribution, and storage. Thus, the protection of foods against such deterioration is of great economic and nutritional importance to the food industry [1, 2]. Therefore, antioxidants may be considered an important tool to protect susceptible products from oxidative deterioration [3]. Furthermore, oxidized polyunsaturated fatty acids (PUFA) may induce premature aging and carcinogenesis [4].

Free radicals cause autoxidation which usually takes place between the atmospheric oxygen and an organic compound. Synthetic antioxidants are generally used to protect foodstuffs against oxidation in the food industry. However, the possible toxicity of synthetic antioxidants has resulted in decreased use of these compounds in foods for human consumption [5]. As a consequence of this and due to the appeal of natural products to consumers, numerous studies have been carried out in order to identify naturally occurring compounds which possess antioxidant activity such as phenolic phytochemicals [2, 6, 7].

Fructus Lycii from *Lycium* (Solanaceae) species has been used as a medicine since ancient times in many countries, especially in China, for its emenagogue, diuretic, antipyretic, tonic, aphrodisiac, hypnotic, and hepatoprotective effects [8, 9]. *Lycium,* which is mainly used for decoration of gardens, comprises 7 species in Turkey [10] and their sedative, diuretic and digestive actions are known in Turkish folk medicine [8]. The plant has been extensively studied in China, India, and South Korea. To the best of our knowledge, there is no previous study on the species growing in Turkey.

The aim of the present article is to determine radical scavenging compounds in different extracts of *Lycium* species, based on in vitro reaction of the antioxidant with the DPPH˙ radical. In addition, these active phenols were analyzed quantitatively using HPLC-UV method.

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TABLE 1. Extract Yields and Total Phenol Amounts of *Lycium* Extracts

Extract	*L. barbarum*		*L. ruthenicum*	
	extraction yield [$mg_{extract}/g_{drog}$]	total phenol [$mg_{GAE}/g_{extract}$]	extraction yield [$mg_{extract}/g_{drog}$]	total phenol [$mg_{GAE}/g_{extract}$]
Petroleum ether	15	28.05±0.06*	8	31.97±0.06
Ethyl acetate	13	97.03±2.21	9	96.97±1.02
Methanol	545	65.44±0.09	537	59.60±0.02
n-Butanol	21	112.69±0.01	7	125.19±0.04
Water	112	106.80±0.46	72	70.57±0.04
70% Ethanol	524	57.87±0.21	550	92.32±0.45

TABLE 2. Free Radical Scavenging Activities of Extracts of *Lycium* Species

Extract	IC_{50} [mg/ml]	
	L. barbarum (LB)	*L. ruthenicum* (LR)
Petroleum ether (1)	>5	>5
Ethyl acetate (2)	3.384±0.290*	2.946±0.356
Methanol (3)	3.980±0.335	1.927±0.079
n-Butanol (4)	0.889±0.023	0.867±0.024
Water (5)	1.633±0.362	1.467±0.043
70% Ethanol (6)	3.448±0.388	2.041±0.049
BHT	0.080±0.002	
BHA	0.054±0.001	
AscA	0.085±0.001	

*mean ±SE

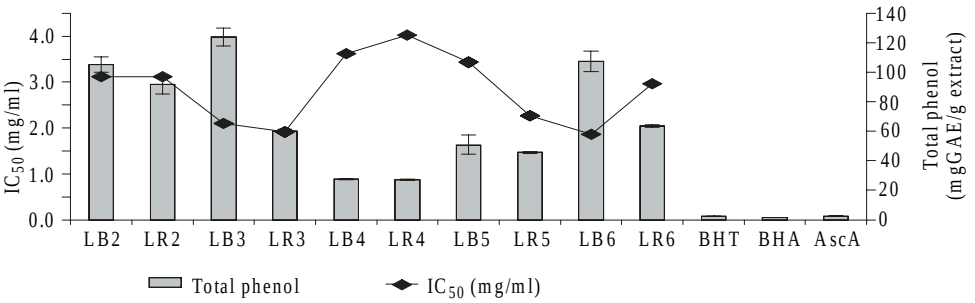

Fig. 1. Relationship between free radical scavenging activities and total phenol amounts of extracts of *Lycium* species.

This study is the first research into the antioxidant action of *Lycium* species of Turkish origin. The extract yields and total phenol amounts of the extracts are shown in Table 1. The total phenol content in each extract was assayed using the Folin-Ciocalteau reagent method [11]. The order of increasing phenolic content expressed as $mg_{gallic\ acid}\ g^{-1}{}_{extract}$ was petroleum ether < 70% ethanol < methanol < ethyl acetate < water < *n*-butanol (Table 1). As can be seen in Fig. 1, a linear relationship was found between total phenol amounts and free radical scavenging activities. In Table 2 the lowest IC_{50} value indicates the highest free radical scavenging activities. Free radicals are involved in the process of lipid peroxidation and are considered to play a cardinal role in numerous chronic pathologies, such as cancer and cardiovascular diseases among others, and are implicated in the aging process. Therefore, the extracts were assessed against $DPPH^{\cdot}$ radicals to determine their free radical scavenging properties. In this assay, the $DPPH^{\cdot}$ radical serves as the oxidizing substrate, which can be reduced by an antioxidant compound to its hydrazine derivative via hydrogen donation, and as the reaction indicator molecule.

TABLE 3. HPLC Quantitative Data of Identified Compounds in *L. barbarum* and *L. ruthenicum* Extracts

	p-Coumaric acid	Chlorogenic acid	Rutin
LB EtOAc	1.802±0.026*	2.810±0.044	N.d
LB MeOH	N.d	1.419±0.002	N.d
LB BuOH	N.d	1.593±0.007	N.d
LB Su	N.d	1.407±0.013	N.d
LB 70% EtOH	N.d	1.127±0.030	N.d
LR EtOAc	6.027±0.003	10.940±0.069	N.d
LR MeOH	0.710±0.001	2.486±0.004	0.216±0.037
LR BuOH	0.518±0.003	1.680±0.000	0.310±0.057
LR Su	N.d	0.251±0.001	N.d
LR 70% EtOH	0.763±0.013	2.474±0.019	0.411±0.013

*mg g^{-1} (dry weight) extract.

TABLE 4. HPLC Qualitative Data for *L. barbarum* and *L. ruthenicum* Extracts

	Σ Hydroxycinnamates[a]	Σ Benzoates[b]	Σ Anthocyanins[c]
LB EtOAc	3.412±0.050[e]	92.224±0.714	Tr.
LB MeOH	1.841±0.009	7.019±0.025	Tr.
LB BuOH	0.000±0.000	118.874±0.066	Tr.
LB Su	5.186±0.113	4.466±0.010	Tr.
LB 70% EtOH	1.988±0.195	0.790±0.098	Tr.
LR EtOAc	2.356±0.003	121.261±0.105	Tr.
LR MeOH	3.608±0.006	5.674±0.010	2.193±0.002
LR BuOH	6.189±0.013	92.983±0.014	0.894±0.001
LR Su	0.243±0.001	Tr.	Tr.
LR 70% EtOH*	0.404±0.000	1.930±0.195	3.804±0.018

[a]Quantitated using chlorogenic acid. [b]Quantitated using gallic. [c]Quantitated using cyanidin-3-glycoside.
[e]mg g^{-1} (dry weight) extract.
Tr.: trace.
Values are presented as mean ± SE for duplicate analyses.
*Σ Flavonoids 0.299 ± 0.001.

All the Lycium extracts were capable of scavenging $DPPH^{\cdot}$ radicals at pH 7.4 in a dose-dependent fashion. From the estimated IC_{50} values, it can be seen that the butanol extracts of both species were the most potent scavengers, in the order LR5 > LB5 > LR3 > LR6 > LR2 > LB2 > LB6 > LB3 (Fig. 1). None of the extracts were as effective $DPPH^{\cdot}$ radical scavengers as the positive controls ascorbic acid, BHA, or BHT in this assay.

The anthocyanins were detected only in methanol and 70% ethanol extracts of *L. ruthenicum*. Total anthocyanin contents of methanol and 70% ethanol extracts were calculated by using the pH differential method [12] (0.033% and 0.07% respectively). Gallic acid was used as a standard in this method.

For the identification of compounds in *Lycium* extracts, the High Pressure Liquid Chromatography-Diode Array Detection technique was used. The identification of known compounds in the extracts was carried out by comparing their t_R values and UV spectra with those of standards. The other phenolics were characterized by their spectral features. *p*-Coumaric acid, chlorogenic acid and rutin were identified by their retention times and UV spectra against those of the standards. In addition, these compounds were also calculated by using the calibration curves of those standards. Other benzoic and

hydroxycinnamic acid, flavonoid, and anthocyanin derivatives within the extracts were identified by UV spectra and calculated by using gallic acid, *p*-coumaric acid, rutin, and cyanidin-3-glycoside as a standard, respectively. In all the extracts, phenolic acid derivatives were found as the main compounds (Table 3, 4). The most active samples, LB4 and LR4, contained the highest amount of total phenols (112.69 ± 0.01 and 125.19 ± 0.04 $mg_{GAE}\ g^{-1}{}_{extract}$, respectively) and benzoic acid (118.874 0.086 and 92.983 0.014 mg $g^{-1}{}_{extract}$, respectively). According to the HPLC results, butanolic extracts of both species revealed an abundance of benzoic and hydroxycinnamic acid derivatives. Both groups of compounds are known strong free radical scavengers [3].

These results constitute the first report on the free radical scavenging activity of any *Lycium* species.

EXPERIMENTAL

Plant Material and Reagents. Fruits of *Lycium barbarum* L. and *L. ruthenicum* Murray were collected from Odunpazarý-Eskisehir (Turkey) and Malatya (Turkey), respectively. Ultrapure water (18.2 MWcm, HPLC grade) was prepared by a Millipore Milli-RO 12 plus system (Millipore Corp., Bedford, MA). All solvents were of analytical grade and purchased from the usual sources. 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$, 95%) was obtained from Aldrich (Steinheim, Germany). All standards were purchased from Sigma (Steinheim, Germany).

Extraction Procedure. Plant material was sieved and 30 g was extracted with petroleum ether, ethyl acetate, methanol, and *n*-butanol in a Soxhlet apparatus, respectively. All the extracts were freeze-dried after evaporation in vacuum and stored at –18°C. A further 30 g ground material was macerated with 200 mL of 70% aqueous methanol for 24 h and then filtered. This maceration procedure was repeated with fresh solvent for 5 days. All the macerates were combined and freeze-dried after removal of methanol and stored at –18°C.

High Performace Liquid Chromatography (HPLC) Set-up. The liquid chromatographic apparatus (Shimadzu LC 10A*vp*) consisted of an in-line degasser, pump and controller coupled to a photodiode array detector equipped with a automatic injector (20 mL injection volume) interfaced to a PC running Class VP chromatography manager software (Shimadzu, Japan). Separations were performed on a reverse-phase Discovery-C18 analytical column (250 × 4.6 mm i.d., particle size 5 mm) (Supelco, PA) operating at room temperature with a flow rate of 1 mL/min. Detection was carried out with a sensitivity of 0.1 a.u.f.s. between the wavelengths of 200 to 550 nm. Elution was effected using a nonlinear gradient of the solvent mixture 2.5% HCOOH in water (solvent A) and 2.5% HCOOH in acetonitrile (solvent B). The composition of B was increased from 5% to 13% in 15 min, increased to 15% in 5, min and to 30% in a further 5 min and held for 3 min, increased to 45% in 4 min and held for 3 min, increased to 90% in 5 min and held for 5 min and then return to initial conditions in 5 min. Components were identified by comparison of their retention times to those of authentic standards under analysis conditions and UV spectra with our in-house PDA library. A 10 min equilibrium time was allowed between injections.

Quantitative Analysis. All the extracts were dissolved in 70% (aq.) methanol at a concentration of 10 mg/mL. For the stock solution of the standards, the polyphenols (gallic acid, *p*-coumaric acid, chlorogenic acid, rutin, cyanidin-3-glycoside) were dissolved in 70% (aq.) methanol at a concentration of 1 mg/mL. The concentration ranges of reference compounds used for calibration of the HPLC analysis were 0.01–0.10 mg/mL. All samples and standards were injected three times and mean values were used.

1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) Scavenging Activity. The ability of the extracts to scavenge $DPPH^{\cdot}$ free radicals was determined by the method of Gyamfi et al. [13]. A 50 mL aliquot of each extract, in Tris-HCl buffer (50 mM, pH 7.4), was mixed with 450 mL Tris-HCl buffer (50 mM, pH 7.4) and 1.0 mL 1,1-diphenyl-2-picrylhydrazyl (0.1 mM, in methanol). After a 30 min incubation time in darkness and at ambient temperature (23°C), the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using equation (1) below and the IC_{50} values were estimated by a nonlinear regression algorithm (SigmaPlot 2001 version 7.0, SPSS Inc., Chicago, IL). Ascorbic acid, BHA, and BHT were used as positive controls. The values are presented as the mean of 5 measurements.

$$\text{Percentage Inhibition} = (Abs_{control} - Abs_{sample}) \times 100/Abs_{control} \qquad (1)$$

Total Phenolics. Total phenols were estimated as gallic acid equivalent according to the Folin-Ciocalteau method [11]. To ca. 6.0 mL H_2O, 100 mL sample was transferred to a 10.0 mL volumetric flask, to which was subsequently added 500 mL

undiluted Folin-Ciocalteau reagent. After 1 min, 1.5 mL 20% (w v^{-1}) Na_2CO_3 was added and the volume made up to 10.0 mL with H_2O. After 2 h incubation at 25°C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. Total phenolics were determined as gallic acid equivalents and are presented as the mean of duplicate analyses.

Total Anthocyanins. Total anthocyanins were estimated by a pH differential method [12] using a spectrophotometer (Shimadzu 1201V). Absorbance was measured at 510 nm and 700 nm in buffers at pH 1.0 and 4.5 using

$$A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$$

with a molar extinction coefficient of 29.600. Results were expressed as milligrams of cyanidin-3-glycoside equivalent per gram of dry extract.

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