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Metabolic profile and biological activities of *Lavandula stoechas* L.

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Abstract: Medicinal and aromatic plants play a significant role in drug discovery. *Lavandula stoechas* L. has been used as folk medicine to treat various diseases. The aim of this work is to investigate the phytochemisty of *Lavandula stoechas* with biological activities. An aerial part of the plant was extracted with methanol. Another sample of plant was boiled in water then aqueous part was extracted with ethyl acetate. Identification and quantification of phenolic compounds, organic acids and flavonoids in methanol extract were carried out by High Performance Liquid Chromatography/Time of Flight/Mass Spectrometry, HPLC-TOF/MS. Rosmarinic acid was found as a chief compound (80.9%). The essential oil was generated by steam distillation and identified by GC-MS. The main constituents were camphor (48.1%) and fenchone (30.5%). The essential oil exhibited good insecticidal activity on *Sitophilus granarius and Sitophilus oryzae* pests as 43.3% and 62.9% mortality, respectively. However, the methanol extract has only insecticidal activity against *S. granaries* (50.0%). The antioxidant activities were investigated using assays of 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging, reducing power (FRAP), and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS$^{\bullet+}$) radical scavenging on both extracts. In addition, total phenolic contents were determined. Total phenolic content of EtOAc extract was found as 2.18 g GAE (Gallic acid equivalent) phenolic compounds/kg plant. Whereas, total phenolic of methanol was 3.33 g GAE/kg plant. EtOAc extract revealed the considerable DPPH$^{\bullet}$ scavenging, ABTS$^{\bullet+}$ scavenging and reducing power activities with the values of 28.71 (IC_{50}, µg/ml), 8.72 (IC_{50}, µg/ml) and 6.99 (µmol trolox equivalent/mg extract) respectively.

Key words: Chemical constituents; essential oil components; antioxidant activity; insecticidal activity; *Lavandula stoechas.*

Introduction

Natural products play a significant role in drug discovery and development process due to the including a large variety of biological active constituents (1-9).

The genus *Lavandula* (Family Lamiaceae) is considered a medicinal and aromatic plant with great economic values, cultivated in many European countries, widely distributed throughout the world and consists of 35 species (10). The *Lavandula* species have been utilized as an alternative medicine to treat diverse diseases such as diabetes, depression, headaches (11). The essential oils of *L. stoechas* are well documented and known to have anti-inflammatory, antifungal, antibacterial, antioxidant, sedative, neuroprotective activities. They have been used for food, cosmetic, perfumery and pharmaceutical industries as well as treatment of wounds, burns and skin injuries (12). The essential oils containing necrodane monoterpenoid inhibited beta secretase activity, so they exhibited the ability to penetrate cell membranes. Therefore, these oils have a potency to be a medicine for Alzheimer's disease (13). Besides essential oils, photochemical researches of *Lavandula* species have brought about the isolation of monoterpenes (14), flavonoids (15), dichotomoside, succinic acid, caffeic acid, ferulic acid, beta-sitosterol, ursolic acid and daucosterol (16). *L. stoechas* consists of acetylated glucoside of luteolin and flavone glucosides (17), triterpenes, steroids (18).

Essential oils (EOs) (volatile oils) are aromatic oily liquids generated from plant materials. They are widely used in pharmaceutical and food industries. EOs have also been reported to display a wide range of biological activities. (19,20).

Insects have been known the most significant problem for agricultural industries. They damage to the crops while growing up and after harvest. Many works have been conducted to find the best solution to these issues. The synthetic chemicals used for insecticide can be harmful to the environment as well as human beings who consume chemically contaminated food (21). Natural products have attracted the great attention of scientists who search to find non-toxic, environmentally safe insecticide (22).

Sitophilus granaries (L.) (Coleoptera: Curculionidae) is one of the most dangerous pests effecting stored wheat kernels. Because of the invasion, nutritional wheat values deteriorate and an increase in hygiene problems can be observed (23).

Sitophilus oryzae (L.) (Coleoptera: Curculionidae) is considered to be one of the most destructive species in stored cereal grains throughout the world. It can attack intact grain. The adults feed on grains and the larva grows inside the grain kernels causing both quantitative and qualitative losses of the stored grain products (24).

Hydroxyl radicals, superoxide and singlet oxygen called the reactive oxygen species (ROS), responsible many chemical reactions in eukaryotic cells can harm cells leading to disease such as DNA damage, cardiovascular diseases and cancer (25). Antioxidants

have been used in food industry to keep the food freshly. The main source of deterioration, nutritional losses and discoloration in foods are free radicals oxidizing the antioxidant compounds. Recently, a great number of scientific investigations have been carried out to find the natural antioxidants for use in food industries to replace synthetic ones which have been restricted on account of their carcinogenicity (26-28).

Herein we extracted aerial part of *L. stoechas* with methanol and ethyl acetate. Chemical constituents of methanol extract were identified by LC-TOF/MS. The essential oil of *L. stoechas* was obtained by steam distillation and identified by GC-MS. Moreover, insecticidal activity of essential oil and methanol extract was carried out on *Sitophilus granarius* and *Sitophilus oryzae* pests and found out that the essential oil exhibited good insecticidal activity. Antioxidant activities of methanol and ethyl acetate extracts were carried out and these extracts exhibited the considerably antioxidant activities.

Materials and Methods

Chemical and Reagents

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Trolox, 1,1-diphenyl-2-picryl-hydrazyl (DPPH$^{\bullet}$), trichloroacetic acid (TCA) were obtained from Sigma. Gallic acid, sodium carbonate, potassium ferricyanide, Folin-Ciocalteu reagent and solvents were bought from Merck.

Plant material

Lavandula stoechas L. was collected from Denizli , Turkey during the flowering season and identified by Prof.Dr. Ahmet Ilcim, Mustafa Kemal University, Faculty of Arts and Sciences, Department of Biology where a voucher specimen was deposited (No: MKUH-1329).

Insects

The cultures of *S. granaries* and *S. oryzae* were supplied from Cankiri Karatekin University, Biology Department. The wheat was filled into the one third of glassware (5.0 L), and then adult males and females were added to lay eggs to get a single aged population. The adults were removed and cultures were incubated at 27 °C and 60% relative humidity in a dark climate chamber after 48 h later. After forty-five days, the new generation of adults appeared to be used in experiments (22).

Extractions

The aerial part of plant material (100 g) was dried at room temperature at shadow. It was powdered then extracted with methanol (150 mL) for 24 h (3 times) to yield the crude extract (5.0 g). *L. stoechas* another sample (80 g) was boiled in water (150 mL) then filtered (whatman filter paper) and liquid part was extracted with ethyl acetate (100 mL ×3) with a separatory funnel to yield the ethyl acetate extract (2.0 g).

LC/TOF/MS Analysis

The quantitative analysis was carried out by LC-TOF/MS, Agilent 62100 with ZORBAX SB-C18, 4.6 × 100 mm, 3.5 µm with injection volume 10 µL. The flow rate was 0.6 mL/min at 35 °C. The mobile phase consisted of eluent A, water with 0.1% formic acid and eluent B, acetonitrile. The gradient programme was as follow: 0-1 min, 10% B; 1-20 min, 50% B; 20-23 min, 80% B; 23-25 min, 10% B; 25-30 min, 10% B. The evaluation time was 30 min. Positive mode was used for TOF analysis and gas temperature was 325 °C. Drying gas flow was 10 mL/min and fragmentor voltage was 175 V (29).

Isolation of the essential oils

Aerial parts of the 25 g plant diluted with 250 mL distillated water were subjected to hydrodistillation for 4 h, using a Clevenger-type apparatus. The oil was stored at +4 ºC for analysis.

GC and GC–MS analysis

GC analyses were carried out on a Perkin–Elmer Clarus 500 Series, in divided mode, 50:1, equipped with a flame ionization detector (FID) and a mass spectrometer-equipped BPX-5 apolar capillary column (30 m × 0.25 mm, 0.25 m i.d.). The injection temperature was fixed and FID was executed at 250 ºC. The carrier gas was helium at a rate of 1.0 mL/min. The initial column oven temperature was 50°C and was raised to 220 °C at a rate of 8 °C/minute. In the mass spectrometer, transfer line temperature was at 250 °C, ionization energy was 70 eV. The standard components were used for the majority of the essential oil constituents and Kovats retention indices (RIs) were determined for all the sample components using the Van den Dool and Kratz equation according to homolog n-alkane series retention times.

Single-dose contact effects assay

The essential oil and methanol extract of plant were diluted with acetone to 100 µL/mL and 100 mg/mL respectively. Diluted essential oil and extract were applied to the dorsal surface of thorax of *S. granarius* and *S. oryzae* at an amount of 1 µL/insect with 50 µl Hamilton syringe. Acetone was used as control at a dozen of 1 µL/insect. For each replication, 20 insects were used and each experiment was repeated for three times. The tested insects were transferred to the 60 mm diameter clean Petri dishes filled with 5 g of wheat and incubated at 27± 2 ºC in a dark climate chamber. The number of dead insects was recorded after 24 h. A randomized block design was operated comprising treatments and blank control (30).

Determination of Total Phenolic Content

Total phenolic contents of *L. stoechas* methanol extract and ethyl acetate extract were estimated by a colorimetric assay (31). An extract solution (100 µL, 1.0 µg/mL), distilled water (4.6 mL) and Folin-Ciocalteu reagent (100 µL) were mixed in a volumetric flask. After adding Na_2CO_3 (200 µL, 2%), reaction mixture was shacked for 2 h. The absorbance was measured at 760 nm in a spectrophotometer (Hitachi U-2900). The concentration of total phenolic compounds in extract was determined as mg gallic acid equivalent using an equation obtained from the standard gallic acid graph:

Absorbance = 0.0051 × Total phenols [Gallic acid eqv.(µg)] – 0.0057

DPPH free radical scavenging activity

The DPPH$^{\bullet}$ assay was used to investigate for the free radical scavenging activity of extracts (32). DPPH$^{\bullet}$ solution (0.26 mM, 1.0 mL) was added to the various concentrations of extracts (3 mL, 20-100 µg/mL). The reaction mixture was stirred for 40 sec. The absorbance was measured at 517 nm with a spectrophotometer. The DPPH$^{\bullet}$ scavenging activity was calculated using the equation:

DPPH$^{\bullet}$ scavenging effect (%) = [(Ac – As) / Ac] × 100

in which, *Ac* is the absorbance of the control and *As* is the absorbance of the sample (33-35).

Reducing power

Reducing power of the *L. stoechas* extracts and standards was determined with the Oyaizu method (36). Potassium ferricyanide [$K_3Fe(CN)_6$] (1.25 mL, 1%) were treated with each extract and at different concentrations (1.25-5 µg/mL) at 50 °C for 30 min and total volume was completed to 2.5 mL with buffer solution (0.2 M, pH 6.7). The reaction mixture was stirred for 20 min. Trichloroacetic acid (1.25 mL, 10%) was added to the reaction mixture and then $FeCl_3$ (0.25 mL, 0.1%) was added. The absorbance was measured at 700 nm in a spectrophotometer. High absorbance value of the reaction mixture determined high reducing capability.

ABTS$^{\bullet+}$ scavenging activity

ABTS$^{\bullet+}$ scavenging activity of extracts, and standards (BHT, BHA, Trolox) were determined according to the literature (37). This method is based on the capability of destroying ABTS radical cations by antioxidants at 734 nm absorption in comparison to BHA, BHT, and Trolox. Initially, ABTS$^{\bullet+}$ was prepared by the reaction of ABTS (2 mM) with potassium persulfate (2.45 mM) then it was stored for 6 h in dark at room temperature. Subsequently, ABTS$^{\bullet+}$ solution (1.0 mL) reacted with each sample solution (3.0 mL) at various concentrations (2.5-40 µg/mL). The inhibition was calculated for each concentration comparative to a blank absorbance. The decolorization rate was calculated as absorbance (734 nm) of reduction percent. The results were calculated as $IC_{50.}$ The capability of ABTS$^{\bullet+}$ was calculated by the equation: *ABTS$^{\bullet+}$ scavenging effect (%) = [(A_c – A_s) / A_c] × 100* in which, A_c is ABTS$^{\bullet+}$ initial concentration and A_s is ABTS$^{\bullet+}$ remaining concentration in the sample.

Results

Two extraction procedures were carried out for *L. stoechas,* one is direct extraction with methanol to yield the methanol extract and the other is the ethyl acetate extraction in which, the plant was boiled in water then aqueous part was extracted with ethyl acetate. The quantification of methanol extract was determined by HPLC/TOF/MS analysis (Table 1).

The relationship between peak area and concentration was found to be linear from 25 to 1000 µg/L (ppb) for each compound. Linearity was evaluated by linear regression analysis of six points for each compound. Linear plot contains of three replicates per point. The correlation coefficients (R^2 values) were found as ≥0.99.

Table 1. Quantitative analysis of *L. stoechas* methanol extract by HPLC/TOF/MS.

Composition (mg/1.0 kg plant)	*L. stoechas*	Linear regression equation	R^2	LOD µg/L	LOQ µg/L	%RSD	Recovery (%)
Gallic acid	trace	y = 611,85x -10726	0.996	5.38	6.78	4.45	96.45
Gentisic acid	7.69	y = 572.41x + 5626.5	0.998	5.78	7.11	3.49	98.41
Catechine	trace	y = 510.75x + 10111	0.998	5.12	6.31	2.85	99.14
Chlorogenic acid	trace	y = 348.27x + 3531.5	0.997	9.04	14.01	4.25	100.76
4-hydroxybenzoic acid	67.06	y = 402.27x - 1951.8	0.998	6.89	9.41	3.12	97.50
Protocatechuic acid	1.49	y = 645.26x + 3051.7	0.999	4.42	5.54	1.25	104.20
Caffeic acid	27.85	y = 1247.2x + 1675.9	0.998	5.45	7.13	2.74	98.55
Vanillic acid	2.30	y = 61.059x + 2701.5	0.995	4.99	6.11	4.12	101.25
4-hydroxybenzaldehyde	trace	y = 4525.7x +109831	0.998	6.90	9.21	2.91	95.90
rutin	trace	y = 966.33x + 1355.3	0.999	5.71	8.79	3.54	105.90
p-coumaric acid	3.25	y = 813.51x + 3442.4	0.999	7.33	11.11	3.14	99.85
Chicoric acid	trace	y = 314.64x - 1519.1	0.999	5.12	7.29	2.97	102.74
Ferulic acid	6.43	y = 329.67x - 1895.4	0.998	6.62	9.00	4.90	100.15
Hesperidin	trace	y = 713.87x + 8548.6	0.996	7.85	11.84	3.65	99.45
Apigenin-7-*O*-glucoside	trace	y = 1125.4x + 15634	0.998	8.75	13.44	2.21	100.86
Rosmarinic acid	80.89	y = 786.7x - 7818.5	0.998	6.25	7.44	3.55	102.50
Protocatechuic acid ethyl ester	trace	y = 3379.4x + 53655	0.998	4.60	7.89	2.66	85.75
Salicylic acid	1.20	y = 1176.7x + 19371	0.996	8.20	13.59	4.66	90.55
Quercetin	trace	y = 2684x - 16457	0.999	6.62	11.59	2.21	99.90
Naringenin	trace	y = 3015.5x + 6664.8	0.998	7.24	10.67	4.10	103.50
Campherol	trace	y = 1853.9x - 2942.5	0.999	5.52	6.29	3.35	98.75
Total phenolic (mg phenolic/kg plant)	198.16						

LOD: Limit of Detection, LOQ: Limit of Quantification, RSD: Relative Standard Deviation, R^2: Regression Coefficient.

Table 2. Chemical composition of the essential oil of *Lavandula stoechas* L.

No.	Compounds	Retention time (min)	Retention index*	Content (%)
1	alpha-Pinene	12.714	939	1.09
2	Camphene	13.254	957	2.42
3	Dehydrosabinene	13.390	961	0.30
4	*p*-Cymene	15.657	1029	0.32
5	Limonene	15.811	1034	0.25
6	Linalool oxide	17.322	1077	0.47
7	Fenchone	18.033	1096	30.5
8	Fenchyl alcohol	18.903	1123	0.42
9	Campholenal	19.286	1134	0.40
10	Camphor	20.129	1158	48.1
11	Acetic acid, [4-(1-hydroxy-1-methylethyl)cyclohex-1-enyl] methyl ester	20.704	1174	1.87
12	*p*-Cymen-8-ol	21.304	1191	1.03
13	alpha-Terpineol	21.564	1197	0.33
14	Verbenone	22.345	1221	1.59
15	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-3-(2-propenyl)-, (1α,2β,3α,5α)	22.517	1227	0.73
16	Carvone	23.437	1254	0.47
17	Borneolacetate	24.825	1294	1.79
18	Carvacrol	25.130	1302	0.51
19	Bisabolol	34.542	1607	0.35
20	tau-Muurolol	36.408	1674	5.72
21	Cadalene	36.988	1694	0.58
22	3-Keto-β-ionone	37.550	1715	0.75

*RI: Retention indices calculated against n-alkanes, % calculated from FID data.

Linear regression equations were given in Table 1. LOD and LOQ were determined using the measurements of reagent blanks spiked with low concentrations of analytic according to Eurachem Guide. The blank solution was spiked to 5 ppb standard. The LOD and LOQ were calculated from the following equations:

LOD = 3 × S_0 and LOQ= 10× S_0.

The repeatability in the intra-day values (relative standard deviation, RSD %) for compounds was calculated using the corresponding peak areas of three replicate analyses at approximately 5 µg/kg concentration level.

The trueness was tested as recovery of each compound from mixed stock standard solutions in spiked plant extracts. The recovery was assessed by means of three replicate measurements in a day. The average recovery data of the compounds were determined from the following equation and the recovery was given in Table 1.

$$Recovery\ (100\%) = \left(\frac{Measured\ concentration}{Spiked\ concentration}\right) \times 100$$

The concentration of compounds in the plant was obtained from either one of the corresponding calibration curves. Finally, the calculated concentrations were converted to mg/kg of plant.

As a result, rosmarinic acid (%80.89) was detected as the main constituent (Figure 1). The other major constituents were 4-hydroxybenzoic acid (%67.06), caffeic acid (%27.85), gentisic acid (%7.69), ferulic acid (%6.43), *p*-coumaric acid (%3.25), vanillic acid (%2.30), protocatechuic acid (% 1.49), and salicylic acid (%1.20). In addition, the total amount of phenolic substance in *L. stoechas* was calculated as 198.16 (mg phenol / kg plant) (Table 1).

Twenty two chemical constituents of essential oil were presented by GC-MS analysis. Camphor (48.1%) and fenchone (30.5%) were indicated as the major components of the essential oil of the *L. stoechas* (Table 2).

Insecticidal activity of essential oil and methanol extract was investigated. The methanol extract showed 50% activity on *S. granarius*. The essential oil exhibited the higher activity on *S. oryzae* (62.9%) than that of the *S. granarius* (43.3%) (Table 3).

Table 3. Insecticidal activity of *L. stoechas* essential oil and methanol extracts on *S. granarius* and *S. oryzae.*

Plant materials	% Mortality ± SD*	
	S.granarius	*S. oryzae*
Control	0.00±0.00^{c1}	0.00±0.00^{c}
Essential oil	43.33±0.0^{b}	62.92±0.04^{b}
methanol extract	50.00±0.2^{b}	0.56±0.42^{c}

[1] Means in column followed by a different lowercase letter are significantly different (Anova P<0.05, Tukey test). *SD=Standard deviation.

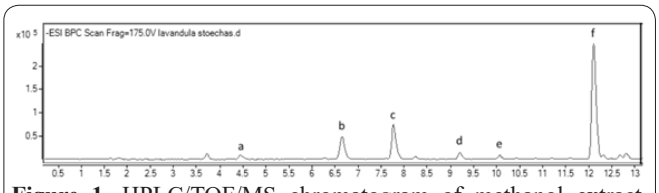

Figure 1. HPLC/TOF/MS chromatogram of methanol extract, gentisic acid (a), 4-hydroxybenzoic acid (b), caffeic acid (c), 4-hydroxylbenzaldehyde (d), ferullic acid (e), rosmarinic acid (f).

Table 4. Antioxidant assays of *L. stoechas* extracts and standards.

Extracts	Total phenolic contents[a]	DPPH$^{\bullet}$ scavenging activity[b]	ABTS$^{\bullet+}$ Cation radical scavenging[b]	Reducing power[c]
MeOH	2.33 ± 0.016	54.60 ± 1.31	14.93 ± 0.27	0.322 ± 0.024
EtOAc	2.18 ± 0.012	28.71 ± 0.61	8.72 ± 0.21	0.699 ± 0.032
BHA		3.47 ± 0.32	3.32 ± 0.19	0.911 ± 0.027
BHT		14.78 ± 0.90	3.33 ± 0.26	0.619 ± 0.044
Trolox		4.88 ± 0.43	6.12 ± 0.18	0.467 ± 0.028

[a]g gallic acid equivanet phenolic compounds/kg plant. [b]IC_{50} values of of extracts and standards (µg /ml). [c]Trolox equivalent activity (5.0 µg/ml).

Antioxidant activities of methanol and ethyl acetate extracts were carried out. Methanol extract consisted of more phenolic compounds (2.33 mg/g plant) than ethyl acetate extract (2.18 mg/g plant) whereas ethyl acetate extract exhibited better DPPH$^{\bullet}$ scavenging activity. This might be due to the active compounds in the ethyl acetate extract. Ethyl acetate extract revealed more reducing power activity than standards, BHT and Trolox as 0.70, 0.62, 0.47 respectively at the concentration of 5.0 µg/ml. Ethly acetate extract revealed good ABTS$^{\bullet+}$ scavenging activity (Table 4).

Discussion

Medical plants containing biologically active compounds are highly successful in developing new pharmaceutical components (38). Phytochemical studies from the aerial parts of *L. stoechas* have found to contain molecules such as glycoside, saponins, oleanolic acid, ursolic acid, vergatric acid, β-sitosterol, α-amylin, α-aminine acetate, lupeol, erythrodiol, flovonoids, luteolin, acacetin, viteksin, coumarin and lavanol (39,40)

Caffeic acid existed in methanol extract in a significant amount (27.9 mg/1.0 kg plant) (Table 1). The caffeic acid derivatives reveal anticancer, anti-influenza activities (41). They act a considerable function in improving ionized-induced oxidative damage to red blood cells in rats, cisplatin-induced oxidative damage to the rat liver, and also regulating superoxide dismutase stress enzyme activities in diabetic rat retinas and inducing apoptosis in human multiple myeloma cells (42). Caffeic acid revealed the considerable antioxidative effect (43).

Rosmarinic acid had a number of interesting and important biological effects such as antioxidant, antiinflammatory , anti-HIV, antimicrobial activity (44). Rosmarinic acid also includes antidepressant properties (45) and it inhibited some metabolic enzymes (46,47). It was reported that *Lavandula pedunculata subsp. lusitanica* included hydroxycinnamic acids(3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, rosmarinic acids) and flavones (luteolin and apigenin) and rosmarinic acid was the major compound (48). There was coherence with present work (Table 1, Figue 1).

The essential oil of *L. stoechas* has attracted the great interest due to its biological activities. The most commonly identified chemotype (fenchone/camphor) to that reported previously is in accordance with the present study camphor (48.1%) and fenchone (30.5%) (Table 2) were the most abundant compounds in essential oils of *L. stoechas* with one exception as different relative amounts (49-51).

Recently, extracts, essential oils, and active compounds derived from the plants gained the great interest due to the exhibiting the significant insecticidal activity. (52) Insects have the ability of developing resistance to the synthetic insecticides and have side effect on human health; therefore, bio-pesticides are preferred due to their non-toxic, biodegradable, environmental friendly effects.(53) The plants consist of bioactive compounds such as flavonoids, alkaloids, terpenoids, organic acids and lipids which are biodegradable with non-residual effects on the ecosystem.(5). Hence, the essential oils and extracts obtained from *L. stoecheas* could be appropriate insecticide for stored products (Table 3).

The antioxidant activity of ethanol and water extracts of *L. stoechas* was investigated using different antioxidant analyses; superoxide anion, free radical scavenging, metal chelating, and reductive potential. The both extracts of *L. stoechas* reported to show strong antioxidant activity (54), which support this work (Table 4). In consequence of revealing the good antioxidant activity of methanol and ethyl acetate extracts, *L. stoechas* could be promising natural antioxidant agent for food industries.

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Interest conflict

No conflict of interest associated with this work.

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