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Chemical characterization and antioxidant activity of *Eryngium pseudothoriifolium* and *E. thorifolium* essential oils

Gülsen TEL-ÇAYAN [1] *, Mehmet Emin DURU [2]

[1] Department of Chemistry and Chemical Processing Technologies, Muğla Vocational School, Muğla Sıtkı Koçman University, Muğla 48000, Turkey.
[2] Department of Chemistry, Faculty of Sciences, Muğla Sıtkı Koçman University, Muğla 48000, Turkey.
* Corresponding Author. E-mail: gulsentel@mu.edu.tr (G.T.Ç.); Tel. +90-252-212 20 38.

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ABSTRACT: The chemical characterization and antioxidant activities of essential oils of *Eryngium pseudothoriifolium* Contandr. & Quézel and *E. thorifolium* Boiss., endemic plant species from Turkey, were investigated. The essential oils obtained by hydro-distillation were analyzed by GC/FID and GC/MS. Thirty and twenty-three compounds representing 99.5% and 99.7% were identified in *E. pseudothoriifolium* and *E. thorifolium* essential oils, respectively. The main component of *E. pseudothoriifolium* oil was falcarinol (84.8%) whereas those of *E. thorifolium* were α-pinene (31.9%), caryophyllene oxide (21.6%) and hexahydrofarnesylacetone (11.1%). The antioxidant capacities of the essential oils were tested by several methods including β-carotene-linoleic acid, radical scavenging (DPPH and ABTS) and reducing power (CUPRAC) assays. The essential oil of *E. pseudothoriifolium* with the high amount of falcarinol exhibited the best antioxidant activity in all assays. The results of the present study supported the possible uses of *Eryngium* essential oils as a valuable source of natural antioxidant in food and pharmaceutical industries.

KEYWORDS: *Eryngium pseudothoriifolium*; *Eryngium thorifolium*; essential oil; chemical characterization; antioxidant activity.

1. INTRODUCTION

The Apiaceae (Umbelliferae) family comprises aromatic plants used as a food, spices, and for their utility in pharmacy, agriculture, and cosmetics [1]. Apiaceae are valuable sources of essential oils which are extracted by steam or hydro-distillation from flowers, seeds, leaves, stems, bark, and roots of plants. The major constituents of the Apiaceae essential oils are monoterpenes, sesquiterpenes and phenylpropanoids [2].

The genus *Eryngium* is an important member of the family Apiaceae and contains about 317 species distributed in temperate regions of every continent, mainly in Eurasia, North Africa, North and South America, and Australia [3, 4]. In Turkey, 23 species (24 taxa) are recorded, and ten of them are reported to be endemic [5]. *Eryngium* species are known as 'boğa dikeni' in Turkish folk medicine and are used to treat a wide range of ailments. The roots of *Eryngium* species are used to treat several inflammatory disorders, sinusitis, goiter, edema, urinary infections and inflammations, snake or scorpion bites, while the roots and leaves are used for infertility, and the herbs are used for wound healing and as food when they are fresh [6-8].

Phytochemical studies performed on *Eryngium* species including several secondary metabolites such as terpenoids, steroids, triterpenoid saponins, polyacetylenes, phenolics, flavonoids, coumarins and rosmarinic acid derivatives [9, 10]. Antioxidant, cytotoxic, anti-inflammatory, antimicrobial, antimalarial, antidiabetic and anti-mutagenic activities have been reported from *Eryngium* species [11, 12].

The aim of the present work was to evaluate the chemical characterization and antioxidant effects of the essential oils from aerial parts of *E. pseudothoriifolium* Contandr. & Quézel and *E. thorifolium* Boiss., which are endemic in Muğla, Turkey.

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2. RESULTS AND DISCUSSION

2.1. Chemical characterization of the essential oils

The yields of the essential oil from aerial parts of *E. pseudothoriifolium* Contandr. & Quézel and *E. thorifolium* Boiss. were 0.65 and 0.48%. The essential oils obtained from *E. pseudothoriifolium* and *E. thorifolium* were analyzed by using the gas chromatography (GC) and GC/mass spectrometry (GC-MS) techniques. The chemical characterization of the essential oils, relative percentage (%) and Kovats index of compounds are given in Table 1. Thirty compounds were identified in the essential oil of *E. pseudothoriifolium* representing 99.5% of the total oil; falcarinol (84.8%) was found as the main compound. A total of twenty-three compounds were found in the essential oil of *E. thorifolium*, representing about 99.7% of total oil. α-Pinene (31.9%), caryophyllene oxide (21.6%) and hexahydrofarnesylacetone (11.1%) were major compounds in the essential oil.

Table 1. Chemical characterization of the essential oils of *E. pseudothoriifolium* and *E. thorifolium.*

No	Compounds[a]	*E. pseudothoriifolium* (%[b])	*E. thorifolium* (%[b])	RI[c]	LRI[d]	Identification Methods[e]
1	(*E*)-3-Nonene	0.1	*tr*	921	924	MS, RI
2	*α*-Pinene	-	31.9	930	939	Co-GC, MS, RI
3	*p*-Cymene	0.1	-	1021	1020	Co-GC, MS, RI
4	Nonanal	0.2	0.8	1075	1073	MS, RI
5	*α*-Isophorone	0.1	-	1090	1088	MS, RI
6	(*E*)-2-Nonenal	0.2	0.4	1118	1112	MS, RI
7	4-Ethyl-o-xylene	*tr*	-	1125	1124	MS, RI
8	Prehnitene	0.1	-	1150	1152	MS, RI
9	2-Hexylfuran	-	0.5	1163	1165	MS, RI
10	2-Nonen-1-ol	0.6	0.4	1168	1171	MS, RI
11	(*E*)-3-Dodecene	0.3	2.2	1218	1215	MS, RI
12	(*Z*)-7-Decenal	0.1	-	1232	1230	MS, RI
13	Thymol	0.1	0.7	1294	1289	Co-GC, MS, RI
14	*α*-Methyl naphtalene	0.2	-	1304	1309	MS, RI
15	Benzocycloheptatriene	0.1	0.8	1351	1354	MS, RI
16	Dimethyl salicylate	-	0.4	1382	1380	MS, RI
17	(*E*)-7-Tetradecene	1.5	-	1421	1425	MS, RI
18	(*Z*)-*β*-Farnesene	0.5	-	1443	1445	MS, RI
19	Dihydroactinidiolide	0.2	0.4	1488	1483	MS, RI
20	Caryophyleine-(13)	*tr*	1.3	1509	1499	MS, RI
21	4-(2-Methy-3-oxocyclohexyl)butanal	0.6	1.8	1517	1515	MS, RI
22	Spathulenol	1.3	8.5	1576	1572	Co-GC, MS, RI
23	Caryophylene oxide	1.5	21.6	1580	1578	Co-GC, MS, RI
24	Isoaromadendrene epoxide	*tr*	3.7	1582	1585	Co-GC, MS, RI
25	Benzophenone	0.1	-	1590	1601	MS, RI
26	1-Hexadecene	1.6	1.8	1615	1602	MS, RI
27	*δ*-Cadinol	-	1.5	1646	1651	MS, RI
28	*cis*-Lanceol	0.2	3.3	1761	1737	MS, RI
29	Hexanoic acid decyl ester	0.3	-	1779	1776	MS, RI
30	Cetyl alcohol	0.4	2.0	1824	1826	MS, RI
31	Hexahydrofarnesyl acetone	3.4	11.1	1923	1935	MS, RI
32	*α*-Hexylcinnamaldehyde	0.8	-	1964	1690	MS, RI
33	Hexadecanoic acid ethyl ester	0.1	-	1976	1968	MS, RI
34	Falcarinol	84.8	-	1987	1906	MS, RI
35	*α*-Kaurene	-	3.2	2045	2044	MS, RI
36	Oleyl alcohol	-	1.4	2072	2061	MS, RI
	Total identified (%)	**99.5**	**99.7**			

[a] Compounds are listed in order of their elution from a DB-5 fused silica column. [b] Percentage concentration. [c] Retention index on DB–5 fused silica column. [d] Linear retention index taken from Adams (2007) and /or NIST 08 (2008). [e] Identification methods: Co-I: Co-injection: based on comparison with authentic compounds; MS: based on comparison with WILEY, ADAMS and NIST 08 MS databases; RI: based on comparison of calculated with those reported in ADAMS and NIST 08.

Çelik *et al*., [13] investigated the chemical composition of the essential oil of *E. thorifolium* by direct thermal desorption (DTD) - GC/MS analyses and α-pinene (58.65±3.52%), 7-exo-ethenylbicyclo [4.2.0] oct-1-ene (10.56±3.62%), limonene (3.14±5.14%) and (E)-Oct-2-enal (3.04±4.68%) were identified as major compounds. Usluer *et al.* [14] were previously studied the chemical compositions of hexane extracts of *E. pseudothoriifolium* and *E. thorifolium* using soxhlet extraction technique by GC-MS. Falcarinol (82.89%), 6,10,14-trimethyl pentadecanone (3.24%) and caryophyllene (2.15%) were identified as major compounds in the hexane extract of *E. pseudothoriifolium* while cis-verbenyl acetate (24.84%), caryophyllene oxide (18.17%), (E)-Farnesene epoxide (12.72%), and Selina 4,11-diene 2-ol (9.56%) were detected as main components in the hexane extract of *E. thoriifolium*. The chemical composition of essential oil of *E. pseudothoriifolium* was described for the first time in this study.

In earlier reports, the chemical contents of essential oils of different *Eryngium* species have been studied. The essential oil of *E. billardieri* was analyzed by Sefidkon *et al*. [15], *α*-muurolene (42.0%), *β*-gurjunene (17.0%), *δ*-cadinene (6.2%) and valencene (5.7%) were identified as major compounds. Klein-Junior *et al*. [16] reported spathulenol (36.0%) and 2, 3, 6-trimethyl benzaldehyde (15.7%) as the major compounds in *E. floribundum*; bicyclogermacrene (17.2%) and cyclocolorenone (14.7%) in *E. nudicaule*; sesquicineole (21.3%), pentadecane (53.5%), globulol (18.6) and aromadendrene (9.1%) in *E. horridum*. In the study of Medbouhi *et al.* [4] the chemical compositions of *E. triquetrum* essential oils from 25 different locations were investigated and falcarinol (63.5-90.6%) was found to be highly dominant in the essential oils, followed by octanal (1.0-12.8%). 2-Dodecen-1-al (46.68%) and caprinic alcohol (14.80%) in *E. foetidum* essential oil and α-bisabolol (32.6%), α-curcumene (6.5%) and α-selinene (5.4%) in *E. tricuspidatum* essential oil were reported as major compounds [17,18]. A comparison of the major compounds of the essential oil from different species of *Eryngium* is depicted in Table 2. The main component of some *Eryngium* species such as *E. campestre* (13.8%) [2], *E. amethystinum* (56.7%) [2], *E. eriophorum* (35.1%) [16], *E. pandanifolium* (28.9%) [16], *E. maritimum* (10.4-15.9%) [19], *E. serbicum* (19.7%) [20] and *E. yuccifolium* (18.3%) [21] was Germacrene D. Falcarinol also was found to be principal components in *E. triquetrum* (86.9%) [4] and *E. palmatum* (34.9%) [9] essential oils.

Our results have similarities and differences in the literature. The obtained and literature results show that not only the different species but also the chemical composition of the same species may be different. These differences originated due to the location of collection area, climatic conditions and genetic factors [30].

2.2. Antioxidant activity

The antioxidant properties of the essential oils of *E. pseudothoriifolium* and *E. thoriifolium* were evaluated using β-carotene-linoleic acid, DPPH free radical scavenging, ABTS cation radical scavenging and CUPRAC assays. The antioxidant activities of the essential oils were compared with the synthetic antioxidants, BHA and α-tocopherol, which were used as standards. The essential oils were tested at different concentrations and the results were given as inhibition percentage (%) at 200 µg/mL concentration of the essential oils in Table 3.

In β-carotene-linoleic acid assay, *E. pseudothoriifolium* essential oil (74.81±1.15%) showed higher lipid peroxidation inhibition activity than *E. thoriifolium* essential oil (41.72±1.30%). The essential oil of *E. pseudothoriifolium* showed the higher radical scavenging activity with inhibition values of 57.96±0.27% and 63.46±1.38% at 200 µg/mL concentration in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, respectively (Table 3).

The essential oil of *E. pseudothoriifolium* (1.22±0.07 absorbance) was active than *E. thorifolium* essential oil (0.44±0.07 absorbance). As shown in Figure 1, *E. pseudothoriifolium* essential oil was found to be higher reductant than α-tocopherol at 100 and 200 µg/mL concentration.

The essential oils of *Eryngium* species are known to be rich in falcarinol. The significant antioxidant activity of *E. pseudothoriifolium* essential oil in all assays can be attributed to high amounts of falcarinol as reported in the literature [4]. This is the first comprehensive study about the antioxidant activities of the essential oils of *E. pseudothoriifolium* and *E. thorifolium*.

According to our knowledge, *Eryngium* genus has approximately 250 species throughout the world, the limited number of essential oils (*E. maritimum*, *E. triquetrum*, *E. tricuspidatum* and *E. foetidum*) was investigated for their antioxidant activities. Radical scavenging activities of the essential oils of *E. maritimum* collected from five different regions were determined by using $DPPH^{\bullet}$ (IC_{50}: 104-141 µg/mL) and $ABTS^{\bullet+}$ (IC_{50}: 39-71 µg/mL) radicals [31]. *E. triquetrum* essential oil was reported to exhibit high $DPPH^{\bullet}$ radical scavenging activity (IC_{50}:

28.68 µg/mL) [4]. In a different study, Thomas *et al*. [22] investigated antioxidant properties the essential oils of leaf, stem, and root of *E. foetidum*. In DPPH assay, IC_{50} values for the leaf, stem and root oils were 56 µg/mL, 46 µg/mL, and 54.5 µg/mL respectively, while the leaf oil showed the highest reducing potential in the FRAP assay. Antioxidant activity of *E. tricuspidatum* essential oil (DPPH assay: IC_{50}: 510 µg/mL; FRAP assay: reducing the power of oil increases from 0.0188 at 5 µg/mL to 0.5016 at 1000 µg/mL) was studied by Merghache *et al*. [18]. Our results are comparable to those of earlier studies.

Table 2. Major compounds of essential oils from *Eryngium* species reported.

Eryngium species	Major compounds	Ref.
E. campestre	Germacrene D (13.8%), allo-Aromadendrene (7.7%)	[2]
E. amethystinum	Germacrene D (56.7%), *β*-Elemene (4.7%), Bicyclogermacrene (3.3%)	[2]
E. triquetrum	Falcarinol (86.9%), Octanal (1.8%)	[4]
E. palmatum	Falcarinol (34.9%), Octanal (31.7%), *a*-Curcumene (5.9%)	[9]
E. creticum	Hexanal (52.90%), Heptanal (13.90%),	[13]
E. thorifolium	*a*-Pinene (58.65%), 7-exo-Ethenylbicyclo[4.2.0]oct-1-ene (10.56%)	[13]
E. billardieri	*a*-Muurolene (42.0%), *β*-gurjunene (17.0%), *δ*-cadinene (6.2%)	[15]
E. floribundum	Spathulenol (36.0%), 2,3,6-Trimethyl benzaldehyde (15.7%)	[16]
E. eriophorum	Germacrene D (35.1%), Bicyclogermacrene (10.4%)	[16]
E. nudicaule	Bicyclogermacrene (17.2%), Cyclocolorenone (14.7%)	[16]
E. horridum	Pentadecane (53.5%), Globulol (18.6), Aromadendrene (9.1%)	[16]
E. pandanifolium	Germacrene D (28.9%), Bicyclogermacrene (12.8%)	[16]
E. foetidum	2-Dodecen-1-al (46.68%), Caprinic alcohol (14.80%)	[17]
E. tricuspidatum	*a*-Bisabolol (32.6%), *a*-Curcumene (6.5%), Ledol (4.8%)	[18]
E. maritimum	Aerial oil: Germacrene D (10.4%), 2,4,5-Trimethylbenzaldehyde (8.3%), Spathulenol (4.5%) Flower oil: Germacrene D (15.9%), 2,4,5-Trimethylbenzaldehyde (6.7%), Bicyclogermacrene (4.7%)	[19]
E. serbicum	Germacrene D (19.7%), *β*-elemene (10.0%), spathulenol (6.9%)	[20]
E. palmatum	Sesquicineole (21.3%), caryophyllene oxide (16.0%), spathulenol (16.0%)	[20]
E. yuccifolium	Germacrene D (18.3%), terpinolene (17.8%), bicyclogermacrene (8.8%)	[21]
E. foetidum	Leaf oil: (*E*)-2-Dodecenal (28.43%), 13-tetradecenal (27.45%), dodecanal (14.59%) Stem oil: Dodecanal (20.21%), 2,4,5-trimethylbenzaldehyde (18.43%) and (*E*)-2-dodecenal (8.27%) Root oil: 2,4,5-trimethylbenzaldehyde (56.08%), 13-tetradecenal (9.26%) (*E*)-2-dodecenal (7.65%)	[22]
E. glomeratum	Aerial oil: *cis*-Chrysanthenyl acetate (27.3%), 14-Hydroxy-*a*-muurolene (19.6%), *a*-Bisabolol (12%) Root oil: *β*-Oplopenone (20%), Di-epi-Cedrenoxide (15.9%), *δ*-Selinene (15.6%)	[23]
E. barrelieri	Aerial oil: *β*-Selinene (20.1%), Di-epi-Cedrenoxide (18.7%), *β*-oplopenone (10.6%) Root oil: Di-epi-Cedrenoxide (26.5%), *β*-oplopenone (20.3%), Acoradiene (10.5%)	[23]
E. caeruleum	Cyclobuta [1,2,3,4] dicyclooctene, hexadecahydro (47.03%), *n*-Hexadecanoic acid (11.16%), Linoleic acid (5.41%)	[24]
E. duriaei	*a*-Neocallitropsene (26.0%), *β*-Betulenal (16.2%), 14-hydroxy-*β*-caryophyllene (13.4%)	[25]
E. paludosum	*γ*-Terpinene (12.9%), *β*-bisabolene (12.2%), germacrene D (7.6%)	[26]
E. rosulatum	*β*-Elemene (16.0%), bicyclogermacrene (12.5%), *δ*-elemene (7.0%)	[27]
E. campestre	*γ*-Cadinen-15-al (23.3%), spathulenol (10.7%), octanoic acid (9.8%)	[28]
E. bungei	Cumin alcohol (55.3%), terpinolene (14.6%), carvacrol (8.9%)	[29]

Table 3. Antioxidant activities of the essential oils of *E. pseudothoriifolium* and *E. thoriifolium* by β-carotene-linoleic acid, DPPH•, ABTS•+ and CUPRAC assays[a].

		β-carotene-linoleic acid assay	DPPH• assay	ABTS•+ assay	CUPRAC assay[b]
Species	***E. pseudothoriifolium***	74.81±1.15	57.96±0.27	63.46±1.38	1.22±0.07
	E. thoriifolium	41.72±1.30	31.02±0.59	49.72±1.34	0.44±0.07
Standards	**BHA[c]**	91.91±0.38	88.39±0.26	86.73±0.10	2.90±0.21
	α-Tocopherol[c]	92.70±0.69	90.15±0.51	85.94±0.14	1.04±0.07

[a]: % inhibition of 200 µg/mL concentration of essential oils represent the means ± SEM of three parallel sample measurements ($p < 0.05$).
[b]: Absorbance values of 200 µg/mL concentration of essential oils
[c]: Reference compounds

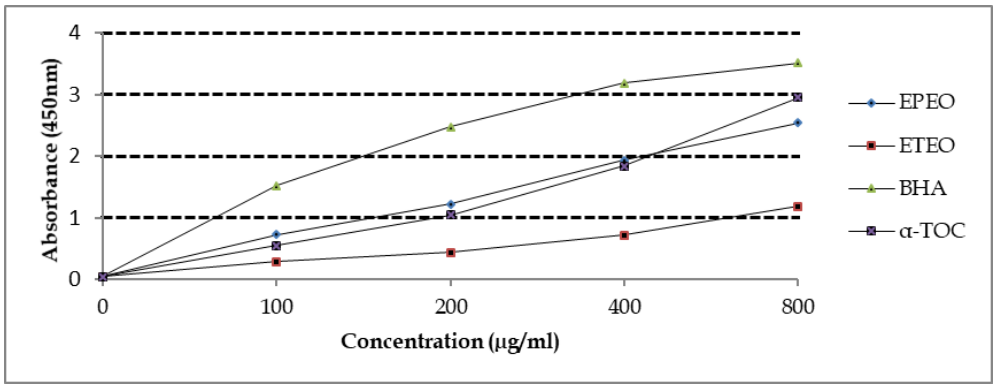

Figure 1. Cupric reducing antioxidant capacity (CUPRAC) essential oils of *Eryngium* species (EPEO, and ETEO), BHA and α-tocopherol using spectrophotometric detection of the Cu^{2+}–Cu^{1+} transformation.

3. CONCLUSIONS

This study is the first report of comprehensive antioxidant effects and detailed chemical characterization of these essential oils. In fact the essential oil of *E. pseudothoriifolium* can be considered as rich source of falcarinol. The high antioxidant activity of *E. pseudothoriifolium* essential oil is the result of high amount of falcarinol. In conclusion, our results indicate that *Eryngium* species could be explored as a natural source of food supplements and therapeutic applications.

4. MATERIALS AND METHODS

4.1. Plant materials

The aerial parts of *E. pseudothoriifolium* and *E. thorifolium* were collected from Muğla, Turkey in 2016. The plants were identified by Dr. Hasan Yıldırım at Ege University, Izmir, Turkey. The voucher specimen has been deposited at the herbarium of Natural Products Laboratory of Muğla Sıtkı Koçman University with voucher no MU1141 (for *E. pseudothoriifolium*) and MU1145 (for *E. thorifolium*).

4.2. Isolation and analysis of the essential oil

The essential oils of dried aerial parts of *E. pseudothoriifolium* and *E. thorifolium* were hydro-distillated in a Clevenger-type apparatus for 4 h. The oils were dried over anhydrous sodium sulfate and stored under +4°C until analyzed.

The essential oils were analyzed by GC-FID and GC-MS techniques. A Flame Ionization Detector (FID) and a DB-5 fused silica capillary non-polar column (30 m×0.25 mm id., film thickness 0.25 µm) were used for GC analyses. The injector temperature and detector temperature were adjusted 250 and 270°C, respectively. Carrier gas was He at a flow rate of 1.4 mL/min. The sample size was 0.1 µL with a split ratio of 20:1. The initial oven temperature was held at 60°C for 5 min, then increased up to 240°C with 4°C/min increments and held at this temperature for 10 min. The percentage composition of the essential oil was determined by the Class GC10 GC computer program.

An Ion trap MS spectrometer and a DB-5ms fused silica non-polar capillary column (30 m×0.25 mm ID, film thickness 0.25 µm) were used for the GC/MS analyses. The carrier gas was helium at a flow rate of 1.4

mL/min. The oven temperature was held at 60°C for 5 min, then increased up to 240°C with 4°C/min increments and held at this temperature for 10 min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. The ion source temperature was 200°C. The injection volume was 0.2 µL with a split ratio of 20:1. EI–MS measurements were taken at 70 eV ionization energy. Mass range was from *m/z* 28 to 650 amu. Scan time 0.5 s with 0.1 inter scan delays. Identification of components of the essential oils was based on GC retention indices and computer matching with the Wiley, NIST-2008 and TRLIB Library as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and whenever possible, by co-injection with authentic compounds [32].

4.3. Antioxidant activity assays

4.3.1. β-carotene/linoleic acid assay

The total antioxidant activity was determined by β-carotene-linoleic acid method based on the measurement of the inhibition of conjugated dien hydroperoxides resulting from linoleic acid oxidation with slight modifications [33]. A β-carotene–linoleic acid mixture was prepared as following: 0.5 mg β-Carotene in 1 mL of chloroform was added to 25 µL linoleic acid and 200 mg Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, was added by vigorous shaking. One hundred-sixty microliters of this mixture was transferred into 40 µL the samples at different concentrations. As soon as the emulsion was added into each tube, the zero time absorbance was measured at 470 nm using a 96-well microplate reader. Absorbance of the emulsion was read again at the same wavelength after incubation of the plate for 2 h at 50 °C. Measurement of absorbance was continued until the colour of β-carotene disappeared. BHA and α-tocopherol were used as antioxidant standards for comparison of the activity. The same procedure was repeated with used antioxidant standards and a blank. The bleaching rate (R) of β-carotene was calculated according to Eq. 1.

$$R = \frac{\ln \frac{a}{b}}{t} \qquad \text{(Eq. 1)}$$

Where: ln=natural log, a=absorbance at time zero, b=absorbance at time t (120 min). Antioxidant activity was calculated in terms of percentage inhibition relative to the control, using Eq. 2.

$$\text{Antioxidant activity (\%)} = \frac{R_{control} - R_{sample}}{R_{control}} \text{x}100 \qquad \text{(Eq. 2)}$$

4.3.2. DPPH free radical scavenging assay

The free radical scavenging activity was determined spectrophotometrically by the DPPH assay described by Blois [34] with slight modification [33]. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. Briefly, 40 µL sample solutions at different concentrations were added to 160 µL 0.4 mM DPPH solution. Thirty minutes later, absorbance was measured at 517 nm by using a 96-well microplate reader. The capability of scavenging the Inhibition activity (I) was calculated using Eq. 3.

$$\text{I (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \text{x}100 \qquad \text{(Eq. 3)}$$

4.3.3. ABTS cation radical scavenging assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ scavenging activity was determined according to the method of Re *et al.* [35] with slight modifications [33]. Briefly, $ABTS^{\bullet+}$ was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The radical cation was stable in this form for more than 2 days when stored in the dark at room temperature. Before usage, the $ABTS^{\bullet+}$ solution was diluted to get an absorbance of 0.708±0.025 at 734 nm with ethanol. Then, 160 µL of $ABTS^{\bullet+}$ solution was added to 40 µL of sample solution in ethanol at different concentrations. After 10

min, by using a 96-well microplate reader, the percentage inhibition at 734 nm was calculated for each concentration relative to a blank absorbance (ethanol). The scavenging capability of $ABTS^{\bullet+}$ was calculated using Eq. 3.

4.3.4. Cupric reducing antioxidant capacity (CUPRAC) assay

The cupric reducing antioxidant capacity was determined according to the method of Apak *et al.* [36] with slight modifications [33]. To each well, in a 96 well plate, 50 µL 10 mM Cu (II), 50 µL 7.5 mM neocuproine, and 60 µL NH_4Ac buffer (1 M, pH 7.0) solutions were added. Forty microliter extract at different concentrations were added to the initial mixture so as to make the final volume 200 µL. After 1 h, the absorbance at 450 nm was recorded against a reagent blank by using a 96-well microplate reader.

4.4. Statistical analysis

All data on antioxidant activity tests were the average of three parallel sample measurements. Data were recorded as mean ± S.E.M. Significant differences between means were determined by student's-t test, p values <0.05 were regarded as significant.

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