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In vitro antioxidant capacities and phenolic contents of four *Erica* L. (Ericaceae) taxa native to Turkey

Ayşegül KÖROĞLU [1], Muhammed Mesud HÜRKUL [1], Gülsen KENDİR [2] *, Nurgün KÜÇÜKBOYACI [3]

[1] Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 06100 Tandoğan, Ankara, Turkey.
[2] İstinye University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 34010 Zeytinburnu, İstanbul-Turkey.
[3] Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, 06330 Etiler, Ankara, Turkey.
* Corresponding Author. E-mail: kendir08@gmail.com (G.K.); Tel. +90-505-848 99 78; ORCID No: 0000-0001-8879-3751

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ABSTRACT: *Erica* species (Ericaceae) are widely used due to their medicinal properties in Turkish folk medicine. In this study, the antioxidant capacities of the extracts with different polarities of the aerial parts of *Erica arborea*, *E. manipuliflora*, *E. bocquetii* and *E. sicula* subsp. *libanotica* native to Turkey were investigated. 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging and the thiobarbituric acid (TBA) test systems used for the determination of antioxidant capacities. Also, total phenol contents in the extracts were determined as spectrophotometric. All extracts exhibited strong scavenging activity against DPPH except the chloroform extracts. Ethyl acetate extracts of *Erica* species had the best scavenging activity against DPPH, as well as the highest DPPH scavenging activity was exhibited by *E. manipuliflora* and *E. bocquetii* with IC_{50} values of 0.002±0.001 and 0.004±0.000 µg/mL, respectively. In TBA test, the highest activity was observed in the ethyl acetate extract of *E. manipuliflora* (IC_{50}= 0.001±0.000 µg/mL), followed by the water extract of this plant (0.03± 0.005 µg/mL). The highest total phenolic content was found in the ethyl acetate extracts ranging from 875.5 to 701.7 mg GAE/g extract. Our results showed that the ethyl acetate extracts of *Erica* taxa are rich in natural antioxidant substances.

KEYWORDS: *Erica*; Turkey; antioxidant capacity; total phenolic content.

1. INTRODUCTION

The family Ericaceae is represented by 100 genus and 3000 species and widely distributed in Himalayas, New Guinea and South Africa [1]. In Turkey, there are 12 genus and about 30 species in the family. The genus *Erica* L. (Ericaceae) is mostly found with more than 700 species in South Africa, West Europe and Mediterranean regions. This genus is represented by five taxa in flora of Turkey and one of them, *E. bocquetii* (Peşmen) P. F. Stevens, is an endemic species for Turkey. *E. arborea* L. and *E. manipuliflora* Salisb., are widely distributed in the coastal regions of Turkey. However, *E. sicula* Guss. subsp. *libanotica* (C. & W. Barbey) P. F. Stevens is presented only in the South West of Anatolia [2] and *E. spiculifolia* Salisb. is a new species, which was separated from the genus *Bruckenthalia* Reichb [3] These species are named "funda", "püren" or "süpürge çalısı" in Turkey [4]. Regarding the International Union for the Conservation of Nature and Natural Resources (IUCN) Red Data Book, *E. sicula* subsp. *libanotica* and *E. bocquetii* are evaluated in "Vulnerable" (VU) status in Turkey [5].

Herba Ericae is a natural drug consisting of dried leaves and flowers of *Erica* species, especially *E. arborea* and *E. manipuliflora*. In Turkish folk medicine, this drug is widely used as diuretic, astringent and treatment for urinary infections [4, 6]. On the other hand, 5% infusion of *E. arborea* is taken after meals for slimming purposes [7]. Furthermore, a decoction of the aerial parts of *E. arborea* is used as diuretic in Italy [8] as well as *E. multiflora* is also used as diuretic and antiseptic agent in Morocco [9]. In addition, *E. multiflora* has been reported that it is used for wound treatment in Spain [10].

Erica species mainly contain flavonoids [11, 12], anthocyanins [13], phenylpropanoid glycosides [14], coumarins [15] and triterpenic compounds [16]. Genus *Erica* has been investigated for their biological acitivites such as antiulcer [17], anti-inflammatory, antinociceptive [18], antimicrobial [16, 19-22], antilithic

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[23] and cytotoxic [21, 24] activities. There are several reports on the antioxidant activity of *Erica* species [21, 22] except *E. arborea* [11, 12, 21, 25-28].

Free radicals are molecules which contain one or more unpaired electrons. These highly reactive molecules which tend to initiate chain reactions with other molecules are also known as oxidants or reactive oxygen particles [29]. It is widely accepted that virtually every state of any disease involves some degree of oxidative stress and free radicals which can be obviated by the administration of either exogenous and endogenous antioxidants or free radical scavengers [30]. Medicinal plants, vegetables and spices are known as the primary sources of naturally occurring antioxidants [31-33]. Antioxidants, used in typical diets, are derived from plant sources, containing various compounds with a wide variety of physical and chemical properties. The most important characteristic of an antioxidant is free radical scavenging or the ability to trap free radicals [33]. Phenolics, including phenols, phenolic acids, flavonoids, tannins and lignans are the main sources of natural antioxidants. Antioxidants due to phenolic groups, which are excellent nucleophiles and lipid peroxidation inhibitors, cease the reaction of oxidation by binding free radicals in several ways. In addition, phenolic compounds act as chelating agents of metal ions that induce oxidation [32]. Significant evidence suggests that foods containing antioxidants and especially antioxidant nutrients may have importance in preventing diseases [34].

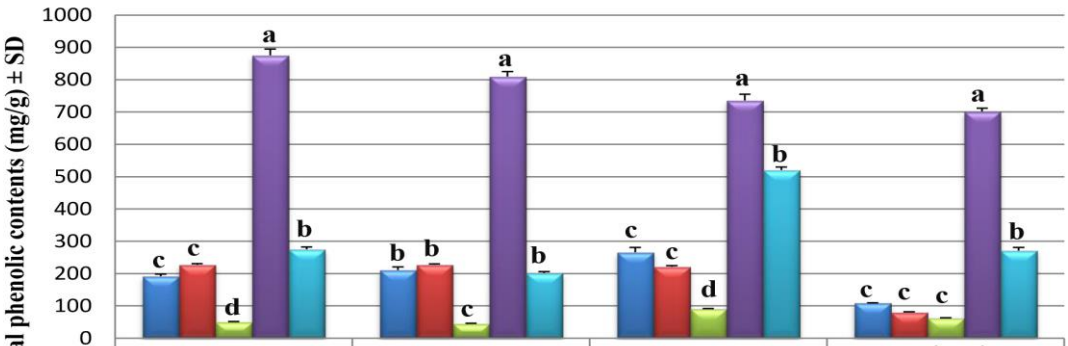
Currently, 19 *in vitro* and 10 *in vivo* methods are used for antioxidant evaluation. DPPH scavenging activity method is the most commonly used for assessing antioxidant activity as *in vitro*, while lipid peroxidation assay has been found as the most commonly used method as *in vivo* [34].

This study was conducted to carry out a comparative study on four *Erica* species native to Turkey in five different solvent extracts to determine their antioxidant capacities as well as total phenolics and flavonoids. Antioxidant effect of the extracts was tested for their scavenging activity against DPPH and anti-lipid capacity using TBA test.

2. RESULTS AND DISCUSSION

This study was carried out to determine the total phenolics and total flavonoids as well as the *in vitro* antioxidant capacity of different extracts prepared from four *Erica* taxa used as natural medicines in Turkish folk medicine. Figure 1 shows an overview of the total phenolic contents of *Erica* taxa. Maximum total phenolic content was found in EtOAc (Ethyl acetate) extracts of *E. arborea* (877.5±19,29 mg gallic acid equivalent (GAE)/g), followed by *E. bocquetii* (810±14,95 mg GAE/g), *E. manipuliflora* (735.5±19,35 mg GAE/g) and *E. sicula* subsp. *libanotica* (701.7±10,2 mg GAE/g) ($p< 0.05$). Furthermore, the plates were sprayed with 1% vanillin-H_2SO_4, antioxidant compounds were detected mainly the flavonoids. Kaempferol and luteolin together with water, EtOAc and *n*-BuOH extracts of *Erica* species have been showed yellow and orange fluorescence in UV light (365 nm) after spraying. The radical scavenging capacity of the extracts from four *Erica* species was evaluated by using qualitative and quantitative DPPH assays. The highest free radical scavenging effects in both systems were found in EtOAc extracts *of E. bocquetii* (IC_{50}= 0.004±0.000 µg/mL) and *E. manipuliflora* (IC_{50}= 0.002±0.001 µg/mL) ($p< 0.05$). *E. sicula* subsp. *libanotica* also displayed the highest effect in quantitative DPPH assays (IC_{50}= 0.002±0.000 µg/mL), similar to *E. manipuliflora*. Water extracts of *Erica* species usually showed higher effect than the propyl gallate used as standard ($p< 0.05$) in this test. The results of the qualitative DPPH test showed that EtOAc extracts of *E. bocquetii* and *E. manipuliflora* was appeared more prominent yellow zones on purple background than the others. The free radical scavenging capacities of *Erica* extracts were in the following order: EtOAc> water> MeOH (methanol)> *n*-BuOH (*n*-butanol)> $CHCl_3$ (chloroform). The IC_{50} values for antioxidant capacity of all the plants are given in Figure 2. All samples inhibited DPPH radicals in a concentration-dependent manner.

The inhibitory capacities of the extracts against lipid peroxidation on liposomes are shown in Table 1. In the TBA method, all the extracts of *Erica* species were shown to possess significant anti-lipid peroxidation capacity in *in vitro* system. The most active extracts were found to be EtOAc and water extracts of *E. manipuliflora* with IC_{50}= 0.001±0.000 µg/mL and 0.03±0.005 µg/mL, respectively. These extracts exhibit a higher activity than the propyl gallate used as standard (IC_{50}= 0.14 ± 0.01 µg/mL).



	Erica arborea	E. bocquetii	E. manipuliflora	E. sicula subsp. libanotica
Water	192	211,3	265,7	109,6
MeOH	227,7	227,3	221	80,2
CHCl3	50	44,9	90,5	62,2
EtOAc	875,5	810	735,5	701,7
n-BuOH	275	202,3	520,7	271,7

Figure 1. Total phenolic contents of the extracts of *Erica* taxa. Means with different letters in the same plant represent significantly difference at the level ($p< 0.05$); n=3.

We usually observed higher values for total phenolic content and antioxidant activity than the literature findings. Turgay and Esen (2015) have been reported that the acidified methanol extract of *E. manipuliflora* showed moderate antioxidant capacity in DPPH scavenging capacity test (IC_{50}= 255.9 mg/mL). Besides, they determined its amount of total phenolic as 260 mg GAE/gr. In that study, the plant material has been purchased from local supermarkets, due to this reason it may not be a fresh plant [22]. In other previous study, the antioxidant activities of some polar extracts such as ethanol, methanol and water of *E. bocquetii* (2.36±0.01 millimolar (mM), 2.65±0.00 mM, 2.6±0.03 mM, respectively) were higher than those of *Erica arborea* (1.63±0.07 mM, 1.92± 0.06 mM, 1.92±0.12 mM) in TEAC assay were demonstrated [21]. In another previous study, the antioxidant capacities of methanol, ethyl acetate, butanol and water extracts of aerial parts of *E. arborea* were determined. The ethyl acetate extract was found to be richest in terms of phenolic (315.52±3.81 microgram (µg) pyrocatechol equivalents/mg extract) and flavonoid (150.42±1.63 µg quercetin equivalents/mg extract) contents which exhibited the highest antioxidant activity in especially DPPH assay (IC_{50}= 5.98±0.09 mg/mL) [25]. In other study, the ethanolic extract of *E. arborea* exhibited notable antioxidant activity in FRAP (Ferric-reducing antioxidant power) (3.55±0.11 millimol Fe^{2+}/g), lipid peroxidation (62.5 mg/ml; 78.97 %) and DPPH free radical scavenging activity (13.40±0.67 µg/mL) tests with the high total polyphenols and tannins values (28.00±0.25 and 21.86±0.09%, respectively) [27]. Water extracts of different parts such as leaves, flowers and branches of *E. arborea* were investigated for their phenolic and flavonoid contents, and antioxidant activity using *in vitro* tests such as total antioxidant activity, FRAP, reducing power, DPPH and ABTS (2-2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). The leaves were found to be have the highest total phenolic (30.6 mg GAE/g) and flavonoid (4 mg quercetin equivalent/g) contents. In all tests, the leaves usually showed the highest antioxidant activity [28]. Furthermore, phenylethanoids (RC_{50}= 11.11±10^{-2} to 2.44±10^{-5} mg/mL) and flavonoids (RC_{50}= 15.6×10^{-3} to 17.2×10^{-3} mg/mL) obtained from the methanol extract of *E. arborea* leaf exhibited significant antioxidant capacity in DPPH assay [11, 12]. The methanol extract of *E. arborea* leaf also displayed considerable antioxidant capacity in DPPH assay (RC_{50}= 9.9±10^{-3} mg/mL) [12]s.

E. manipuliflora is widely used as herbal tea in folk medicine in Turkey [4]. However, regarding the results from the TBA, $CHCl_3$ extracts of studied taxa have low antioxidant capacity compared with propyl gallate. These results are in agreement with the results of the free radical scavenging capacity.

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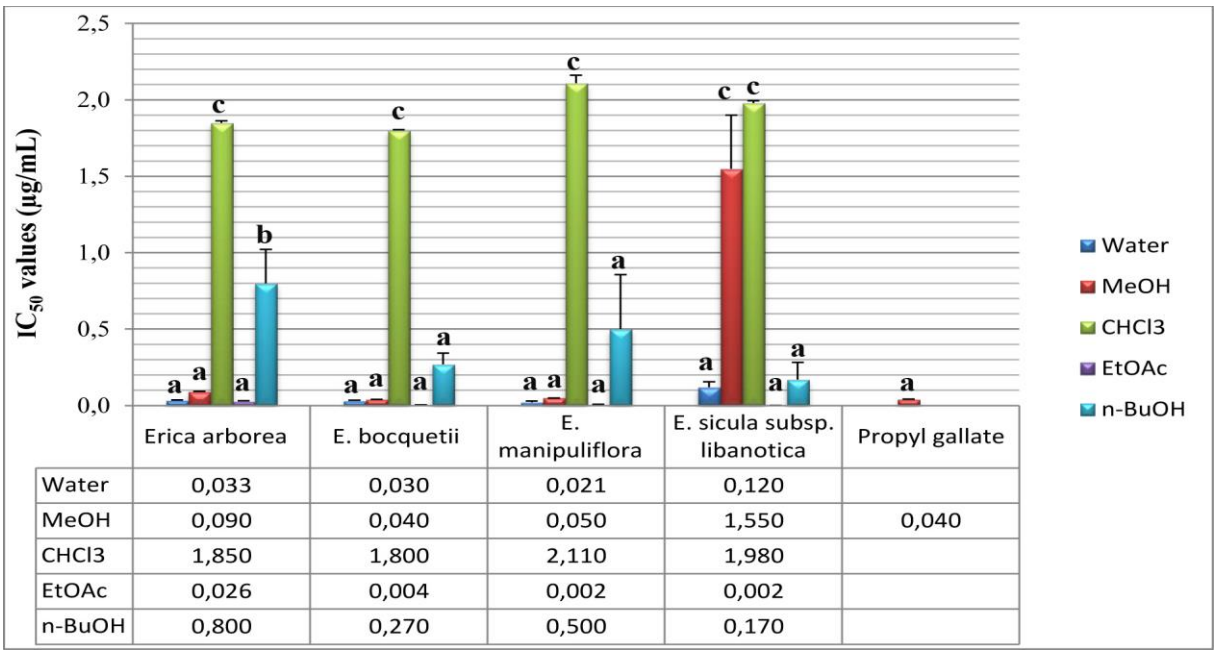

	Erica arborea	E. bocquetii	E. manipuliflora	E. sicula subsp. libanotica	Propyl gallate
Water	0,033	0,030	0,021	0,120	
MeOH	0,090	0,040	0,050	1,550	0,040
CHCl3	1,850	1,800	2,110	1,980	
EtOAc	0,026	0,004	0,002	0,002	
n-BuOH	0,800	0,270	0,500	0,170	

Figure 2. Free-radical scavenging activity by quantitative DPPH of the extracts of *Erica* taxa. Means with different letters in the same plant represent significantly difference at the level ($p< 0.05$); n=3.

Phenolic substances have been shown responsible for the antioxidant capacity of plants [31, 32, 35]. EtOAc extracts of *Erica* taxa were found to have significantly high content of total phenols. These results are in a good agreement with the literature [18, 20, 25]. *Erica* species contain flavonoids, anthocyanins, coumarins and terpenoid compounds [10, 13, 15, 16, 25]. As soon as the plates were sprayed with 1% vanillin-H_2SO_4, antioxidant compounds were detected mainly the flavonoids, especially in the EtOAc extracts, because of the observance of the characteristic yellow and orange bands in the fluorescence under UV light (365 nm) after the spraying process [36]. In our study, after the development procedure following the spraying process on TLC (thin layer chromatography), distinct yellow and orange zones were observed in the fluorescence under UV light. These results are in good accordance with the results of total phenolic compounds (Figure 1). Furthermore, the highest free radical scavenging capacity (Figure 2) and inhibition of lipid peroxidation (Table 1) were observed in EtOAc extracts. It is reported that there is a strong correlation between total phenolic contents, antiradical and anti-lipid peroxidative capacities of the compounds. Scavenging and inhibition of lipid peroxidation capacities of EtOAc extracts of *Erica* taxa may be attributed to their phenolic contents, especially flavonoids. The EtOAc extract of *E. arborea* has been found to have the richest phenolic and flavonoid content [(-)-epicatechin and quercitrin] and showed the highest antioxidant capacity [25]. The antioxidant capacity of phenolics is mostly related to their reduction properties, which allow them to act as hydrogen donors and singlet oxygen quenchers. In recent years the powerful antioxidant capacity of the flavonoids has been attracting much attention [37].

Table 1. Antioxidant activities of the *Erica* taxa in the TBA test.

Species	IC_{50} value (µg/mL) ± SEM				
	Water extracts	MeOH extracts	$CHCl_3$ extracts	EtOAc extracts	*n*-BuOH extracts
Erica arborea	1.02 ± 0.01	2.65 ± 0.15	≥ 50 ± 0.9	0.22 ± 0.04	13.54 ± 0.2
E. bocquetii	19.92 ± 0.06	4.05 ± 0.01	≥ 50 ± 0.6	2.65 ± 0.02	25.98 ± 0.06
E. manipuliflora	0.03 ± 0.005	3.03 ± 0.6	≥ 50 ± 1.8	0.001 ± 0.000	2.77 ± 0.06
E. sicula subsp. *libanotica*	10.15 ± 0.01	29.1 ± 0.5	≥ 50 ± 1.8	0.31 ± 0.02	15.04 ± 0.01
Propyl gallate	0.14 ± 0.01				

SEM: Standard error mean; TBA: Thiobarbituric acid; IC_{50}: Inhibitory concentration.

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3. CONCLUSION

The data obtained are to verify the ethnopharmacological use of these Turkish *Erica* taxa. *E. arborea* and *E. manipuliflora* are used as infusion or decoction form in folk medicine in Turkey. The results obtained in this study are expected to establish a firm basis for the future studies in this area. It was observed that these plants have great potential as the natural antioxidant compounds. Further bioassay-guided fractionation procedures are necessary to characterize and isolate the active constituents for *E. manipuliflora*. *In vivo* and clinical studies could be conducted on the active constituents of this species and, based on the results obtained, these active constituents could be a source of pharmaceutical preparations. This work is the first study for evaluating of antioxidant capacity of *E. sicula* subsp. *libanotica* growing in Turkey.

4. MATERIALS AND METHODS

4.1. Plant materials

Plant materials were collected from different regions of Turkey in their natural habitats. Voucher samples were kept in the Ankara University Faculty of Pharmacy Herbarium (AEF), Turkey. Localities, collection periods and herbarium numbers of the species studied are as follows: *E. arborea*: A3 Bolu: Akçakoca, ca. 300 m, 19.05.2006, *A. & U. Güvenç, M. Coşkun* (AEF 23874); *E. manipuliflora*: C6 Hatay: Dörtyol, 1100 m, 07.09.2003, *G. Kendir* (AEF 23013); *E. sicula* subsp. *libanotica*: C3 Antalya: Kemer Beldibi, 42 m, 10.05.2003, *R. S. Göktürk, G. Kendir* (AEF 23009); *E. bocquetii*: C2 Antalya: Çığlıkara, 1750 m, 19.07.2003, *A. & U. Güvenç, R. S. Göktürk* (AEF 23016).

4.2. Chemicals

Methanol (Merck 1.06008-extra pure), Chloroform (Merck 1.02431-extra pure), Ethyl acetate (Merck 1.00864-extra pure), *n*-Butanol (Merck 16904588), Folin-Ciocalteu reagent (Molychem 31740), Na_2CO_3 (Sigma-Aldrich 13418-1KG-R), Gallic acid (Merck), DPPH• reagent (Aldrich D211400), Phosphate buffer (Biomatik A3602), Ferric cloride (Sigma F1513), Ascorbic acid (Aldrich 25,556-4), Thiobarbituric acid (Sigma-Aldrich T5500), Butylated hydroxytoluene(Sigma-Aldrich B1378), Brain extract (Sigma-Aldrich B3635), Proply gallate (Aldrich P5,330-6), kaempferol (Sigma K0133), luteolin (Sigma L9283), TLC plates (Merck).

4.3. Preparation of extracts

After drying the aerial parts (including of stem, leaves and flowers) of plant materials under shade, the materials were powdered to a fine grade in a mechanical grinder.

4.3.1. Water extracts

Twenty gram of aerial parts of plants were extracted by refluxing with distilled water (100 mL) for 30 min. The extracts were filtered when hot and then the resultant extracts were lyophilized.

4.3.2. Methanol (MeOH) extracts

Twenty gram of aerial parts of the plants were macerated with methanol (MeOH; 3×250 mL) for 8 h at 60 ˚C. The methanol extracts were filtered and then evaporated *in vacuo* at 40 ˚C.

4.3.3. Solvent fractionation

Forty gram powdered herbs of the species studied were macerated with MeOH (3×250 mL) for 8 h at 60 ˚C. Following filtration, the combined methanol extract was evaporated to dryness *in vacuo* at 40 ˚C. The crude methanol extract was dissolved in 50 mL of H_2O:MeOH (9:1, v/v) and then this solution was extracted successsively with chloroform ($CHCl_3$; 3×150 mL), ethyl acetate (EtOAc; 3×150 mL) and *n*-butanol saturated with distilled water (*n*-BuOH; 3×150 mL). Each extract was evaporated to dryness under reduced pressure.

4.4. Determination of total phenolic content

Total phenolic content of the extracts was determined according to the Folin-Ciocalteau method, referring to the calibration curve of gallic acid which a phenolic compound used as a standard [38]. The extracts in the concentration of 2 mg/mL was used in the analysis. According to this method, 50 µL samples were mixed with 250 µL of Folin-Ciocalteu's reagent and 500 µL of 20% (w/v) aqueous Na_2CO_3. The volume was made up to 5 mL with distilled water. After incubation of the samples at room temperature for 30 min,

the absorbances of the samples were read at 765 nm on a Shimadzu UV-1800 Spectrophotometer. Total phenolic content was measured as gallic acid equivalent (GAE) and expressed in mg GAE/g extract (dw) ± standard error mean (SEM). All experiments were performed in triplicate.

4.5. Qualitative determination of flavonoid

5 microliter of 1 milligram/milliliter *Erica* extracts prepared from the different polarity solvents were applied to silica gel TLC plates (Merck 5554, Darmstadt, Germany) by the use of Wiretrol II micropipettes. The TLC plate was developed with the mixture of $CHCl_3$:MeOH:distilled water (80:20:2) and then sprayed with 1% vanillin-H_2SO_4 solution. The plate was heated for 5 min at 110 ˚C before being examined under UV light (365 nm). Flavonoids appeared as yellow and orange zones [31, 36]. Kaempferol (Sigma K0133) and luteolin (Sigma L9283) were used as reference substances.

4.6. Antiradical capacity

The free radical scavenging activity of the extracts has been investigated by using qualitative and quantitative DPPH• tests.

4.6.1. Qualitative DPPH•

DPPH• assay was used as a rapid TLC screening method for evaluation of the free radical scavenging capacity of MeOH, $CHCl_3$, EtOAc, *n*-BuOH and water extracts of four *Erica* species. When TLC plate is sprayed with DPPH• solution, the antioxidant compounds in samples are observed as yellow zones on a purple background. 2 µg/mL of all extracts were applied to the TLC plate. The TLC plate was sprayed with 0.2% DPPH (Aldrich D 211400) solution in MeOH and left for 30 min at 20 ˚C and then it was examined [31]. Propyl gallate was used as a positive control.

4.6.2. Quantitative DPPH•

DPPH radical scavenging capacity was evaluated by the method described by Brand-Williams *et al.* (1995) [39]. The extracts were dissolved in methanol, then 0.1 mL of each extract at different concentrations (0.016–1 mg/mL) was mixed with 2.9 mL of freshly prepared 10^{-4} M DPPH solution and left in a water bath for 30 min at 30 ˚C. Following incubation, the decrease in absorbance at 517 nm was measured using a Shimadzu UV-1800 Spectrophotometer. Propyl gallate was used as a positive control. The inhibition of DPPH radicals was calculated as follows:

$$\% \text{ DPPH radical scavenging capacity} = [(Ao - As)/Ao] \times 100$$

Ao= the absorbance of the control and As= the absorbance of the sample or reference.
The IC_{50} value of the extracts was calculated by linear regression analysis. All analyses were performed triplicate and the results were expressed as mean values ± standard error mean (SEM).

4.7. Anti-lipid peroxidation capacity

The lipid peroxidation capacity was measured by the use of thiobarbituric acid (TBA) test described by Conforti *et al.* (2002) [31, 35]. Different concentrations of the extracts (0.016-1 mg/mL) and propyl gallate (0.000064-1 mg/mL) as reference were tested for their lipid peroxidation capacity against liposomes prepared from bovine brain. The absorbance was measured at 532 nm by a Shimadzu UV-1800 Spectrophotometer. The inhibition of lipid peroxidation was calculated as follows:

$$\% \text{ Inhibition} = 100 \times \frac{(A_o - B) - (As - B - EA)}{(A_o - B)}$$

A_o= the absorbance of control reaction, B= the absorbance of the blank mixture (liposomes only), As= absorbance of the sample, EA= the absorbance due to the extract alone.

The IC_{50} value of the extracts was calculated by linear regression analysis. Four replicate experiments were performed for each extract and the results were given as mean values ± standard error mean (SEM).

4.8. Statistical analysis

Statistical analysis were conducted by using SPSS Statistics 20 software (IBM Corporation, Armonk, NY) and one-way analysis of variance (one-way ANOVA, Tukey's test). A statistical significance of $p < 0.05$ was considered to be significant.

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