Investigation of the composition and antioxidant activity of acetone and methanol extracts of *Daphne sericea* L. and *Daphne gnidioides* L.

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Abstract The compositions of methanol and acetone extracts obtained from *Daphne sericea* L. and *Daphne gnidioides* L. were investigated. The antioxidant activities of each extract were determined by various test methods. Phenolic composition profile analysis by HPLC–DAD showed that *D. gnidioides* extracts contain more phenolic compounds than *D. sericea* extracts. Among the components, syringic acid was found to be the most abundant one in *D. gnidioides* extracts (42.8 and 38.4 mg per g dry extract of methanol and acetone, respectively). Total flavonoid, total phenolic and total carotenoid contents of methanolic *D. gnidioides* extracts were found to be 244.5 mg rutin/g dry weight of extract, 1219.3 mg GAE/g dry weight of extract and 11.9 mg/g dry weight of extract, respectively. DPPH[•], ABTS^{•+} and O₂^{•−} assays were applied to plant extracts as radical scavenging activity tests. Methanolic extracts of *D. sericea* and *D. gnidioides* showed the highest radical scavenging activities according to DPPH[•] and ABTS^{•+} tests (61.6 and 147.2 in terms of IC₅₀, respectively). Antioxidant activity tests for measuring lipid oxidation inhibiting capacity were carried in low density lipoprotein (LDL) and bovine brain extract (BBE). Methanolic extracts of *D. gnidioides* and *D. sericea* demonstrated remarkable lipid oxidation inhibiting capacity in LDL and BBE tests.

Keywords *Daphne sericea* · *Daphne gnidioides* · Antioxidant activity · Lipid oxidation · Phenolic compound · HPLC–DAD

Introduction

Many source of endogenous and exogenous reactive oxygen species (ROS) can be found in our oxygen-rich environment. The human body may be exposed to some ROS such as superoxide anion (O₂^{•−}), hydroxyl radical (OH[•]) and hydrogen peroxide (H₂O₂) in daily activities. As a natural defense system, our body is protected against these free radicals by antioxidant molecules and antioxidant enzymes. When level of ROS exceeds the capacity for defense or antioxidant systems, our body may be faced with health problems such as induction of aging, abnormal physiological functions or various human diseases (Gao et al. 2014).

Plants are potential source of compounds having antioxidative activity to counteract or minimize oxidative stress caused by photons and reactive oxygen species. Since traditional plants are known as sources of modern drugs, medicine formulations consist of components isolated from natural sources, together with synthetic compounds. Earlier phytochemical studies have shown that essential oils, flavonoids, terpenoids and mono- and sesquiterpenes are among the major chemical constituents of the plants (Kilani et al. 2005). Among the reasons for the preference of plant-origin drugs are their being safer, relatively cheaper, highly tolerated and convenient for many patients (Cai et al. 2007). Healing effects of the constitutive chemical compounds in plant-origin medicines were typically associated to phenolic compounds, most of which are flavonoids and phenolic acids, together with coumarins, chalcones, anthocyanins, tannins, lignins, terpenes and terpenoids (Amaral et al. 2009). It is known that phenolic compounds affect as anti-allergic, anti-atherogenic, anti-inflammatory, antimutagenic, antioxidant, antimicrobial agents and metal chelators (Vichapong et al. 2014).

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Flavonoids, the groups of compounds that most of the antioxidant activity of plants comes from, contain phenolic structure and are widely distributed in photosynthesizing cells (Kumar and Pandey 2013). Flavonoids can be subdivided into several classes: flavones, flavonols, flavanones, isoflavones, flavans, flavanols, and anthocyanins. These compounds differ in the number of phenolic hydroxyl groups, structure and their position, leading to variation in their antioxidative capacity. Investigation of the presence and activity of flavonoids as antioxidants in tea and herbs has been performed in various studies (Erkan et al. 2008; Atoui et al. 2005). It is known that extracts obtained with organic solvents from dried leaves of such plants are of much interest due to the high capacity of these solvents in extracting antioxidant compounds. The driving force for the natural and/or organic additive market is the consumer's demand for the products which are perceived as being healthier, organic and ecological. Products of external usage such as personal care products usually contain plant extracts, waxes, essential oils, lipids, plant carbohydrates or vitamins, antioxidants and purified plant components having biological activity. The variety of plants providing these ingredients includes edible plants (legumes, fruits, plants, roots, spices, etc.), herbs, teas and exotic plants.

The *Daphne* L. species (Thymelaeaceae) are evergreen shrubs native to Asia, Europe, and North Africa. Among the 70 species distributed worldwide, seven species grow in Turkey, namely, *D. glomerata*, *D. gnidioides*, *D. mezereum*, *D. mucronata*, *D. oleoides*, *D. pontica* and *D. sericea* (Suntar et al. 2012). *Daphne* species have shown a variety of pharmacological actions. Various species of *Daphne* are used in several folk medicines to treat gonorrhea and cutaneous affections, rheumatoid arthritis, wound healings, malaria and anti-inflammations. They exhibit few bioactivities such as antimalarial, antiviral, antitumor-promoting, antifertility and antibacterial activities (Suntar et al. 2012, Kupeli et al. 2007, Mansoor et al. 2013). *Daphne gnidioides* is semi-woody, evergreen shrub plant distributed in South Anatolia (Ari et al. 2014). Apigenin-7-glucoside, luteolin-7-glucoside, luteolin-4-glucoside, isovitexin, and quercetin-3-glucoside are among the phenolic compounds detected in the plant. Another member of *Daphne* family, *D. sericea* L. Vahl is distributed in Northwest, West and South Anatolia. The aerial parts of *D. sericea* was reported to contain luteolin 7-methyl ether 5- β -D-glucoside and luteolin 7,3'-dimethyl ether 5- β -D-glucoside, as well as luteolin 7-methyl ether, isovitexin, apigenin and its 7- β -D-glucoside (Tosun 2006). The origin of these species is Eastern Mediterranean.

The latter two *Daphne* species mentioned above have been used both internally and externally for their medicinal effects, with precautions regarding their toxicity, especially

during internal usage (Tosun 2006). Some species of the *Daphne* family have also been used for cancer treatment (Kupeli et al. 2007). Although there are reported works on some properties and use of *Daphne* species in literature as outlined above, no detailed study could be found about the antioxidant activities of *D. gnidioides* and *D. sericea* which are expected to be closely related to their pharmacological activities. The present study provides information on antioxidant properties of the two plants in their external usage. The assays applied are comprised of methods testing radical scavenging and lipid peroxidation preventing capacities of methanol and acetone extracts of the two plants, together with their free phenolic acid and flavonoid profiles analyzed by HPLC–DAD.

Materials and methods

Chemicals and instruments

All phenolic compounds (> % 95 purity), ($\pm$)- α -tocopherol, chlorophyll a&b, gallic acid, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS⁺), trolox, potassium persulphate, nicotinamide adenine dinucleotide (NADH), aluminum chloride hexahydrate, phenazinmethosulfate (PMS), phosphate buffered saline (PBS), lipoprotein, bovine brain extract (BBE) was purchased from Sigma Aldrich (St. Louis, Missouri, USA); methanol (HPLC grade $\geq$ 99,9%), acetone (HPLC grade $\geq$ 99,9%), Folin-Ciocalteu reagent, sodium hydroxide, iron (III) chloride hexahydrate, o-phosphoric acid, iron (II) chloride, copper (II) chloride were obtained from Millipore Corporation (Billerica, Massachusetts, USA); ethylenediaminetetraacetic acid (EDTA), 2-thiobarbituric acid (2-TBA), 2-deoxy-D-ribose, sodium dodecyl sulphate (SDS), trichloroacetic acid (TCA), nitrobluetetrazolium (NBT) were purchased from Fluka (St. Louis, Missouri, USA). All chemicals used were analytical grade.

Liquid chromatography system was used for detection and quantification of phenolic compounds. The system consists of Agilent (Santa Clara, California, USA) 1100 series HPLC and diode array detector (DAD). Chromatographic separation of phenolic compounds were succeeded with Agilent 250 mm $\times$ 4.6 mm ID 5 μ m particle size Hypersil ODS C18 column. For spectrophotometric analyses Thermo Scientific (Waltham, Massachusetts, USA) Multiskan FC Microplate Photometer and Varian (Palo Alto, California, USA) Cary 100 Bio series spectrophotometer were used.

Plant material

D. gnidioides and *D. sericea* were harvested from their natural growth area (*Quercus coccifera* maquis, limestone) of the University campus in their flowering time of May, 2014. The collected plants were placed in separate labeled plastic bags and brought immediately to the laboratory. Voucher specimens (Erkan 1001, 1002 & Cinbilgel) have been deposited at Organic Agriculture Laboratory, Vocational School of Technical Sciences of the University. Both plants were dried in air at ambient temperature (25–27 °C) for 10 days. Then the leaves were separated from the branches, blended in a blender and stored at – 20 °C in sealed bags. Samples (10.502 and 10.499 g *D. sericea* and 10.499 and 10.504 g *D. gnidioides* for acetone and methanol extractions, respectively) from each plant species were agitated in 200 mL of methanol or acetone using a laboratory shaker for three days at room temperature. Mixtures were filtered and solutions were evaporated for the removal of their solvents with the use of a rotary evaporator under reduced pressure and temperature. They were then kept in a vacuum oven at 30 °C for 2 days for removal of any solvent residue. The residues of methanol and acetone extracts of *D. gnidioides* EGM and EGA, respectively, and those of *D. sericea* ESM and ESA, respectively, were washed with hexane repeatedly to remove chlorophyll components of the plants as much as possible.

The reason for choosing methanol and acetone, instead of water, for extraction is the possibility of recovering more phenolic compounds from the plant materials into these organic solvents. All precautions were taken for the complete removal of these solvents from the extracts, such as evaporation under reduced pressure and keeping in vacuum oven for a long time. It is to be noted that these extracts can further be washed with water and then freeze-dried before their use in real food processing operations.

HPLC–DAD analysis for quantification of free phenolic acids and flavonoids

An acid hydrolysis step was applied to the extracts to release aglycones of phenolic compounds for simplification of peak identification in HPLC analysis as described previously (Erkan et al. 2011a). HPLC–DAD analyses of the acid-hydrolyzed extracts were performed according to the procedure given in our previous work (Erkan et al. 2011b). All the plant extracts were dissolved in and diluted to a certain volume with methanol prior to analysis. Standard reference compounds were chlorogenic, caffeic, syringic, p-coumaric, ferulic and rosmarinic acids, myricetin, quercetin, apigenin, rhamnetin, isorhamnetin, kaempferol and

luteolin. Their solutions were also prepared in methanol. Peak identification was performed by comparison of retention time and UV spectra of reference standards with those of extract samples. The chromatograms were acquired at 280, 330 and 370 nm. Peak areas in chromatograms were utilized in quantification of individual phenolics in the extract and reported as mg/g dry weight (DW) of extract.

Chlorophyll a, chlorophyll b and total carotenoid contents

Portions from each plant extract were homogenized in diethyl ether and the mixtures were centrifuged. Supernatants were separated and the absorbances for chlorophyll *a*, chlorophyll *b* and total carotenoid content (TCC) were measured at 662, 646 and 470 nm, respectively. Amounts of these pigments in the plants were calculated according to the formulations given in the study of Ramazzina et al. (2015).

Total phenolic and total flavonoid contents of plant extracts

Total phenolic contents (TPCs) of plant extracts were determined using Folin-Ciocalteu (FC) reagent according to the spectrophotometric method given previously (Erkan et al. 2011b) which is a slightly modified form of the method originally reported by Singleton et al. (1999). The results were expressed in gallic acid equivalent (GAE) as mg GAE/g DW of extract, utilizing an absorbance versus concentration calibration curve for gallic acid (1.7–42.5 µg/mL). Total flavonoid content (TFC) was estimated by the spectrophotometric method which is reported by Quettier-Deleu et al. (2000). Results were expressed as mg rutin/g DW of extract using standard rutin solutions (5–100 µg/mL).

In vitro antioxidant activity assays

DPPH[•] and ABTS^{•+} radical scavenging assays were carried out as described in our previous study (Erkan et al. 2011b). Superoxide anion radical (O₂^{•−}) scavenging activities were tested using a method reported by Valentão et al. (2001). Antioxidant activities of plant extracts were expressed as IC₅₀, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH[•], ABTS^{•+} or O₂^{•−} concentration.

Lipid peroxidation inhibiting capacities in low density lipoprotein (LDL) and bovine brain extract (BBE)

Lipid peroxidation assay in bovine brain extract (BBE) was performed as described by Tang et al. (2004). Lipid peroxidation assay in human low density lipoprotein (LDL) was performed according to the procedure reported by Yu et al. (2005). Incubation period for BBE was 120 min at 37 °C, while 4, 12 and 36 h incubation times were applied for LDL. The results in both assays were given as % inhibition of lipid peroxidation. Ferulic acid, quercetin, α -tocopherol and BHT were used as positive controls in all assays as long as proper dissolution was ensured.

Statistical analysis

All measurements were made in triplicate and the values were reported as means of the measurements with standard deviations (SD) in tables and SD bars in figures using SAS systems (Windows Release Version 7). Duncan's multiple comparison test was performed to determine significant differences at $\alpha = 0.05$, for all experiments.

Results and discussion

Phenolic compounds

The results of TFC, TPC, TCC, chlorophyll *a* & *b* contents of crude extracts from *D. gnidioides* and *D. sericea* are given in Table 1. Typical chromatograms obtained at 330 nm for acid hydrolyzed extracts of *D. gnidioides* and *D. sericea* are shown in Figs. 1 and 2, respectively. Some components whose concentrations were found to be low in hydrolyzed samples, possibly due to degradation during

hydrolysis process, were analyzed in untreated (unhydrolyzed) samples and shown as insets in Figs. 1 and 2. Plant extracts from the two plants were labeled based on the extraction solvent; ESA: extract of *D. sericea* in acetone, ESM: extract of *D. sericea* in methanol, EGA: extract of *D. gnidioides* in acetone, EGM: extract of *D. gnidioides* in methanol.

The free phenolic compound contents of plant extracts are listed in Table 2. The sums of detected phenolic compound contents are given in the last row of this table. When these sums are compared with the TPC values in Table 1, it is seen that the sum of individually detected phenolic compounds comprise only about 10% of the TPCs. However, it is interesting to see that the order of TPCs given in Table 1 is the same as that of sums of the amounts of individually detected phenolic compounds given in the last row of Table 2 for the four extracts; EGM > EGA > ESA > ESM.

Radical scavenging activities

Phenolic compounds which are sorted as 'chain breaking antioxidants' are reported to quench free radicals by donating a hydrogen atom and/or an electron to free radicals (Gaikwad et al. 2010). The solubility, polarity, reducing potential and hydroxylation position and its degree are the major parameters effectuating the radical scavenging, i.e. antioxidant activity of phenolic compounds (Millic et al. 1998; Karadag et al. 2009; Dawidowicz and Olszowy 2012). DPPH[•], ABTS^{•+} and O₂^{•−} assays were applied to evaluate the radical scavenging capacities of extracts EGM, EGA, ESA and ESM together with positive controls α -tocopherol, butylated hydroxytoluene (BHT), quercetin and ferulic acid. The results were expressed as IC₅₀ value and given in tabular form in Table 3 and graphical form in Fig. 3. It is known that the lower the IC₅₀

Table 1 TFC, TPC, TCC, chlorophyll *a* content and chlorophyll *b* content of crude extracts from *D. sericea* and *D. gnidioides*

Sample	TFC (mg rutin/g DW of extract)	TPC (mg GAE/g DW of extract)	TCC ^{a,b} (mg/g DW of extract)	Chlorophyll <i>a</i> content ^b (mg/g DW of extract)	Chlorophyll <i>b</i> content ^b (mg/g DW of extract)
ESA	39.1 ± 4.9f	667.6 ± 88.8c	n.d.	1.6 ± 0.0a	4.2 ± 0.1a
ESM	121.3 ± 19.7c	602.4 ± 23.6c	6.1 ± 2.6a	1.4 ± 0.0b	2.0 ± 0.0c
EGA	144.2 ± 8.2b	954.7 ± 67.3b	n.d.	0.5 ± 0.0c	2.7 ± 0.0b
EGM	244.5 ± 19.5a	1219.3 ± 25.2a	11.9 ± 2.6a	0.3 ± 0.0d	0.6 ± 0.0d
Quercetin	93.2 ± 2.0d	328.5 ± 11.6d	n.a.	n.a.	n.a.
Ferulic acid	82.4 ± 1.4ed	322.1 ± 15.4d	n.a.	n.a.	n.a.
α -tocopherol	38.8 ± 1.6f	101.9 ± 8.6f	n.a.	n.a.	n.a.
BHT ^c	71.2 ± 1.6e	241.9 ± 10.7e	n.a.	n.a.	n.a.

^an.d.: not detected

^bn.a.: not applicable

^cBHT: Butylated hydroxytoluene

Fig. 1 The chromatograms of acid-hydrolyzed **a** aceton extract of *D. gnidioides* (EGA) and **b** methanol extract of *D. gnidioides* (EGM) at 330 nm

value the stronger is the antioxidant activity of the test material. Plant extracts have shown activities in DPPH[•] and ABTS^{•+} assays at levels shown in Fig. 3a. It is interesting to note that ABTS^{•+} scavenging assay displays a strikingly high IC₅₀ value for EGA. Radical scavenging activity ranking of *D. gnidioides* and *D. sericea* extracts together with positive controls is found as α -tocopherol $\geq$ quercetin $>$ ESM $>$ ferulic acid $>$ EGM $\geq$ ESA $\geq$ EGA according to DPPH[•] and as α -tocopherol $\geq$ quercetin $>$ ferulic acid $>$ EGM $\geq$ ESM $>$ ESA $>$ EGA according to ABTS^{•+} (Fig. 3a). BHT was not sufficiently soluble in the polar test media of DPPH[•] and ABTS^{•+} assays and thus, it was not included in the two assays. Methanolic extracts, ESM and EGM, appear to be more successful than the acetone extracts, ESA and EGA, in scavenging the synthetic radicals.

Figure 3b shows superoxide (O₂^{•-}) radical scavenging activities expressed as IC₅₀ values of the plant extracts, together with those of quercetin and ferulic acid. The

activities of BHT and α -tocopherol were given as % inhibition of O₂^{•-} radicals as a function of concentration (inset of Fig. 3b). O₂^{•-} scavenging tendency of BHT is almost constant up to 50 μ g/mL, and then it drops at 100 μ g/mL. On the other hand, O₂^{•-} scavenging tendency of α -tocopherol displays a regular decrease with concentration from 10 to 100 μ g/mL. The decrease in scavenging tendency with concentration is probably due to the fact that these strong antioxidants begin to act as prooxidants in this concentration range. As this concentration range seems to be inappropriate for calculation of IC₅₀ for both antioxidants, only their tendency in inhibiting O₂^{•-} radicals in this range were shown in inset of Fig. 3b. The ranking for the test materials in scavenging O₂^{•-} anion is quercetin $>$ ferulic acid $>$ EGM $>$ EGA $>$ ESA $>$ ESM. ESM with quite high IC₅₀ value shows almost no antioxidant activity according to this assay. From these results, it can be seen that there is no close correlation between total individual phenolics contents (Table 2), and radical scavenging

Fig. 2 The chromatograms of acid-hydrolyzed **a** acetone extract of *D. sericea* (ESA) and **b** methanol extract of *D. sericea* (ESM) at 330 nm

activities based on $\text{DPPH}^\cdot$ and $\text{ABTS}^{+\cdot}$ (Fig. 3a), for the materials tested. On the other hand, the superoxide scavenging capacity order (Fig. 3b) is in accord with that for total of individual phenolics calculated from the HPLC results (last row of Table 2).

Lipid oxidation preventing capacities in LDL and BBE

Figure 4a shows % inhibition of lipid oxidation values in LDL for the plant extracts and the compounds studied. It can be seen that the extracts investigated have lipid oxidation inhibiting capacities in LDL to a certain degree during 4 and 12 h of oxidation. As the period of oxidation is increased from 12 to 36 h, considerable increases in %inhibition values are observed, especially with EGM, α -tocopherol and BHT. Thus, it will be convenient to interpret LDL assay results mostly based on the data obtained for 36 h of oxidation. Accordingly, a ranking for the capacity in retarding lipid oxidation in LDL can be given as α -tocopherol > EGM $\geq$ BHT > ferulic

acid > quercetin > ESA > EGA > ESM. It is not surprising to see α -tocopherol to exhibit the highest activity in LDL as being a very powerful lipid oxidation chain breaking antioxidant. It is immediately followed by EGM and BHT. Thus, we can evaluate methanolic extract of *D. gnidioides* (EGM) as a far more efficient plant extract in stabilizing LDL, compared to the other extracts tested. This order is almost consistent with the order obtained based on the total concentration of individual phenolics found from the HPLC results (Table 2), also on TFCs and TPCs (Table 1) with the exception of ESA in the order. EGA, ESA and ESM inhibited the oxidation of LDL at proportions ranging from ~ 20 to $\sim 40\%$ under the same conditions (36 h of oxidation). The protection capacity in this lipid substrate exhibited by the plant extracts may primarily arise from their chlorogenic acid, ferulic acid, rosmarinic acid and quercetin contents, especially in case of EGM. The results obtained from the study of Natella et al. (1999) showed that the antioxidant efficiency of phenolic compounds in lipid systems such as LDL is strongly enhanced by the introduction of a second hydroxyl group ortho to each

Table 2 Contents of free phenolic compounds detected in acid-hydrolyzed crude extracts from *D. sericea* and *D. gnidioides*

Phenolic compound	Concentration in plant extract (mg/g DW of extract) ^{a,b}			
	ESA	ESM	EGA	EGM
Chlorogenic acid	0.2kJC	<i>trace</i>	10.7dB	36.9bA
Caffeic acid	0.9iC	0.9hC	1.4iB	12.4fA
Syringic acid	0.3kJC	n.d.	38.4aB	42.8aA
p-coumaric acid	4.5eD	9.5cB	5.7fC	32.5cA
Ferulic acid	2.9gC	6.4eB	n.d.	8.8hA
Rosmarinic acid	2.3hC	1.9gD	7.5eB	20.4dA
Quercetin	9.3bC	11.0bB	11.4cB	14.6eA
Luteolin	3.7fC	6.6eB	1.0iD	7.9iA
Kaempferol	6.1dB	n.d.	26.3bA	n.d.
Apigenin	0.4jD	22.0aA	4.2gC	10.2gB
Isorhamnetin	6.8cB	3.9fC	2.3hD	10.0gA
Rhamnetin	28.2aA	8.4dB	1.0iD	4.3jC
Total	65.6	34.3	109.9	200.8

n.d. not detected

^aResults given in boldface-italic form were from analysis of untreated plant extracts

^bMeans (n = 3) followed by the same lowercase letter within each column and the same uppercase letter within each row are not significantly different [Duncan's test, ($p < 0.05$)]

Table 3 Radical scavenging capacities of extracts and positive controls, expressed as IC₅₀

	IC ₅₀ DPPH ^a	IC ₅₀ ABTS ^a	IC ₅₀ O ₂ ^{-a}
ESM	61.6 ± 0.1d	154.1 ± 0.5c	839.9 ± 2.2a
ESA	151.1 ± 3.9b	262.5 ± 0.4b	283.8 ± 1.7b
EGM	150.8 ± 2.0b	147.2 ± 0.4c	215.6 ± 0.9c
EGA	236.3 ± 0.9a	607.9 ± 0.4a	227.3 ± 0.8c
Quercetin	39.3 ± 0.3e	64.4 ± 0.4e	71.1 ± 0.2d
Ferulic acid	116.6 ± 1.1c	91.3 ± 0.6d	123.0 ± 0.4d
α-tocopherol	35.5 ± 0.2e	33.9 ± 0.4f	N.A. ^b

^aMeans (n = 3) followed by the same letter with in each column are not significantly different [Duncan's test, ($p < 0.05$)]

^bα-tocopherol did not respond to superoxide radical scavenging activity test

other (as in the case of chlorogenic acid, quercetin or luteolin), or one of the two methoxy substitutions in position ortho to the –OH group (as in the case of ferulic acid and syringic acid). Our results seem to be consistent with this finding. Phenolic and flavonoid compound contents of EGM (Tables 1, 2) appear to be greater than those of the other extracts studied. Furthermore, during lipid oxidation over longer periods, some phenolic antioxidants were reported to degrade into some products which are considered to exhibit even higher antioxidative activity than their initial forms (Cassidy et al. 2000; Ren et al. 2003; Kamiyama et al. 2015). These antioxidants may have carry-through properties which may confer their protective effects in the lipid substrates they are incorporated with (Nanditha et al. 2009). Thus, one may propose that the

additives become more protective as the time of storage is increased.

Figure 4b shows the preventing capacity of lipid oxidation in bovine brain extract (BBE) liposomes exhibited by the plant extracts and the positive controls, at 5 µg/mL. It is seen that, the capacities of *D. sericea* extracts in stabilizing BBE are higher than the *D. gnidioides* extracts. If the methanolic and acetone extracts of *D. sericea* are compared, methanolic extracts are seen to be more successful in lipid oxidation prevention. α-tocopherol is found to exert the highest capacity in retarding lipid oxidation in BBE, among all materials tested. Similar results were found in LDL for both *D. gnidioides* extracts, as they exhibited activities very close to each other statistically. However, their activities are found to be considerably higher than those of quercetin, ferulic acid and BHT. Thus, lipid oxidation preventing capacity ranking in BBE, tested at a rather low concentration (5 µg/mL), can be given as α-tocopherol > ESM > ESA > EGM ≥ EGA > BHT ≥ quercetin > ferulic acid. It is recognized that this order is not consistent with that for the total of individual phenolics content (Table 2), TPC and TFC (Table 1). As mentioned above, *D. gnidioides* extracts were found to contain most of the phenolic compounds of interest at higher amounts compared to the extracts obtained from *D. sericea*.

Correlation of results obtained with different methods for the activities of plant extracts

D. gnidioides extracts seem to exhibit more success in scavenging O₂⁻ radicals compared to *D. sericea* extracts as

Fig. 3 **a** DPPH· and ABTS·+ radical scavenging assay results of extracts **b** superoxide anion radical (O₂^{·-}) scavenging activities of extracts

reflected in their lower IC₅₀ values (Fig. 3b). On the other hand, methanolic extracts of both plants (EGM and ESM) are more efficient in scavenging DPPH· and ABTS·+, than the acetone extracts (Fig. 3a). Furthermore, activity orders of different plant extracts and even control compounds are found to be somewhat different according to different test methods applied; phenolic content, scavenging assays (DPPH·, ABTS·+, O₂^{·-}), LDL and BBE. There are some variations in the trends for antioxidative protection of plant extracts and also for positive control antioxidants, based on the results of radical scavenging, LDL and phenolic compounds content assays (HPLC, TPC and TFC test results). One of the reasons for this moderate inconsistency is probably the composite and complex nature of the plant extracts (Dawidowicz and Olszowy 2012). It is possible that there are many components in a plant extract beside the class of components of interest, many of which are uncharacterized. The interactions between these components may alter the behaviors of some components towards a test substrate compared to the case when they are tested alone. Furthermore, different test substrates (LDL and BBE) have different structures, thus they interact with samples at different centers with different mechanisms. For

example LDL is known to contain four different types of oxidizable targets. These are free or esterified cholesterol, polyunsaturated fatty acids (PUFA) bound to surface phospholipids, triacylglycerols containing PUFA and apolipoprotein B (Yu et al. 2005). On the other hand, liposomes of BBE are globular structures organized between two aqueous compartments that are surrounded by one or more phospholipid bilayers (Laguerre et al. 2007). Therefore, plant extracts tested may have differing affinities towards LDL and BBE. There are obviously other nonphenolic components in a plant extract beside the components of interest and they may also have some varying effects on the interactions with the test substrate. Thus, the results obtained from the plant extracts reflect the overall activity of phenolic and nonphenolic components existing in their complex structure. Although the extracts are attempted to be enriched as much as possible, apparently they still maintain their complex nature.

There are quite a few methods for measuring the efficiency of antioxidants which show an alteration in the applied reactive oxidant, reaction mechanisms and reaction conditions in which the antioxidant assay is performed (Dawidowicz and Olszowy 2011). Regardless of the

Fig. 4 % inhibition of **a** LDL oxidation of extracts and **b** lipid peroxidation in BBE at 5 µg/mL extract concentration

analysis method, a deficiency of correlation between determined antioxidant activities on the same material using different experiments is very often observed in literature (Zhang et al. 2006; Millic et al. 1998).

Correlation of activities with phenolic contents of plant extracts

Figures 1 and 2 show the HPLC–DAD signals obtained at 330 nm for acid-hydrolyzed extracts of *D. gnidioides* extracts and *D. sericea* extracts, respectively. It is seen from both figures that there are many compounds not identified. Thus, these uncharacterized compounds, beside the identified ones, should also contribute to the antioxidant protection of the plant extracts evaluated by the assays. Therefore, it is not convenient to relate the activity of the extracts strictly to the phenolics detected. However, there are some interesting findings arising from the correlation of the activities of plant extracts with the antioxidative phenolic component contents detected. Methanolic extract of *D. gnidioides*, EGM, was found to contain most of the

phenolic compounds of interest at higher amounts than the other extracts studied. Cinnamic acid derivatives were reported to have more efficient antioxidative capacities than their benzoic acid counterparts, due to the participation of double bond of propenoic derivatives in stabilizing the radical by resonance (Natella et al. 1999). Strong antioxidative phenolic acids, chlorogenic, caffeic, ferulic and rosmarinic acids and a very effective antioxidant flavonoid quercetin (Rice-Evans et al. 1996; Wojdylo et al. 2007; Erkan et al. 2011a, b) are all determined to exist at the highest amounts in EGM, followed by EGA. *D. sericea* extracts are usually found to contain lesser amounts of the phenolic components of interest. EGM seems to contain also the antioxidants of moderate strength, apigenin, syringic acid and p-coumaric acid (Rice-Evans et al. 1996; Wojdylo et al. 2007; Rice-Evans et al. 1997) at considerable amounts. Rhamnetin, an *o*-methylated antioxidative flavonol, was found in *D. sericea* extracts, especially in ESA, at much higher amounts than *D. gnidioides* extracts. On the other hand, quercetin was detected in similar amounts in all extracts, with the highest amount in EGM.

Conclusion

It is found that methanol and acetone extracts of *D. gnidioides* and *D. sericea* contain significant amounts of phenolic compounds and display considerable radical scavenging activities. They have lipid oxidation inhibiting effect comparable to some of the positive control antioxidants; BHT, α -tocopherol, ferulic acid and quercetin. Methanolic crude extract of *D. gnidioides* was found to exhibit in vitro antioxidant activity and lipid oxidation inhibiting properties similar to one or more of the positive controls.

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