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Antioxidant activity and proline content of leaf extracts from *Dorystoechas hastata*

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ABSTRACT

Dorystoechas hastata (*D. hastata*) is a monotypic plant endemic to Antalya province of Turkey. *D. hastata* leaves are used to make a tea locally called "çalba tea". Diethyl ether (E), ethanol (A), and water (W) were used for the sequential preparation of extracts from dried *D. hastata* leaves. A hot water extract (S) was also prepared by directly boiling the powdered plant in water. The antioxidant activities of the extracts were tested by ferric thiocyanate (FTC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging methods. E extract exhibited the greatest antioxidant activity with FTC method, whereas S extract exhibited the lowest IC_{50} value (6.17 ± 0.53 μg/ml) for DPPH radical scavenging activity. Total phenolic contents of the extracts were estimated by Folin–Ciocalteu method and S extract was found to contain the highest amount (554.17 ± 20.83 mg GAE/g extract) of phenolics. Extract A contained highest flavonoid content and there was a inverse linear correlation ($R^2 = 0.926$) between IC_{50} values for DPPH radical scavenging activity and flavonoid contents of all extracts. Reducing power of extracts increased in a concentration-dependent manner. S extract was found to possess higher reducing power than equivalent amount of ascorbic acid at 20 and 25 μg/ml concentrations. Linear correlation between reducing power and concentration of E, A, and W extracts ($R^2 > 0.95$) was observed. A, W, and S extracts contained relatively high levels of proline. The results presented suggest that *D. hastata* may provide a natural source of antioxidants and proline.

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1. Introduction

Plants produce an extensive range of chemicals, including "secondary metabolites", which may exert beneficial health effects when consumed by man. Many of the plant secondary metabolites act as antioxidants in animals. On the other hand, these metabolites of plant origin may be used to prevent food deterioration *via* inhibition of lipid oxidation. Antioxidant action is a combination of several distinct chemical events such as metal chelation; quenching free radicals by hydrogen donation from phenolic groups; oxidation to a non-propagating radical; redox potential, enzyme inhibition (Williamson, Rhodes, & Parr, 1999). When oxidation caused by free radicals and reactive oxygen species emerges in food or in biological systems, antioxidants can prevent or delay this process *via* single or combination of aforementioned mechanisms. Hence, antioxidants may help the body to protect itself from various types of oxidative damage which are linked to diseases such as cancer, diabetes, cardiovascular disorders and aging (Halliwell, 1991). Consequently, search for food and drinks with high antioxidant content and enhancement of their antioxidant properties for nutritional purposes are currently of major interest. Food such as common vegetables and fruits which are consumed all over the world as well as wild plants that are consumed by local inhabitants are screened for their antioxidant capacity. However, scientific information on antioxidant properties of various wild plants, particularly those that are less widely used due to their endemic nature, is still rather scarce. The assessment of antioxidant capacity of such wild plants remains an interesting and useful task for finding new sources of natural antioxidants.

Dorystoechas hastata Boiss. and Heldr. Ex Bentham (Lamiaceae = Labiatae; *D. hastata*) is a wild monotypic plant endemic to Southwest Anatolia, Turkey (Davis, 1982). Fresh or dried *D. hastata* leaves is used to make an aromatic tea, locally called "çalba (chalba) tea", with a pungent taste which is used as a healing beverage against common cold or as a health drink by the local inhabitants. Many of the folk medicine in the Mediterranean subdivision of Turkey are investigated and extensive use of plants belonging to Labiatae family as a tea, as a tonic or as remedies for various disorders is reported (Yeşilada, Honda, Sezik, Tabata, & Ikeshiro, 1993). Previous research on *D. hastata* is limited to its phytochemical properties. Venturella, Venturella, Marino, Meriçli, and Çubukçu (1988) extracted ground leaves of *D. hastata* sequentially with petroleum ether and ethanol and reported that major components of ether extract were diterpenoids namely carnosol and rosmanol whereas ethanol fraction contained flavonoids such as luteolin, luteolin

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7-glucoside, 6-methoxyluteolin 7-glucoside and phenolic acids such as caffeic acid and chlorogenic acid. Oil extract obtained by water-distillation from *D. hastata* leaves was also analyzed and major components were identified to be monoterpenes such as α-terpineol, camphor and terpinene-4-ol (Meriçli & Meriçli, 1986). Another study reported the composition of essential oils obtained by water distillation from *D. hastata* leaves to be a mixture of monoterpenes and sesquiterpenes (Başer & Öztürk, 1992). Many of the aforementioned chemicals may behave as antioxidants individually but to our knowledge no data exists on *D. hastata's* antioxidant capacity as a food and/or traditional drug source. Therefore, the main objective of this study was to investigate the antioxidant activities of extracts of *D. hastata*. Additionally, determination of total phenolics contents and proline levels of extracts as indicative of antioxidant capacity was aimed.

2. Materials and methods

2.1. Chemicals

Ethyl alcohol, diethyl ether, HCl, $FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, glacial acetic acid, methanol and toluene were obtained from Merck (Darmstadt, Germany). Phosphate buffer solution (PBS), ammonium thiocyanate, Folin and Ciocalteu's phenol reagent (FCR), butylated hydoxytoluene (BHT) and proline were obtained from Sigma (Steinheim, Germany). Linoleic acid, Na_2CO_3, $NaH_2PO_4 \cdot 2H_2O$, Na_2HPO_4, $K_3Fe(CN)_6$, 2,2-diphenyl-1-picrylhydrazyl (DPPH), rutin trihydrate, 5-sulphosalicylic acid dihydrate and ninhydrin were obtained from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) and phosphoric acid was from Carlo Erba (Rodano, Italy); gallic acid, NaOH and $AlCl_3.6H_2O$ were from Riedel-de Haen (Seelze, Germany). Ascorbic acid was from Panreac (Barcelona, Spain). Deionized double distilled water was used for all assays.

2.2. Plant material

D. hastata was collected in the flowering stage from the vicinity of Antalya Province of Southwest Anatolia, Turkey in July and identified as *Dorystoechas hastata* Boiss. & Heldr. Ex Bentham (Lamiaceae; *D. hastata*) by botanist Dr. Özkan Eren, in the Department of Biology at Adnan Menderes University. A sample of the whole plant was dried according to the botanical procedure and stored in the herbarium with voucher herbarium specimen number: AYDN 124.

2.3. Plant extracts

Dried leaves of *D. hastata* were ground in a coffee grinder (Type 591, Moulinex, Paris, France). The powder was passed through a sieve in order to maintain particle size unity (300 μm). Three solvents (diethyl ether, ethanol and water) with distinct polarity (dielectric constants 4.3, 24.3, and 78.5, respectively) were employed sequentially for the preparation of plant extracts. Ten grams of powdered plant were mixed with 250 ml of diethyl ether and extracted in a Soxhlet apparatus for 8–10 h. The extraction unit was covered in order to prevent light deterioration during extraction. The resulting solution was decanted to the round-bottom flask of a rotary evaporator (RE, IKA RV 05 basic 1B, Staufen, Germany, D-79219) and the solvent was removed under vacuum at +30 °C. This diethyl ether extract was coded as (E). The plant residue obtained from diethyl ether extraction was shaked at 150 rpm with 3 × 100 ml aliquots of 95% ethanol for 2 h using a bench top shaker (Promax 2020, Heidolph, Heidolph instruments GmbH & Co KG, Kelheim, Germany). The aliquots were pooled, and filtered using Whatman filter paper (No. 1) and the filtrate was evaporated down to dryness under vacuum using rotary evaporator at +40 °C. This ethanol extract was coded with letter (A). The solid residue on filter paper was transferred to a beaker with 200 ml of distilled water and was heated for 2–3 min and filtered immediately through Whatman filter paper (No. 1). The filtrate obtained from water extraction was lyophilized for 96 h at −50 °C and 0.04 mbar (Freezone 6, Model 77520; Labconco Co. Kansas City, MO). This extract was coded with letter (W).

D. hastata is traditionally consumed as a tea prepared by boiling fresh or dried leaves for 5–10 min in hot water. Therefore, it was decided to include a direct hot water extract in this study. For this, 10 g of plant powder were mixed with 200 ml water and boiled for 10 min and hot filtered. The resulting solution was frozen and lyophilized for 96 h at −50 °C and 0.04 mbar. The residue was coded with letter (S).

All extracts were stored at +4 °C in airtight vials for immediate use. Samples that were not to be used within 24 h were transferred to a freezer and stored at −20 °C.

2.4. Total antioxidant activity determination

Total antioxidant activity was measured by ferric thiocyanate (FTC) method described by Saha et al. (2004). Briefly, 4 mg of plant extract were dissolved in appropriate solvent and mixed with 4.1 ml linoleic acid emulsion (2.5%, v/v). Eight milliliters of PBS (0.04 M; pH 7.4) and 3.9 ml of water were added to the mixture and incubated at +40 °C and assayed with 24 h intervals for 96 h. For the assay, 100 μl of the reaction mixture were mixed with 9.7 ml of ethyl alcohol, 100 μl of NH_4SCN (30%, v/w) and 100 μl of $FeCl_2$ (20 mM in 3.5% hydrochloric acid) solutions. The mixture was vortexed (REAX top tube shaker, Heidolph Instruments, Schwabach, Germany) and absorbance at 500 nm was read after 3 min using a Shimadzu UV–vis spectrophotometer (Model 1601; Shimadzu Scientific Instruments, Kyoto, Japan). Higher absorbance values indicated higher linoleic acid oxidation. Absorption of reaction mixture with no plant extract was measured as control. BHT was used for positive control as representative of high antioxidant capacity synthetic antioxidant. Total antioxidant activity was calculated as % inhibition according to the following equation: Percent inhibition = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance of the sample.

2.5. DPPH radical scavenging assay

Radical scavenging activity of plant extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined spectrophotometrically. Method first introduced by Blois (1958); developed by Brand-Williams, Cuvelier, and Berset (1995) and criticized by Molyneux (2004) was employed. The principle of the assay is based on the color change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant. Briefly, 1 ml of 0.1 mM DPPH in ethanol was mixed with 3 ml of extract solution with differing concentrations (5–250 μg/ml) and the mixture was vortexed. The samples were kept in the dark for 30 min at room temperature and then the decrease in absorbance at 517 nm was measured. Absorbance of DPPH solution in the absence of plant extract was measured as the control. Ascorbic acid, BHT and rutin were used as positive controls. DPPH radical scavenging activity was expressed using the formula: % DPPH radical scavenging activity = $[(A_0 - A_1)/A_0] \times 100$ where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

2.6. Determination of total phenolic content

Total phenolic contents (TPC) of the extracts were assayed according to Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, stock solutions were prepared by

dissolving 10 mg of plant extract in 10 ml ethyl alcohol or water. This stock solution (300 μl) was added to 45.9 ml of water in a 100 ml flask. Then, 1 ml of undiluted FCR was added to the mixture. After 3 min 3 ml of Na_2CO_3 (2% w/v) solution were added to each flask. The flasks were shaked at 150 rpm for 2 h in the dark at room temperature. Absorption at 760 nm was measured. Major phenolics detected in *D. hastata* were reported to be flavones and phenolic acids, previously (Table 1). Therefore gallic acid was employed as a representative of both type of phenolics and the results were expressed as mg gallic acid equivalents (GAE)/gram extract.

2.7. Determination of flavonoid content

The flavonoid content was measured using the method of Quettier-Deleu et al. (2000). Briefly 1 ml of extract solution was mixed with 1 ml of 2% (w/v) methanolic solution of $AlCl_3 \cdot 6H_2O$. After 10 min the absorbance was measured at 430 nm. Major flavonoids that are present in *D. hastata* were reported to be luteolin and derivatives (Table 1). Luteoline derivatives and rutin belong to the same flavonoid subclass, namely flavones (Heim, Tagliaferro, & Bobilya, 2002). Therefore, in accordance with the earlier work (Miliauskas, Venskutonis, & van Beek, 2004) rutin was thought to best represent flavonoids present in the extracts and was employed as the standard. The results were expressed as mg rutin equivalents (RtE)/gram extract.

2.8. Reducing power of the extracts

Reducing power of *D. hastata* extracts was measured according to the method of Oyaizu (1986). Stock solutions were prepared by mixing 10 mg of extract with 10 ml of extraction solvent. Stock solution (1 ml) was mixed with 2.5 ml of phosphate buffer (0.2 M; pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v). The mixture was incubated at +50 °C for 20 min and 2.5 ml of TCA (10% w/v) were added. After centrifugation for 10 min at 1000 g (Universal 32 R, Hettich, Tuttlingen, Germany), 2.5 ml of the supernatant was added to the tubes containing 2.5 ml distilled water and 0.5 ml $FeCl_3 \cdot 6H_2O$ (1% w/v). The absorbance of resulting solution was measured at 700 nm using water as blank. A control was also prepared replacing water with plant extract. Ascorbic acid which possesses relatively strong reducing power was used as a standard and reducing power was expressed as % ascorbic acid.

2.9. Proline analysis

Proline analysis was performed according to Bates (1973). Diethyl ether extract was exempted from this test since proline is practically insoluble in diethyl ether. Briefly, 50 mg of extract were homogenized in 10 ml sulphosalicylic acid (3% w/v) and filtrated through filter paper. Two milliliters of the filtrate were mixed with 2 ml of acid ninhydrin solution (1.25 g ninhydrin + 30 ml glacial acetic acid + 20 ml 6 M H_3PO_4) and 2 ml of glacial acetic acid and kept at +100 °C for 1 h. Then the reaction was stopped by transferring the mixture to an ice bath. Four milliliters of toluene were added to the mixture and vortexed for 15–20 s. The toluene phase was aspirated and absorbance at 520 nm was measured using pure toluene as reference. A calibration curve was prepared with pure proline. Results were expressed as μg proline/gram extract.

2.10. Statistical analysis

All results were means ± SD of three parallel measurements. Analysis of variance was tested by two-way ANOVA procedure. Significant differences between means were determined by Mann–Whitney test. P values < 0.05 were regarded as significant.

3. Results and discussion

The major components of leaf extracts prepared from *D. hastata* leaves were investigated previously as listed in Table 1. Essential oil of the plant was mainly composed of mono-, sesqui-, and diterpenes whereas ethanol fraction contains phenolics, mostly flavonoids. Water soluble components were phenolic acids such as caffeic and chlorogenic.

Major antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays (Huang, Ou, & Prior, 2005). In addition, metal chelation potential as a measure of antioxidant activity may be assayed (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). For this investigation, FTC and DPPH methods were chosen as representatives of HAT and ET categories, respectively. FTC method evaluates the ability of the antioxidant to inhibit lipid peroxidation which is, in turn, a measure of antioxidant activity. Fig. 1 displays total antioxidant activities of *D. hastata* extracts determined by FTC method and expressed as % inhibition at the end of 72 h. Diethyl ether extract (E) exhibited the highest (89.3%) inhibition of linoleic acid peroxidation. Ethanol extract (A), water extract (W) and hot water extract (S) caused 83.2%, 61.5% and 76.6% inhibition, respectively. Equivalent amount (4 mg) of standard synthetic antioxidant BHT caused 97.4% inhibition of linoleic acid peroxidation. In brief, total antioxidant activity of *D. hastata* extracts and positive control determined by FTC method decreased in the order of BHT > E > A > S > W.

The synthetic nitrogen-centered DPPH radical is not biologically relevant but DPPH assay is often used to evaluate the ability of antioxidant to scavenge free radicals which are known to be a major factor in biological damages caused by oxidative stress. This assay is known to give reliable information concerning the antioxidant ability of the tested compounds (Huang et al., 2005). Fig. 2 demonstrates DPPH scavenging activity, expressed in percents, caused by different concentrations of *D. hastata* extracts. In a scale of 5–250 μg of extracts in a total volume of 4 ml, the highest percent scavenging activity (95.6%) was observed with 50 μg/ml ether extract (E) in the presence of 0.025 mM DPPH (final

Table 1
Major components of leaf extracts of *D. hastata*

Extract	Major components	Chemical class	References
Leaf oil obtained by water-distillation	α-Terpineol	Monoterpene	Meriçli and Meriçli (1986)
	Camphor	Monoterpene (ketone)	
	Terpinene-4-ol	Monoterpene(alcohol)	
	α-Phellandrene	Monoterpene	
	d-Limonene	Monoterpene	
	Camphene	Monoterpene	
	α-Pinene	Monoterpene	
Aerial parts extracted with petrol ether	Carnosol	Diterpene	Venturella et al. (1988)
	Rosmanol	Diterpene	
Aerial parts extracted with ethanol	Luteolin	Flavonoid	Venturella et al. (1988)
	Luteolin 7-glucoside	Flavonoid	
	6-Methoxyluteolin 7-glucoside	Flavonoid	
	Caffeic acid	Phenolic acid	
	Chlorogenic acid	Phenolic acid	
Essential oil obtained by water distillation from leaves and stems	1,8-Cineole	Monoterpene	Başer and Öztürk (1992)
	α-Pinene	Monoterpene	
	Borneol	Monoterpene	
	Guaiol	Sesquiterpene	
	Camphor	Monoterpene (ketone)	
	Caryophyllene	Sesquiterpene	

concentration). However, DPPH scavenging activity is best presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Therefore, extract concentrations providing 50% inhibition (IC_{50}) were calculated using the data plotted in Fig. 2, and were presented in Table 2. Lower IC_{50} value reflects better DPPH radical scavenging activity (Molyneux, 2004). The hot water extract (S) of *D. hastata* provided the highest radical scavenging activity with the lowest IC_{50} value of 6.17 ± 0.53 μg/ml. DPPH scavenging activity of extracts increased in the order of S > Ascorbic acid > E > W > A > Rutin > BHT. Ascorbic acid (Brand-Williams et al., 1995) and rutin (Ordoñez, Gomez, Vattuone, & Isla, 2006) were used as standards.

Data obtained from FTC and DPPH assays revealed that all extracts isolated from *D. hastata* carry the antioxidative potential for chain-breaking inhibition of lipid peroxidation and for free radical scavenging. However, the extracts did not follow the same order of efficiency with FTC and DPPH methods. In FTC method, albeit all extracts exhibited high total antioxidant activities, none of them surpassed the efficiency of BHT. On the other hand, in DPPH method all extract were stronger radical scavenger than BHT. This is not unusual since an effective antioxidant in one assay system is not necessarily an effective antioxidant in another (Miliauskas et al., 2004). Moreover, ascorbic acid, rutin and BHT were tested for their DPPH radical scavencing activity recently, and the study reported that these antioxidants showed 92.8%, 90.4% and 58.8% inhibition respectively (Yang, Guo, & Yuan, 2007). In our study IC_{50} values for ascorbic acid, rutin and BHT followed similar sequence (i.e ascorbic acid < rutin < BHT). These findings indicate that BHT is not a very strong scavenger for DPPH radical. Data obtained in this study showed that S extracts demonstrated higher radical scavenging activity than both ascorbic acid and rutin. Major water soluble compounds of *D. hastata* were reported to be caffeic and chlorogenic acids (Venturella et al., 1988). Since S extracts were found to contain high amount of total phenolics but low amount of total flavonoids (Table 2) it is likely that caffeic acid and chlorogenic acid are responsible from the high radical scavenging activity of S extracts. High DPPH scavenging activity of caffeic acid (Gülçin, 2006) and chlorogenic acid (Xiang Z. & Z., 2007) were reported recently. Moreover, caffeic acid was shown to have DPPH scavenging activity very close to that of ascorbic acid (Villano, Fernandez-Pachon, Moya, Troncoso, & Garcia-Parrilla, 2007) and higher than that of rutin (Bandonien, Murkovic, Pfannhauser, Venskutonis, & Gruzdiene, 2002). Relatively high DPPH radical scavenging activities of chlorogenic acid compared to ascorbic acid (Xiang & Ning, 2007) and to rutin (Dall'Acqua and Innocenti, 2004) were also reported. The high antioxidant capacity of S extracts measured by both FTC and DPPH methods may be attributed to the high total phenolics content (more than 50%; Table 2) and cooperative effect of phenolics and/or synergistic effect (Bilia, Giomi, Innocenti, Gallori, & Vincieri, 2008) of phenolic acids.

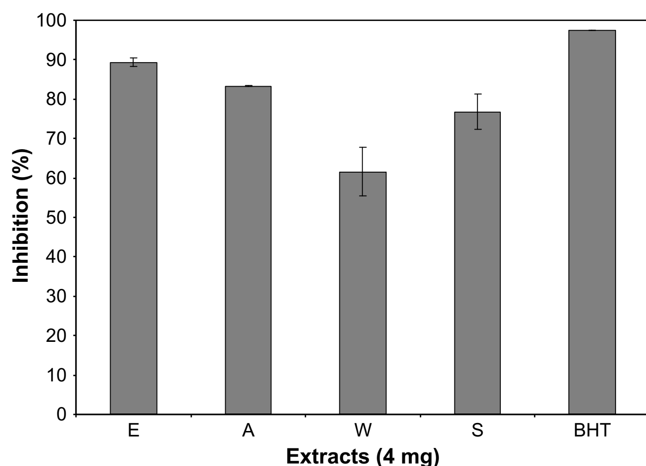

Fig. 1. Total antioxidant activities of *D. hastata* extracts determined by FTC method. E: diethyl ether extract; A: ethanol extract; W: water extract; S: hot water extract; BHT: butylated hydroxytoluene. Data present means ± SD ($n = 3$). Results demonstrate the inhibition at 72 h.

E, W, and A extracts showed higher radical scavenging activity than rutin and IC_{50} values followed the order of E < W < A < rutin (Table 2). Although the identification of individual compounds was not the subject of this work, data from previous studies displayed in Table 1 show that *D. hastata* leaves constitute an array of chemicals with various polarity. Consequently, it is thought that these compounds were partitioned among extracts during extraction procedure and they contribute to the antioxidant activities of the extracts. An additional explanation to the different order of antioxidant activity of the extracts may be brought by the nature of the methods applied. It was argued that, in FTC method, the hydrophobicity and thus solubility of the antioxidant in linoleic acid emulsion effects the test (Erkan, Ayranci, & Ayranci, 2008) whereas in DPPH method the results were independent of substrate polarity (Koleva, van Beek, Linssen, de Groot, & Evstatieva, 2002). Therefore, the high antioxidant activities of E, A and W extracts may be attributed to the overall actions of compounds listed in Table 1. High antioxidant activities of some of these single compounds such as carnosol and rosmanol (Chang et al., 2008), luteolin (Joubert, Winterton, Britz, & Ferreira, 2004), luteolin7-glucoside (Dall'Acqua and Innocenti, 2004), caffeic acid (Gülçin, 2006) and chlorogenic acid (Xiang & Ning, 2007) measured by either FTC and/or DPPH methods were reported.

Plant phenolics exhibit an array of solubility in solvents with different polarity and they constitute one of the major groups of compounds acting as primary antioxidants of free radical terminators (Rice-Evans, Miller, & Paganga, 1997). The assay used for the determination of total phenolics content employs Folin and Ciocalteu's phenol reagent which response depending on the chemical structure of phenolics (i.e. the higher the number of functional –OH group the higher the total phenolics content). The major phenolics of *D. hastata* were reported to be mono- and di- terpenes; some with phenolic –OH group (i.e. carnosol, rosmanol, terpinene-4-ol). Flavonoids and phenolic acids with varying number of functional –OH groups were also present (Table 1). Therefore gallic acid was used as the standard to represent all phenolics present in *D. hastata* extracts. Fig. 3 exhibits gallic acid equivalents of total phenolic contents of all extracts. As displayed, S extract carried highest total phenolics and the rest of the extracts followed the order of E > A > W. It is accepted, although not necessarily that, plants with higher total phenolic content provide higher antioxidant activity (Moure et al., 2001). Total phenolic contents of extracts calculated from regression equation of calibration curve ($A_{760\text{nm}} = 0.0008[\text{Gallic acid}]_{\text{mg}} + 0.0001$) and expressed in mg GAE/g extract were 554.17 ± 20.83, 302.78 ± 22.95, 277.78 ± 15.77, 191.67 ± 4.17 for S, E, A and W as listed in Table 2. There were no linear correlation between total antioxidant activity measured by FTC method and total phenolic content of the extracts ($R^2 < 0.95$). The correlation between DPPH radical scavenging activity (IC_{50}) and total phenolic content was not linear ($R^2 < 0.95$) either. Flavonoid content of the extracts was listed in Table 2. In agreement with the findings of Venturella et al. (1988), ethanol fraction possessed the highest flavonoid content. However, there was an inverse linear correlation ($R^2 = 0.926$) between flavonoid content and IC_{50} of DPPH scavenging activity indicating low contribution of flavonoids to the radical scavenging activity. It was difficult to interpret these results since high (Trouillas et al., 2003), moderate (Lu & Foo, 2001) or low (Miliauskas

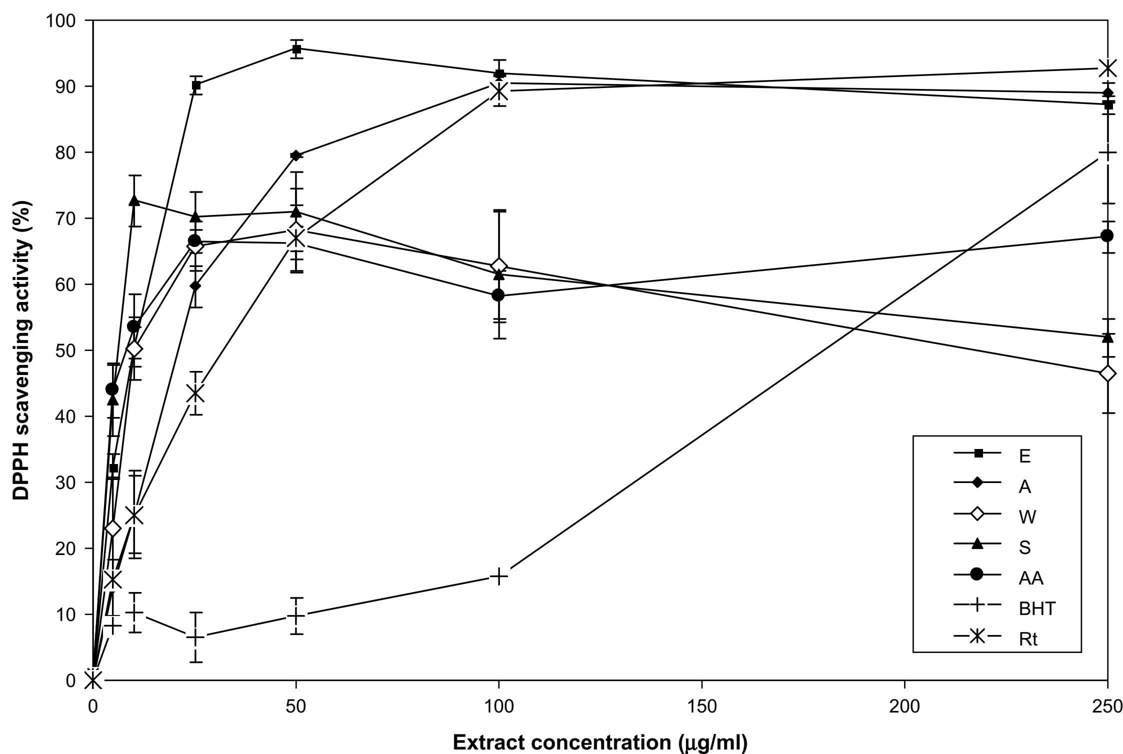

Fig. 2. Radical scavenging activities of *D. hastata* extracts and standards measured by DPPH method at different concentrations. E: diethyl ether extract; A: ethanol extract; W: water extract; S: hot water extract; AA: Ascorbic acid; BHT: Butylated hydroxytoluene; Rt: Rutin. $[DPPH]_{final}$ = 0.025 mM.

Table 2
IC_{50} values, total phenolics and proline content of *D. hastata* extracts

Sample	IC_{50} (µg/ml)[d]	Total phenolics[e] (mg GAE/g extract)	Total flavonoids (mg RtE/g extract)	Proline Content[e] (µg/g extract)
Diethyl ether extract (E)	9.99 ± 0.84[a,b]	302.78 ± 22.95	29.83 ± 0.78	–
Ethanol extract (A)	20.90 ± 0.37[a,b]	277.78 ± 15.77	69.34 ± 4.72	7412.79 ± 29.40
Water extract (W)	11.30 ± 2.37[a,b]	191.67 ± 4.17	47.38 ± 0.37	3116.19 ± 102.91
Hot water extract (S)	6.17 ± 0.53[a,b,c]	554.17 ± 20.83	24.21 ± 0.24	7767.38 ± 45.74
Ascorbic acid (AA)	9.00 ± 2.26	–	–	–
Butylated hydroxytoluene (BHT)	180.90 ± 9.28	–	–	–
Rutin (Rt)	31.70 ± 2.51	–	–	–

[a] Significantly lower ($p < 0.05$) than BHT.
[b] Significantly lower ($p < 0.05$) than Rutin.
[c] Significantly lower ($p < 0.05$) than Ascorbic acid.
[d] Final DPPH concentration was 0.025 mM.
[e] Correlation coefficient between total phenolics and proline content was R^2 = 0.536.

et al., 2004) DPPH radical scavenging activities of flavonoids namely luteolin and luteolin derivatives were reported. It is generally stated that only flavonoids of certain structure and particularly hydroxyl position in the molecule determine antioxidant properties (Miliauskas et al., 2004). Surely, the antioxidative potential of a plant does not depend solely on phenolic content (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003). As discussed in the previous paragraph, terpenes are a major group of chemicals present in plants and they have been shown to possess antioxidative properties particularly against lipid peroxidation (Grassmann, Hippeli, & Elstner, 2002) and DPPH radical scavenging activity (Elzaawely, Xuan, Koyama, & Tawata, 2007).

Reducing power is one mechanism for action of antioxidants (Jayaprakasha, Negi, Sikder, Rao, & Sakariah, 2000). Fig. 4 shows the absorbance values as a measure of the reducing power of *D. hastata* extracts compared to ascorbic acid in a concentration range of 2.5–25 µg/ml. The correlation between the extract concentration and the reducing power defined as % ascorbic acid is demonstrated in Fig. 4. The concentration-power correlation coefficients for S, E, A, and W extract were relatively high (R^2 = 0.8857; 0.9736; 0.9900; 0.9678, respectively). Moreover, S extract displayed higher reducing power than ascorbic acid at higher concentration (i.e. 20 and 25 µg/ml). This result is in agreement with the report of Kim et al. (2006) that heat treatment during extraction enhances the release of antioxidants.

Proline is one of the twenty amino acids found in proteins. It has a sweet taste and it finds usage as an ingredient in drinks etc. Stress-induced proline accumulation in plants is reported (Hare & Cress, 1997). Exogenous proline introduced to shoot cultures of oregano (a member of Lamiaceae family) significantly enhanced rosmarinic acid and endogenous proline contents which may be indicative of the stimulation of pentose phosphate pathway. The very early precursor of rosmarinic acid is erythrose-4-P which is one of the end-products of pentose phosphate pathway. Pentose phosphate pathway is regulated by synthesis of cytosolic proline which oxidizes $NADPH_2$. Thus, high proline synthesis induces

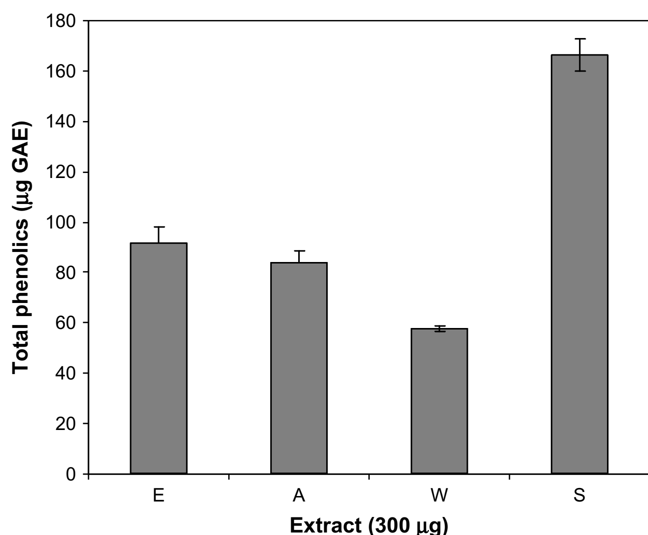

Fig. 3. Total phenolics content of *D. hastata* extracts expressed as gallic acid equivalent (GAE). E: diethyl ether extract; A: ethanol extract; W: water extract; S: hot water extract. Data present means ± SD ($n = 3$).

pentose phosphate pathway and synthesis of shikimate, rosmarinic acid and other phenolics (Shetty, 1997; Yang & Shetty, 1998). Furthermore, the role of proline in proline-linked pentose phosphate pathway in plants is studied and it is concluded that plants with high proline content exhibit high concentration of phenolic compounds (Shetty, 2004). Therefore, proline content of edible plants may be accepted as a measure of its antioxidant capacity. The proline content of *D. hastata* extracts is listed in Table 2. Although not linear, there was a positive correlation ($R^2 = 0.536$) between total phenolic and proline contents of A, S and W extracts. Research on proline content of plants or food is a newly arising area therefore there is not much data available to compare the results of this study. However, proline levels of *Leptadenia hastata* leaves (14.0 mg/g dry wt) (Cook et al., 2000); of 27 honey samples (highest 2169.4 ± 18.4 mg/kg) (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005) and of nectar of ornamental tobacco (2 mM) (Carter, Shafir, Yehonatan, Palmer, & Thornburg, 2006) were reported and all were lower than that of *D. hastata* extracts measured in this study.

D. hastata is a monotypic endemic plant which makes it difficult to compare its contents with another plant from the same genus. However, *D. hastata* belongs to Lamiaceae = Labiatae family which have many representatives as high antioxidant capacity plants that are being used as traditional medicine or health food. One of them is garden sage (*Salvia officinalis,* Lamiaceae) which is taken as a reference due to its well-known and widely documented antioxidant properties. Miliauskas et al., 2004 reported that different (methanol, ethyl acetate and acetone) extracts of *Salvia officinalis* have over 90% DPPH absorption inhibition values when the ratio of mass of plant extract to DPPH was 3:1. This may be interpreted that 3 mass units of *Salvia officinalis* extract scavenges 90% of one mass of DPPH. In the present study, 50 µg of W, S, A, and E extracts exhibited 68–96% DPPH radical scavenging activity in the presence of 0.025 mM (ca. 9.85 mg) of DPPH. Drinking tea made from garden sage that have similar taste qualities to that of *D. hastata* which is also used by local inhabitants

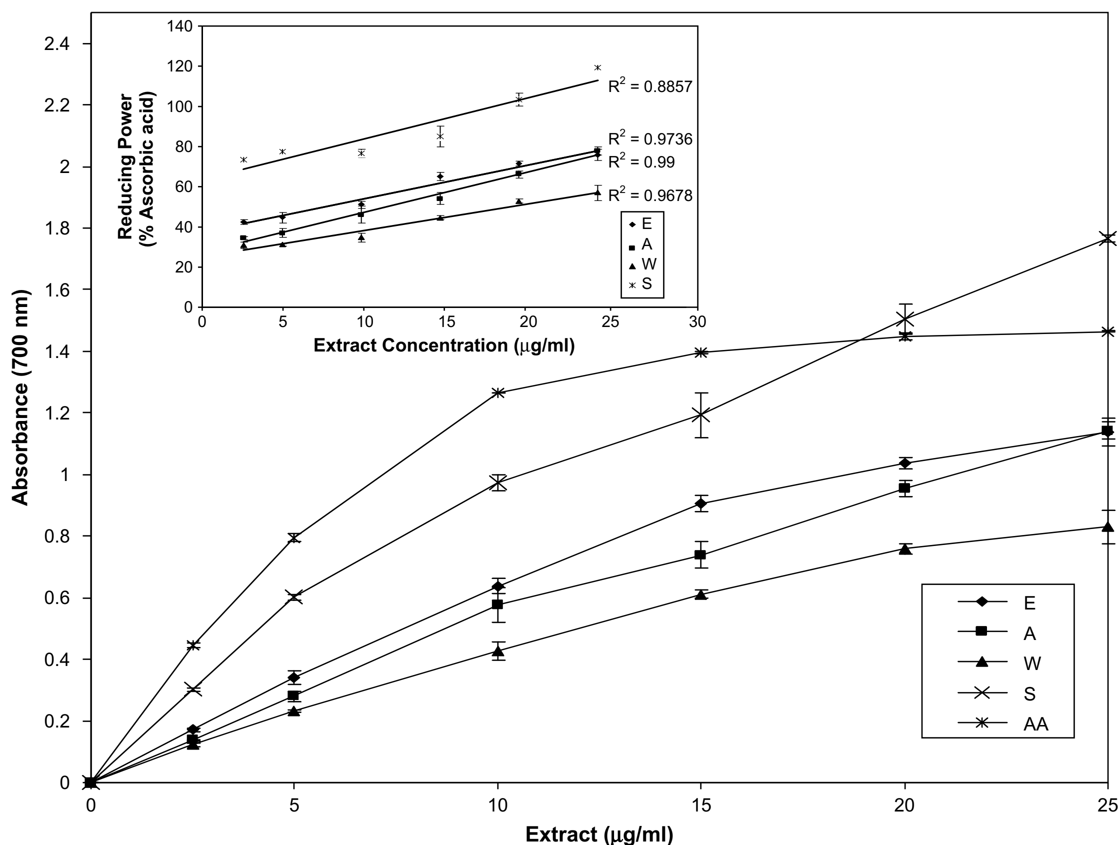

Fig. 4. Reducing power of different concentrations of *D. hastata* extracts compared to ascorbic acid. E: diethyl ether extract; A: ethanol extract; W: water extract; S: hot water extract; AA: ascorbic acid. Data present means ± SD ($n = 3$). Inset shows the correlation between concentration of *D. hastata* extracts and their reducing powers presented as % ascorbic acid.

where *D. hastata* is grown. Our results suggest that *D. hastata* tea may be a more powerful radical scavenger than garden sage. An additional comparison could be made with extract obtained from *Ginkgo biloba* L. leaves which is one of the oldest herbal medicines used as a health supplement. Aqueous ethanol extracts of *Ginkgo biloba* leaves contained 60.0 mg/100 ml total phenolics, 39.6 ± 2.2% inhibition of linoleic acid oxidation, and IC_{50} value of 30.6 ± 1.1 for DPPH radical scavenging activity (Lugasi, Horvahovich, & Dworschák, 1999). Compared with the results presented here it may be stated that *D. hastata* may have higher antioxidant capacity than *Ginkgo biloba*.

4. Conclusions

Antioxidant properties are among the first links between chemical reactions and biological activity. However, it is well known that after digestion antioxidant compounds lead to the formation of metabolites with different antioxidant activities. The results presented here strongly suggest that the antioxidant capacity of *D. hastata* should be considered as an important feature and examined with additional *in vivo* research. High proline content should also be evaluated. *D. hastata* may be a good source of components that would help to increase the overall antioxidant capacity of an organism and protect it against lipid peroxidation, induced by oxidative stress. Hence, *D. hastata* which is consumed as a tea by local inhabitants may provide a health benefit beyond basic nutrition. However, before recommending the plant as a good source of antioxidant, its toxicity and antioxidant action *in vivo* should be checked.

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