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Antioxidant activity of hot water extract from the fruit of the Doum palm, *Hyphaene thebaica*

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Abstract

A hot water extract from the fruit of *Hyphaene thebaica* was examined for its (i) hydrogen donating activity, (ii) Fe^{2+}-chelating activity, (iii) hydroxyl radical-scavenging activity, (iv) inhibition of substrate site-specific hydroxyl radical formation, (v) superoxide radical-scavenging activity, and (vi) reducing power. The total phenolic content of the fruit extract was also determined in order to quantitate antioxidant activity as gallic acid equivalent (GAE) per ml reaction. The total phenolic content of the Doum fruit is low, but the extract exhibited potent antioxidant activity in terms of GAE. The activities expressed as mmol pure compound equivalent per g GAE content of extract are: (i) 2.85 mmol ascorbic acid equivalent, (ii) 1.78 mmol ethylenediamine tetraacetic acid equivalent, (iii) 192 mmol gallic acid equivalent, (iv) 3.36 mmol gallic acid equivalent, (v) 1.78 mmol gallic acid equivalent and (vi) 3.93 mmol ascorbic acid equivalent. These values were of the same magnitude as antioxidant activity in black tea except for Fe^{2+}-chelating activity which was about 14 times more potent. The results show that the fruit of *Hyphaene thebaica*'s fruit is a source of potent antioxidants.

Keywords: *Hyphaene thebaica*; Doum; Antioxidant activity; Reactive oxygen species; DPPH; Hydroxyl radical; Superoxide; Reducing power

1. Introduction

Free radicals play a prominent role in human health. Their implication in the etiology of a large number of diseases is well documented (for some of the latest reviews with nutritional relevance, see Halliwell, 2001; Honda, Casadesus, Paterson, Perry, & Smith, 2004; Miquel & Romano-Bosca, 2004; Youdim, Spencer, Schroeter, & Rice-Evans, 2002). The most important free radicals in the body are the reactive oxygen and reactive nitrogen species (ROS and RNS), such as superoxide, hydroxyl and nitric oxide radicals. Non-radical ROS and RNS, such as singlet oxygen, hydrogen peroxide, hypochlorous acid and peroxynitrile are also important. They are generated in the body as a consequence of cellular and metabolic activities and also arise from exogenous sources (exposure to ionizing radiation, injury, oxidative drugs and pollutants). Some of these ROS and RNS have various important roles in vivo, such as in energy production, phagocytosis, regulation of cell growth and intercellular signalling and, in some biosynthetic pathways, excess production and "leakage" from their sites of generation are damaging to cells and tissues, due to their reactivity with other biologically functional compounds. The body maintains a balance in ROS and RNS by various scavenging mechanisms (Halliwell & Gutteridge, 1999). These include a number of antioxidant enzymes and antioxidant molecules.

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Oxidative stress occurs, when an imbalance occurs, leading to potentially serious health consequences. Many clinical studies have shown that the consumption of fruit and vegetables can exert positive effects upon human health and the aging process. Evidence points to these foodstuffs as being rich in antioxidant phytochemicals, such as vitamins and, in particular, the flavonoids, coumarins, hydroxycinnamates and lignin components which act to prevent or reduce oxidative stress by scavenging free radicals (Bravo, 1998; Sohal, Mockett, & Orr, 2002). In addition, many flavonoids and hydroxycinnamates exhibit various beneficial pharmacological properties, such as vasoprotective, anti-carcinogenic, anti-allergic, anti-viral, anti-ischemic, anti-inflammatory and anti-proliferative activity in cell studies. For example, the numerous biochemical studies point to the health-promoting properties of water extracts of the leafy shoots of the tea plant *Camellia sinensis* (Higdon & Frei, 2003; Trevisanato & Kim, 2000), which contain large quantities of polyphenolic materials (over 30% of the dry weight, Dreosti, 1996). These compounds are the source of tea's potent antioxidant activity and also some beneficial pharmacological properties, relevant to a number of diseases. Several epidemiological studies have found positive correlations between tea consumption and decreased risk of atherosclerosis, strokes, coronary heart disease, certain cancers and liver disorders (Ahmad & Mukhtar, 1999; Imai & Nakachi, 1995; Knekt, Jarvinen, Reunanen, & Maatela, 1996; Laurie, Miller, Grant, Kris, & Ng, 2005; Tavani & La Vecchua, 2004).

Hyphaene thebaica is a desert palm native to Egypt, sub-Saharan Africa and West India. It is known in Egypt as the Doum or gingerbread palm which grows to 6 or 9 m and usually has forked stems with fan-shaped leaves, 65–75 cm long. It is listed as one of the useful plants of the world (Fletcher, 1997). The trunk of the palm is used for construction, as well as for manufacture of various domestic utensils and the leaves are used to make mats, bind parcels and writing paper. The oblong, yellow-orange apple sized fruit has a red outer skin, a thick, spongy and rather sweet fibrous fruit pulp (mesocarp) that tastes like gingerbread and a large kernel.

The fruit pulp is used in cooking, in various ways, and the different varieties differ in their edibility. While the unripe kernel is edible, the ripe kernel is hard and used only as a vegetable ivory (Doren, 1997). The rind from the kernel is used to make molasses and ground kernels are used to dress wounds (Cunningham, 1990; Hadiwigeno & Harcharik, 1995). To the peoples of the desert where Doum palms are found, it is life-sustaining and is listed as a famine food. There is a report by USAID (1995) that communities in the Turkana region of Kenya were becoming dependent on wild Doum during the drought years from 1992 to 1994.

Previous studies on Doum had focussed on the fruit because, besides its nutritional value, the fruit drink brewed from hot water infusion of the dried fruit pulp is widely consumed as a health tonic and has been valued in the region, for its many anecdotal medicinal properties, for centuries (Martin, 1999). Research on the fruit pulp showed that it contains nutritional trace minerals, proteins and fatty acids, in particular the nutritionally essential linoleic acid (Cook et al., 2000). Identification of compounds, by thin-layer chromatography, showed that the fruit contains significant amounts of saponins, coumarins, hydroxycinnamates, essential oils and flavonoids (unpublished data), and the fruit also lowers blood pressure in animal models (unpublished data; Sharaf, Sorour, Gomaa, & Youssef, 1972).

There is a single report on the antioxidant activity of the Doum fruit, measuring only hydrogen-donating activity as trolox equivalents (Cook et al., 1998). This study extends the investigation by identifying and quantifying the nature of the antioxidant activity of the fruit so that its nutritional value as a health food supplement and as a source of potent antioxidants can be more fully evaluated.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents used were of analytical grade. Butylated hydroxyanisole, ethylenediamine tetraacetic acid (Na_2EDTA, disodium salt), gallic acid, potassium ferricyanide (III), hydrogen peroxide (3% solution in water), nitro-blue tetrazolium (NBT), reduced β-nicotiamide adenine dinucleotide (β-NADH, dipotassium salt), phenazine methosulfate (PMS), ascorbic acid, Folin-Ciocalteu's phenol reagent (2 N solution), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), iron(III) chloride hexahydrate, iron(II) chloride tetrahydrate and 2-thiobarbituric acid were purchased from Sigma–Aldrich Inc. (St Louis, MO, USA). Trichloroacetic acid (TCA) and 2-deoxy-D-ribose were from Fluka Chemical Co (Buchs, Switzerland). Sodium carbonate, potassium dihydrogen orthophosphate and potassium hydroxide were from Merck Pty Ltd. (Victoria, Australia). All water used was of Millipore® HPLC Deionized Water grade.

2.2. Equipment and apparatus

UV absorption measurements were performed with a Cary® UV–visible spectrophotometer. Solvent was removed using a RotaVapor RE111 rotary evaporator fitted with a BUCHI 461 temperature-controlled water bath. Solid phase extraction was performed using an

Alltech High Capacity C_{18} cartridge (1.5 cm × 2 cm; Supelco, USA).

2.3. Plant materials

The Doum fruit was imported from a local supplier in Cairo, Egypt, where it was sourced from the arid Aswan desert region of southern Egypt. It came as a dried crushed fruit obtained from the dried fleshy mesocarp of the fruit, excluding the seed. The crushed fruit was milled into a course powder with a mortar and pestle for hot water extraction. Lipton® black tea was purchased from a local supermarket.

2.4. Doum fruit extract

Ten grams of Doum fruit powder were put into a glass bottle, containing 600 ml boiling deionized water, with continuous stirring for 30 min. The fruit extract was filtered by Whatman® number 1 filter paper, using a Buchner funnel and water suction. The clear filtrate was evaporated to dryness under vacuum using the RotaVapor at 35–40 °C, and further dried by freeze–drying. The yield was 2.39 g (23.9% by weight). The freeze–dried fruit extract was redissolved in methanol:water (1:1, v/v) to 50 mg/ml concentration and aliquots were kept at −20 °C until used. Sample extract was warmed to redissolve precipitated materials prior to use.

2.5. Black tea extract

5.1 g of Lipton® black tea leaves were put into a glass bottle, containing 200 ml of boiling deionised water with continuous stirring for 30 min. The tea extract was filtered by Whatman® number 1 filter paper, using a Bushner funnel and water suction. The clear filtrate was evaporated to dryness under vacuum in the RotaVapor at 35–40 °C, and further dried by freeze–drying. The yield was 1.5 g (29.4% by weight). The freeze–dried tea extract was re-dissolved in methanol to 10 mg/ml concentration and was kept at room temperature until used.

2.6. Determination of total phenolic content

The total phenolic contents of the Doum fruit and tea extracts were determined by reaction with phosphomolybdotungstate acid, as described by Singleton and Orthofer (1999), after removing interfering reducing substances by solid phase extraction (SPE), using C_{18} solid phase. Gallic acid was used as standard for the absorbance standard curve. Two high capacity C_{18} cartridges (1.5 cm width × 2 cm height in solid phase) were prepared by washing with methanol, followed by extensive rinsing with 4 × 20 ml 0.1% acetic acid solution. 25 mg of Doum fruit extract and 5 mg tea extract were diluted to ~12 mg/ml and ~1 mg/ml, respectively, with 0.1% acetic acid solution. Samples were loaded onto separate cartridges, followed by extensive washing with 0.1% acetic acid solution to remove interfering substances. Bound phenolic and other materials were eluted by 70% methanol, followed by 100% methanol wash. The eluant and wash were collected and pooled from each separate cartridge and dried by evaporation under vacuum with the RotaVapor. The dried materials were dissolved in 1 ml (Doum) or 4 ml (tea extract) methanol and assayed for phenolic content.

The reaction mixture, in a 20.0 ml volumetric flask, contained 20 µl Doum fruit C_{18} extract, 20 µl black tea C_{18} extract or gallic acid (0–12.0 mg) and Folin-Ciocalteu Reagent® (100 µl). The reaction was left for 10 min at RT followed by the addition of 300 µl of Na_2CO_3(20% w/v), mixed well and incubated at 40 °C for 30 min. The contents of the volumetric flask were cooled in cold water for 1 min and the absorbance measured at $\lambda_{760\,nm}$ against a blank which contained all components except for extract. The amount of phenolic material was estimated from a standard plot using gallic acid as standard.

2.7. Evaluation of antioxidant activity

For spectroscopic quantitative determination of antioxidant activity, the reaction time for each activity assay was selected, such that maximum formation or disappearance of a particular chromogen occurred at a fixed concentration of test material. All the antioxidant activity assayed, except for reducing power, was measured as decrease in absorbance. Scavenging effects (%) or chelating effects (%) were calculated from the absorbance data using the equation

$$\text{Scavenging or Chelating Effect}(\%) = \left(1 - \frac{A_{\text{Sample}}^{\lambda_{\text{Activity Assay}}}}{A_{\text{Control}}^{\lambda_{\text{Activity Assay}}}}\right) \times 100,$$

where A is absorbance and $\lambda_{\text{Activity Assay}}$ the wavelength of absorbance measurement for the particular assay. Four replicate reactions were performed for each concentration point and the mean ± 1 S.D percentage scavenging or chelating effect values were plotted against concentration of test material in mg per ml reaction volume. The reactions in these assays were concentration-dependent and were allowed to reach saturation (maximum inhibition in assay) to accurately determine EC_{50} (amount of test material by mass required to produce 50% maximum inhibition per ml reaction volume) by fitting data into a non-linear regression algorithm (GraphPad Prism®, version 4).

The reducing power assay measures increase in absorbance in the presence of a large excess of oxidized substrate (Fe^{3+}). Four replicate absorbance values were

calculated as means ± 1 S.D and were also plotted against concentration of test material in mg per ml reaction volume. The reducing power, $RP_{0.5\,AU}$ (defined as amount of material in μg or mg per ml reaction volume that produces 0.5 absorbance unit at $\lambda_{700\,nm}$), was obtained from the plot of the absorbance data (mean ± 1 S.D.) fitted into a straight line by linear regression method.

2.8. Hydrogen-donating activity

Hydrogen-donating activity was measured by direct hydrogen donation to DPPH radical, as described by Yamaguchi, Matoba, and Junji (1998). The disappearance of the free radical can be tracked by absorbance measurement at $\lambda_{517\,nm}$. The reaction mixture (1.10 ml) contained 100 μl of test material [Doum extract (0–5 mg), black tea extract (0–0.05 mg) or ascorbic acid (0–0.02 mg)] and 1.00 ml of DPPH solution (0.1 mM in methanol). The control contained all the reaction reagents except test material. The reaction mixture was shaken well and allowed to react for 20 min at room temperature. The remaining DPPH free radical was determined by absorbance measurement at $\lambda_{517\,nm}$ against methanol blanks. The percentage scavenging effect was calculated from the decreased in absorbance against control (without added test material).

2.9. Fe^{2+}-chelating activity

Iron(II)-chelating activity was measured by inhibition of the formation of iron(II)-ferrozine complex after pre-incubation of the reaction mixture with test material according to the method of Decker and Welch (1990). The reaction mixture (1.50 ml) contained 500 μl test material (Doum extract [0–15 mg], tea extract [0–3 mg] or Na_2EDTA [0–0.07 μg]), 100 μl $FeCl_2$ (0.6 mM) and 900 μl methanol. The control contained all the reaction reagents except test material. The mixture was shaken well and left at RT for 10 min; 100 μl of ferrozine (5 mM in methanol) were then added, mixed and left for another 5 min to complex the residual Fe^{2+}. The absorbance of the Fe^{2+}–ferrozine complex was measured at $\lambda_{562\,nm}$ against methanol blanks. The percentage chelating effect was calculated from the decreased in absorbance against control (without added test material).

2.10. Hydroxyl radical-scavenging activity

Hydroxyl radical-scavenging activity was determined by measuring the formation of thiobarbituric acid-reactive substances (TBARS) using 2-deoxy-D-ribose as substrate, as described by Aruoma (1994). An EDTA–Fe^{2+} complex was employed to generate hydroxyl radical in situ by the decomposition of H_2O_2, using ascorbic acid as the reducing agent for EDTA–Fe^{3+}, to initiate the decomposition reaction (non-site specific method). Because the reaction is incompatible with methanol, the latter was removed from the Doum fruit and tea extracts by multiple rotary evaporations under vacuum and each test sample redissolved in water at a lower concentration for solubility before use.

The reaction mixture (1.00 ml) contained 250 μl of test material (Doum extract [0–12.5 mg], black tea extract [0–2.5 mg] or gallic acid [0–1.25 mg]), 250 μl of potassium phosphate buffer, pH7.4 (100 mM), 100 μl of 2-deoxy-D-ribose (28 mM), 200 μl of Fe^{3+}–EDTA (a mixture of 100 μM $FeCl_3$ + 104 μM Na_2EDTA in 1:1 ratio, v/v), 100 μl of H_2O_2 (1 mM) and 100 μl of ascorbic acid (1 mM). H_2O_2 and ascorbic acid were added last in that order to initiate the generation of hydroxyl radical. The control contained all the reaction reagents except test material. The reaction mixture was mixed by vortex and then incubated for 1 h at 37 °C. To terminate the reaction and form TBARS chromogen, 50 μl of butylated hydroxyanisole (2%, w/v in methanol), 1 ml of trichloroacetic acid (2.8%, w/v in water) and 1 ml of 2-thiobarbituric acid (1%, w/v in water) were added, tubes capped and the mixture heated in a boiling bath for 20 min. The reaction was stopped by a short incubation period in an ice water bath. 2.0 ml of 1-butanol were then added and the tubes mixed before centrifugation at 3000 rpm for 5 min to clarify the 1-butanol phase for absorbance measurement at $\lambda_{532\,nm}$ against 1-butanol blanks. The percentage scavenging effect was calculated from the decrease in absorbance against control (without added test material).

2.11. Inhibition of substrate site-specific hydroxyl radical formation

Inhibition of substrate site-specific hydroxyl radical formation was determined by direct absorbance measurement of the formation of TBARS using 2-deoxy-D-ribose as substrate and site of hydroxyl radical formation as described by Aruoma (1994). The procedure was the same as that described for measuring non-site specific hydroxyl radical-scavenging activity (above) except that EDTA was left out of the reaction mixture. The percentage scavenging effect was calculated from the decreased in absorbance against control (without added test material).

2.12. Superoxide radical-scavenging activity

Superoxide radical-scavenging activity was determined by absorbance measurement of the blackish blue formazan product of superoxide addition to nitro blue tetrazolium (NBT) substrate, according to the method of Nishikimi, Rao, and Yagi (1972). Superoxide was

generated chemically by the reduction of phenazine methosulphate (PMS), using β-NADH as the electron donor in the presence of dissolved molecular oxygen in the reaction solution. The reaction mixture (1.00 ml) contained 700 µl of test material [Doum extract (0–25 mg), tea extract (0–0.64 mg) or gallic acid (0–0.5 mg)] in methanol, 100 µl of β-NADH (1 mM in water), 100 µl of NBT (1 mM) in 1 M-phosphate buffer, pH 7.8, and 100 µl of PMS (120 µM in water) added in that order and the mixture allowed to react at RT for 10 min. The control contained all the reaction reagents except the test material. The reaction was terminated by adding 40 µl of concentrated HCl (10 M) and absorbance measured at $\lambda_{560\,nm}$ against blanks that contained all components except test material and PMS. The percentage scavenging effect was calculated from the decrease in absorbance against control (without added test material).

2.13. Reducing power

Reducing power was measured by direct electron donation in the reduction of $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$ as described by Yen and Chen (1995). The product was visualized by the addition of free Fe^{3+} ions after the reduction reaction, by forming the intense Prussian blue colour complex, $Fe_4^{3+}[Fe^{2+}(CN^-)_6]_3$, and quantitated by absorbance measurement at $\lambda_{700\,nm}$.

The reaction mixture (1.16 ml) contained 160 µl of test material [Doum extract (0–4 mg), tea extract (0–120 µg) or ascorbic acid (0–32 µg)], 500 µl of potassium ferricyanide $K_3Fe^{3+}(CN^-)_6$, (1% w/w in water) and 500 µl of 0.2 M phosphate buffer, pH 6.6. The control contained all the reaction reagents except the test material. The mixture was incubated at 50 °C for 20 min and was terminated by addition of 500 µl of 10% (w/v) TCA, followed by centrifugation at 3000 rpm for 10 min. Five hundred microliter of the supernatant upper layer was mixed with 500 µl water and 100 µl ferric chloride ($Fe^{3+}Cl_3$, 0.1% w/v in water) and the absorbance was measured at $\lambda_{700\,nm}$ against blanks that contained distilled water and phosphate buffer. Increased absorbance of the reaction mixture indicates increased reducing power of sample.

3. Results and discussion

3.1. Extract yields and total phenolic contents

The amount of materials that can be extracted from a plant depends on the vigour of the extraction procedure and the possibility exists of sample-to- sample variation in extracted materials . We employed a single extraction of Doum fruit and black tea as an example extraction for the study. For a hot water infusing procedure that is commonly employed in preparing drink from the dried Doum fruit (30 min infusing with boiling water but with continuous stirring), 23.9% (w/w) bulk materials was extracted from the fruit (Table 1). A similar infusion procedure extracted 29.4% (w/w) bulk materials from black tea leaves.

Since most antioxidant activities from plant sources are derived from phenolic-type compounds (Bravo, 1998), the total phenolic content of the fruit and tea extract was measured and was expressed as gallic acid equivalents (GAEs). Interfering hydrophilic reducing substances were removed from the fruit and tea extracts by solid phased extraction (SFE) using a C_{18} solid phase column before assay, due to the oxidizing nature of the assay reaction. This is especially necessary for the fruit extracts which contain a high proportion of reducing sugars (unpublished data). A gallic acid standard curve was obtained with a linear coefficient value of 0.9891 and was used for the calculation of GAE after SPE (Table 1). The total phenolic content in the fruit extract was low, yielding only 1.3% (w/w) of phenolic materials. This was not unexpected because the extract comes from the mesocarp of the fruit. A more vigorous hot water extraction procedure at boiling temperature for 30 min did not significantly increase the amount of phenolic materials recovered by SFE (data not shown). Not all of the C_{18}-recoverable materials are simple phenol or phenolic compounds. TLC showed the presence of saponins, coumarins, hydroxycinnamates, essential oils and flavonoids, in that order of abundance (unpublished data). Tea leaves, which are fibrous materials, contain a much higher proportion of phenolic materials with 36.8% (w/w) yield. This high level of phenolic content in tea is consistent with several other reports of hot water extract of black tea (Atoui, Mansouri, Boskou, & Kefalas, 2005; Dreosti, 1996).

We evaluated antioxidant activity of the hot water extract of the Doum fruit by comparing it with that of black tea using the same reaction protocol. This has the advantage of allowing direct comparison and the tea also serves to validate individual activity assay as positive crude extract material control. In addition, the nutritional comparison is more relevant with tea as it is a popular beverage. The activity for some pure compounds (ascorbic acid, EDTA and gallic acid) was also measured.

Table 1
The contents of hot water extracts of Doum fruit and black tea

Extract	Raw materials (% yield by weight)	Phenolic content (mg GAE[a]/mg extract; % w/w)
Doum fruit extract	23.9	1.3
Black tea extract	29.4	36.8

[a] GAE, Gallic acid equivalent.

Table 2
EC_{50}^{i-v} and $RP_{0.5\,AU}^{vi}$ values

Antioxidant activity	Doum fruit	Black tea	Ascorbic acid	Na_2EDTA	Gallic acid
			EC_{50}		
(i) Hydrogen-donating activity	308 (154)[a] 4 (2)[b]	7 (3.50)[a] 3 (1.50)[b]	2[c]	–	–
(ii) Fe^{2+}-chelating activity	419 (105)[a] 6 (1.50)[b]	230 (58)[a] 85[b]	–	4[c]	–
(iii) Hydroxyl radical-scavenging activity	314 (2.4)[a] 4 (0.03)[b]	8 (0.06)[a] 3 (0.02)[b]	–	–	131[c]
(iv) Inhibition of substrate site-specific hydroxyl radical formation	529 (132)[a] 7 (1.8)[b]	7 (1.75)[a] 3 (0.75)[b]	–	–	4[c]
(v) Superoxide radical-scavenging activity	7316 (252)[a] 96 (3.31)[b]	100 (3.45)[a] 37 (1.28)[b]	–	–	29[c]
			$RP_{0.5\,AU}$		
(vi) Reducing power	978 (109)[a] 13 (1.44)[b]	30 (3.33)[a] 11 (1.22)[b]	9[c]	–	–

The EC_{50} and $RP_{0.5\,AU}$ values for Doum fruit and black tea were calculated from data presented in Figs. 1–6 and Table 1. The values for the pure compounds (ascorbic acid, EDTA and gallic acid) were calculated from data obtained from similar experiments and scavenging effect (%), chelating effect (%) or reducing power (absorbance) *versus* test material plots (data not shown). Values shown are in μg extract[a], μgGAE[b] of extract or μg pure compound[c] per ml reaction volume. All values were rounded to the nearest μg. Values in () are relative to pure compound for the particular assay. –, Not determined.

Because of the multiple ways in which an antioxidant can protect biological molecules against oxidative damage, we measured different reactions to assess antioxidant activity, so as to determine the true antioxidant potential of the fruit (Aruoma, 2003). These reactions included electron and hydrogen donation, transition metal chelation and direct scavenging of reactive oxygen species. EC_{50} or $RP_{0.5\,AU}$ values were obtained (Table 2) for the fruit extract, tea extract and a pure compound assayed under the same reaction conditions for comparison. Since these values depend on the concentration of the chemical substrate (EC_{50}) and/or volume of reaction ($RP_{0.5\,AU}$) in an assay, quoting these values by themselves is not useful. By converting activity into mmol pure compound equivalents per gramme of test material, comparison with other literature values is possible (Table 3). The EC_{50} and $RP_{0.5\,AU}$ values are convenient points in the reactions to calculate the mmol equivalent values.

Table 3
Antioxidant activities of Doum fruit and black tea extracts

Antioxidant activity	Doum fruit	Black tea
(i) Hydrogen-donating activity	0.04 (0.03)[a]	1.63[a]
	2.85 (0.75)[b]	3.80[b]
(ii) Fe^{2+}-chelating activity	0.03 (0.60)[a]	0.05[a]
	1.78 (14)[b]	0.13[b]
(iii) Hydroxyl radical-scavenging activity	2.45 (0.03)[a]	96.2[a]
	192 (0.75)[b]	257[b]
(iv) Inhibition of substrate site-specific hydroxyl radical formation	0.04 (0.01)[a]	3.36[a]
	3.36 (0.43)[b]	7.83[b]
(v) Superoxide radical-scavenging activity	0.02 (0.01)[a]	1.71[a]
	1.78 (0.39)[b]	4.62[b]
(vi) Reducing power	0.05 (0.03)[a]	1.70[a]
	3.93 (0.85)[b]	4.65[b]

The EC_{50}^{i-v} and $RP_{0.5\,AU}^{vi}$ values for Doum fruit and black tea extracts from Table 2 were converted to (i and vi) mmol ascorbic acid equivalent, (ii) mmol EDTA equivalent and (iii–v) mmol gallic acid equivalent per g extract[a] or per gGAE[b] of extract. Values in ( ) are relative to tea, rounded to two significant figures.

3.2. Hydrogen donating activity

The role of an antioxidant is to remove free radicals. One mechanism through which this is achieved is by donating hydrogen to a free radical in its reduction to an unreactive species. Addition of hydrogen would remove the odd electron feature which is responsible for radical reactivity. The hydrogen-donating activity, measured using DPPH radical as hydrogen acceptor, showed that the fruit extract contained 0.04 mmol ascorbic acid equivalent/g extract of activity (Fig. 1(a) and Table 3i). This activity is only about 0.03 times that in black tea extract (1.63 mmol ascorbic acid equivalent/g extract; Fig. 1(b) and Table 3i).

3.3. Ferrous ion-chelating activity

Iron and copper are essential transition metal elements in the human body for the activity of a large range of enzymes and for some proteins involved in cellular respiration, O_2 transport and redox reactions. But, because they are transition metals, they contain one or more unpaired electrons that enable them to participate in one-electron transfer reactions. Hence, they are powerful catalysts of autoxidation reactions, such as participation in the conversion of H_2O_2 to $OH^{\cdot}$ in the Fenton reaction and in the decomposition of alkyl peroxides to the highly reactive alkoxyl and hydroxyl radicals (Lloyd,

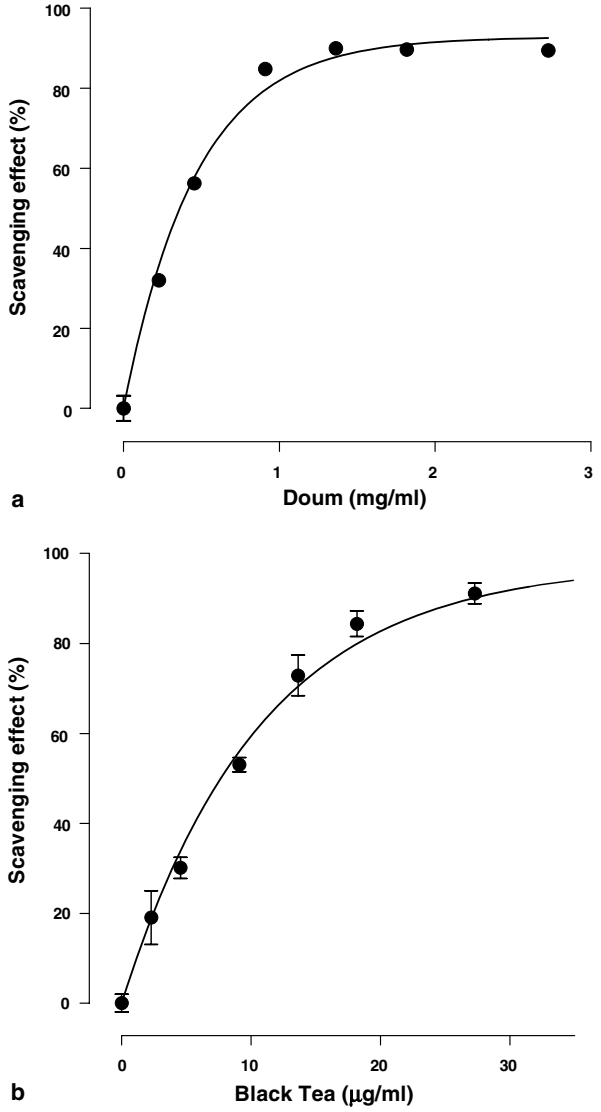

Fig. 1. Hydrogen-donating activities of: (a) Doum fruit extract and (b) black tea extract. The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effects (%) ± 1 S.D ($n = 4$) against extract concentration in weight extract per ml reaction volume. The data were fitted into a non-linear regression algorithm.

Hanna, & Mason, 1997). Due to this property, transition metal chelation to form low redox potential complexes is an important antioxidant property (Halliwell, Aeschbach, Loliger, & Aruoma, 1995) and measuring chelation of iron(II) is one method for assessing this property. Since the reaction is dependent on the affinity of an antioxidant towards iron(II) in relation to ferrozine, the assay is affected by both binding constant and concentration of antioxidant and thus only strong iron antioxidant chelator is detected.

With this assay, the fruit was found to contain iron(II)-chelating activity, approaching that of black tea, at 0.03 and 0.05 mmol EDTA equivalent/g extract (Fig. 2(a/b) and Table 3, ii), respectively.

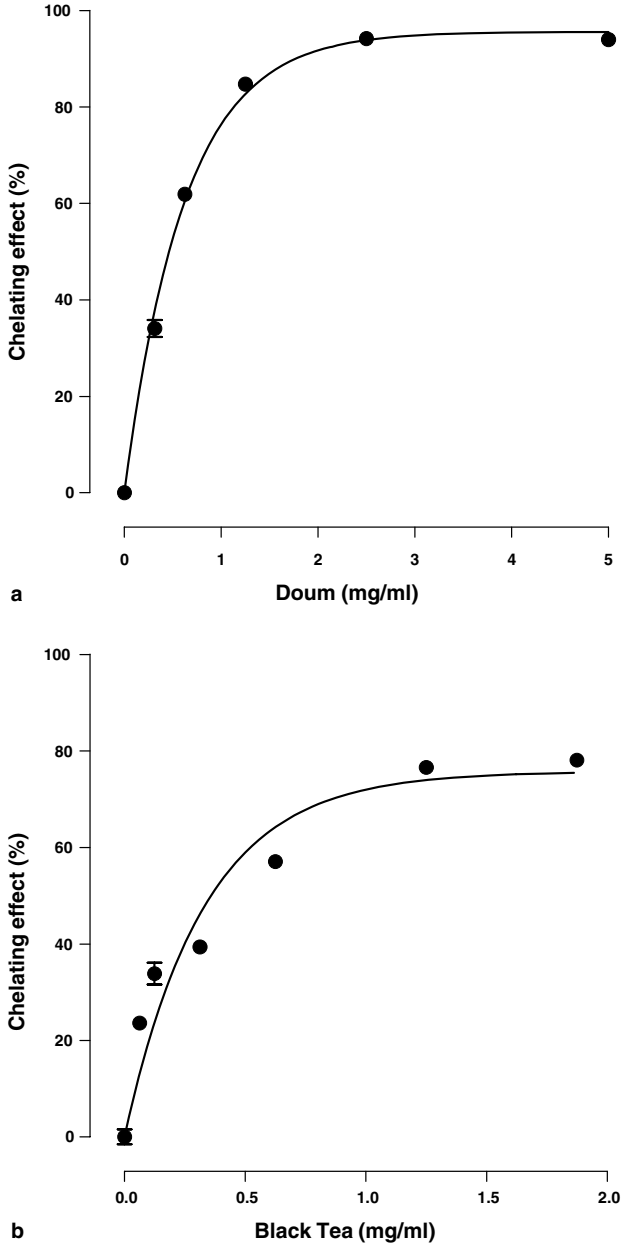

Fig. 2. Fe^{2+}-chelating activities of: (a) Doum fruit extract and (b) black tea extract. The absorbance values were converted to chelating effects (%) and data plotted as the means of replicate chelating effects (%) ± 1 S.D. ($n = 4$) against extract concentration in mg extract per ml reaction volume.

3.4. Hydroxyl radical-scavenging activity

An antioxidant ability to scavenge hydroxyl radical is an important antioxidant activity because of the very high reactivity of hydroxyl radical that enables the radical to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids and nucleotides. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is the Fenton reaction where a transition

metal is involved as a pro-oxidant in the catalyzed decomposition of superoxide and hydrogen peroxide (Stohs & Bagchi, 1995). These radicals are intermediary products of cellular respiration, phagocytic outburst and purine metabolism. Hydroxyl radical can be generated in situ by decomposition of hydrogen peroxide by high redox potential EDTA–Fe^{2+} complex (non-site specific) and, in the presence of 2-deoxy-D-ribose substrate, it forms TBARS which can be measured (Aruoma, 1994). Antioxidant activity is detected by decreased TBARS formation, which can come about by antioxidant donation of hydrogen or electron to the radical or by direct reaction with it.

With this assay the fruit was found to contain 2.45 mmol gallic acid equivalents/g extract in hydroxyl radical-scavenging activity (Fig. 3(a) and Table 3, iii). This activity in the fruit extract was relatively poor compared to that of black tea, which contained 96.2 mmol gallic acid equivalent/g extract in terms of bulk mass (Fig. 3(b) and Table 3, iii).

3.5. Inhibition of substrate site-specific hydroxyl radical formation

Due to the high reactivity of hydroxyl radical, it was recognized that measurement based on scavenging hydroxyl radical, such as the non-site specific method above, does not relate to antioxidant protection of an antioxidant molecule in vivo (Halliwell et al., 1995). This is because the radical is more likely to be scavenged by direct reaction with other surrounding molecules before it can attack its target molecule. Damage to a target molecule, such as lipid and cholesterol in low density lipoprotein and cell membranes, by hydroxyl radical is more likely to arise from generation of the radical in close proximity to or on the molecule surface itself and subsequent immediate reaction with it. The 2-deoxy-D-ribose assay, when performed in the absence of EDTA, forms hydroxyl radical on the surface of the ribose substrate in the presence of H_2O_2 and ascorbic acid (Aruoma, Grootveld, & Halliwell, 1987). This is due to decomposition of peroxide, catalyzed by the higher redox potential Fe^{2+}-ribose complex after reduction of bound Fe^{3+} by ascorbic acid to initiate the reaction. This "site-specific" method offers a more relevant measurement of an antioxidant activity towards hydroxyl radical (Aruoma, 1994), but in reality is a measure of an antioxidant ability to remove iron from complexation by ribose and from the iron–ribose complex itself to form an iron-antioxidant complex of lower redox potential that does not contribute to hydroxyl radical production in the system. Thus, the assay is more appropriately termed "inhibition of substrate site-specific hydroxyl radical formation".

Using this inhibition assay, the fruit was found to contain 0.04 mmol gallic acid equivalents/g extract of activity (Fig. 4(a) and Table 3, iv), which was rather poor. Black tea, which contained 3.36 mmol gallic acid equivalent/g extract (Fig. 4(b) and Table 3, iv), exhibited a much more potent activity for this reaction in terms of bulk mass.

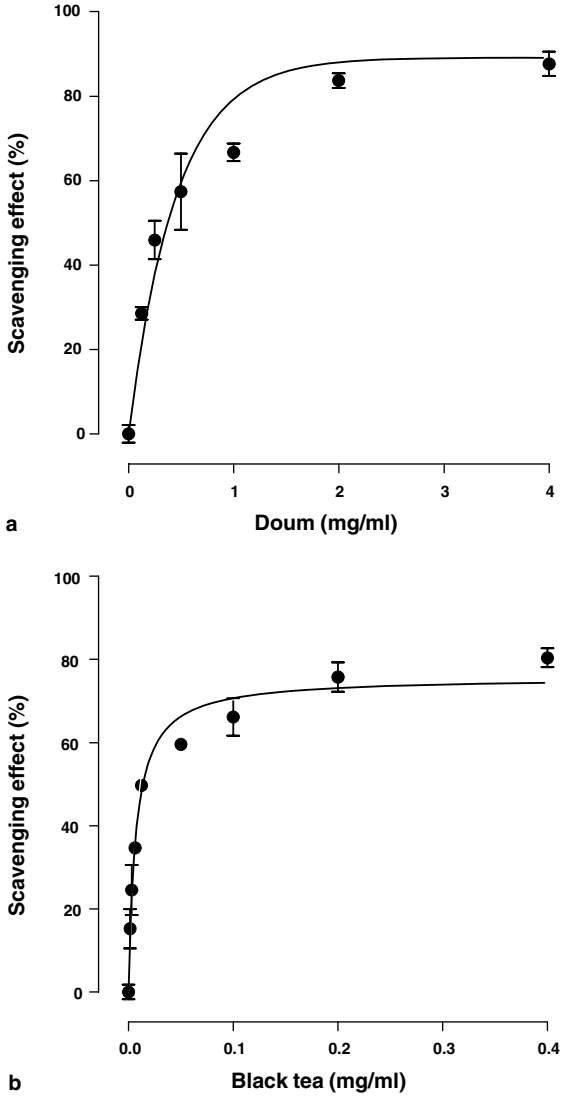

Fig. 3. Hydroxyl radical-scavenging activities of: (a) Doum fruit extract and (b) black tea extract. The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effect (%) values ± 1 S.D. ($n = 4$) against extract concentration in mg extract per ml reaction volume.

3.6. Superoxide radical-scavenging activity

Although the reactivity of superoxide is not high, it is a harmful radical in the body because it is a precursor of hydroxyl radical in the Fenton reaction mentioned above and in its participation in lipid peroxidation as an allylic hydrogen abstractor. Superoxide radical can be generated in vitro and assayed by the PMS/β-NADH/NTB system. Again, an antioxidant activity can come about by antioxidant donation of hydrogen or electron to superoxide or by direct reaction with it.

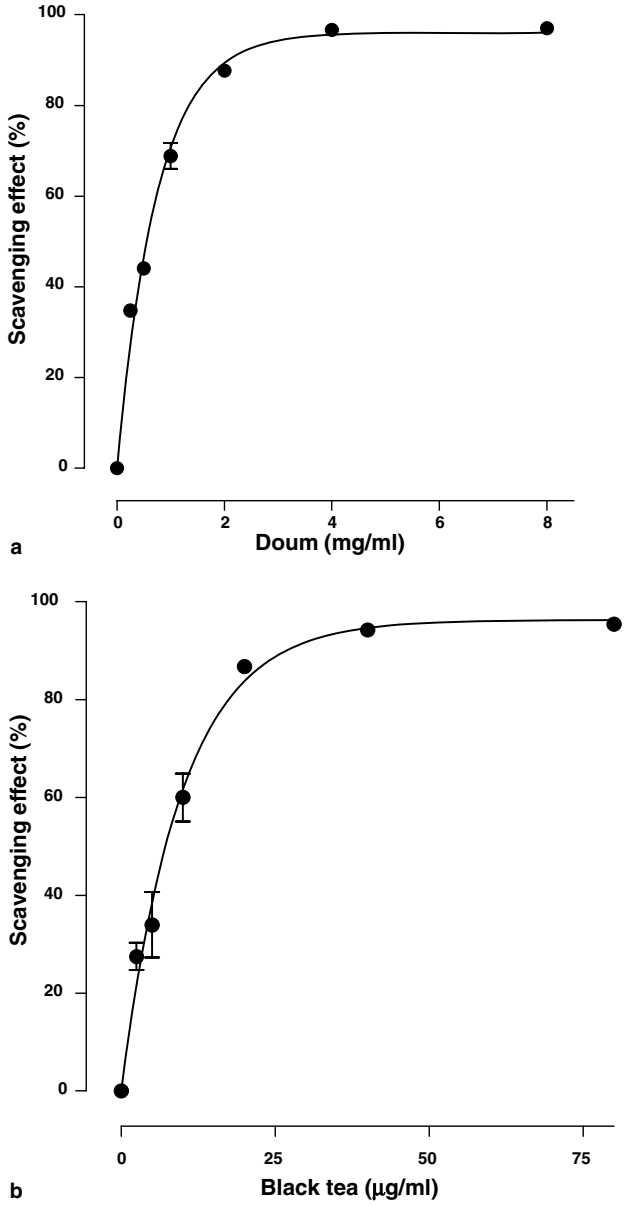

Fig. 4. Inhibition of substrate site-specific hydroxyl radical formation of: (a) Doum fruit extract and (b) black tea extract. The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effects (%) values ± 1 S.D. ($n = 4$) against extract concentration in mg extract per ml reaction volume.

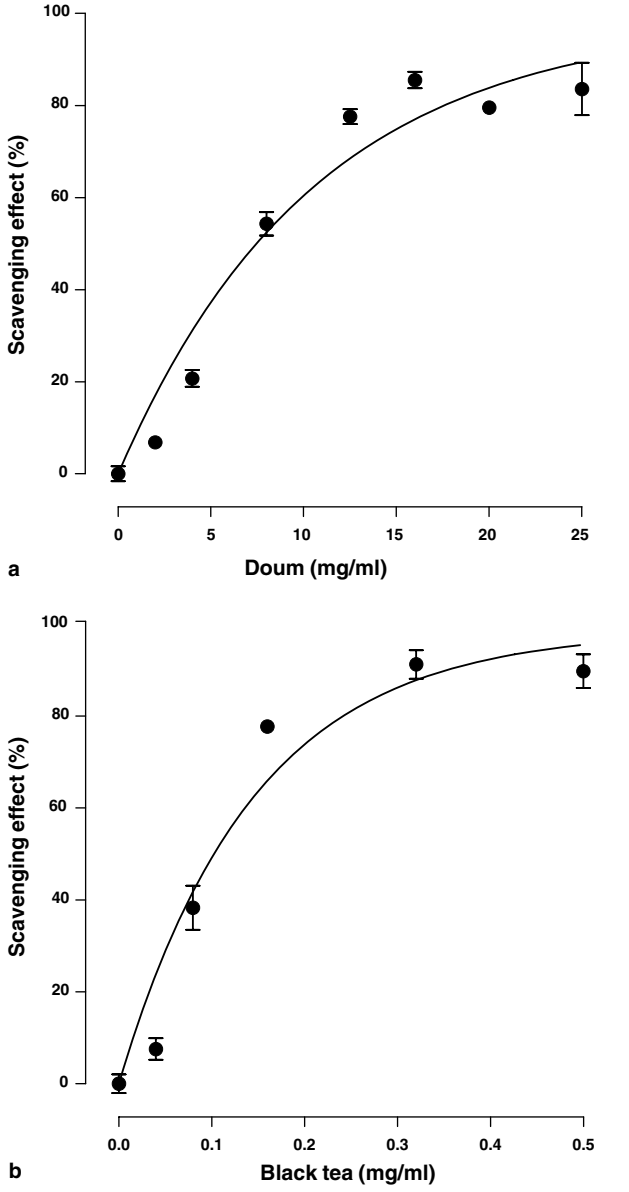

Fig. 5. Superoxide radical-scavenging activities of: (a) Doum fruit extract and (b) black tea extract. The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effects (%) ± 1 S.D. ($n = 4$) against extract concentration in mg extract per ml reaction volume.

Using this assay, the fruit was found to contain 0.02 mmol gallic acid equivalents/g extract (Fig. 5(a) and Table 3, v). This is a very poor activity toward superoxide, considering black tea contained 1.71 mmol gallic acid equivalents/g extract in terms of bulk mass (Fig. 5(b) and Table 3, v).

3.7. Reducing power

An electron-donating reducing agent contributes to antioxidant activity by its capacity to donate an electron to free radicals, which results in neutralization of the reactivity of the radical, and the reduced species subsequently acquires a proton from solution. Another reaction pathway in electron donation is the reduction of an oxidized antioxidant molecule to regenerate the "active" reduced antioxidant. The later antioxidant property is best illustrated by the synergistic action of ascorbic acid and vitamin E on cell membranes, where regeneration of α-tocopheroxyl radical to antioxidant α-tocopherol occurs in the presence of ascorbic acid (Burton, 1990).

Using an assay to directly measure transfer of electrons to Fe^{3+}, the fruit was found to contain 0.05 mmol ascorbic acid equivalents/g extract of activity (Fig. 6(a) and Table 3, vi). This activity was also much weaker

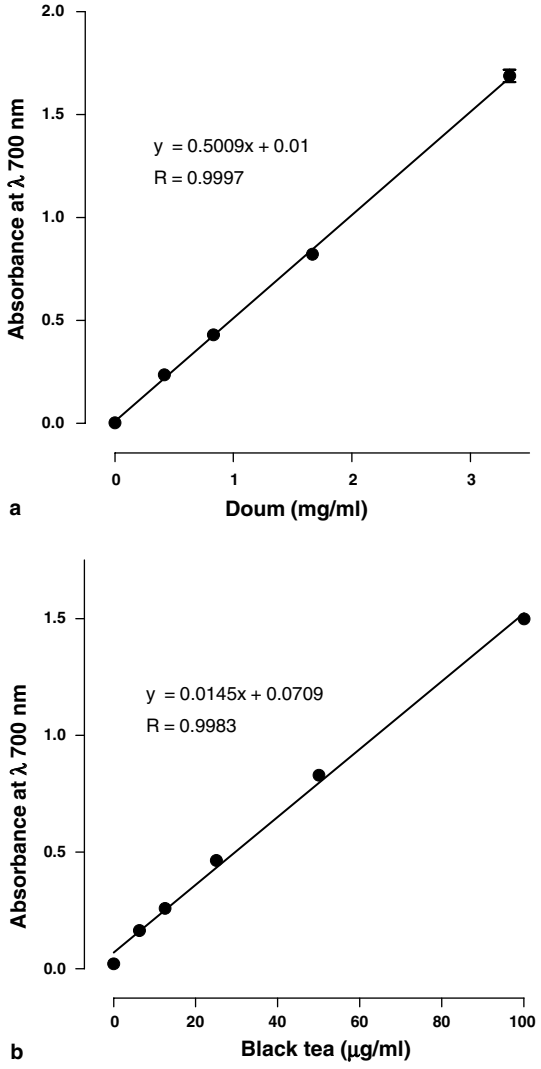

Fig. 6. Reducing power of: (a) Doum fruit extract and (b) black tea extract. The absorbance values were directly plotted as the means of replicate absorbance values ± 1 S.D. ($n = 4$) against extract concentration in mg extract per ml reaction volume.

than that of black tea extract, which contained 1.70 mmol ascorbic acid equivalents/g extract (Fig. 6(b) and Table 2, vi), in terms of bulk mass.

3.8. Enhanced antioxidant potency in terms of phenolic content

Since it was expected that antioxidant activity from fruit and tea could derive largely from phenolic and polyphenolic compounds (Bravo, 1998; Cai, Luo, Sun, & Corke, 2004), the true antioxidant potential is more accurately revealed by expressing antioxidant activity in terms of phenolic content, which can be measured as gallic acid equivalents (GAEs). Due to the low phenolic content in Doum fruit (1.35% GAE, w/w) the antioxidant activity of the fruit was much higher in terms of GAE. The results revealed that the hydrogen donating activity and reducing power were very potent in the fruit extract as these reactions were of the same magnitude as black tea extract, expressed in mmol ascorbic acid equivalent per gGAE of extract (Table 3, i and vi). In fact, these two activities in the fruit were as effective as ascorbic acid itself, which is an efficient organic electron and hydrogen donor. Their $RP_{0.5\,AU}$ (obtained under the same assay conditions) values were similar (Table 2, vi). Again, by comparing EC_{50} values obtained under the same assay conditions, results showed that the fruit and tea extracts contained better hydroxyl radical-scavenging than did gallic acid ($EC_{50} = 4$ and 3 μgGAE/ml, respectively, versus 131 μg/ml for gallic acid; Table 2, iii), which is a structural component of (±)catechin and (±)catechin gallate that is known to possess antioxidant activity (Aruoma, Murcia, Butler, & Halliwell, 1993). Comparing EC_{50} values as GAE also showed that the superoxide radical-scavenging activity and inhibition of substrate site-specific hydroxyl radical formation were of the same magnitude in the fruit and tea extract, and also with gallic acid (Table 2, iv and v).

It is not surprising that black tea has such excellent antioxidant activity. It has been shown, in numerous studies, that this property comes from flavonols (mainly epicatechin, epicatechin gallate, theaflavins and thearubigin) and hydroxycinnamates present in tea. These molecules are efficient transition metal chelators, hydrogen and electron donors and by direct reaction with radicals (Bravo, 1998; Pietta, 2000; Rice-Evans, Miller, & Paganga, 1996). We assume that the observed antioxidant activity in the Doum fruit is also derived from the coumarin, hydroxycinnamate and flavonoid present (unpublished data). Several coumarin, hydroxycinnamate and ferulic acid species have been shown to process potent antioxidant activity (Cai et al., 2004).

3.9. Potent iron(II)-chelating activity in the fruit

The most significant result is the Fe^{2+}-chelating activity in the fruit. Even in terms of bulk materials, the fruit iron-chelating activity approaches that of black tea (0.03 and 0.05 mmol EDTA equivalent/gGAE of extract, respectively; Table 3, ii). When the reaction was expressed as phenolic content the fruit was about 14 times more potent than black tea (1.78 and 0.13 mmol EDTA equivalent/gGAE of extract, respectively; Table 3, ii). That the iron-chelating components interact with C_{18} was confirmed by activity in the "phenolic" (C_{18} interacting) fraction after SFE (data not shown).

Iron has been implicated in the pathogenesis of certain central nervous system disorders, such as Alzhermer's disease, Huntington's disease and Parkinson's disease (Honda et al., 2004). In fact, several of cell-permeable synthetic iron chelators have been studied for their therapeutic potential in preventing these diseases (Richardson, 2004). We speculate that a new chemical

entity might be responsible for the very potent iron-chelating property in the fruit. The fact that the EC_{50} value per phenolic content of the fruit is comparable to that for EDTA, measured under the same assay conditions (Table 2, ii), further indicates the high affinity of the antioxidant(s) for iron. Further research into identifying the metal-chelating antioxidant(s) in the fruit might lead to the discovery of a new natural antioxidant entity with superior metal-chelating property. One successful example of such an approach is the discovery of the non-flavonoid quinone-like antioxidant, Kinobeon A, from safflower that has an antioxidant activity higher than that of quercetin (Kanehira et al., 2003).

4. Conclusion

The worldwide interest in food and food supplements, as sources of antioxidants, is growing, because it is becoming increasingly apparent that antioxidants are important in health and disease prevention. This study has examined various reactions that might contribute to antioxidant activity present in Doum fruit and which could play an important nutritional role in the diet of adults and children alike in some of the poorest regions of the world (Egypt, India and sub-Saharan Africa). The results showed that Doum fruit contains less antioxidant activity than black tea in terms of bulk, presumably due to a high proportion of sugar in the fruit extract. Nonetheless, most of the bulk material antioxidant activity of the fruit was comparable in magnitude to a number of food sources, such as potato peels (Nandita & Rajini, 2004), honey (Aljadi & Kamarudin, 2004) and quince fruits (Silva et al., 2004) which are rich in antioxidants. However, in terms of phenolic content, the antioxidant activity was comparable to black tea, except for the iron chelation which was about 14 times more potent.

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