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Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (*Murraya koenigii* L.) extracts

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Abstract

The *in vitro* antioxidant properties of different extracts (water, alcohol, alcohol:water, hexane or chloroform extract) of curry leaves (*Murraya koenigii* L.) were evaluated using various assays. The alcohol:water (1:1) extract of curry leaves (AWEC) showed the highest antioxidant and free radical scavenging activity. It inhibited membrane lipid peroxidation by 76%, at 50 μg/ml, scavenged 93% of superoxides at 200 μg/3 ml and scavenged approximately 90% of hydroxyl and 1,1-diphenyl-2-picrylhydrazayl radicals at 4–5-fold lower concentrations compared to the other tested extracts. In addition, the alcohol:water extract reduced cytochrome *c* and ferric ion levels, chelated ferrous ions and inhibited ferrous sulfate:ascorbate-induced fragmentation and sugar oxidation of DNA. These results establish the antioxidant potential of AWEC, which could be used as natural antioxidant source.
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Keywords: Curry leaves (*Murraya koenigii* L.); Antioxidant activity; Free radical scavenging activities; Various extracts

1. Introduction

Reactive oxygen species (ROS) and free transition metal ions cause extensive oxidative damage to cellular biomolecules such as DNA, proteins and lipids. Consequently, they contribute to the pathogenesis of oxidative stress-related diseases (Droge, 2002; Hippeli & Elstner, 1999). Although synthetic antioxidants seem to be promising, their toxicity and unwanted side effects rules out their extensive prescription. Hence, there is great interest in the use of naturally occurring antioxidants for treatment or prophylaxis of various oxidative stress-related diseases (Maxwell, 1995). The administration of an antioxidant source comprising of multiple components could offer protection against cancer (Black et al., 1995) and combat oxidative stress-induced physiological malfunctions.

With the above scenario, different extracts of curry leaves (*Murraya koenigii* L.) were subjected to various assays in order to assess their antioxidant properties.

M. koenigii L. Spreng, a member of the family Rutaceae is used as a spice in India for its characteristic flavour and aroma. Curry leaves can be used as antioxidants in high fat diets (Khan, Abraham, & Leelamma, 1997) as they contain the antioxidants tocopherol, β-carotene and lutein (Palaniswamy, 2001).

Earlier studies in our laboratory reported that dietary components, such as turmeric and vegetable extracts, effectively modulate ROS-induced lipid peroxidation and DNA damage (Shalini & Srinivas, 1987; Sujatha & Srinivas, 1995). However, there is no available information relating to the antioxidant properties of curry leaves under *in vitro* conditions.

In this study, the curry leaves were extracted in various solvents or solvent mixtures, of different polarities, to

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optimise the best conditions that lead to an efficient natural nutraceutical or antioxidant drug. The antioxidant properties of the extracts of curry leaves were assessed with a special emphasis on the ethyl alcohol:water (1:1) extract.

2. Materials and methods

2.1. Plant material

Curry leaves (*M. koenigii* L.) were obtained from the garden maintained by Adichunchanagiri Biotechnology and Cancer Research Institute (ABCRI), B.G. Nagara 571448, Karnataka state, India in the months of July to September 2003. The identity of the plant was confirmed by G.R. Shivamurthy, Taxonomist, University of Mysore, India. The herbarium of the plant was deposited in the ABCRI against voucher no. ABCRI 7/2003. One hundred grams of curry leaves were dried at ambient temperature for 15–20 days. After complete drying, the leaves were grounded into a fine powder using a domestic electric grinder (MASTER (CM/L-736889), Soni appliances, Mumbai, India).

2.2. Preparation of extracts of curry leaves

One gram of the curry leaves powder was added to 50 ml of double distilled water, ethyl alcohol:water (1:1), ethyl alcohol, hexane and chloroform, respectively ethyl alcohol, hexane and chloroform were analytical grade (AR) and procured from Merck (Dermastadt, Germany) and double distilled water was used.

The solution was homogenised and the resultant suspension was centrifuged at 10,854*g* in Kubota 6800 (Kubota Co., Osaka, Japan) for 10 min at 4 °C. The supernatants were filtered using Whatman No. I filter paper, followed by 0.045 μm microbial filter (Sartorius minisart, Hannover, Germany).

The water extract of curry leaves (WEC) was lyophilised while the ethyl alcohol water (1:1) extract of curry leaves (AWEC) was rotary evaporated and freeze-dried. Similarly, the ethyl alcohol extract of curry leaves (AEC), the hexane extract of curry leaves (HEC) and the chloroform extract of curry leaves (CEC) were concentrated separately under vacuum using the rotary evaporator. Ten milligrams of each dried extract was dissolved in 0.1 ml of the respective extracting solvent or solvent mixture and made up to 10 ml with water. The solution was filtered using a 0.45 μm microbial filter (Sartorius minisart, Hannover, Germany) and stored at −20 °C for further studies. The efficacy of the extracts reported here was quantified based on the dry weight of the whole extract per volume of assay solution.

2.3. Determination of total phenolics

Total content of phenolics was determined according to the method of Folin–Ciocalteu (Kujala, Loponen, Klika, & Pihlaja, 2000) with minor modifications, using gallic acid as standard. Gallic acid was procured from Sigma (St. Louis, USA) and methanol (AR) was procured from Merck (Dermastadt, Germany). The extract of curry leaves (1 mg) was dissolved in 1 ml of a methanol:water mixture (50:50 v/v). The solution containing various concentrations of the extract, ranging from 0 to 100 μg, was added to a series of tubes and the volume was made up to 100 μl with the methanol:water mixture (50:50 v/v). Five hundred microlitres of 50% Folin–Ciocalteu reagent was added to each tube and mixed. The mixture was then allowed to stand for 10 min followed by the addition of 1.0 ml of 20% sodium bicarbonate. After 10 min incubation at ambient temperature, the mixture was centrifuged at 10,854*g* in Kubota 6800 (Kubota Co., Osaka, Japan) for 5 min and the absorbance of the supernatant was measured at 700 nm. The total phenolics content was expressed as gallic acid equivalents (GAE) in mg per gram of dry sample.

2.4. Inhibition of lipid peroxidation

Lipid peroxidation was induced in an erythrocyte ghost by ferrous sulfate–ascorbic acid solution (Fenton, 1984; Shalini et al., 1994) according to the procedure of Shimazaki, Ueta, Mowri, and Inoue (1984). An assessment of oxidation was achieved by measurement of thiobarbituric acid reactive substances (TBARs), according to the procedure of Dahle, Hill, and Holman (1962). The erythrocyte ghost was isolated according to the procedure of Dodge, Mitchell, and Hanahan (1963). Ferrous sulfate, ascorbic acid, thiobarbituric acid (TBA), butylated hydroxy anisole (BHA) and α-tocopherol were procured from Sigma (St. Louis, USA). Phenol and trichloroacetic-acid (TCA) were procured from Merck (Dermastadt, Germany).

A hundred microlitres of ghost suspension (300 μg membrane protein equivalent) was subjected to peroxidation by ferrous sulfate and ascorbic acid (10:100 μmole) in a final volume of 1 ml of Tris buffered saline (10 mM Tris, pH 7.4,150 mM NaCl), with or without extracts of curry leaves (25–200 μg/ml). BHA and α-tocopherol, at concentrations ranging from 25 to 200 μg/ml, were used as positive controls while the negative control was without any antioxidant or extract. The contents were incubated for 1 h at 37 °C. The reaction was terminated by the addition of 10 μl of 5% phenol and 1 ml of 1% TCA. To each system, 1 ml of 1% TBA was added, the contents were kept in a boiling water bath for 15 min, cooled and centrifuged at 5319*g* in a Kubota 6800 (Kubota Co., Osaka, Japan) for 10 min. The absorbance of the supernatants was measured colorimetrically at 535 nm in a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). Appropriate blanks were included for each measurement. The percent inhibition of lipid peroxidation was determined accordingly by comparing the absorbance of the test samples with the negative control.

2.5. Superoxide scavenging activity

Superoxide radical (O_2^-) scavenging activity was measured according to the method of Lee, Kim, Kim, and Jang (2002) with minor modifications. Hypoxanthine, xanthine oxidase, superoxide dismutase, disodium ethylenediamine tetraacetic acid (EDTA) and nitroblue tetrazolium were procured from Sigma (St. Louis, USA). Different extracts of curry leaves, at concentrations ranging from 50 to 300 μg, were added to the reaction mixture containing 100 μl of 30 mM EDTA, 10 μl of 30 mM hypoxanthine in 50 mM NaOH and 200 μl of 1.42 mM nitroblue tetrazolium and the volume was made up to 2.9 ml with 20 mM phosphate buffer (pH 7.4). After the solution was pre-incubated at ambient temperature for 3 min, 100 μl of xanthine oxidase solution (0.5 U/ml) was added to the mixture. The solution was incubated at ambient temperature for 20 min and the absorbance of the solutions was measured colorimetrically at 560 nm. Superoxide dismutase (SOD) (50–300 μg/3 ml) served as a positive control while the negative control was without any test compound or extract. The percent superoxide scavenging activity was determined accordingly by comparing the absorbance of test samples with the negative control.

2.6. Hydroxyl radical scavenging activity

The deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium (Halliwell, Gutteridge, & Aruoma, 1987). 2-Deoxy-D-ribose, $FeCl_2$, $FeCl_3$, EDTA, H_2O_2, ascorbic acid, TBA, BHA were procured from Sigma (St. Louis, USA). The reaction mixture containing $FeCl_3$ (100 μM), EDTA (104 μM), H_2O_2 (1 mM), 2-deoxy-D-ribose (2.8 mM) were mixed with or without various concentrations of different extracts (10–100 μg) in 1 ml final reaction volume, made with 20 mM potassium phosphate buffer, at pH 7.4 and incubated for 1 h at 37 °C. The mixture was heated to 95 °C in a water bath for 15 min followed by the addition of 1 ml each of TCA (2.8%) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA). Finally the reaction mixture was cooled on ice and centrifuged at 5319*g* in a Kubota 6800 (Kubota Co., Osaka, Japan) for 15 min. The absorbance of the supernatant was measured at 532 nm. All readings were corrected for any interference from the brown colour of the extract or antioxidant by including appropriate controls. The negative control was without any antioxidant or extract. The percent hydroxyl radical scavenging activity of extracts was determined accordingly in comparison with the negative control.

2.7. Protective effect of AWEC on hydroxyl radicals mediated DNA damage

Calf thymus DNA (procured from Sigma, St. Louis, USA) was subjected to oxidation with Fenton reactants as for the deoxyribose assay (Halliwell et al., 1987) and the extent of DNA oxidation was analysed on agarose gel. Calf thymus DNA (1 mg was mixed well in 20 mM phosphate buffer, at pH 7.4, 150 mM NaCl and left at 4 °C for 24 h for complete solubilisation) was sheared using a 21-gauge needle to obtain low molecular weight species. Immediately, 100 μg of calf thymus DNA was treated with ferric chloride (100 μM), ascorbate (100 μM), H_2O_2 (1 mM) and EDTA (104 μM) with or without AWEC (40, 60 μg)/BHA (80 μg) in 100 μl final volume of 20 mM potassium phosphate buffer, at pH 7.4. The reaction mixture was incubated at 37 °C for 30 min and then placed on ice for 10 min to stop the reaction. The reaction mixture was mixed with 10 μl of loading buffer (0.5% bromophenol blue, 50% glycerol in water). Ten microlitres (5 μg) of DNA was run on 1% agarose with ethidium bromide (1 μg/ml). The electrophoresis was conducted in TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, 18 mM NaCl, pH 8) at 60 V for 6 h and DNA was visualised under a UV transilluminator (Bio-Rad, Sydney, Australia).

2.8. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was assessed according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992). DPPH, BHA, α-tocopherol, curcumin, β-carotene and L-ascorbic acid were procured from Sigma (St. Louis, USA). Different extracts of curry leaves and ascorbic acid, at various concentrations ranging from 10 to 100 μg, were mixed in 1 ml of freshly prepared 0.5 mM DPPH ethanolic solution and 2 ml of 0.1 M acetate buffer at pH 5.5. The resulting solutions were then incubated at 37 °C for 30 min and measured colorimetrically at 517 nm in a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). Standard antioxidants such as BHA, α-tocopherol, curcumin, β-carotene, and L-ascorbic acid, all at 400 μM, were used as positive controls under the same assay conditions. Negative control was without any inhibitor or extract. Lower absorbance at 517 nm represents higher DPPH scavenging activity. Percent DPPH radical scavenging activity of the extracts was calculated accordingly from the decrease in absorbance at 517 nm in comparison with the negative control.

2.9. Cytochrome c reduction

The cytochrome *c* reducing capacity of AWEC was determined according to the method of Suter and Richter (2000). Cytochrome *c*, BHA and L-ascorbic acid were procured from Sigma (St. Louis, USA). Unaltered cytochrome *c* has a characteristic spectra with λ_{max} of 550 nm, due to its active heme group, which contains a ferrous ion. When subjected to oxidation by oxygen saturated phosphate buffer (0.1 mM, pH 7), the peak at 550 nm will diminish quantitatively. In the assay, cytochrome *c* (15 μM) was subjected to oxidation by oxygen saturated phosphate

buffer (0.1 mM, pH 7), then AWEC (5–30 μg)/BHA (800 μM)/ascorbic acid (1 mM) was added and incubated at ambient temperature for 30 min. Absorbance at 550 nm was measured using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). Absorbance increases with increase in reduction of oxidised cytochrome *c*. Appropriate controls were maintained.

2.10. Test for ferric ion reducing capacity (Fe^{3+} to Fe^{2+})

The ferric ion reducing capacity was determined according to the method of Wang, Yen, Ling, and Wu (2003) with minor modifications. Potassium ferricyanide, ferric chloride and BHA were procured from Sigma (St. Louis, USA). A hundred microlitres of potassium ferricyanide solution (4 mM) was mixed with 200 μl of 20 mM phosphate buffer, pH 6.5, with or without AWEC/BHA at various concentrations ranging from 5 to 100 μg. The contents were incubated at 50 °C for 20 min. Two hundred microlitres of 10% trichloroacetic acid was added to the reaction mixture and centrifuged at 5319*g* in a Kubota 6800 (Kubota Co., Osaka, Japan). The resulting supernatant was taken and mixed with 100 μl of ferric chloride solution (2 mM) and final volume was made up to 1 ml with water and then incubated at ambient temperature for 10 min. The absorbance was recorded at 700 nm using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). Absorbance increases with an increase in ferric ion reducing capacity.

2.11. Test for ferrous ion chelating activity (binds Fe^{2+})

Ferrous ion chelating activity was measured according to the method of Suter and Richter (2000) with minor modifications. Potassium ferricyanide, ferric chloride and EDTA were procured from Sigma (St. Louis, USA) and double distilled water was used. The reaction solution containing ferrous chloride (200 μM) and potassium ferricyanide (400 μM), with or without AWEC/EDTA at various concentrations ranging from 20 to 100 μg, was made to 1 ml with double distilled water and mixed. The reaction mixture was incubated at 20 °C for 10 min. Formation of the potassium hexacyanoferrate complex was measured at 700 nm using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). The assay was carried out at 20 °C to prevent Fe^{2+} oxidation. Lower absorbance indicated a higher iron chelating capacity. The negative control was without any chelating compound or test sample. The percent ferrous ion chelating activity was calculated accordingly by comparing the absorbance of the test samples with that of the negative control.

2.12. Protective effect of AWEC on Fenton reactant-induced DNA sugar damage

Oxidative DNA sugar damage was determined according to the method of Sultan, Perwaiz, Iqbal, and Athar (1995) with minor modifications. Calf thymus DNA was procured from Sigma (St. Louis, USA). The reaction mixture, in a total volume of 1000 μl of potassium phosphate buffer (20 mM, pH 7.4), which contained 1 mg of calf thymus DNA was treated with ferrous sulfate and ascorbate (5:50 μmole) as the standard Fenton reagent (Fenton, 1984; Shalini et al., 1994) with or without AWEC at the concentrations ranging from 20 to 100 μg/ml. The reaction mixture was incubated at 37 °C for 1 h in a water bath shaker. The colour was developed by adding 1 ml of 2.8% TCA and 1 ml of 1% TBA and boiled for 20 min, cooled on ice and centrifuged at 5319*g* in a Kubota 6800 (Kubota Co., Osaka, Japan) for 10 min. The absorbance was read at 535 nm using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). The negative control was without any test sample. The percent inhibition of DNA oxidation was calculated accordingly by comparing the absorbance of the test sample with a negative control.

2.13. Statistical analysis

Statistical analysis was done in SPSS (Windows Version 10.0.1 Software Inc., New York) using a one-sided student's *t*-test. All results refer to means ± SD. $P < 0.05$ was considered as statistically significant when compared to relevant controls.

3. Results and discussion

3.1. General

Ten milligrams of each dried extract was dissolved in 0.1 ml of respective extracting solvent or solvent mixture and made up to 10 ml with water, making the final concentration of the solvent to 1%. This was further diluted about 10 times in the assay mixture (which is usually 1 ml) making the effective concentration of the solvent to 0.1%. This concentration is assumed not to interfere with the outcome of the assays. To clearly rule out the possibility of solvent affecting the activity in the assay, appropriate solvent controls were included. The AWEC comprised of a high polyphenolic content and also contained significant amounts of carotenoids, carbohydrates and proteins (data not shown) (Palaniswamy, 2001). Thus a bulk of the AWEC constitutes polyphenols and the antioxidant activity may be a result of the synergistic action of all the components, rather than of a single entity of the extract. Nevertheless, the exact nature and the quantity of each of the constituents are yet to be characterised.

3.2. Extraction yield and total polyphenols

The dried material obtained after concentrating the curry leaves extract (yield) was found to be 8, 10, 12, 6.8, 7 g% of the starting material for the water extract, the ethyl alcohol extract, the ethyl alcohol:water (1:1) extract, the hexane extract and the chloroform extract, respectively. Phenolic substances have been shown to be responsible

for the antioxidant activity of plant materials (Rice-Evans, Miller, & Paganga, 1996). Therefore, the amount of total polyphenols in the extracts was determined by the Folin–Ciocalteu method. The content of total phenols is expressed as gallic acid equivalents (mg gallic acid/g extract). As shown in Table 1, AWEC about 168 mg/g extract, which was found to be higher than WEC, AEC, HEC, CEC which showed phenolic content of about 54, 104, 18, 21 mg/g extract, respectively. Therefore, the higher phenolics content of AWEC might be responsible for the enhanced antioxidative activities compared with the other extracts. In similar reports, the enrichment of phenolic compounds within plant extracts is correlated with their enhanced antioxidant activity (Lee et al., 2002).

3.3. Inhibition of lipid peroxidation

In order to determine if the extracts were capable of reducing *in vitro* oxidative stress, the traditional lipid peroxidation assay that determines the production of malondialdehyde and related lipid peroxides in RBC membrane was carried out. Thiobarbituric acid reactive substances are produced as by-products of lipid peroxidation – induced by the ferrous sulfate:ascorbate system. The inhibitory effect of various extracts of curry leaves against ferrous sulfate and ascorbic acid-induced ghost lipid peroxidation are shown in Fig. 1a. AWEC showed the highest inhibition of ghost lipid peroxidation, about $76.4 \pm 3\%$ at a lower concentration (50 μg). While AEC (100 μg) showed significant antioxidant activity of $64.3 \pm 4\%$. However, the inhibitory effect of HEC, CEC and WEC on ghost lipid peroxidation was significantly raised with increasing concentration and these extracts, at 200 μg, showed inhibitory effects of $58.6 \pm 3\%$, $57.5 \pm 2.5\%$ and $51 \pm 2\%$, respectively. These results indicated that the 1:1 ratio of ethyl alcohol:water used to make an extract of curry leaves exhibits maximum antioxidant activity compared with the water, ethyl alcohol, hexane and chloroform extracts of curry leaves.

Table 1
Extraction yield and total polyphenols in different extracts of curry leaves

Extract	% of staring material	Polyphenol content (mg GAE[a]/g curry leaf extract)
Water extract curry leaves	8 ± 0.51	54 ± 4.4
Ethyl alcohol extract curry leaves	10 ± 0.44	104 ± 5.2
Ethyl alcohol:water (1:1) extract curry leaves	12 ± 0.37	168 ± 5.6
Hexane extract curry leaves	6.8 ± 0.52	18 ± 2.5
Chloroform extract curry leaves	7 ± 0.54	21 ± 2.8

Each value is expressed as mean ± standard deviation ($n = 6$).
[a] GAE, gallic acid equivalents.

3.4. Superoxide scavenging activity

Superoxide anions are the most common free radicals *in vivo* and are generated in a variety of biological systems and the concentration of superoxide anions increases under conditions of oxidative stress (Lee et al., 2002). Hence, a NBT assay was carried out to test whether extracts of curry leaves scavenge superoxide anions. As shown in Fig. 1b, the superoxide scavenging activity of different extracts of curry leaves was found to be in the order of AWEC (93%) at 200 μg, AEC (75%) at 250 μg, WEC (71%) at 300 μg, HEC (61%) at 300 μg and CEC (46%) at 300 μg. SOD was used as the positive control that showed 86% superoxide scavenging activity at 250 μg. These results indicate that extraction of curry leaves with the ethyl alcohol:water (1:1) mixture probably gives a larger number of different antioxidants which could exhibit synergistic effects, as compared to other solvent extracts. Similar *in vitro* studies, by other researchers, have reported that ginger could also significantly scavenge superoxide anions and act as a dietary antioxidant *in vivo* (Lee & Lim, 2000).

3.5. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect

The indirect evidence for the scavenging activity of AWEC on Fe^{3+} dependent hydroxyl radical generation was further confirmed using a direct approach with DPPH radicals Chang et al. (2001). The results obtained are shown in Table 2, AWEC exhibited powerful DPPH radical scavenging activity of 92% at 20 μg, which was 5-fold lower in concentration than AEC, WEC, HEC and CEC, which showed DPPH radical scavenging activity of 64%, 41%, 56% and 48%, respectively. Known antioxidants such as BHA (72 μg), α-tocopherol (85.5 μg), Curcumin (73.5 μg), β-carotene (107.33 μg) showed 86%, 82%, 76% and 61% DPPH radical scavenging activity, respectively. The results indicate that AWEC is a powerful free radical scavenger compared to other extracts and known antioxidants.

3.6. Hydroxyl radical scavenging activity

Hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and are thought to initiate cell damage *in vivo* (Rollet-Labelle et al., 1998). Further, the effect of curry leaf extracts on hydroxyl radicals generated by Fe^{3+} ions was measured by the extent of deoxyribose degradation, an indicator of TBA–MDA adducts formation. Among the various extracts tested (Fig. 2a), AWEC showed maximum hydroxyl radical scavenging activity by 91% at 20 μg which was 5-fold lower in concentration than AEC (74%), WEC (66%), HEC (58%) and CEC (51%) whereas, BHA at 80 μg showed 80% hydroxyl radical scavenging activity. This implies that AWEC could be an effective hydroxyl radical scavenger

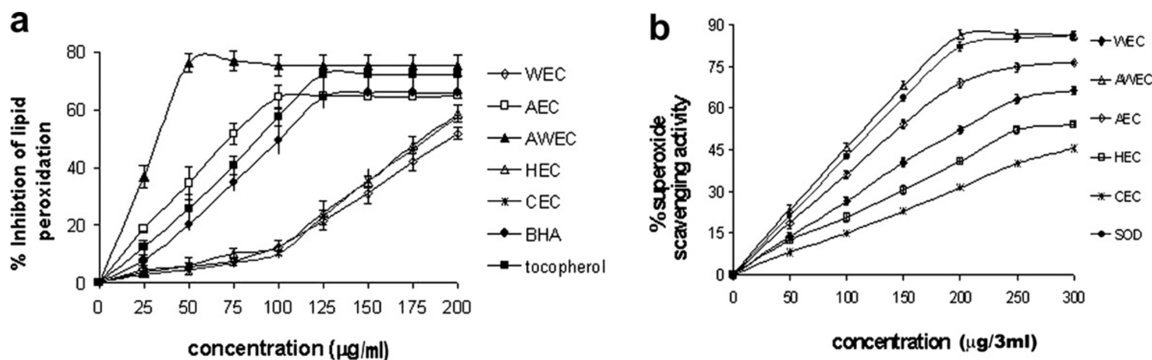

Fig. 1. (a) Inhibition of human RBC membrane lipid peroxidation by various extracts of curry leaves. The negative control was without any antioxidant or extract. Results are shown as mean ± SD ($n = 3$). (b) The superoxide scavenging activity of various extracts of curry leaves. The negative control was without any antioxidant or extract. Results are shown as mean ± SD ($n = 3$). WEC, water extract of curry leaves; AWEC, alcohol:water (1:1) extract of curry leaves; AEC, alcohol extract of curry leaves; HEC, hexane extract of curry leaves; CEC, chloroform extract of curry leaves.

Table 2
DPPH radical scavenging activity of different extracts of curry leaves

Antioxidant	Concentration (µg/ml)	% DPPH[a] radical scavenging activity
Negative control	No antioxidant	0
Water extract of curry leaves	100	41 ± 1.7
Ethyl alcohol extract of curry leaves	100	64 ± 2
Ethyl alcohol:water (1:1) extract of curry leaves	20	92 ± 1.7
Hexane extract of curry leaves	100	56 ± 2.2
Chloroform extract of curry leaves	100	48 ± 2.3
Ascorbic acid	100	82 ± 2.1
BHA	72 (400 µM)	86 ± 2.4
α-Tocopherol	85.5 (400 µM)	82 ± 1.5
Curcumin	73.5 (400 µM)	76 ± 2.6
β-Carotene	107.38 (400 µM)	61 ± 2

Each value is expressed as mean ± standard deviation ($n = 3$).
[a] DPPH, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.

at very low concentration, compared to other extracts of curry leaves and the individual standard antioxidant.

3.7. Protective effect of AWEC on hydroxyl radicals mediated DNA damage

Oxidative DNA damage has been implicated to be involved in various degenerative diseases (Halliwell & Gutteridge, 1981). When calf thymus DNA was treated with Fenton reactants as in the deoxyribose assay for 30 min, extensive DNA fragmentation due to oxidation by hydroxyl radicals was observed on agarose gel, by the enhanced mobility (Fig. 2b, lane 2) as compared to the untreated DNA (lane 1). Known antioxidants such as BHA (80 µg) protected DNA damage as assessed by the similar mobility of the DNA (lane 3) in comparison to the untreated DNA (lane 1). Interestingly, AWEC is also shown to exhibit DNA protectant activity at 20 µg (lane 4) and 40 µg (lane

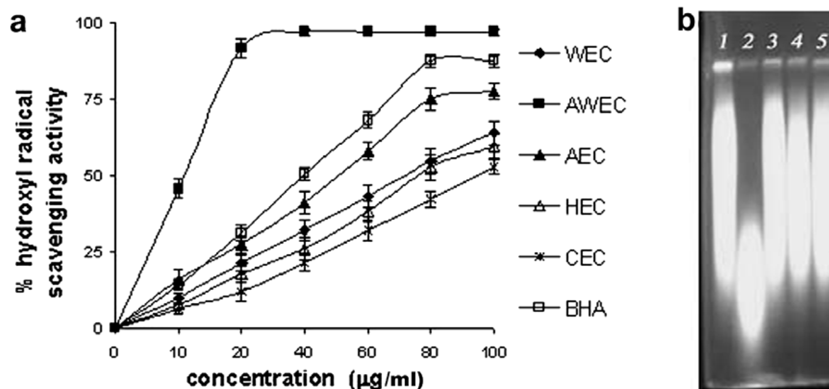

Fig. 2. (a) Hydroxyl radical scavenging activity of various extracts of curry leaves in comparison with butylated hydroxy anisole (BHA). The negative control was without any antioxidant or extract. Results are shown as mean ± SD ($n = 3$). (b) Inhibition of hydroxyl radicals-mediated DNA degradation by the polyphenol-enriched alcohol:water extract of curry leaves. Lane 1: calf thymus DNA untreated; lane 2: Calf thymus DNA + Fenton reactants solution ($FeCl_3$ (100 µM), H_2O_2 (1 mM), EDTA (104 µM), ascorbate (100 µM)); lanes 3–5 show the results for calf thymus DNA + Fenton reactants solution + BHA (100 µg), AWEC at 20 µg and 40 µg, respectively. BHA is a standard antioxidant. WEC, water extract of curry leaves; AWEC, alcohol:water (1:1) extract of curry leaves; AEC, alcohol extract of curry leaves; HEC, hexane extract of curry leaves; CEC, chloroform extract of curry leaves.

5), respectively. This effectiveness of the AWEC to prevent oxidative DNA damage was concomitant to its hydroxyl radical scavenging activity as observed by deoxyribose assay (Fig. 2a). This suggests that AWEC may combat free radical mediated oxidative DNA damage.

3.8. Cytochrome c reduction

Cytochrome *c*, a major electron transport protein of the respiratory chain, was used as a model protein to investigate the direct reductive capacity of AWEC as one of the reaction mechanisms of antioxidant activity. AWEC significantly reduced the oxidised cytochrome *c*, up to 100%, in a time dependent manner at 15 min (data not shown) and in a concentration dependent manner (Fig. 3a). The direct cytochrome *c* reductive capacity of AWEC was comparable to BHA (400 μM) and ascorbate (1 mM) (strong reducing agents). Similar studies have reported that the aqueous extract of herbal remedy PADMA 28, derived from traditional Tibetan medicine, contains reducing compounds that reduce ferric cytochrome *c* to ferrocytochrome *c* (Suter & Richter, 2000). These results indicate that AWEC is a potent reducing agent for the active heme group of cytochrome *c*.

3.9. Test for ferric ion reducing capacity

The extracts of spices and herbs may well act as electron donors and they can react with free radicals to convert them into more stable products and terminate radical chain reactions. Also, it has been shown that the antioxidation effect exponentially increased as a function of the development of the reducing power (Tanaka, Kuie, Nagashima, & Taguchi, 1988). As shown in Fig. 3b, the maximum absorbance for AWEC was up to 1.2, at 5-fold lesser concentration (20 μg), compared to BHA which showed absorbance of 1.2. Similar studies have reported that natural antioxidants are involved in the termination of free radical reactions and exhibit reducing capacity (Wang et al., 2003). The reducing capacity might be due to their hydrogen donating ability (Shimada et al., 1992).

3.10. Test for ferrous ion chelating activity

It is self-evident that the strong reductive capacity of antioxidants may also affect ions, especially Fe^{2+} and Cu^{2+}. Iron is an essential mineral for normal physiology, but an excess of it may result in cellular injury. If they undergo the Fenton reaction, these reduced metals may form highly reactive hydroxyl radicals and thereby contribute to oxidative stress (Hippeli & Elstner, 1999). The resulting oxy radicals cause damage to cellular lipids, nucleic acids, proteins, carbohydrates and lead to cellular impairment. Since ferrous ions are the most effective pro-oxidants in food system, the good chelating effect would be beneficial and removal of free iron from circulation could be a promising approach to prevent oxidative stress-induced diseases. When iron is chelated, it may lose pro-oxidant properties. Hence, we herein tested the chelation of Fe^{2+}, by the extract of curry leaves in a competition assay with potassium ferricyanide. Interestingly, as seen in the Fig. 4a, the antioxidant factors of the AWEC were found to be capable of binding Fe^{2+}ions by 92% at 100 μg, as evidenced by the loss of absorption at 700 nm (iron–hexacyanoferrate complex) and its chelating effect is comparable to EDTA (60 μg) which showed a chelating effect of 95%.

3.11. Inhibitory effect of AWEC on Fenton reactant-induced DNA sugar damage

To confirm the chelating effect, the inhibitory effect of AWEC against iron dependent oxidation of calf thymus DNA sugar was tested by a TBARs assay. As shown in

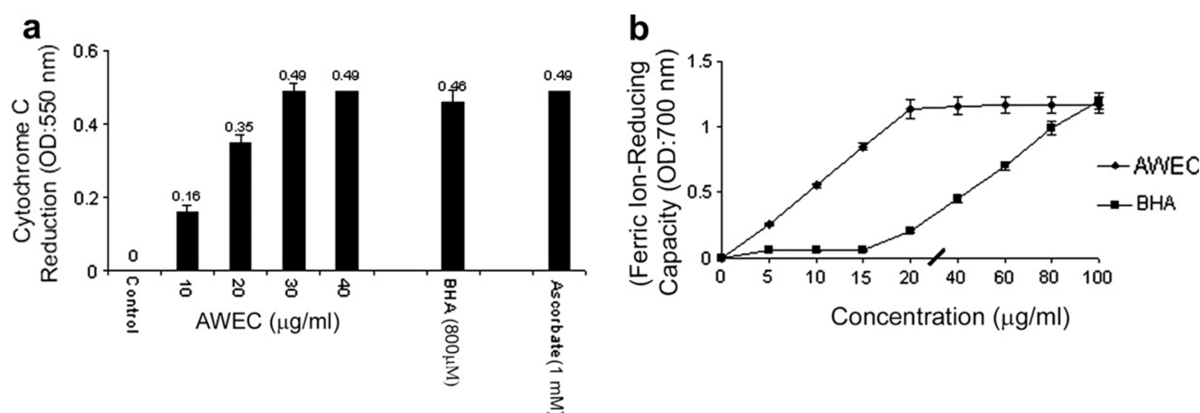

Fig. 3. (a) Cytochrome *c* reduction capacity of the polyphenol-enriched ethyl alcohol:water extract of curry leaves The positive control was ascorbic acid (100% cytochrome *c* reduction). Results are shown as mean ± SD ($n = 3$). (b) Dose-dependent ferric ion-reducing capacity of the polyphenol-enriched alcohol:water extract of curry leaves. Results are shown as mean ± SD ($n = 3$). AWEC, alcohol:water (1:1) extract of curry leaves; BHA, butylated hydroxy anisole. The positive control was BHA, which is a known antioxidant.

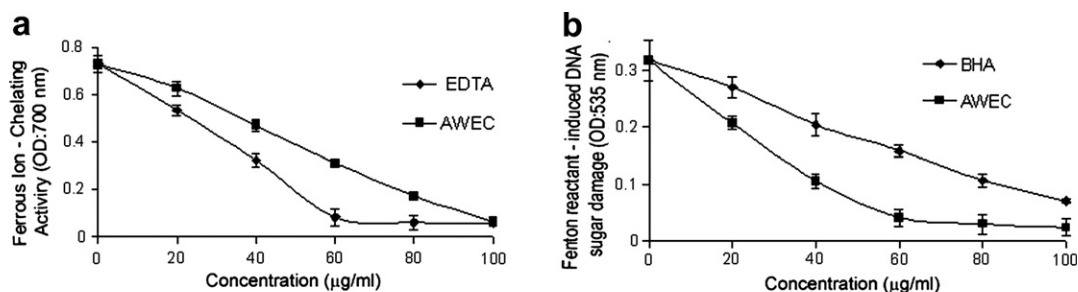

Fig. 4. (a) Dose-dependent ferrous iron chelating activity of the polyphenol-enriched alcohol:water extract of curry leaves. The negative control was without any antioxidant or extract. Results are shown as mean ± SD ($n = 3$). (b) Dose-dependent inhibition of Fenton reactant-induced DNA sugar damage by polyphenol-enriched alcohol:water extract of curry leaves. The negative control was without any antioxidant or extract. Results are shown as mean ± SD ($n = 3$). AWEC, alcohol:water (1:1) extract of curry leaves; AEC, alcohol extract of curry leaves; HEC, hexane extract of curry leaves; CEC, chloroform extract of curry leaves; BHA, butylated hydroxy anisole.

Fig. 4b, AWEC offered effective inhibition by 87% at 60 μg against ferrous sulfate:ascorbate-induced (Fenton, 1984) DNA sugar damage, when compared to BHA, which showed 77.5% at 100 μg. The results (Fig. 4a and b) imply that the effective chelating activity of AWEC is probably due to synergism of both water-soluble and ethyl alcohol soluble antioxidants and this may explain the higher antioxidant activity of AWEC, as compared to other extracts of curry leaves. Similar studies have reported that extracts of mushroom exhibit chelating effect of ferrous ions and a reducing capacity (Mau, Lin, & Song, 2002). The importance of iron chelation for the antioxidant activity of flavonoids has been documented recently (Van Acker, Van Balen, Van den Berg, Bast, & van der Vijgh, 1998). Iron deprivation is now considered to be a promising strategy to ameliorate or prevent oxidant-induced human diseases (Polla, 1999).

4. Conclusion

This work highlights the importance of curry leaves which have been traditionally used in the Indian culinary system from time immemorial, as a rich source of antioxidants. More specifically, the extraction of curry leaves in a 1:1 ethyl alcohol:water mixture, compared to other solvent systems, showed maximum antioxidant and free radical scavenging activities under *in vitro* conditions. Thus, the ethyl alcohol:water (1:1) extract of curry leaves could be a very good lead for extraction of an effective natural nutraceutical or antioxidant drug. Further investigation into ethyl alcohol:water (1:1) extract of curry leaves for its lead active compounds and *in vivo* antioxidant mechanisms is warranted.

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