Free Radical Scavenging Activity of Aqueous and Ethanolic Extract of Brassica oleracea L. var. italica

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ORIGINAL PAPER

Free Radical Scavenging Activity of Aqueous and Ethanolic Extract of *Brassica oleracea* L. var. *italica*

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Abstract In this study, antioxidant activities of aqueous and ethanolic extracts of *Brassica oleracea* L. var. *italica* were investigated. The antioxidant properties of both extracts of *Brassica oleracea* L. var. *italica* were evaluated using different antioxidant tests, including 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, superoxide radical scavenging, inhibition of microsomal lipid peroxidation, reduction of power, and metal ion chelating activities. Inhibition of superoxide scavenging by aqueous and ethanolic extracts showed an IC_{50} of 0.93 and 0.25 mg/ml, respectively. Metal ion chelation showed an IC_{50} of 0.35 mg/ml of both the extracts and was equipotent to positive control, ethylenediamine tetra-acetic acid. The ethanolic extract of *Brassica oleracea* L. var. *italica* exhibited higher antioxidant activity in DPPH radical and superoxide anion scavenging than that of aqueous extract. The results obtained in the in vitro models clearly suggest that, *Brassica oleracea* L. var. *italica* is a natural source for antioxidants, which could serve as a nutraceutical with potential applications in reducing the level of oxidative stress and related health benefits. However, comprehensive studies need to be conducted to ascertain the in vivo safety of such extracts in experimental animal models.

Keywords *Brassica oleracea* L. var. *italica* · Antioxidant activity · Reactive oxygen species · Lipid peroxidation · Metal ion chelation

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Introduction

Reactive oxygen species (ROS) are generated by many redox processes that normally occur in the metabolism of aerobic cells. If not eliminated, ROS can attack important biological molecules, such as lipids, proteins, enzymes, DNA, and RNA (Jung et al. 1999; Pietta et al. 1998). Moreover, it has been suggested that a chronic imbalance between formation of ROS and antioxidant defenses has been implicated in the development of chronic diseases, such as cancer, atherosclerosis, nephritis, diabetes mellitus, rheumatism, ischemic, cardiovascular diseases, and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Behl and Moosmann 2002; Pulido et al. 2000; Coulson et al. 2004; Rekha et al. 2008). There is convincing epidemiological evidence that the consumption of fruits and vegetables are beneficial for health, due to the protection provided by the antioxidant phytonutrients contained in them (Guthrie and Kurowska 2001; Avila-Sosa et al. 2008). Vitamin C, vitamin E, carotenoids, and dietary flavonoids can play specifically important roles in human nutrition, and foods rich in these compounds should be included in an optimal diet (du Toit et al. 2001). The assessment of the antioxidant properties of therapeutic molecules and their potential use in preventing or limiting the damage induced by free radicals appears to be of particular value (Bansal et al. 2005). Therefore, there is a

great deal of interest in edible plants/vegetables that contain antioxidants and health promoting phytochemicals, in view of their health implications.

Brassica oleracea L. var. *italica* (family: Cruciferae) is commonly called broccoli, brocks, or calabrese. Cao et al. (1996) and Chu et al. (2002) have reported the antioxidant activity of 26 common vegetables; among them, broccoli also possessed the highest activity. Biologically active compounds including polyphenols such as hydroxycinnamic acid esters, kaempferol, and quercetin glucosides and traces of isorhamnetin have been reported (Moreno et al. 2006). Broccoli also contains high concentrations of selenium and glucosinolates, particularly glucoraphanin and isothiocyanate sulforaphane (Finley et al. 2005) which have anticarcinogenic properties (Jeffery and Jarrell 2001). *Brassica oleracea* L. var. *italica* confers protection against cancer (Matusheski et al. 2006) and heart disease (Subhendu et al. 2008). Antioxidant activity of broccoli on cooked food and juices has also been reported (Chun and Chi 2004; Ting et al. 2007). The present work studied the antioxidant potential of the aqueous and ethanolic extracts of the *Brassica oleracea* L. var. *italica* employing various in vitro assay systems, such as 1, 1-diphenyl-2-picrylhydrazyl (DPPH)/superoxide radical scavenging, inhibition of lipid peroxidation, reducing power, and metal ion chelating activity.

Materials and Methods

Chemicals

Butylated hydroxylanisole (BHA), nitroblue tetrazolium (NBT), DPPH, phenazine methosulphate (PMS), thiobarbituric acid (TBA), and ethylenediamine tetra-acetic acid (EDTA) were purchased from M/s Sigma Chemicals Co. (St. Louis, MO, USA). Ferrozine, Nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), potassium ferricyanide, and ferric chloride were purchased from M/s Sisco Research Laboratories, Mumbai, India. All reagents were of analytical grade.

Preparation of Plant Extract and Extraction

The *Brassica oleracea* L. var. *italica* was purchased from the local suppliers in Mysore, India. The plant was identified by a botanist at the Department of Botany, University of Mysore, Mysore. Aqueous extract was obtained as described by Gulcin et al. (2003) with slight modifications. Forty grams of sample was homogenized with 50 ml of water and the homogenate was kept in a shaker at 40°C for 24 h. Then, the extract was filtered over Whatman No. 1 paper. The filtrates were frozen and lyophilized in lyophilizer at 5 μm Hg pressure at −50°C. For ethanol extraction, 20 g of sample was put into a fine powder in a mill and was mixed with 50 ml of ethanol. The residue was re-extracted until extraction solvents became colorless. The resulting extracts were filtered over Whatman No. 1 paper and the filtrate was collected, and then ethanol was removed by a rotary evaporator at 50°C to obtain dry extract. Both extracts were placed in a plastic bottle, and then stored at −20°C until used.

DPPH Radical Scavenging Assay

The DPPH test was carried out as described by Guohua et al. (1997) with some modifications. Different concentrations of both aqueous and ethanolic extracts were mixed with of 1-ml DPPH solution (0.1 mmol/l, in 95% ethanol (*v*/*v*)) and the reaction mixture incubated for 30 min at room temperature. The absorbance was read at 517 nm in a spectrophotometer against a blank. BHA was used as a positive control. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and calculated using the following equation:

$$\%\ \text{Inhibition} = \left(\frac{1 - A_{\text{sample(517nm)}}}{A_{\text{control(517nm)}}}\right) \times 100$$

Superoxide Radical Scavenging Activity

Measurement of superoxide radical scavenging activity of the extracts was based on the method described by Nishikimi et al. (1972). Superoxide radicals are generated by the reaction of NADH and PMS couple to the reduction of NBT. The reaction mixture, containing *Brassica oleracea* L. var. *italica* (0.3–1.5 mg/ml for aqueous extract and 0.1–0.3 mg/ml for ethanolic extract), PMS (0.1 mmol/l), NADH (1 mmol/l), and NBT (1 mmol/l) in phosphate buffer (0.1 mol/l, pH 7.4), was incubated at room temperature for 5 min and the absorbance was measured at 560 nm against a blank. Decreased absorbance of the reaction mixture indicates increased superoxide radical scavenging activity. The scavenging effect was calculated using the equation described as in the case of DPPH.

Inhibition of Microsomal Lipid Peroxidation

Liver excised from adult male Wistar rats, was homogenized (20–25 g) in 0.02 mol/l tris buffer (pH 7.4). Microsomes were isolated by the calcium aggregation method (Kamath and Rubin 1972). To 100 μl of liver microsomal suspension, 1 mmol/l each of $FeSo_4$ and ascorbic acid were added, with or without *Brassica oleracea* L. var. *italica* (0.05–0.5 mg/ml for both aqueous

and ethanolic extracts) in a total volume of 1 ml in 0.1 mol/l phosphate buffer (pH 7.4) and incubated at 37°C for 1 h. After incubation, the reaction mixture was heated in a boiling water bath with 20 g/100 ml TCA (2 ml) and 1 g/100 ml TBA (2 ml) for 10 min, cooled and centrifuged. Formation of malondialdehyde (MDA) in the mixture was measured at 535 nm (Buege and Aust 1978). BHA was used as a positive control. Percent inhibition was calculated using the equation described for DPPH.

Measurement of Reducing Power

The reducing power of the extracts was quantified by the method described by Oyaizu (1986). One milliliter of reaction mixture containing extracts (0.5–2.5 mg/ml for both aqueous and ethanolic extract) in phosphate buffer (0.2 mol/l, pH6.6), was incubated with potassium ferricyanide (1 g/100 ml) at 50°C for 20 min. The reaction was terminated by adding TCA solution (10 g/100 ml) and the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was mixed with distilled water (2.5 ml) and ferric chloride solution (0.1 g/100 ml) and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power.

Metal Ion Chelating Assay

The ferrous ion chelating potential of the extract was investigated according to the method described by Decker and Welch (1990), by measuring the ferrous iron–ferrozine complex at 562 nm. The reaction mixture, containing *Brassica oleracea* L. var. *italica* (0.1–0.5 mg/ml for both aqueous and ethanolic extract), $FeCl_2$ (2 mmol/l), and ferrozine (5 mmol/l), was adjusted to a total volume of 0.8 ml with methanol, mixed well and incubated for 10 min at room temperature. The absorbance of the mixture was read at 562 nm against a blank. EDTA was used as positive control. The ability of the extract to chelate ferrous ion was calculated using the equation described for DPPH.

Phenolic Content

Total phenolic content was estimated by Folin–Ciocalteau method (Singleton and Rossi 1965). One milliliter of extracts was added to 5 ml of deionized distill water and 0.5 ml of Folin–Ciocalteau reagent followed by the addition of 1 ml of Na_2CO_3 (20 g/100 ml). The reaction mixture was incubated for 30 min at 25°C, the absorbance was measured at 765 nm in spectrophotometer, and the phenolic content was calculated with guaicol as the standard and expressed as milligram of guaicol equivalent per gram dry weight.

Statistical Analysis

Data were expressed as mean±standard error of three separate experiments and IC_{50} values were calculated by regression analysis.

Results and Discussion

DPPH Radical Scavenging Activity

DPPH is a free radical compound and has been widely used to test the free radical-scavenging ability of various samples

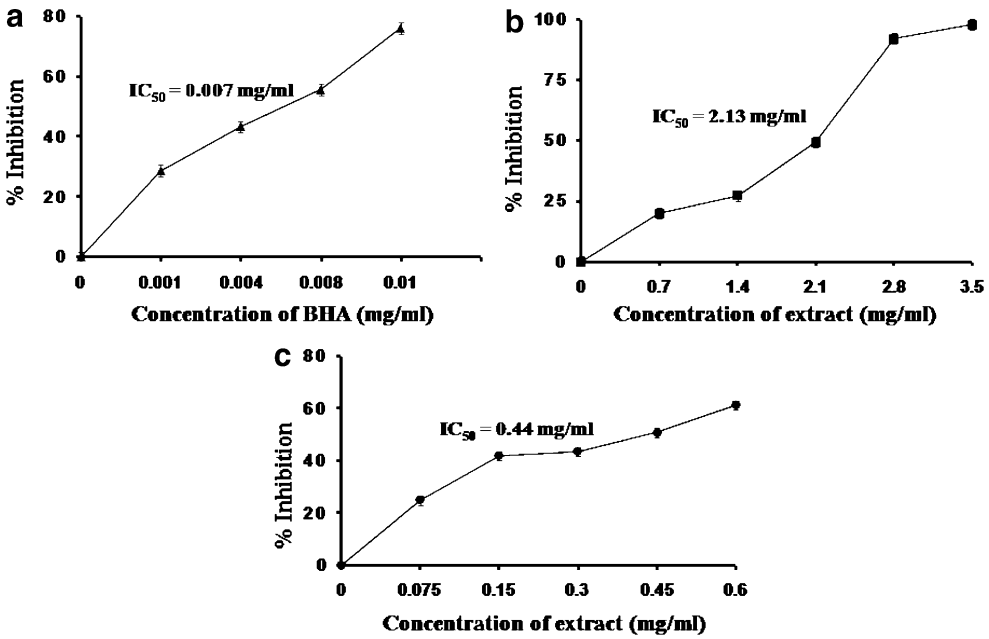

Fig. 1 DPPH radical scavenging by the extracts of *Brassica oleracea* L. var. *italica* (**a** BHA; **b** aqueous extract; **c** ethanolic extract). Values are means±SE of three replicates

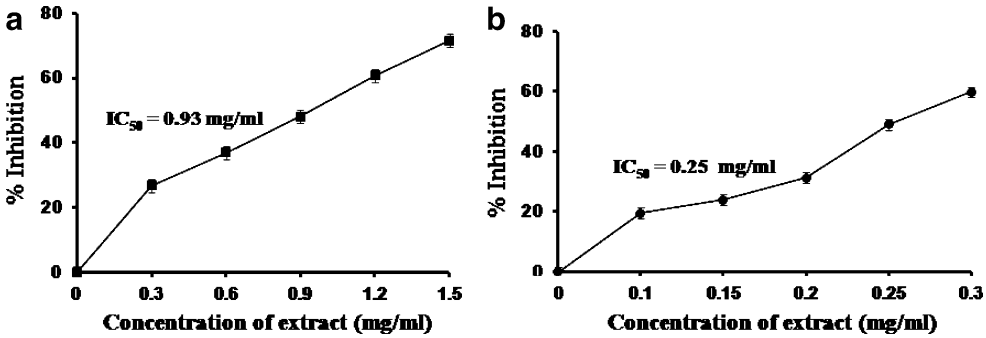

Fig. 2 Superoxide radical scavenging by the extracts of *Brassica oleracea* L. var. *italica* (**a** aqueous extract; **b** ethanolic extract). Values are means±SE of three replicates

(Hatano et al. 1997). The ethanolic extract was more active than the aqueous extract with IC_{50} values of 0.44 and 2.13 mg/ml, respectively (Fig. 1). DPPH is one of the compounds that possesses a proton free radical with characteristic absorption maxima at 517 nm, which decreases significantly on exposure to proton radical scavengers. The free radical scavenging by the antioxidant samples are credited to their hydrogen-donating ability (Yamaguchi et al. 1998). In the present study, *Brassica oleracea* L. var. *italica* showed a concentration-dependent scavenging of the DPPH radical, which may be attributable to its hydrogen-donating ability.

Scavenging of Superoxide Radical

Superoxide anion plays an important role in the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induces oxidative damage in lipids, protein, and DNA (Halliwell and Gutteridge 1989; Pietta 2000). In the present study, *Brassica oleracea* L. var. *italica* effectively scavenged superoxide in a concentration-dependent manner with IC_{50} values for the aqueous and ethanolic extracts being 0.93 and 0.25 mg/ml, respectively (Fig. 2). Superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Meyer and Isaksen 1995). These results clearly suggest that the antioxidant activity of *Brassica oleracea* L. var. *italica* is also related to its ability to scavenge superoxides.

Lipid Peroxidation Assay

Lipid peroxidation inhibitory activity was observed in both aqueous and ethanolic extract of *Brassica oleracea* L. var. *italica*. IC_{50} values for the aqueous and ethanolic extract were 0.40 and 0.44 mg/ml, respectively (Fig. 3). Lipid peroxidation (oxidative degradation of polyunsaturated fatty acid in the cell membranes) generates a number of degradation products, such as Malondialdehyde (MDA), which is found to cause cell membrane destruction and cell damage, leading to liver injury, atherosclerosis, kidney damage, aging, and susceptibility to cancer (Rice-Evans and Burdon 1993). MDA, one of the major products of lipid peroxidation, has been extensively studied and measured as an index of lipid peroxidation and as a marker of oxidative stress (Janero 1990). Our extracts showed that inhibition of lipid peroxidation (LPO) was concentration dependent, which in turn suggest that extracts may have a mixture of biomolecules with hydroxyl groups that prevent the abstraction of hydrogen atom from the double bond of lipid bilayer thereby avoiding the damage of lipid membrane.

Reducing Power

The reducing power of *Brassica oleracea* L. var. *italica* correlated well with increasing extract concentrations (Fig. 4). However, reducing power of aqueous extract was higher than the ethanolic extract. The antioxidant activity of

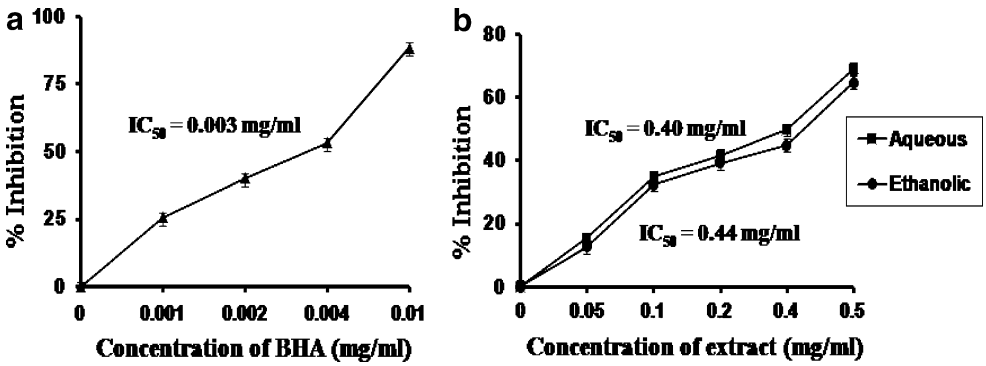

Fig. 3 Inhibition of Lipid peroxidation by the extracts of *Brassica oleracea* L. var. *italica* (**a** BHA; **b** aqueous and ethanolic extract). Values are means±SE of three replicates

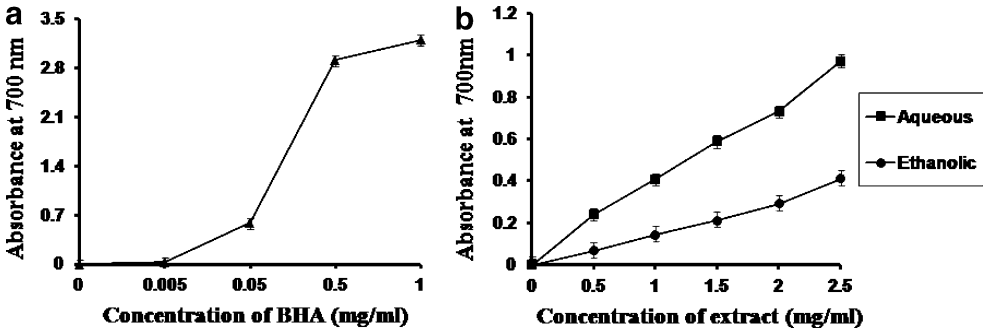

Fig. 4 Reducing power by the extracts of *Brassica oleracea* L. var. *italica* (**a** BHA; **b** aqueous and ethanolic extract). Values are means±SE of three replicates

putative antioxidants has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging (Diplock 1997). The reducing properties are generally associated with the presence of reductones (Pin-Der-Duh 1998), which have been shown to exert antioxidant action by donating a hydrogen atom and braking the free radical chain (Gordon 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Yen and Chen 1995).

Metal Ion Chelating Activity

The ferrous ion chelating effect was shown by both of aqueous and ethanolic extracts of *Brassica oleracea* L. var. *italica* with IC_{50} values of 0.35 mg/ml (Fig. 5). Iron, a transition metal, is capable of generating free radicals from peroxides by the Fenton reaction and is implicated in many diseases (Halliwell and Gutteridge 1990). Fe^{2+} has also been shown to produce oxyradicals and lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl (Halliwell and Gutteridge 1986). Ferrozine forms complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is inhibited, resulting in a decrease in the red color of the complex. Measurement of color reduction is proportional to metal chelating activity of the coexisting chelator (Yamaguchi et al. 2000). Our results showed that both the extracts of *Brassica oleracea* L. var. *italica* inhibited the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are able to capture ferrous ion before ferrozine.

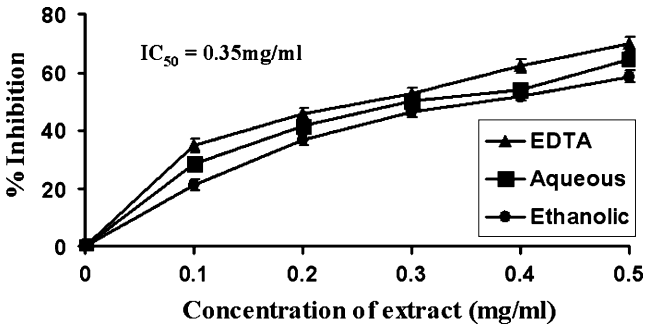

Fig. 5 Metal ion chelating activity by EDTA and the aqueous and ethanolic extracts of *Brassica oleracea* L. var. *italica*. Values are means±SE of three replicates

Phenolic Content

Phenolic content in the aqueous extract of *Brassica oleracea* L. var. *italica* was higher than that of the ethanolic extract (21.20±5.4 and 13.96±3.7 mg guaicol equivalent per gram, respectively). Phenols are very important plant constituents because of their radical scavenging ability resulting from their hydroxyl groups (Hatano et al. 1989). The phenolic compounds may contribute directly to the antioxidative action (Duh et al. 1999). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al. 1998). In our study, there is little correlation between antioxidant activity and phenol content. Ethanolic extract with less phenol content showed higher activity in DPPH radical, superoxide anion scavenging compared to aqueous extract, which may have been due to nonphenolic compounds present in the extract. There are reports that antioxidant activity could also be from nonphenolic compounds (Mariko et al. 2005). The results obtained in the present study clearly demonstrate that the aqueous and ethanolic extracts of *Brassica oleracea* L. var. *italica* may contain certain nonphenolic compounds in addition to the molecules such as selenium, glucosinolates, particularly glucoraphanin, and isothiocyanate sulforaphane (Finley et al. 2005), which could serve as antioxidants and may effectively scavenge various reactive oxygen species/free radicals under in vitro conditions. The multiple antioxidant activity of aqueous and ethanolic extracts of *Brassica oleracea* L. var. *italica* demonstrated in this study clearly indicates the potential application value of the *Brassica oleracea* L. var. *italica*. The potency of the crude extracts (aqueous and ethanolic) of *Brassica oleracea* L. var. *italica* in free radical scavenging and the inhibition of ROS and lipid peroxidation has not been reported earlier.

Conclusion

Broccoli is used in many food preparations for a variety of uses, though their potential components were not explored for health benefits. The results obtained in the present study clearly demonstrate that the aqueous and ethanolic extracts of Broccoli are effective scavengers of various reactive oxygen species/free radicals under in vitro conditions. The broad range of antioxidant activity of the extracts indicates the potential of Broccoli as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits.

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