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Bidens biternata (Lour.) Merr. and Sheriff. – A wild leafy vegetable with significant antioxidant potential

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ABSTRACT

The antioxidant potential of Bidens biternata (Family- Asteraceae), a wild leafy vegetable used by Paniya, Chetti and Kattunaayika tribes of Waynadu District of Kerala were investigated. Different antioxidants properties like DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging, and TBA (Thiobarbituric acid) free radical scavenging abilities were analyzed. Non-enzymatic antioxidants like total phenols, carotenoids, lycopene, proanthocyanidins, flavonoids, flavonols, total polyphenol (TP), proline and enzymatic antioxidants like glutathione peroxidase (GPX), glutathione S transferase (GST), ascorbate oxidase (EC 1.10.3.3), polyphenol oxidase (PPO) and lipoperoxides (LPX) were also measured. The obtained data suggest that B. biternata has tremendous potential to destroy the free radicals that damage the cells.

INTRODUCTION

Generation of Reactive Oxygen Species (ROS) or free radicals during cellular metabolism cause oxidative stress and oxidizes nucleic acids, proteins or lipids that trigger a variety of health disorders such as cancer, cardiovascular diseases, cataract, diabetes, asthma and inflammation [5]. Oxidation of lipid free radicals (lipid peroxidation) in particular has been shown to be associated with human degenerative diseases, since it alters the fluidity of biological membranes and causes cell degradation affecting the biological defense mechanism [2]. All living organisms possess complex network of antioxidant enzymes and chemicals such as oxidative phosphorylation and the formation of disulfide bonds to combat such oxidative damage to cellular components [37]. Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. [20, 34].

A large number of plant sources including many vegetables and fruits have been reported to have high antioxidant activities [25, 39]. *Bidens biternata* of the family Asteraceae is one such candidate herb. It is an erect annual herb that occurs widely as a weed in Western Ghats regions of Kerala state in India. It is used as a leafy vegetable by *Paniya*, *Chetti* and *Kattunaayika* tribes of Waynadu Districts in Kerala with a folklore usage to cure hepatitis, cold, cough, asthma and dysentery. Various phytochemicals like reducing sugar, glycosides, flavonoids, alkaloids, tannins, steroids, terpenoids, coumarins and saponins have been reported from *B. biternata* [26]. The synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary

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butylhydroquinone used for treatment of arthritis, cancer, diabetes are expensive, chemically derived and have undesirable side effects. So for providing modern health care to poor and rural people it becomes imperative to look for alternatives in locally available flora to treat common health disorders [35]. Hence attention has to be given to the commonly available leafy plants, which possess potential to provide health can benefits to society. In this context, an attempt has been made in the present study to evaluate the antioxidant properties of a green leafy vegetable *Bidens biternata* used by the tribes of Waynadu District of Kerala in India.

MATERIALS AND METHODS

Collection of plant material and preparation of samples

Bidens biternata plants were collected from Western Ghats of Kerala state in India where the plant grows in laterite soil under moist, shady and cool environment with an annual rainfall of 2, 786 mm. Fresh plant samples were used for TBA free-radical scavenging assay and phenols, carotenoids, lycopene, total flavanoids, total flavanols, proline and enzymatic antioxidants analysis. Dried powdered samples of the mature leaves, young leaves, stem and roots were used for the analysis of DPPH free-radical scavenging assay and other antioxidants. The fine powder was used for extraction by methanol in Soxhlet apparatus for 8 hrs at 70^0 C. The extract was collected and evaporated in oven at 37^0 C - 40^0 C. The crude concentrated extract was again weighed and used for further studies. Experiments were done in triplicate, graph pad Instat DTCG was employed followed by ANOVA for the calculation and comparison.

Estimation of antioxidant potential

The analysis were performed for estimation of DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging ability, TBA (Thiobarbituric acid) free radical scavenging ability, total phenols, carotenoids, lycopene, proanthocyanidins, flavonoids, flavonols, total polyphenols (TP), proline and antioxidant enzymes like glutathione peroxidase (GPX), glutathione S transferase (GST), ascorbate oxidase, polyphenol oxidase (PPO) and lipoperoxides (LPX) as per following methods.

DPPH free-radical scavenging assay

The effect of extracts on DPPH radicals was estimated using the method of Liyana-Pathirana and Shahidi [15] using 1 ml of 0.135 mM DPPH in methanol mixed with 1.0 ml of extract. The reaction mixture was vortexed and the absorbance of the mixture was measured spectrophotometrically at 517 nm after a dark period for 30 minutes. Ascorbic acid was used as reference and the ability to scavenge DPPH radical was calculated.

Thiobarbituric acid (TBA) free-radical scavenging assay

The method of Ottolenghi [24] was followed to determine the TBA free-radical scavenging activity of the plant samples. Two ml of 20% trichloroacetic acid (TCA) aqueous solution and 2 ml 0.67% thiobarbituric acid (TBA) aqueous solution were added to 1 ml of sample solution, boiled, cooled and centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 532 nm against a blank.

Total phenols

Total phenols were estimated by Folin – Ciocalteau method [17]. A known quantity of fresh tissue was chopped, refluxed, homogenized and centrifuged at 10,000 rpm for 10 minutes. To an aliquot of supernatant which was made up to a known volume, 0.5 ml Folin – Ciocalteau reagent was added and kept for 30 min, followed by addition of 2ml of 20% sodium carbonate. The tubes were kept in a water bath for 2 min, cooled, centrifuged and absorbance was read at 650 nm against a blank. The amount of total phenol was calculated against standard value of catechol and expressed in mg/g.

Carotenoids estimation

Total carotenoids content was measured by the method of Arnon [38]. To 0.5 ml plant extract 4.5 ml 80% acetone was added and the OD was observed at varying wavelengths like 490 nm, 638 nm 645 nm and 663 nm. The amount of carotene was calculated.

Lycopene estimation

The level of lycopene in the plant samples was estimated by the method of Zakaria *et al*., [40]. The samples were saponified for about 30 min in a shaking water bath at 37ÚC after extracting the sample in 12% alcoholic KOH. The lycopene pigments were separated in the petroleum ether layer followed by addition of anhydrous sodium sulphate to remove turbidity. After noting final volume of the petroleum ether extract, absorbance at 450 and 503 nm was noted in a spectrophotometer using petroleum ether as a blank.

Total proanthocyanidins assay

Determination of total proanthocyanidin was based on the procedure reported by Sun *et al*., [36]. A known volume of extract was mixed with of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid. The absorbance was measured at 500 nm after 15 min. Total proanthocyanidin contents were expressed as catechin equivalents (mg/g).

Total flavonoids estimation

The total flavonoids (TF) content was measured according to the method outlined by Meda *et al*, [18] with slight modifications. Fresh sample was refluxed in 80% methanol, homogenized, filtered and centrifuged at 10000 rpm. The supernatant was made up to a known volume by 80% methanol and an aliquot of 0.5 ml of sample was mixed with 0.1 ml of 10% aluminium nitrate and 0.1 ml of potassium acetate (1M). The mixture was vortexed and the absorbance was measured after 40 min at 415 nm and flavonoids content was calculated using quercetin as standard.

Total flavonols measurement

Total flavonols in the plant extracts were estimated using the method of Kumaran and Karunakaran [13]. To an aliquot of refluxed, homogenized and centrifuged sample, 2 ml of 2% aluminium nitrate, ethanol and 3 ml (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 hrs at 20^{0}C and calculated using quercetin as standard.

Total polyphenol (TP) assay

The total phenolic content was determined by the Folin – Ciocalteau assay [9]. A sample aliquot was mixed with 200 µl of phenol reagent (1M) followed by 1.8 ml of distilled water. After 3 min 400 µl of Na_2CO_3 (10% v/v) was added and vortexed. Final volume was made to 4 ml and absorbance was measured at 725 nm after incubation for 1 hr at room temperature. The TP content was expressed as Gallic acid equivalents (GAE) and expressed in mg/100 g (d/w) of sample.

Proline estimation

Proline in the sample was estimated by the method of Bates *et al*, [7]. A known quantity of fresh tissue was homogenized in 10 ml of 3% aqueous sulphosalicylic acid. To the filtrate 2 ml of glacial acetic acid and 2 ml of acid ninhydrin were added. Reaction mixture was heated in boiling water bath for 1 hr and the reactions were terminated by transferring the tubes to an ice bucket. 4 ml of toluene was added to the reaction mixture. Separated toluene layer warmed to room temperature and the absorbance was read at 520 nm against a blank and expressed in mg/g.

Glutathione peroxidase (GPx) assay

The activity of GPx was meaured by the method of Rotruck *et al*., [30]. A known quantity of fresh tissue was homogenized in phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 15 minutes. To an aliquot of supernatant 10 mM sodium azide, 0.2 ml of reduced glutathione 0.1 ml of 2.5 mM H_2O_2, were added. The reaction mixture was incubated for 0, 30, 60, 90 seconds. The reaction was terminated by the addition of 0.5 ml 10% TCA. To determine the glutathione content, 3.0 ml of disodium hydrogen phosphate solution and 1.0 ml of DTNB reagent were added after centrifugation. The absorbance was read at 412 nm and the enzyme activity was expressed in terms of µm/µg protein.

Glutathione-S-transferase (GST) assay

The method by Habiq *et al*., [12] was employed to estimate GST activity. A known quantity of fresh

tissue was homogenized in phosphate buffer (pH 6.5) and centrifuged at 10,000 rpm for 10 min. To an aliquot of made up supernatant, 1.0 ml of phosphate buffer, 0.1 ml of 1-chloro-2, 4-dinitrobenzene (30 mM CDNB in 95% ethanol) were added and incubated at 37 ^{0}C for 5 min. After incubation, 0.1 ml of 30 mM reduced glutathione was added. The increase in optical density was measured against that of a blank at 340 nm. The enzyme activity was calculated and expressed in µm/µg protein.

Ascorbate oxidase measurement

The sample was assayed for ascorbate oxidase (EC 1.10.3.3) by the method of Oberbacher and Vines [21]. Homogenized fresh tissue in phosphate buffer (pH 6.8) was centrifuged at 15,000 rpm for 15 min. The assay mixture consisted of 1.0 ml of 100 µM ascorbic acid, 1.0 ml of 500 µM phosphate buffer and 2.0 ml of sample. After incubation at 37^{0}C for 25 min the reaction was stopped by the addition of 0.4 mM DCPIP (dichlorophenol indophenols). The optical density was measured at 620 nm. The enzyme activity was calculated and expressed in µm/µg protein.

Polyphenol oxidase assay

The determination of polyphenol oxidase (PPO) was done by the method of Esterbauer *et al*., [11]. A known quantity of fresh tissue was homogenized in phosphate buffer (pH6) and to 2.0 ml of sample in 3.0 ml of phosphate buffer 1.0 ml of 0.01 M catechol was added and the change in absorbance was recorded at 412 nm, every 30 sec for 3 min. The enzyme activity was calculated and expressed in µm/µg protein.

Lipid peroxidase (LPX) measurement

Lipid peroxidase activity was measured by the method of Zhang and Kirkham [41]. A known quantity of fresh tissue was homogenized in 5 ml of 0.1% TCA, centrifuged at 7400 rpm and made up to a known volume with TCA. An aliquot was pipetted out and reaction mixture (0.5% Thiobarbituric acid solubilized in 20% TCA) was added, incubated in boiling water bath for 30 min and the absorbance were measured at 512 nm against a blank. The amount of lipid peroxidase was calculated and expressed in µm/µg protein.

RESULTS AND DISCUSSION

Non-enzymatic antioxidants

Plant phytochemicals expressed their antioxidant property by preventing the production of free radicals or by neutralizing/scavenging free radicals produced in the body or by reducing/ chelating the transition metal composition of foods [19, 23]. Therefore, prevention of the electron transport chain initiation step by scavenging various reactive species, such as free radicals, is considered to be an important antioxidant mode of action [8]. The DPPH free radical scavenging ability (175%) in mature leaves of *Bidens* was higher compared to 141% in younger leaves, 41% in stem and 25% in root (Fig. 1). These values were higher compared to reported leafy vegetables like *Bidens pilosa* and *Chenopodium album* of families Asteraceae and Chenopodiaceae, respectively [1]. TBA free radical scavenging ability (Fig. 2) were also found to be higher in mature leaves (0.016 mg/ g) of *B. biternata* compared to other plant parts such as young leaves (0.015 mg/g), stem (0.005 mg/g) and root (0.004 mg/g). Phenolic compounds can protect the human body from free radicals, whose formation is associated with the natural

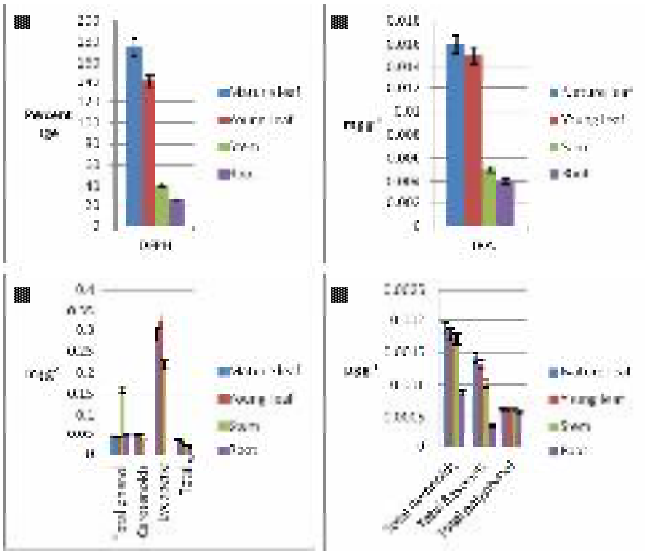

Fig. 1-4: DPPH free radical scavenging (1), TBA free radical scavenging (2), Antioxidant factors (3) and Total flavonoids, flavonols and polyphenols in levels *B. biternata* (4).

aerobic metabolism of cells. The antiradical activity of flavonoids and phenols are principally based on the structural relationship between different parts of their chemical structure [29]. Plant phenolics have the potential to function as antioxidants by trapping free radicals which then undergo coupling reactions leading to formation of colored polymeric or oligomeric products [14]. Data on total phenol content in different parts of *B. biternata* (Fig.3) suggested their higher accumulation in stem (0.16 mg/g) compared to mature leaves (0.042 mg/g), young leaves (0.04 mg/g) or root (0.045 mg/g). This was much higher than their accumulation in other wild green leafy vegetables like *Solanum macrocarpon* and *Amaranthus cruentus* [22].

The highest content of carotenoids (Fig. 3) in *B. Biternata* was recorded in young leaves (0.048 mg/g) compared to other green leafy vegetables like *Centella asiatica, Murayya koenigii, Trigonella foenum* and *Amaranthus* species [33]. Carotenes occurring in different chemical forms and their levels are altered during physiological and pathological conditions [28]. High amount of lycopene was present in young leaves (0.3221 mg/g) of *Bidens* (Fig 3) compared to other leafy vegetables [34] but mature leaves of *Bidens* had high amount of total proanthocyanidins (0.0321 mg/g) compared to other plant parts such as young

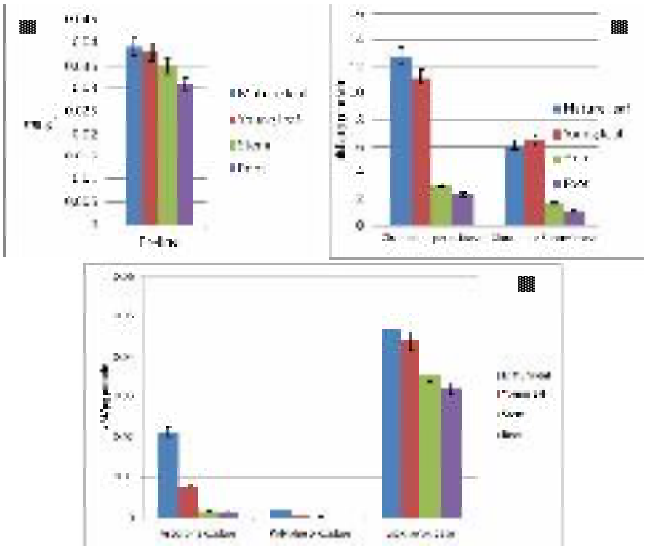

Fig. 5-7: Proline content (5), Glutathione peroxidase and Glutathione S transferase activity (6), Ascorbate oxidase, Polyphenol oxidase and Lipid peroxidase activity in *Bidens biternata* (7).

leaves, stem and root. Flavonoids have antioxidant activity and could therefore lower cellular oxidative stress, which has been implicated in the pathogenesis of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's diseases and amyotrophic lateral sclerosis [3]. Mature leaves of *Bidens* (Fig.4) were found to have higher amount of total flavonoids (TF), total polyphenol (TP) and total flavonols 0.001866 µg/g, 0.000613 µg/g, and 0.00142 µg/g, respectively compared to green leafy vegetables like *Bidens pilosa* and *Chenopodium album* [1]. The high flavonoid contents of leaves might be responsible for its medicinal properties. Proline which is a basic amino acid with important role under stress conditions was found to be present in higher amount in mature leaves (0.039 mg/g) of *B. biternata* compared to other plant parts such as young leaves, stem and root as shown in Fig 5.

Enzymatic antioxidants

Glutathione reduces the formation of toxic lipid peroxide and hydrogen peroxide in biological system by acting as substrate for glutathione peroxidase [6] and is an effective anticarcinogen and antioxidant [27, 16] by acting directly as free radical scavenger. It has been well established that glutathione peroxide, a selenium enzyme, plays a major role in regulating the concentration of hydrogen peroxide and wide variety of organic peroxides [31]. Synergistic action of glutathione with glutathione S- transferase detoxifies a wide range of xenobiotics and hence exhibit important functions in cellular protection [32]. *Bidens* mature leaves showed presence of high amount of glutathione peroxidase (12.826 µM/µg protein) and glutathione S transferase (6.149 µM/µg protein; Fig 6). Ascorbate oxidase is a key enzyme in fine control of hydrogen peroxide concentration is also present in high amount in mature leaves of *Bidens* (0.02139 µM/µg protein; Fig 7) compared to common reported leafy vegetables like *Amaranthus spinosus*, *Spergula arvensis* and *Spinacia oleracea* [4]. The polyphenol oxidase (PPO) comprises of catechol oxidase laccase and are important with regard to plant defense mechanism and palatability [10]. Mature leaves of *Bidens* has high amount of

polyphenol oxidase (0.00183 µM/µg protein; Fig. 7) compared to common reported leafy vegetables like *Amaranthus spinosus*, *Spergula arvensis* and *Spinacia oleracea* [4]. The amount of lipid peroxidase (LPX) was also high in mature leaves (0.047 µM/µg protein) of *Bidens* than other plant parts such as young leaves, stem and root as shown in Fig. 7. Mature leaves of this plant are also the rich sources of many nutrients and form a major category of vegetable groups that have been designated as 'nature anti-aging wonders' [33].

CONCLUSION

The present investigation analyzed the antioxidant potential of *B. biternata,* a wild leafy vegetable of Western Ghats region of Kerala. Present study revealed that this plant has numerous antioxidant factors in mature leaves compared to young leaves, stem or root that can help to destroy the free radicals, that damage the cells. The observations could enhance potential interest in this wild vegetables as nutraceutical and pharmacological candidate. This leafy vegetable like other non-conventional vegetables presently grow in the wild and deserves more scientific attention towards its domestication and planned cultivation.

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