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EFFECT OF HEATING RESEMBLING COOKING ON ANTIOXIDANT PROFILE AND PHYTOCHEMICAL CONSTITUENTS OF MALABAR SPINACH (*BASELLA ALBA*) FRUITS OF DIFFERENT MATURITY STAGES

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ABSTRACT

Malabar spinach (*Basella alba*) fruits of different maturity stages were compared for their antioxidant and pharmacognostic properties upon thermal treatments resembling cooking. It was found that the antioxidant capacities of two variants differed considerably with respect to their maturity stages. Red variant showed significantly higher antioxidant activity than green variant in all assay parameters, both in raw condition and also after thermal processing that resembled cooking. Among all thermal treatment process, antioxidant activity is maximum upon microwave treatment. This might be due to the radiation which breaks down the complex structure of molecules into smaller particle to enhance their antioxidant property. Enhanced activity of the fruits shown after thermal processing in water might be due to enhanced extraction of polyphenols compound like anthocyanins, flavonoid, carotene, xanthopylls which usually have less solubility in normal water but enhanced solubility in hot water which also depends on pH.

KEYWORDS

Malabar spinach, *Basella alba*, Antioxidant, Polyphenols and Cooking.

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INTRODUCTON

The rural people in India are still largely devoid of the modern healthcare due to their economic constraints, and depend mainly on naturally available plant materials to cure various health disorders[1]. The green leafy vegetables (GLV) are also considered as important protein sources as they are cheap, available locally and can be cooked fairly easily in the local households, and can easily be coined as 'poor man's vegetables[2]. They also help in reducing the malnutrition problem since they

provide essential minerals, vitamins and amino acids that are absent in the rice-based diet[3]. Taking the lead from such traditional knowledge, the modern research is paying attention on exploring plant sources to find pharmacophores that are beneficial to humans. It is now well known that GLVs' are good sources of natural antioxidants for the human diet, which provide protection against harmful free radicals and strongly associated with reduced risk of chronic diseases, such as cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts and age-related functional decline in addition to other health benefits[4]. A diet rich in fresh vegetables reduces the risk of cancer, cardiovascular diseases and mortality, mainly attributable to antioxidants such as ascorbic acid, vitamin E, carotenoids, lycopenes and polyphenols[5].

Malabar spinach (*Basella alba* L.) is an important green leafy vegetable found commonly in the tropical regions of the world and is extremely heat-tolerant[6]. It is a monotypic genus of the family Basellaceae[7]. The plant is sometimes used as a substitute for true spinach (i.e. *Spinacea Oleracea L.*) in cookery, especially in Africa and South-east Asia[8]. The plant has prominent ethno medical importance as the leaves and stem are rich in vitamin A and vitamin C along with flavonoids, saponins, carotenoids, many amino acids and organic acids. Young leaves and stem are eaten usually as salads, steamed with tofu and ginger[9]. Fruits of different maturity stages (i.e. red and green) are used to cure intestinal disorders, earache, carminative, itch, scabies, colic, sore throat, liver diseases and as a blood producer[10]. Regarding their use in cuisines, the boiled seeds are also used in the preparation of pulses, commonly known as 'Dahl', a high protein-rich food in Indian subcontinent[11].

Cooking of vegetables by heat treatments is common in the different societies and cultures that might bring some physico-chemical changes in them, sometimes towards betterment of their nutritive values[12,13]. Since red and green variants of the Malabar spinach fruits might contain different bioactives, the present study dealt with the *in vitro* antioxidant profile of them before and after thermal processing with water to decipher the effects on their antioxidant potentials. The extractions in the present study were done specifically in water in order to ascertain their pharmacognostic behavior during cooking. To our knowledge, it was one of the very few studies that dealt with human consumable water extractives of foodstuffs for their radical scavenging abilities, and probably the first with Malabar spinach fruits of different maturity stages. In this way, we would be able to know how different cooking methods could retain the most effectiveness of the substance for human consumption. The present study reports the achievement of the aim through some common *in vitro* antioxidant assays.

MATERIAL AND METHODS

Reagents and chemicals

2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), ABTS, were obtained from Sigma, USA. 2, 2′-Diphenyl-1-picryl hydrazyl (DPPH) were obtained from Himedia, India. Analytical grades of 2-Deoxy-D-ribose, thiobarbituric acid (TBA), ascorbic acid, gallic acid, Folin-Ciocalteau's solution, sodium hydroxide and sodium carbonate were obtained from Merck, India. All other reagents and chemicals used were of analytical grade procured from local sources. Deionized distilled water was used in the entire study.

Preparation of samples

Green and pink fruits of Malabar spinach were procured from local markets of Sodepure, Kolkata. The samples were checked for dirt or any visible damages prior to the study. Such samples were discarded. 5 gms each of the samples were suspended in 50 ml double distilled water, separately. The samples were extracted by the following procedures:

i) Heating at 80^{o}C for 10 mins (coded as - HT),

ii) Treating in a microwave oven at high power for 5 mins (coded as - MW), and

iii) Putting in a pressure cooker for 10 mins (coded as - PC).

To estimate the optimum antioxidant capacity, the samples were warmed separately at 60^{o}C for 10 mins, after suspended in 60% methanol in water. After extraction, all the samples were centrifuged at 8000 rpm for 10 mins. The clear supernatants were used for *in vitro* antioxidant and antimicrobial assays.

ABTS radical decolorization assay

The ABTS assay was performed using a previously described procedure[14]. The oxidant was generated by per sulfate oxidation of 2, 2′-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid. This solution was diluted with phosphate buffer (pH 7.4) until the absorbance reached 0.7 to 0.8 at 734 nm in a Systronics spectrophotometer (model - 2202). The oxidant solution was mixed with the sample solutions in such a way that total volume of the solution reached 1 ml. The absorbance was read at room temperature, 4 minutes after mixing. The concentration that causes a decrease in the absorbance of initial radicals by 50% is defined as IC_{50} of the samples. Gallic acid was used as positive control and comparing with its' IC_{50} and the results were expressed as Gallic acid equivalents (µg/gm fresh fruit).

DPPH radical decolorization assay

The DPPH assay was performed using a previously described procedure[14]. 1 ml DPPH solution (0.1 mM) was mixed with 0.5 ml sample solution and the decrease in absorbance of the mixture after 20 minutes of incubation in the dark was monitored at 517 nm in a Systronics spectrophotometer (model - 2202). The concentration that causes a decrease in the absorbance of initial radicals by 50% is defined as IC_{50} of the samples. Gallic acid was used as positive control and comparing with its' IC_{50} and the results were expressed as Gallic acid equivalents (µg/gm fresh fruit).

Estimation of total phenolics content

Total phenolics compound contents were determined by the Folin-Ciocalteau method[15]. The samples (0.5 ml) were mixed with Folin-Ciocalteau reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous sodium carbonate (4 ml, 1 M) was then added. The absorbance of the reaction mixture was then measured at 765 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid was used as standard. The results were expressed in terms of µg gallic acid equivalent/gm fresh fruit.

Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power of the samples was estimated with a previously described procedure[16]. Briefly, a maximum of 100 µl of extract solution or standard was mixed with 1.9 ml of FRAP reagent and incubated at 37^{o}C for 30 mins. FRAP reagent was prepared by mixing 50 ml of 0.1 M acetate buffer (pH 3.6), 5 ml of 10 ml TPTZ solution and 5 ml of 20 ml ferric chloride solution. After the stipulated time period, absorbance was measured at 593 nm in a UV-Vis spectrophotometer (model - Systronics 2202). Gallic acid is used as standard. Results are expressed as Gallic acid equivalents (GAE) in terms of µg gallic acid equivalent/gm fresh fruit.

Estimation of total flavonoids content

Total flavonoid content was determined with a previously described procedure with minor modifications[17]. Briefly 0.5 ml sample was mixed with 2 ml of distilled water and 0.15 ml of aqueous sodium nitrite solution (5% w/v), allowed to stand for 6 min, 0.15 ml aqueous aluminium trichloride solution (10% w/v) was added and allowed to stand again for 6 min, followed by addition of 2 ml of aqueous sodium hydroxide (4% w/v) solution. The final volume was made up to 5 ml by distilled water. The reaction mixture was mixed thoroughly and allowed to stand for another 15 min. The absorbance of the reaction mixture was then measured at 510 nm with a UV-Vis spectrophotometer (model - Systronics 2202). Quercetin was used as standard. The results are expressed in terms of quercetin equivalent (µg/gm fresh fruit).

Estimation of total monomeric anthocyanin content

Determination of monomeric anthocyanin content was conducted by pH-differential method[18]. Sample absorbance was read against a blank cell at 700 nm and 510 nm and at pH 1.0 and 4.5.The absorbance (A) of the sample was then calculated according the following formula:

$$A = (A_{510} - A_{700})_{\text{pH 1.0}} — (A_{510} - A_{700})_{\text{pH 4.5}}$$

Where A is the net absorbance of samples at the wavelengths mentioned in the subscript. The monomeric anthocyanin pigment content in the sample will be calculated according the following formula:

$$\text{Anthocyanin content (mg/L)} = (A \times MW \times DF \times 1000) / (\varepsilon \times l)$$

Where DF was dilution factor, MW was molecular weight of cyanidin-3-glucoside (449.2) and ε was molar absorptivity (26,900). The anthocyanin content in the sample extractive was converted into

cyanidin-3-glucoside content per gram of fresh fruit and reported.

Estimation of ascorbic acid content

Estimation of ascorbic acid content of the samples was estimated with a previously described method[16]. Briefly, a maximum of 100 µl sample (or standard) was mixed with 400 µl 5% metaphosphoric acid solution. Then another 500 µl of 10% metaphosphoric acid solution was added followed by 300 µl of citrate buffer (pH 4.15) and 300 µl of 2, 6-DCP-IP solution. Absorbance was read at 520 nm in a UV-Vis spectrophotometer (model - Systronics 2202) within 1 min.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging potentials of the samples were estimated with a previously described procedure[16]. Briefly, 10 mM each of $FeSO_4.7H_2O$, EDTA, 2-deoxy-D-ribose and H_2O_2 solutions were prepared in water. 0.2 ml each of above four and 0.2 ml sample and/or standard solution was mixed in a test tube and incubated at 37ºC for 90 mins. H_2O_2 solution was added last. After the incubation, 1 ml of 2.8% (w/v) aqueous TCA solution and 1 ml of 1% (w/v) aqueous TBA solution were added to the reaction mixture and kept at boiling water bath for 20 mins. Development of the pink chromophore was measured at 532 nm in a UV-Vis spectrophotometer (model - Systronics 2202). Gallic acid is used as standard. Results were expressed as Gallic acid equivalents (GAE) in terms of µg gallic acid equivalent/gm fresh fruit.

Inhibition of lipid peroxidation *in vitro*

A 10% (w/v) of fresh chicken liver homogenate was prepared using ice-cold KCl (0.15 M) in a Teflon tissue homogenizer and the test system containing homogenate of protein content was adjusted to 500µg/ml. In the control system to 1ml of tissue homogenate, the lipid peroxidation was initiated by the addition of 0.1ml of $FeSO_4$ (25 µg), 0.1ml of ascorbic acid (100 µM) and 0.1ml of KH_2PO_4 (10 mM). The volume was made up to 3 ml with distilled water and incubated at 37ºC for one hour. Then 1 ml of 5% TCA and 1 ml of 0.67% TBA in 50% acetic acid was added to this reaction mixture and the tubes were boiled for 20 mins in a boiling water bath. The extent of inhibition of lipid peroxidation was evaluated by the estimation of Thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance at 532nm. Gallic Acid was used as standard positive control and the results were expressed as gallic acid equivalents (GAE) in terms of µg gallic acid equivalent/gm fresh fruit.

Statistical analyses

Values were expressed as mean ± standard deviation of four replicates of each experiment. The analyses were done using the software - SPSS Statistics 17.0 (IBM Corporation).

RESULTS AND DISCUSSION

ABTS radical decolorization assay

The result of this assay is furnished in Figure No.1. It was observed that ABTS radical neutralization potential of red fruit was greater than the green. The antioxidant potential of both samples increased after thermal treatment and it was maximum after pressure cooking for red variant and after boiling for green variant.

DPPH radical decolorization assay

The result of this assay is given in Figure No.2. It was observed that DPPH radical neutralization potential of red fruit was greater than the green. The neutralization potential of both fruits increased after thermal treatment.

Estimation of total phenolics content

The result of this assay is furnished in Figure No.3. It was observed that total phenolics content in fresh red fruit was greater than green variant. The amount increased after thermal treatment and it was maximum after microwave treatment in case of red fruit. In case of green variant, the change was not so prominent with slight increase after boiling.

Ferric reducing antioxidant power (FRAP)

The result of this assay is shown in Figure No.4. It was observed that the red fruit showed greater reducing power than green variant. The reducing power of both fruits increased after thermal treatment and it was maximum after microwave irradiation. Reducing power of a sample provide significant reflection of the antioxidant activity *in vitro*. Compounds possessing reducing power are usually electron donors and can reduce the oxidized intermediates of lipid peroxidation process, ultimately minimizing adverse health condition. FRAP indicated greater amounts of electron donating bioactives in red fruits than green ones.

Estimation of total flavonoids content

The result of this assay is depicted in Figure No.5. The results indicated that flavonoids content in fresh red fruit was greater than green fruit although the difference is within standard deviation limit. The levels were almost constant after thermal treatment, clearly indicating sustenance of the bioactive even after harsh actions.

Estimation of total monomeric anthocyanin content

Monomeric anthocyanin contents of the fruits are given in Figure No.6. The assay indicated that the amount of anthocyanin in red fruit was far greater than green variant. However, the level of anthocyanin in red fruit significantly decreased after microwave treatment and pressure cooking. In case of green fruit, the amount increased after thermal treatments.

From the above results, it was quite clear that the radical scavenging activities of the fruit extracts were mainly due to the presence of anthocyanins. Relatively greater gallic acid equivalent for red fruits in case of DPPH radical scavenging (*ca*. 1300 µg/gm sample as in Figure No.2) in comparison to its' ABTS radical scavenging ability (*ca*. 490 µg/gm sample as in Figure No.1) substantiated the above fact. The radical scavenging activities like ABTS assay and DPPH assay is generally used to indicate antioxidant potential of plant extracts with different polarities[14].

However, ABTS assay is performed in aqueous medium, whereas DPPH assay is based on non-aqueous less polar medium (i.e. alcohol). Since monomeric anthocyanins are soluble in less polar solvents, they have endowed better results in case of DPPH radical scavenging assay as observed in the present study.

Estimation of ascorbic acid content

Ascorbic acid contents of the fruits are given in Figure No.7. The differences in the contents were negligible in both fruits. However, after thermal treatments, ascorbic acid contents in the green fruits reduced significantly, whereas the changes after thermal treatment in case of red fruits were within standard deviation range.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activities of the fruits are given in Figure No.8. It was observed that hydroxyl radical neutralization potential of red fruits was greater than green variant. The neutralization potential of both fruits increased after thermal treatment and it was maximum after microwave treatment.

Inhibition of lipid peroxidation *in vitro*

Lipid peroxidation inhibition assay results indicated that raw red fruit showed much greater reducing power than green variants (Figure No.9). The protecting power of both fruits increased after thermal treatments and it was maximum after microwave treatment.

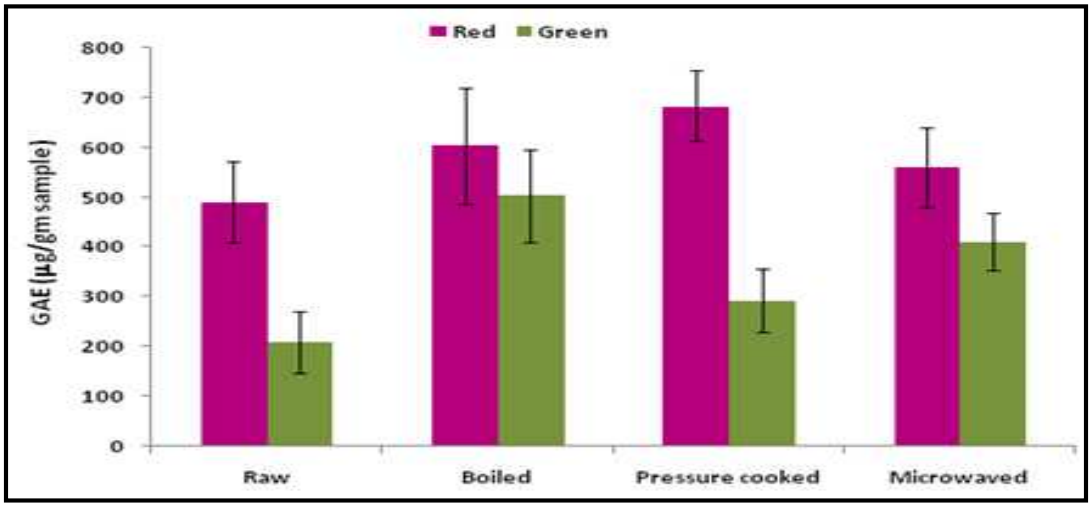

Figure No.1: Comparative ABTS radical scavenging potential of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking [GAE = Gallic acid equivalent (µg gallic acid/gm sample)]

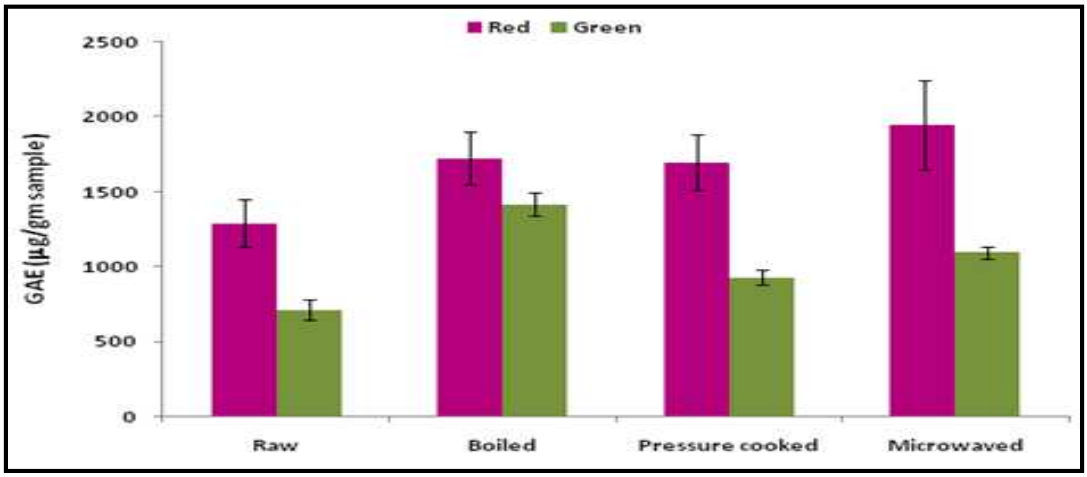

Figure No.2: Comparative DPPH radical scavenging potential of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking [GAE = Gallic acid equivalent (µg gallic acid/gm sample)]

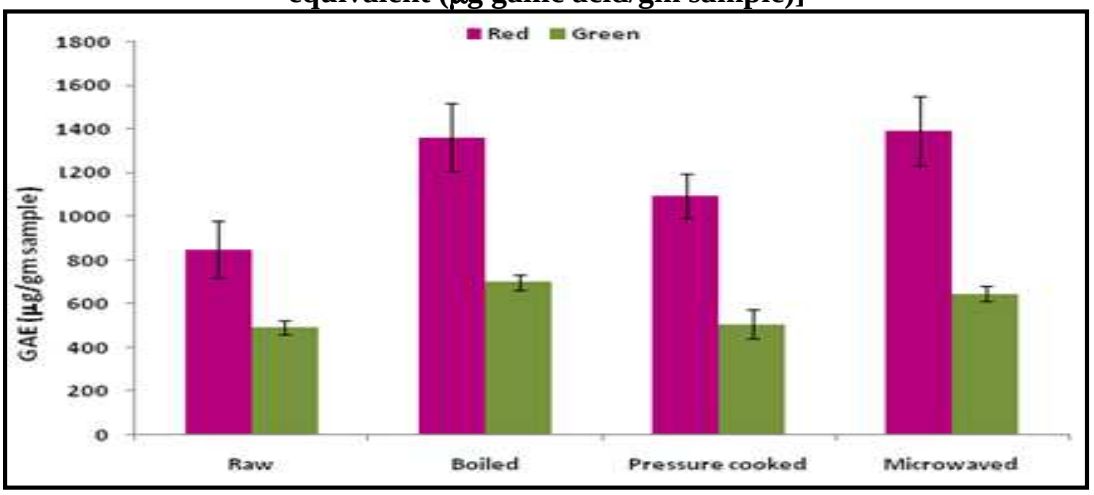

Figure No.3: Comparative total Phenolic contents of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking [GAE = Gallic acid equivalent (µg gallic acid/gm sample)]

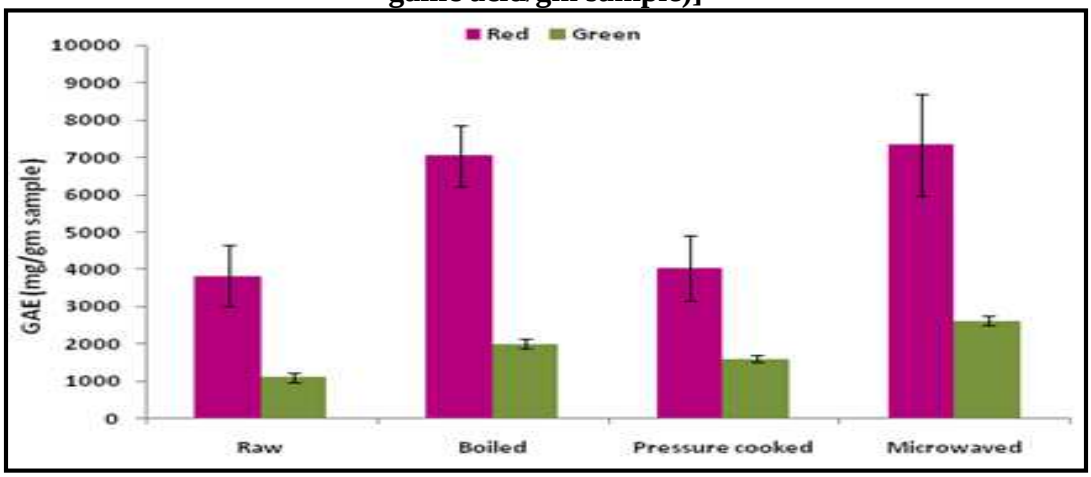

Figure No.4: Comparative ferric reducing antioxidant power (FRAP) of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking [GAE = Gallic acid equivalent (µg gallic acid/gm sample)]

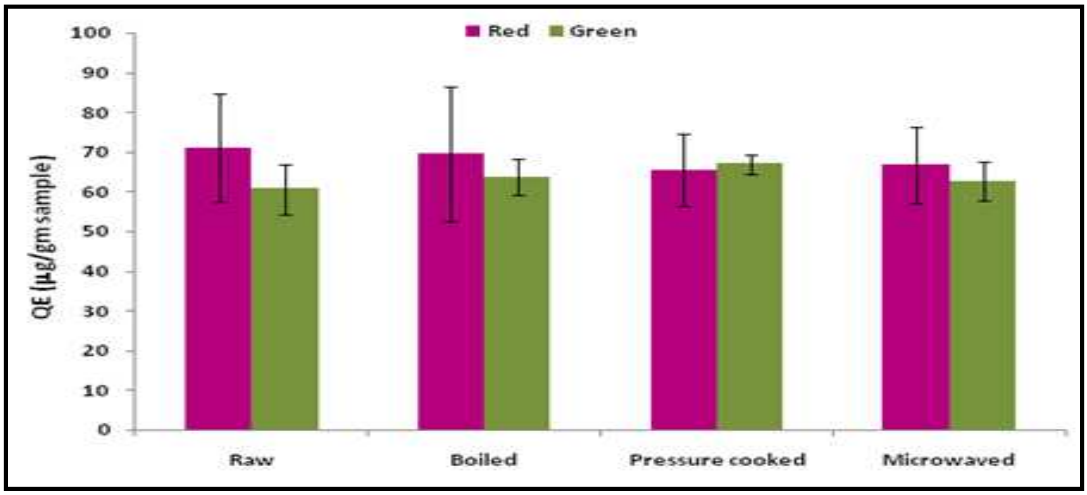

Figure No.5: Comparative flavonoids contents of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking [QE = quercetin equivalent (µg quercetin/gm sample)]

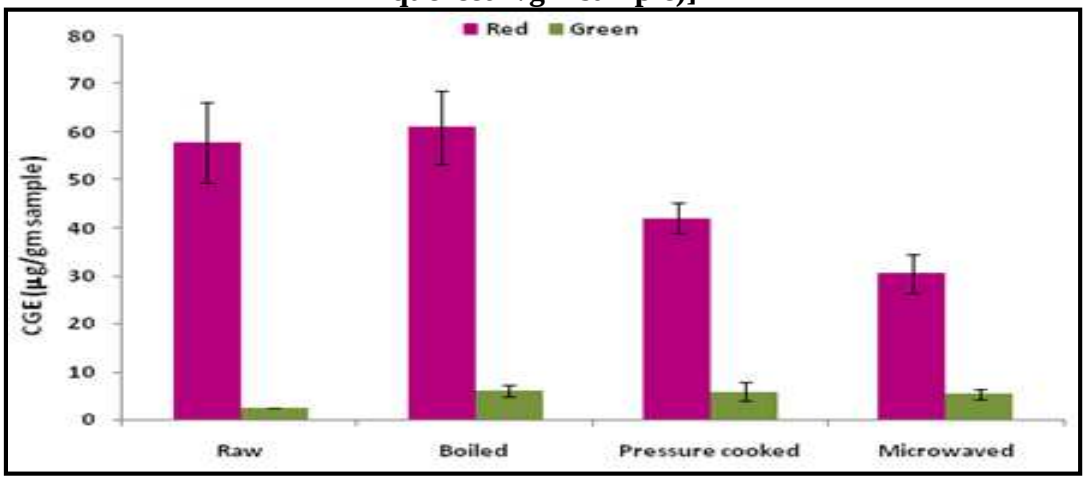

Figure No.6: Comparative monomeric anthocyanin contents of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking [CGE = cyanidine-3-*O*-glucoside equivalent (µg/gm sample)]

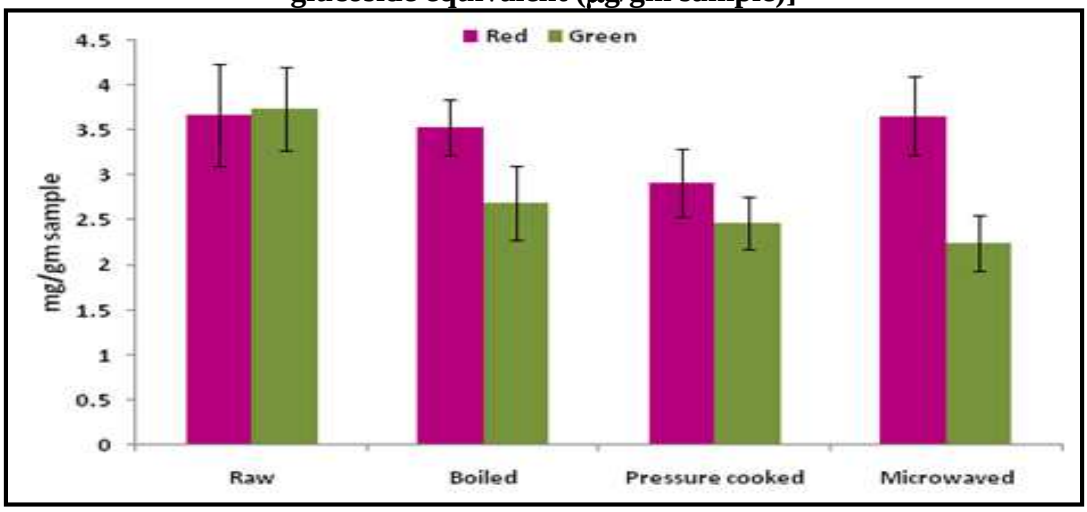

Figure No.7: Comparative ascorbic acid contents of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking

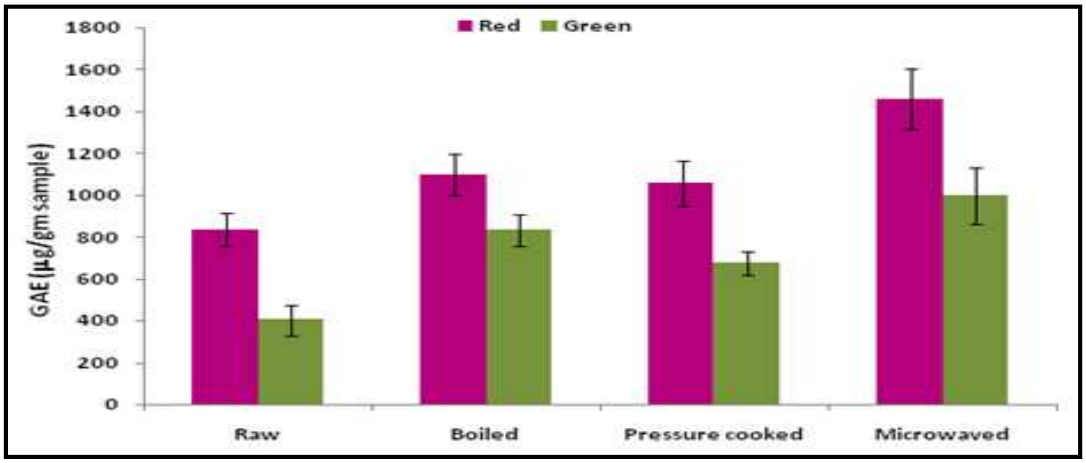

Figure No.8: Comparative hydroxyl radical scavenging activities of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking [GAE = Gallic acid equivalent (µg gallic acid/gm sample)]

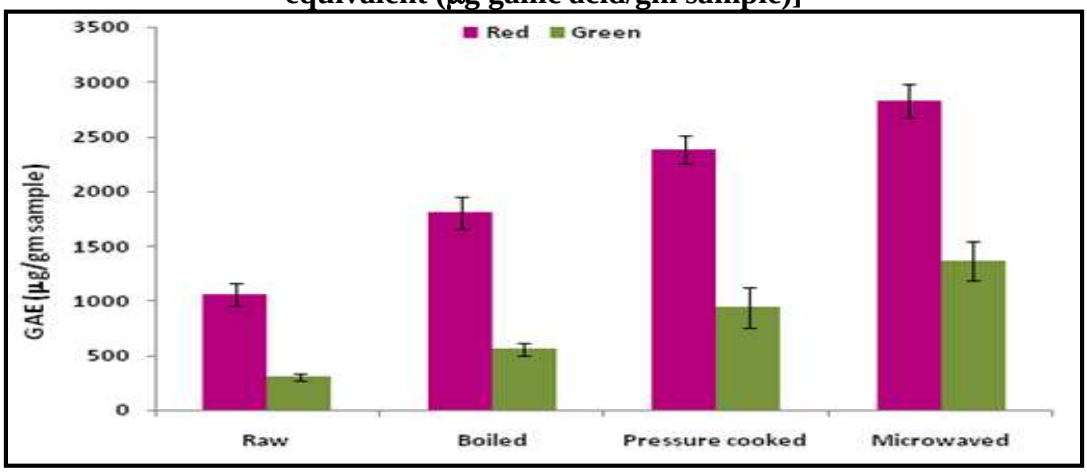

Figure No.9: Comparative inhibition of lipid peroxidation activities of fresh red and green fruits of Malabar spinach (*Basella alba*) before and after thermal treatments resembling cooking [GAE = Gallic acid equivalent (µg gallic acid/gm sample)]

CONCLUSION

The major conclusions arising out of this research was that the antioxidant capacities of the two fruit variants differ considerably with respect to their maturity stages. Enhanced activities of the fruits shown after thermal processing in water might be due to enhanced extraction of polyphenolic bioactives like flavonoids and anthocyanins that usually have less solubility in normal water but enhanced solubility in hot water. There was a strong positive correlation between the antioxidant capacities and the total phenolics contents, which indicated that the antioxidant activities of the fruits were mainly due to the polyphenolics extracted in the water, both before and after thermal processing.

The enhanced DPPH radical scavenging activity of two fruits may be due to greater extraction of anthocyanins in non-polar solvent. However presence of other water soluble bioactives like ascorbic acid produced excellent effects in the assay protocols like ABTS radical scavenging activity, inhibition of lipid peroxidation, reducing power activity, hydroxyl radical scavenging activity, indicating that the polar bioactives are also important in the beneficial health effects produced by the fruits.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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