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ORIGINAL PAPER

Studies on Indian Green Leafy Vegetables for Their Antioxidant Activity

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Abstract To identify the potential of green leafy vegetables (GLV) as antioxidants, methanolic extracts of *Amaranthus* sp., *Centella asiatica*, *Murraya koenigii* and *Trigonella foenum graecum* were studied for their antioxidant activity in different systems at multiple concentrations. Total antioxidant activity assessed by phosphomolybdenum method, free radical scavenging activity by 1,1-diphenly-2-picryl hydrazyl (DPPH), reducing power and ferrous ion chelating activity were determined. The GLV were analyzed for ascorbic acid, total and β-carotene and total polyphenol contents. The ascorbic acid, total carotene, β-carotene and total phenolic content (tannic acid equivalents) of the GLV ranged between 15.18–101.36, 34.78–64.51, 4.23–8.84 and 150.0–387.50 mg/100 g GLV, respectively. The extracts were found to have significantly different levels of antioxidant activities in the systems tested. The total antioxidant activity was highest in *Murraya koenigii* (2,691.78 μmol of ascorbic acid/g sample) and least in *Centella asiatica* (623.78 μmol of ascorbic acid/g sample). The extract concentration causing 50% inhibition of DPPH (IC_{50}) was determined (*M. koenigii*<*C.asiatica*< *Amaranthus* sp.<*T. graecum*). The maximum DPPH scavenging activity and reducing power was exhibited by *Murraya koenigii*. Multiple regression analysis showed that the relationship of total antioxidant activity, free radical scavenging activity, and reducing power with polyphenol and total and β-carotene was highly significant.

Keywords Antioxidant activity · Ascorbic acid · β-Carotene · Free radical scavenging activity · Green leafy vegetables · Polyphenol · Reducing power

Introduction

In present times, it is believed that the regular consumption of dietary antioxidants may reduce the risk of several serious diseases [1]. Regular consumption of fruits and vegetables has always been associated with health benefits, but their mechanism has become clear only in the recent decades. Fruits and vegetables contain a wide variety of biologically active, non-nutritive compounds known as phytochemicals. These phytochemicals impart health benefits beyond basic nutrition [2]. The role of food constituents as essential nutrients to one of preventing or delaying the premature onset of chronic disease late in life has now been generally accepted.

Researchers have estimated that every serving increase in fruit and vegetable consumption reduces the risk of cancer by 15%, cardiovascular disease by 30% and mortality by any cause by 20%. This has been confirmed by epidemiological studies [3, 4]. This is often attributed to different antioxidant components in fruits and vegetables such as ascorbic acid, vitamin E, carotenoids, lycopenes, polyphenols and other phytochemicals [5]. Studies have shown these antioxidant components to lower the risk of several diseases [6–9]. Special emphasis has been on antioxidant activity of polyphenols, which have many potent biological properties and beneficial effects on human health [10–12].

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Green leafy vegetables (GLV) are rich sources of many nutrients and form a major category of vegetable groups that have been designated as 'natures anti-aging wonders'. Gupta et al. [13] have reported that several GLV are rich sources of antioxidant vitamins. Brahmi (*Centella asiatica*), curry leaf (*Murraya koenigii*), fenugreek (*Trigonella foenum graecum*) and keerae (*Amaranthus* sp.) are used in Indian culinary and are also known for their medicinal value. Therefore, the objective of the present study was to determine the antioxidant activity of these GLV using *in vitro* models and their correlation with their total polyphenol, ascorbic acid, total and β-carotene contents.

Materials and Methods

Plants and Extract Preparation

Four GLV namely *Centella asiatica*, *Murraya koenigii*, *Trigonella foenum graecum* and *Amaranthus* sp. were procured from the local market of Mysore city, India. The edible portion was separated and washed under running water followed by double glass-distilled water. They were drained completely, dried over filter paper and analyzed for ascorbic acid, total and β-carotene, polyphenols and antioxidant activities. All chemicals used for the study were of analytical grade and double glass-distilled water was used for entire analysis.

One gram of GLV in 30 ml of methanol was homogenized in a blender. After recovery of the homogenate, 15 ml methanol was used to wash the blender and then pooled with the first homogenate. The mixture was centrifuged at 4,500 rpm for 15 min at room temperature (27 °C). Supernatants were filtered using Blue Ribbon No. 589 filter paper and volume was made up to 50 ml with methanol [14]. The extracts were prepared in duplicate and all analysis was carried out in triplicates.

Determination of Antioxidant Vitamins and Total Polyphenols

15 g of fresh GLV was blended with 3% metaphosphoric acid, made up to 100 ml and filtered. 5 ml of metaphosphoric acid extract was taken and titrated with 2,6-dichlorophenol indophenol dye to get a pink end-point that persisted for at least 15 s. The dye factor was calculated by titrating the standard ascorbic acid against the dye. Ascorbic acid in the GLV was calculated using the titre value and the dye factor taking into account the suitable dilutions that were carried out. Total carotene was extracted in acetone, transferred to petroleum ether phase and read colourimetrically at 452 nm using petroleum ether for baseline correction. β-carotene was separated by column chromatography using neutral aluminium oxide as the adsorbent in a 10 cm length adsorbent column. β-carotene, which moves down the column prior to all the pigments, is collected till the desired pigments have moved off the column and the eluent is colorless. Eluent is made up to a known volume and the intensity of color is measured in a spectrophotometer (Systronics 108, Ahmedabad, India) at 452 nm using 3% acetone in petroleum ether as blank [15]. The entire analysis was carried out in four replicates and the average values are reported on as-is basis.

A sample of methanolic extract (0.2 ml) was mixed with 1 ml of Folin–Ciocalteau reagent (ten fold dilutions) and 0.8 ml of 2% Na_2CO_3. The volume was made to 10 ml with 4:6 water/methanol as the solvent. This was allowed to stand for 30 min and absorbance read at 740 nm using a specrotphotometer (Systronics 108, Ahmedabad, India) [16]. Concentration was calculated using tannic acid as standard and the results were expressed as mg tannic acid equivalents/100 g sample.

Analysis of Antioxidant Activity

Total Antioxidant Activity by Phosphomolybdenum Method This assay is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of green phosphate/Mo(V) complex at acidic pH [17]. An aliquot of 0.1 ml methanolic extract was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95 °C for 90 min. After the samples had cooled to room temperature (27 °C), the absorbance was measured at 695 nm against blank. A typical blank contained 1 ml of reagent solution and an appropriate volume of the same solvent used for the sample and was incubated under the same conditions as the rest of the samples. For samples of unknown composition, water-soluble antioxidant capacities were expressed as equivalents of ascorbic acid (μmol/g of sample).

Free Radical Scavenging Activity Using 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) DPPH, a commercial oxidizing radical is reduced by antioxidants. The disappearance of the DPPH radical absorption at a characteristic wavelength is monitored by decrease in optical density [18]. Different concentrations of the methanolic extract were taken in different test tubes and the volume made to 1 ml with methanol. 4 ml of 0.1 mM methanolic solution of DPPH was added. The tubes were shaken vigorously and allowed to stand for 20 min at room temperature. A control was prepared as above without any sample and methanol was used for base line correction. Changes in absorbance of samples were measured at 517 nm. Free radical scavenging

activity was expressed as inhibition percentage and was calculated using the following formula:

$$\%\ \text{Free radical scavenging activity} = \frac{(\text{Control OD} - \text{Sample OD})}{\text{Control OD}} \times 100$$

Reducing Power In this assay, Fe^{3+}/ferricyanide complex is reduced to the ferrous form by antioxidants. The Fe^{2+} formed is monitored by measuring the formation of Perl's Prussian blue at 700 nm [19]. Different amounts of methanolic extracts in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. About 2.5 ml of 10% TCA was added and centrifuged at 3,000 rpm for 10 min. Upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.1% $FeCl_3$ (0.5 ml), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Ferrous Ion Chelating Capacity This method is based on the principle of the Fe^{2+}-chelating ability of the antioxidant extract by measuring the ferrous iron-ferrozine complex formed at 562 nm [20]. To different concentrations of sample extracts (10–50 mg) were added 0.1 ml of 2 mM $FeCl_2$. 4 H_2O, 0.2 ml of 5 mM ferrozine and methanol to make up the volume to 5 ml. The solutions were mixed and reacted for 10 min. The absorbance at 562 nm was measured; a lower absorbance indicated a higher ferrous iron chelating capacity, which was calculated as follows:

$$\text{Ferrous ion chelating capacity}\ \% = 1 - \left(\frac{A_{\text{sample 562 nm}}}{A_{\text{control 562 nm}}}\right) \times 100$$

Statistical Analysis

The data were subjected to statistical analysis using ANOVA and Duncan's multiple range tests to verify the differences between the antioxidant activities of GLV, determined by different methods. Multiple regression analysis was done to evaluate the relationship between antioxidant activities and the antioxidant components (polyphenols, ascorbic acid, total and β-carotene).

Results and Discussion

Antioxidant Vitamins and Total Polyphenols

The moisture content of the green leafy vegetables was found to be highest in *T. graecum* (87.92%) followed by *Amaranthus* sp. (86.54%), *C. asiatica* (85.67%) and lastly *M.koenigii* (77.12%). GLV are known to be rich sources of ascorbic acid. The ascorbic acid content of the GLV ranged between 15.18 mg/100 g for *C. asiatica* to 101.36 mg/100 g for *T. graecum*. GLV are also good sources of total carotene and β-carotene (precursor of vitamin A). *M. koenigii* was found to be the richest source of total and β-carotene (64.51 and 8.85 mg/100 g, respectively) among the GLV analyzed followed by *C. asiatica*>*Amaranthus* sp.>*T. graecum*, respectively (Table 1). Highly significant differences were observed in the ascorbic acid, total and β-carotene content of the GLV (P=0.000).

Phenolics are aromatic secondary plant metabolites widely spread throughout the plant kingdom and associated with colour, sensory qualities and nutritional and antioxidant properties of food. The antioxidant activity of phenolic compounds is mainly due to redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, heavy metal chelators and hydroxy radical quenchers [21]. The total polyphenol content of four GLV is presented in Table 1. The GLV were found to have varying levels of polyphenols, ranging from 150 mg tannic acid/100 g for *C. asiatica* to 387 mg of tannic acid/100 g for *M. koenigii*. *T. graecum* and *Amaranthus* sp. had similar amounts of total polyphenols (158.33 mg of tannic acid/100 g). Kaur and Kapoor [22] reported the total phenolic content of *Trigonella foenum graecum* to be 217.5 mg of catechol/100 g of fresh vegetable. Salvatore et al. [23] in their study on antioxidant characterization of some Sicilian edible greens stated that the frequently consumed greens in the Mediterranean areas were very rich in antioxidants such as flavonoids and carotenoids. The total polyphenol content of some common Indian leafy vegetables was found to be in the range of 5–69.5 mg of tannic acid/g of extract [24]. Therefore it can be said that the total polyphenol content of vegetables varies widely depending on the variety of vegetable and a comparison is difficult, as different standard compounds have been used for their analysis. ANOVA revealed highly significant differences in the total polyphenol content of the GLV analyzed (P=0.000).

Antioxidant Activity

The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid [17]. The results are presented in Table 1. Among the GLV, total antioxidant capacity was found to be high in *M. koenigii* (2,691.78 μmol of ascorbic acid/g of sample) and low in *C. asiatica* (623.78 μmol of ascorbic acid/g of sample). In *T. graecum* and *Amaranthus* sp., the total antioxidant capacity was found to be 1,294.78 and 908.09 μmol of ascorbic acid/g of sample, respectively. The antioxidant capacities were in the order of *M. koenigii*>

Table 1 Moisture, ascorbic acid, total and β-carotene, total polyphenol content and total antioxidant activity of green leafy vegetables

Green leafy vegetable	Moisture (%)	Ascorbic acid (mg/100 g)	Total carotene (mg/100 g)	β-Carotene (mg/100 g)	Total polyphenol[a]	Total antioxidant activity (%)[b]
Centella asiatica	85.67±0.59	15.18±0.80	36.40±0.01	5.33±0.04	150.00±0.00	623.78±34.46
Murayya koenigii	77.12±0.57	29.31±0.00	64.51±0.00	8.84±0.02	387.50±30.62	2,691.78±91.73
Trigonella foenum graecum	87.92±0.07	101.36±0.00	34.78±0.01	4.23±0.14	158.33±20.41	1,292.28±92.86
Amaranthus sp.	86.54±0.59	64.95±0.00	41.47±0.01	5.31±0.35	158.33±20.41	908.09±45.80
F-ratio	186.61[c]	18,683.06[c]	10,985.37[c]	230.06[c]	182.49[c]	–

[a] Expressed as mg of tannic acid/100 g sample
[b] Expressed as μmol of ascorbic acid/g of sample
[c] Statistically significant at 5% level

T. graecum>*Amaranthus* sp.>*C. asiatica*. There are very few studies on the antioxidant capacity of vegetable measured by the phosphomolybdenum method. Dasgupta and De [25] studied the antioxidant capacity of three varieties of *Piper betle* leaves and reported that the Kauri variety had higher total antioxidant capacity (equivalents of gallic acid) in comparison with tea. ANOVA showed highly significant differences in the total antioxidant activity of the GLV (P=0.000).

DPPH· is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The methodology involves reaction of specific compounds or extracts with DPPH· in methanol solution. In the presence of hydrogen donors, DPPH· is reduced and a free radical is formed from the scavenger. The reaction of DPPH is monitored by the decrease of the absorbance of its radical at 517 nm, but upon reduction by an antioxidant, the absorption disappears [26]. DPPH radical scavenging activity of the GLV extracts at varying concentrations (4–20 mg/ml) were measured and the results are depicted in Fig. 1. All the GLV studied showed appreciable free radical scavenging activities. *M. koenigii* had the strongest radical scavenging activity compared to the other GLV (83.44%).

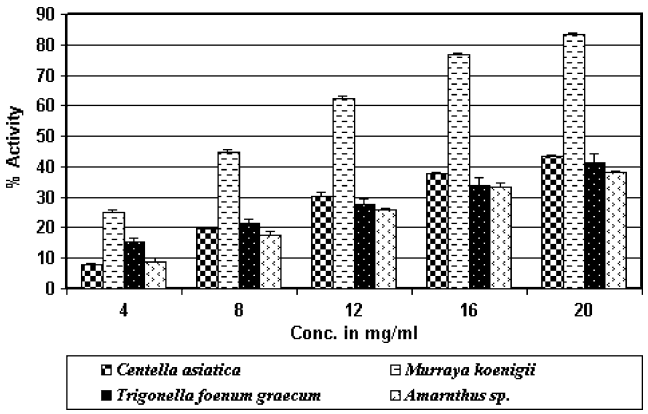

Fig. 1 Free radical scavenging activity of various amounts of extracts of GLV determined by DPPH method (values are mean±SD of six determinations)

However, *Amaranthus* sp. showed the lowest free radical scavenging activity (38.18%). A dose-response relationship was found in the DPPH radical scavenging activity; the activity increased with an increase in the concentration of each individual GLV. At concentrations from 4 to 20 mg/ml, *M. koenigii* had free radical scavenging activity ranging from 25.13% to 83.44%, *T. graecum* from 15.23% to 41.46%, *C. asiatica* from 7.86% to 43.61% and *Amaranthus* sp. from 8.71% to 38.18%. ANOVA revealed significant differences in the free radical scavenging activity among the fresh GLV (P=0.013).

The IC_{50} value, a measure of the extract concentration which is required for 50% inhibition of the free radical DPPH, was determined (IC_{50}). The IC_{50} values for *M. koenigii* was 9.62 mg/ml, for *T. graecum* was 27.69 mg/ml, for *C. asiatica* was 19.89 mg/ml and for *Amaranthus* sp. was 27.27 mg/ml. Shyamala et al., [24] reported free radical scavenging activities of >70% for three green leafy vegetables namely *Spinacia oleracea*, *Coriandrum sativum* and *Alternanthera sessilis* at 100 ppm levels. Chu et al., [27] also reported similar free radical scavenging activity in various GLV. The involvement of free radical, especially their increased production, appears to be a feature of most, if not all human diseases, including cardiovascular disease and cancer. Therefore, such dietary antioxidants from fresh GLV may be particularly important in fighting these diseases by conferring protection against free radical damage to cellular DNA, lipids and proteins.

The antioxidant effect exponentially increases as a function of the development of the reducing power, indicating that the antioxidant properties are concomitant with the development of reducing power [19]. Okuda et al., [28] have reported that the reducing power of tannins from medicinal plants prevents liver injury by inhibiting formation of lipid peroxides. Reductones are believed not only to react directly with peroxides but also prevent peroxide formation by reacting with certain precursors. Among the GLV, *M. koenigii* had the highest reducing power as estimated by using the potassium hexacyanoferrate method (Fig. 2). At concentrations of 2–10 mg/ml, reducing power

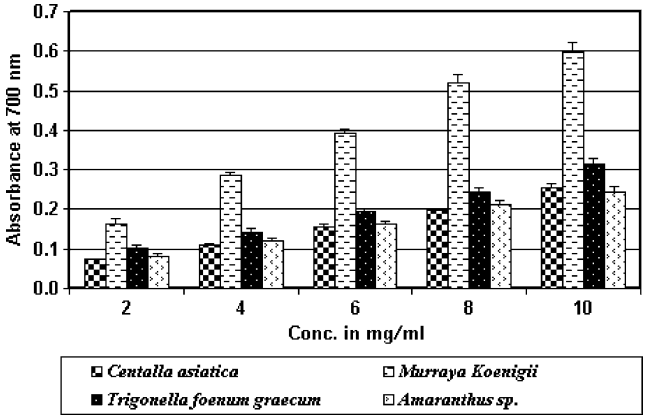

Fig. 2 Reducing powers of various amounts of extracts of GLV (values are mean±SD of six determinations)

of GLV followed the order *M. koenigii*>*T. graecum*> *Amaranthus* sp.>*C. asiatica*. The reducing power of the methanolic extracts of GLV increased with increasing concentrations of the extracts. ANOVA revealed significant differences in the reducing power of the GLV (P=0.011).

Ferrous ion, commonly found in food systems, is well known as an effective pro-oxidant [20]. The purpose of the test of ferrous ion chelating activity was to determine the capacity of GLV to bind the ferrous ion catalyzing oxidation. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour of the complex decreases. A measure of the rate of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator [29]. In this assay, the extracts of GLV interfered with the formation of a ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ions before ferrozine. As can be seen from Fig. 3, *T. graecum* had the highest ferrous ion chelating activity (38.14–76.95%) at concentrations of 10–

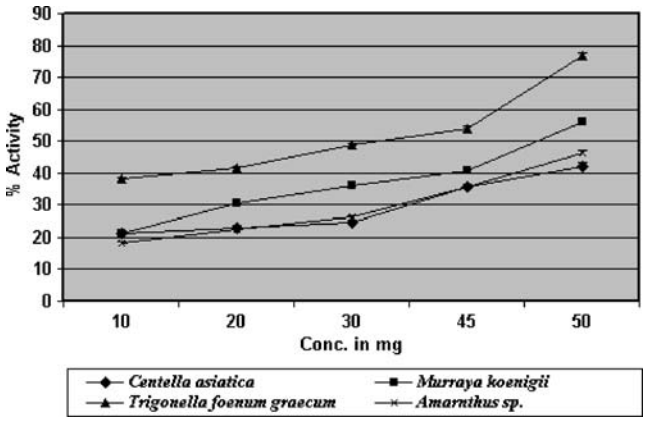

Fig. 3 Chelating effect of ferrous ions by various amounts of extracts of GLV (values are mean±SD of six determinations)

Table 2 Significant differences between antioxidant activities of fresh GLV on application of Duncan's multiple range tests

Green Leafy Vegetable	Total antioxidant activity	Free radical scavenging activity (DPPH)	Reducing power	Ferrous ion chelating activity
A Vs. B	–[a]	–[a]	–[a]	ns
A Vs. C	–[a]	ns	ns	ns
A Vs. D	–[a]	ns	ns	ns
B Vs. C	–[a]	–[a]	–[a]	ns
B Vs. D	–[a]	–[a]	–[a]	–[a]
C Vs. D	–[a]	ns	ns	–[a]

A *Centella asiatica*, B *Murraya koenigii*, C *Trigonella foenum graecum*, D *Amaranthus* sp., *ns* not significant
[a] Significantly different at 5% level

50 mg followed by *M. koenigii* (21.23–55.86%), *Amaranthus* sp. (18.13–46.09%) and *C. asiatica* (21.22–42.18%). A dose dependent relationship was observed in the ferrous ion chelating activity of the green leafy vegetables. ANOVA revealed significant differences in the ferrous ion chelating activity of GLV (P=0.035).

Green leafy vegetables are generally consumed in the cooked form apart from the salads. Therefore there is a need to assess the changes that occur in the antioxidant activity on cooking. Few works have reported the effect of cooking on antioxidant activity of vegetables. Oboh [30] studied the effect of blanching on Nigerian GLV and concluded that the blanching of vegetables reduces their antioxidant properties drastically. Similarly Tarwadi and Agte [31] have reported that uncooked GLV had significantly higher values of TBARS in comparison to the cooked vegetables. A reduction in total phenols and total antioxidant activity in vegetables and culinary herbs by different processing and storage technologies has been reported elsewhere [32, 33]. In another study by Gitanjali et al., [34], a significant reduction was found in the total polyphenol content and antioxidant activity in shallow fried spinach, amaranth and potato, while it increased in shallow fried carrot, tomato and brinjal. However, an overall

Table 3 Regression coefficients (r^2 value) for antioxidant activity analyzed by different methods and the relative influence of antioxidant components in GLV

Methods of analysis of antioxidant activity	Polyphenol	Ascorbic acid	Total carotene	β-carotene
Total antioxidant activity	0.93	0.01	0.80	0.71
Free radical scavenging activity	0.98	0.20	0.87	0.91
Reducing power	0.97	0.07	0.82	0.80
Ferrous ion chelating activity	0.00	0.62	0.02	0.06

increase in the trolox equivalent antioxidant activity, total radical-trapping antioxidant potential and ferric reducing antioxidant power has been reported in differently cooked carrots, courgettes and broccoli and the authors cite that matrix softening and increased extractability of compounds could be the reasons for this increase [35]. Therefore the notion that processed vegetables offer lower nutritional quality can be defied and it can also be said that for each vegetable a different cooking method would be preferred to preserve the phytochemical properties.

Analysis of Variance and Multiple Regression Analysis

The data was subjected to statistical analysis using ANOVA and Duncan's multiple range tests to check for differences between the antioxidant activities of GLV in different systems. The results are presented in Table 2. No significant differences were observed between *C. asiatica* and *M. koenigii* in the ferrous ion chelating activity. The differences between the antioxidant activities of *C. asiatica* and *T. graecum*; *C. asiatica* and *Amaranthus* sp. were not significant in all the analyzed methods of antioxidant activity except for the total antioxidant activity. The differences between the antioxidant activities of *T. graecum* and *Amaranthus* sp. were not significant when free radical scavenging activity and reducing power were considered. The antioxidant activity of GLV analyzed by all the other methods was found to be statistically significant as by Duncan's multiple range tests.

Multiple regression analysis was done to test the relationship between antioxidant activity in different systems and the antioxidant components (polyphenols, ascorbic acid, total and β-carotene). The results are presented in Table 3. The relationship between free radical scavenging activity and polyphenol, total and β-carotene was found to be highly significant. Similar observations were seen for total antioxidant activity and reducing power wherein the relationship between the antioxidant activity and all the antioxidant compounds other than ascorbic acid was found to be highly significant. No significant relationship was observed between the ferrous ion chelating activity and the antioxidant components with the exception of ascorbic acid. Stratil et al., [36] in their study have concluded that the phenolic contents and the antioxidant activities of vegetables correlated very well with the methods used for analysis. Very high antioxidant activities were found in intensely coloured vegetables (red cabbage, red onion, etc.,) and the values were very low in watery vegetables such as potato, marrow and cucumber. Conversely, Hassimotto et al., [37] have reported no relationship between total phenolics content, vitamin C and antioxidant activity suggesting that the antioxidant activity is a result of a combination of different compounds having synergistic and antagonistic effects.

It can be seen that GLV exhibited antioxidant activities at varied levels in different systems. The results and inferences from different methods may differ substantially because each complex chemical reaction generates unique values. Therefore it is difficult to compare the antioxidant capacity of different foods. Hence, an index needs to be developed, which does not represent a specific antioxidant property but can rank the antioxidant capacity of the foods.

Conclusions

Among the GLV analyzed, *Murraya koenigii* exhibited the highest antioxidant activity as analyzed by different methods. These observations enhance potential interest in the GLV for improving the efficacy of different products as neutraceutical and pharmacological products. The consumption of these vegetables may play a role in preventing human disease in which free radicals are involved, such as cancer, cardiovascular diseases and aging. However, further investigations on individual components, their *in vivo* antioxidant activity, and the different antioxidant mechanisms are warranted.

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