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Evaluation of *in vitro* Antioxidant Potential of Red Amaranth (*Amaranthus tricolor*) and Green Amaranth (*Amaranthus viridis*) leaves extracted at different temperatures and pH

Purbasha Pramanik, Ratna Bhattacharjee and *Sauryya Bhattacharyya

Department of Food & Nutrition, Ramakrishna Vivekananda Mission Sarada Ma Girls' College, Barasat, Kolkata 700126, India

Correspondence: sauryya.b@gmail.com

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ABSTRACT

Red Amaranth (Amaranthus tricolor) and green Amaranth (Amaranthus viridis) are two very important leafy vegetables consumed in most parts of India, not only for their flavours, but also for their pharmacological activities. The cooking processes are different in the different part of this country, and therefore, the present study was designed to analyze their in vitro antioxidant profile before and after thermal processing in water at different temperatures and pH. Thermal processing was done at 60°C, 80°C and 100°C, whereas pH 5.0 and 9.0 were also used for extraction. The assays performed included DPPH radical decolorization assay, reducing power assay and assay for total phenolic contents. It was observed that the antioxidant activities and total phenolic content improved in case of the two vegetables after thermal processing and pH dependent extraction. Phenolic contents were nearly doubled after extraction at 100°C and at pH 9.0, separately, probably due to better solublization of the antioxidants in hot water and different acid-base conditions. Improvement in the total phenolic contents substantiated the radical scavenging abilities of the two subject vegetables after aqueous extraction.

Keywords: Antioxidant, Red amaranth, *Amaranthus tricolor*, Green amaranth, *Amaranthus viridis*

INTRODUCTION

The rural people in India are still largely devoid of the necessities of modern healthcare due to their economic constraints, even in this early twenty-first century [1]. That is why, rural people mainly depend on the locally available edible plant materials to cure various health disorders as those plants possess many health promoting bioactives [2]. Taking the lead from such traditional knowledge, the modern research is paying attention towards exploring plant sources for substances that provide nutritional as well as pharmacological advantages to humans. It is now well known that fruits and vegetables are good sources of natural antioxidants for the human diet, containing many different antioxidant components 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 [3]. Among the different plant-based food sources, green leafy vegetables are a good source of antioxidants, minerals and vitamins [4]. The antioxidants of these vegetables play an important role in the maintenance of health and prevention of disease. A number of vitamins such as vitamins A, C, E, as well as carotenes are excellent antioxidants, which also contribute to good health by acting as co-factors for certain enzymes and by involvement in oxidation-reduction reactions [5]. It has also been established that increase in vegetable consumption reduces the risk of cancer, cardiovascular diseases and mortality, mainly attributable to antioxidants such as ascorbic acid, vitamin E, carotenoids, lycopenes and polyphenols [6,7,8].

Usually, starchy foods are the staple foods utilized mostly in developing countries as they provide both energy and proteins [9]. However, in India, the green leafy vegetables are the other important protein sources as they are cheap, available locally and can be cooked fairly easily in the local households. 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 [10]. Among these green vegetables, amaranthus plants (family – Amaranthaceae) are spread throughout the world, growing under a wide range of climatic conditions and they are known to produce useful feed and food products. Amaranthus plants or amaranths are defined as "never-fading flowers" in Greek [9]. Several species of amaranths are often considered as weeds, although people worth amaranth as leafy vegetables to be utilized in daily cuisines in most parts of India. The flavor of the raw or cooked amaranths is reported to be as equal to or better than the spinach or other leafy vegetables [11]. Among the amaranths, red amaranth (*Amaranthus tricolor*) is especially nutritious, as they are rich in minerals like iron and calcium, vitamin C, pigments like carotenoids and betalains and flavonoids [12,13]. Green amaranth (*Amaranthus viridis*), on the other hand, is replete with flavonoids and these compounds are known to involve in anti-inflammatory, anti-ulcerogenic, anti-allergic, antiviral and hepatoprotective responses [14]. It also contains minerals, vitamin C, carotenoids and phenolic acids in substantial quantities [15].

For the past few years, a number of studies have been published to determine the antioxidant potentials of amaranths [16,17]. In such studies, extraction with solvents like raw and aqueous alcohols was a common practice for the determination of bioactives as well as radical scavenging abilities. A few studies were also found where water was used to extract the vegetables for adjudication of antioxidant profile [18]. However, standard pharmacological extraction procedures were followed in such reports, in view of retaining the integrity of the bioactive principals. As it is common that vegetables are usually cooked by heat treatments which would certainly bring some physico-chemical changes in them, the nutritional quality could change irreversibly [19]. The present study deals with the *in vitro* antioxidant profile of the two amaranth species before and after thermal processing with water. Since different cooking processes employ different thermal treatments, the extractions in the present study were done in different temperatures in order to ascertain the difference in antioxidant capacities. Again, the extractions were done at different pH as different cooking processes sometimes utilize different pH conditions (i.e. use of lime juices or use of food grade sodium bicarbonates). 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 where different temperature and pH conditions for extraction were employed. In this way, we would be able to know how different cooking methods could retain the most effectiveness of these natural foods for human consumption. The present study reports the achievement of the aim through some common *in vitro* antioxidant assays.

MATERIALS AND METHODS

Chemicals

2,2′-Diphenyl-1-picryl hydrazyl (DPPH) were obtained from Himedia, India. Analytical grades of trichloroacetic acid, ascorbic acid, Folin-Ciocalteau's solution, citric acid, 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.

Selection of samples

Fresh samples of two amaranths commonly used as food in rural households of this part of the country, namely, red Amaranth (*Amaranthus tricolor*) and green Amaranth (*Amaranthus viridis*) were obtained from local markets in Barasat, West Bengal, and authenticated by a Botanist. The vegetables were checked for dirt or any visible damages, which were discarded. The samples were utilized for extraction afresh, without preservation.

Extraction of the samples at different temperatures

Fresh leaves were washed and cut into small pieces. 1 gm of wet sample was crushed in a mortar-pestle to obtain a fine mixture of homogenous material. The extractions were done using deionized distilled water with a solid-to-solvent ratio of 1:10 (w/v). All samples were heated at temperatures 60°C, 80°C and 100°C for about 10 minutes, separately. A control with extraction done at room temperature (32±2^{o}C) and at pH 7.2 was also prepared for comparative purpose. Then the mixtures were centrifuged at 6000 rpm for 5 minutes to get a clear supernatant. The supernatants were utilized for antioxidant studies.

Extraction of the samples at different pH

Fresh samples were washed and cut into pieces. 1 gm of wet sample was crushed in a mortar-pestle to obtain a fine mixture of homogenous material. The extractions were done at room temperature using deionized distilled water with a solid-to-solvent ratio of 1:10 (w/v). Prior to extraction, the pH of the deionized distilled water was set at 5.0 and 9.0 using citric acid and sodium bicarbonate, respectively. A control with extraction done at room temperature

(32±2°C) and at pH 7.2 was also prepared for comparative purpose. Then the mixtures were centrifuged at 6000 rpm for 5 minutes to get a clear supernatant. The supernatants were utilized for antioxidant studies.

DPPH radical decolorization assay

The DPPH assay was performed using a previously described procedure [20]. 1 ml DPPH solution (3 mg DPPH powder in 25 ml ethanol) 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 oxidants by 50% is defined as IC_{50} of the samples. Ascorbic acid was used as positive control and comparing with its' IC_{50} and the results were expressed as ascorbic acid equivalents (mM/gm spices).

Reducing Power assay

The assay was performed using a previously described procedure with minor modifications [21]. Briefly, 0.5 ml of sample solutions was mixed with phosphate buffer (pH 7.4, 2.5 ml) and aqueous potassium ferricyanide solution (2.5 ml). This mixture was kept at 50±2ºC in water bath for 20 minutes. After cooling, 2.5 ml of 10% (w/v) trichloroacetic acid was added and centrifuged at 3000 rpm for 5 min. 2.5 ml of the supernatant was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm in a Systronics spectrophotometer (model – 2202). Control was prepared in similar manner excluding samples. Gallic acid was used as positive control and comparing with its' IC_{50} and the results were expressed as gallic acid equivalents (µM/gm spices).

Total Phenolic Content assay

The assay was performed using a previously described procedure with minor modifications [22]. Briefly, 0.5 ml of sample solution was mixed with 1.5 ml Folin-Ciocalteau's solution (1:10 v/v diluted with distilled water) and allowed to stand for 28±2°C for 5 min. Then 2 ml of 7% (w/v) aqueous sodium carbonate solution was added and the mixture were allowed stand for another 90 min and at darkness. The absorbance of the blue color that developed was measured at 725 nm using spectrophotometer (Systronics, Model – 2202). Gallic acid was used to prepare the standard curve (20–100 µg/ml) and the total phenolic concentration in the spice extract was expressed as mg of gallic acid per gram of dry weight of the spices (µM/gm spice).

Statistical analysis

Data were expressed as mean ± standard deviation of four independent samples. Data were analyzed by Student's *t*-test using the software 'Prism 4.0' (GraphPad Inc., USA).

RESULTS

Extraction of red amaranth at different temperatures

It was observed that there was an indication of improvement of DPPH radical scavenging potential of red amaranth on thermal treatment compared to the control (Table 1). The activity was maximum (from 85.28 to 95.12 µM ascorbic acid equivalent/gm sample, Table 1) upon extraction at 60°C. In higher temperatures, there was deterioration in the activity, probably due to the decomposition of the bioactives. However, significant improvement was observed in the reducing power assay and maximum activity was found upon extraction at 80°C (from 25.41 to 28.71 µM/gm sample, Table 1). The improvement in the total phenolic content was also in consonance with the reducing power assay, where significant improvement was observed in case of all three extraction conditions (Table 1).

Table 1: Antioxidant potential of red amaranth (*Amaranthus tricolor*) after processing with water at different temperatures. Results for DPPH assay are expressed as ascorbic acid equivalents (µM/gm sample) and as gallic acid equivalents (µM/gm sample) for the rest assays.

Processing temperatures	DPPH assay	Reducing power assay	Total phenolics Content
Control	85.28±5.60	25.41±1.70	0.91±0.07
60°C	95.12±8.13	27.59±1.69	1.18±0.09
80°C	80.68±7.69	28.71±1.76	1.53±0.05
100°C	81.08±7.16	28.24±1.63	1.71±0.04

Data are expressed as Mean ±SD (n=4), Control: Processing temperature 32±2°C and pH 7.2

Extraction of red amaranth at different pH

Reducing power of plant bioactives provides a significant indication of the antioxidant activity *in vitro*. Phytochemicals possessing reducing power are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, ultimately minimizing undesirable health conditions [21]. It was observed that there was

significant improvement of the reducing power and maximum activity was found upon extraction at pH 5.0 (from 25.41 to 36.19 µM/gm sample, Table 2). However, there was deterioration in the DPPH radical scavenging activity upon extraction at different pH, probably due to the structural changes occurred in the bioactives at different acid-base conditions (Table 2). The improvement in the total phenolic content was also in consonance with the reducing power assay, where significant improvement was observed in case of the two extraction conditions, maximum being at pH 9.0 (Table 2).

Table 2: Antioxidant potential of red amaranth (*Amaranthus tricolor*) after processing with water at two different pH. Results for DPPH assay are expressed as ascorbic acid equivalents (µM/gm sample) and as gallic acid equivalents (µM/gm sample) for the rest assays.

pH	DPPH assay	Reducing power assay	Total phenolics Content
Control	85.28±5.60	25.41±1.70	0.91±0.07
5.0	84.43±4.89	36.19±2.16	1.76±0.08
9.0	75.45±7.26	31.08±2.05	1.93±0.09

Data are expressed as Mean ±SD (n=4), Control: Processing temperature 32±2°C and pH 7.2

Extraction of green amaranth at different temperatures

It was observed that there was deterioration of DPPH radical scavenging potential of green amaranth on thermal treatment compared to the control (Table 3). However, significant improvement was observed in the reducing power assay and maximum activity was found upon extraction at 100°C (from 25.06 to 29.76 µM/gm sample, Table 3). The improvement in the total phenolic content was also in consonance with the reducing power assay, where significant improvement was observed in case of all three extraction conditions with maximum at 100°C (Table 3).

Table 3: Antioxidant potential of green amaranth (*Amaranthus tricolor*) after processing with water at different temperatures. Results for DPPH assay are expressed as ascorbic acid equivalents (µM/gm sample) and as gallic acid equivalents (µM/gm sample) for the rest assays.

Processing temperatures	DPPH assay	Reducing power assay	Total phenolics Content
Control	97.22±6.76	25.06±1.76	0.88±0.03
60°C	93.75±7.33	27.18±1.52	1.12±0.06
80°C	81.82±5.63	28.41±1.95	1.24±0.07
100°C	74.15±6.93	29.76±1.27	1.41±0.08

Data are expressed as Mean ±SD (n=4), Control: Processing temperature 32±2°C and pH 7.2

Extraction of green amaranth at different pH

It was observed that there was significant improvement of the reducing power and maximum activity was found upon extraction at pH 9.0 (from 25.06 to 30.88 µM/gm sample, Table 4). However, there was deterioration in the DPPH radical scavenging activity upon extraction at different pH, again probably due to the structural changes occurred in the bioactives at different acid-base conditions (Table 4). The improvement in the total phenolic content was also in consonance with the reducing power assay, where significant improvement (from 0.88 to 1.53 µM/gm sample, Table 4) was observed in case of the two extraction conditions, maximum being at pH 5.0 (Table 4).

Table 4: Antioxidant potential of green amaranth (*Amaranthus tricolor*) after processing with water at two different pH. Results for DPPH assay are expressed as ascorbic acid equivalents (µM/gm sample) and as gallic acid equivalents (µM/gm sample) for the rest assays.

pH	DPPH assay	Reducing power assay	Total phenolics Content
Control	97.22±6.76	25.06±1.76	0.88±0.03
5.0	77.06±7.47	32.18±2.18	1.53±0.07
9.0	97.06±7.59	30.88±2.12	1.51±0.09

Data are expressed as Mean ±SD (n=4), Control: Processing temperature 32±2°C and pH 7.2

Comparison of red and green amaranths

It was revealed from the present study that the green amaranth showed better DPPH radical scavenging activity than red variant in control conditions (Fig. 1). However, after thermal treatment, the two vegetables showed comparative DPPH radical scavenging profile. The improvement was prominent in green amaranth when the extraction was done at pH 9.0.

It was, however, also observed that red amaranth scored better over green amaranth in reducing capability and total phenolic content (Figs. 2 and 3). Thermal treatments improved their antioxidant profile. Although there were no significant differences in reducing power of the two species after thermal treatment, there were significant improvements of the total phenolic content after thermal processing.

Fig. 1: Comparative DPPH radical scavenging capacities of red and green amaranth at different extraction conditions.

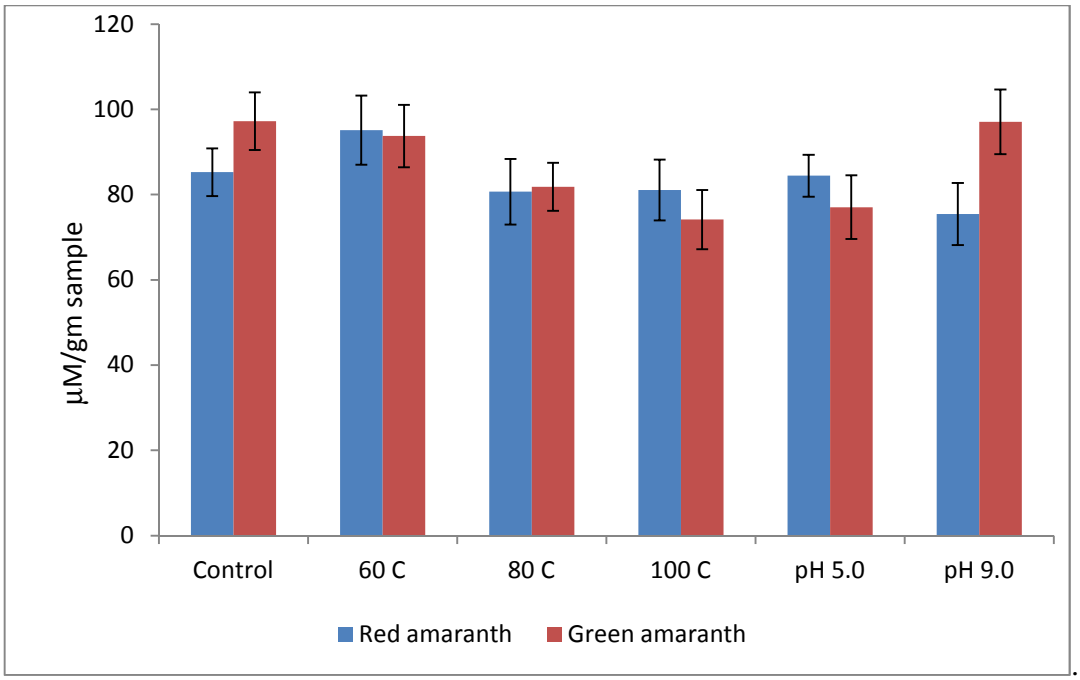


Fig. 2: Comparative reducing power of red and green amaranth at different extraction conditions.

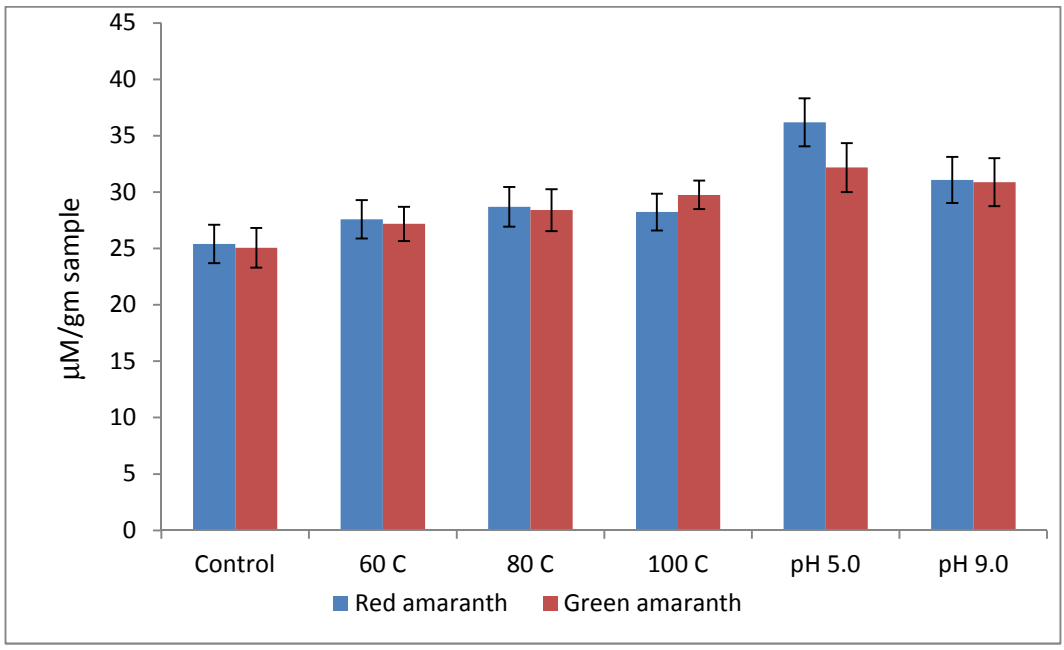
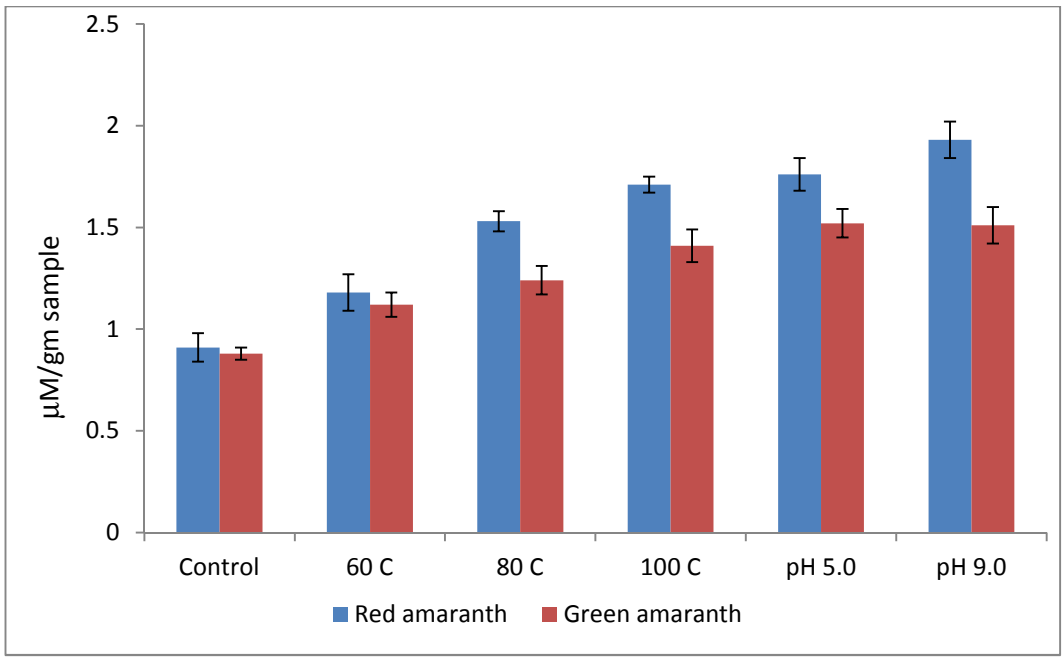


Fig. 3: Comparative total phenolic contents of red and green amaranth at different extraction conditions.



DISCUSSION

Cooking processes would bring about a number of changes in physical characteristics and chemical composition of the vegetables. Many studies have shown that various cooking methods affected the content of phytochemicals, in particular, antioxidant present in the vegetables, however little is known in this regard in depth [23,24]. The determination of antioxidant potential of plant based substances is still being an unresolved problem and not a single assay would be sufficient for the assessment [25]. In the present study, the methods for adjudication of the antioxidant potential of the red and green amaranth leaves were chosen specifically in view of the above proclamation. DPPH assay is one of the most popular radical scavenging assay methods and is based on non-aqueous less polar medium (i.e. alcohol). The phytochemicals that neutralize DPPH radicals are usually hydrogen atom donors and this neutralization reaction is temperature and pH sensitive [26]. The results indicated that there might be some transformation in the hydrogen atom transferring molecules of the two amaranths upon thermal and pH treatment which could be unfavorable for their DPPH radical scavenging abilities. On the other hand, reducing power assay implicated effectiveness of the substances in aqueous (i.e. polar) medium. This can be correlated directly with improvement in phenolic contents of the two amaranths shown after thermal processing in water. The observed enhanced antioxidant profile in some treatment regimens as well as greater extraction of polyphenols might be due to enhanced solubility of the polyphenols in hot water which otherwise have less solubility in water at room temperature.

The bioactives commonly present in the amaranths were reported to be effective against various types of toxic oxidants *in vitro* although not much research have been conducted in this sphere [27, 28]. Phenolic compounds of plants having one or more aromatic rings with one or more hydroxyl groups can potentially quench free radicals by forming resonance-stabilized phenoxyl radicals which play a role in their antioxidant properties [20]. The effectiveness of them against the most harmful ROS after thermal processing that closely resemble cooking methods employed in India, however, was not explicitly studied earlier [21]. In this context, the present study indicated some positive effects of thermal and pH treatment upon antioxidative potential of the two amaranths on extraction, which would provide knowledge about their potential as functional food during human consumption.

CONCLUSION

The foremost conclusion arising out of the present work was that the antioxidant capacities of the two subject amaranth species could be improved by thermal processing at different temperatures or by extraction at different pH conditions that resemble cooking. This could be understood form the improvement of their reducing powers as well as total phenolic contents. Enhanced activities shown after thermal processing in water might be due to enhanced extraction of polyphenols, which might have less solubility in normal water but enhanced solubility in hot water. There was a strong correlation between the improvement of reducing power and the total phenolic contents, which

indicated that the antioxidant activities of the amaranths were mainly due to the polyphenolics extracted in the water by thermal processing as well as pH dependent extraction. The study also indicated that there were marginal differences in the antioxidant potential of the red and green amaranths without any treatment. The improvements in the antioxidative potential of red and green amaranths on heat or pH-dependent treatment with water implied their role as functional foods, even after cooking.

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