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Evaluation of *in vitro* Antioxidant Potential of Capsicum (*Capsicum annuum*) of Different Ripening Stages Extracted at Different Temperature and pH

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ABSTRACT

Capsicum (*Capsicum annuum*) of different ripening stages contains different biochemicals that might be altered by the cooking processes practiced in the different part of this country. 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^{o}C, 80^{o}C and 100^{o}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 reducing power and total phenolic content improved in case of all the three different maturity stages of the vegetable after thermal processing and pH dependent extraction. DPPH radical scavenging ability, however, was diminished. This indicated that non-polar antioxidants were not extracted in the extraction conditions. Improved antioxidant profile in aqueous assay systems was probably due to better solublization of the polar antioxidants in hot water and different acid-base conditions. Improvement in the total phenolic contents substantiated the radical scavenging abilities of the three variants after aqueous extraction.

Keywords: Antioxidant, Capsicum, *Capsicum annuum*, DPPH, Polyphenols

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INTRODUCTION

The antioxidative and antimicrobial properties of many plant extracts are of great interest in both academia and in the food industry, since they can be utilized as natural additives because there is a growing tendency to replace synthetic antioxidants with natural ones[1]. In this regard, colour is one of the most important quality properties of food products, next to texture and aroma, and coloured biomolecules like anthocyanins, flavonoids and carotenoids play a crucial role not only in maintaining physiological homeostasis, but also providing pharmacological surveillance in various disease conditions that show a high worldwide prevalence; such as cancer, rheumatoid arthritis, asthma, diabetes, cardiovascular and neurodegenerative diseases, including atherosclerosis, Alzheimer's disease, and other age-related degenerative disorders[2]. In recent years, however, a focus has been given on the nutrient quality of most of the vegetables that are consumed after cooking, as cooking involves heating protocols[3]. It is known that cooking induces significant changes in chemical composition, influencing the concentration and bioavailability of bioactive compounds in many food ingredients. Since cooking processes are different in the different part of this country, there could be a substantial difference in the nutrient quality as well as antioxidant profile of vegetables consumed as they undergo different processing conditions. This point of view was not properly addressed in recent past. The name capsicum is derived from the Greek word 'Kapsimo', meaning – to bite. It is a part of human diet since 7500 BC[4]. Capsicum (*Capsicum annuum*) is a genus of plant under the family of *Solanaceae*, and they have varieties of names according to their location and type. This species includes both sweet cultivars eaten mainly as vegetables and hot varieties, often used as a spice[5]. They are considered a good source of bioactive compounds, such as vitamins, pro-vitamins, and antioxidant compounds[6]. Depending upon the nature of ripening, color of these bell peppers ranges from green, red, yellow and even purple. Green peppers ripen to yellow and finally red if left on the vine. Most of the differences in color stem from time of harvest and degree of ripening[7]. However, the level of antioxidants differs between cultivars and the hot peppers are a better source of them than the sweet ones[8]. They are one of the most popular fresh vegetables in the world, because of the combination of color, taste, and nutritional value. Many factors affect pepper colour loss during storage, and the most important is oxidative degradation of carotenoids, caused by exposure to heat, light or oxygen. For the past few years, a number of studies have been published to determine the antioxidant potentials of capsicum[9,10,11]. 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. Recently, a report has been published where capsicum of different ripening stages was extracted following methods that resemble cooking and their antioxidant activities were determined[12]. 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[13]. The present study deals with the *in vitro* antioxidant profile of capsicum of different ripening stages 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 their 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, and probably the first with capsicum. 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 METHOD

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

The green, yellow and red variants of fresh capsicum were collected from a local market of Barasat, Kolkata. The samples were checked for dirt or any visible damages, and were discarded if found.

Extraction of the samples at different temperatures

The extraction was done following a published method[14]. 1 gm of fresh 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°C) and at pH 7±0.4 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

The extraction was done following a published method[14].1 gm of fresh 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/or sodium bicarbonate, respectively. A control with extraction done at room temperature (32±2°C) and at pH 7±0.4 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[15]. 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[16]. 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[17]. 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 analyses

The results are presented as the mean of quadruplicates ± SD. Significant differences between means were detected by one-way analysis of variance (ANOVA), followed by multiple comparisons using Tukey's post-hoc test. Differences were considered significant when $p <$ 0.05 compared to control. The analyses were done with the software 'Prism 4.0' (GraphPad Inc., USA).

RESULTS AND DISCUSSION

Extraction of green capsicum at different temperatures and pH

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[18]. It was observed that there was an indication of deterioration of DPPH radical scavenging potential of green capsicum on thermal treatment compared to the control (Table 1). Even extraction at different pH diminished the radical scavenging potential of the extracts. However, improvement was observed in reducing power assay at different extraction conditions and significant improvement was observed at pH 9. The improvement in the total phenolic content was also in consonance with the reducing power assay, where significant improvement was observed at 80^{o}C and 100^{o}C as well as two pH-dependent extraction conditions (Table 1). 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.

Extraction of yellow capsicum at different temperatures and pH

It was observed that DPPH radical scavenging potential of yellow capsicum was deteriorated significantly on thermal treatment compared to the control (Table 2). Even extraction at different

pH diminished the DPPH radical scavenging potential of the extracts. However, improvement was observed in the reducing power assay although it was not significant. The improvement in the total phenolic content was also in consonance with the reducing power assay, where significant improvement was observed only in case of pH-dependent extraction conditions (Table 2).

Table 1: Antioxidant potential of green capsicum after extraction with water at different temperatures and 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.

Assay method	Control	Temperature			pH	
		60°C	80°C	100°C	5.0	9.0
DPPH assay	22.20±1.09	21.5±1.29	16.16±1.26**	15.22±1.43**	8.07±1.36**	18.05±1.39**
Reducing power assay	3.13±0.28	3.29±0.28	3.31±0.28	3.48±0.28	3.29±0.28	4.23±0.3**
Total phenolics Content	0.07±0.01	0.11±0.01	0.14±0.01**	0.16±0.01**	0.14±0.01**	0.16±0.02**

Data are expressed as Mean ± SD (n=4), Control: Processing temperature 32±2°C and pH 7±0.4

* $p<0.05$ and ** $p<0.01$ compared to control.

Table 2: Antioxidant potential of yellow capsicum after extraction with water at different temperatures and 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.

Assay method	Control	Temperature			pH	
		60°C	80°C	100°C	5.0	9.0
DPPH assay	37.25±1.33	33.72±1.39*	28.22±1.43**	25.11±1.01**	22.81±1.45**	36.17±1.24
Reducing power assay	2.35±0.22	3.07±0.28	3.58±0.28	3.61±0.28*	2.35±0.22	3.55±0.28
Total phenolics Content	0.12±0.01	0.19±0.01	0.21±0.01	0.26±0.01	0.28±0.01*	0.31±0.01**

Data are expressed as Mean ± SD (n=4), Control: Processing temperature 32±2°C and pH 7±0.4

* $p<0.05$ and ** $p<0.01$ compared to control.

Extraction of red capsicum at different temperatures and pH

It was observed that DPPH radical scavenging potential of red capsicum was deteriorated significantly on thermal treatment compared to the control (Table 3). Even extraction at different pH diminished the DPPH radical scavenging potential of the extracts. Reduction of DPPH radical scavenging capacities of all three variants indicated that non-polar antioxidant phytochemicals were not effectively extracted by thermal stress as DPPH assay is performed in less polar conditions[15]. However, like the other two variants, significant improvement was

observed in some of the extraction procedures in the reducing power assay. 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 extraction conditions that showed significant improvement in reducing power assay (Table 3). The observed enhanced antioxidant profile in the aforesaid assays 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.

Table 3: Antioxidant potential of red capsicum after extraction with water at different temperatures and 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.

Assay method	Control	Temperature			pH	
		60°C	80°C	100°C	5.0	9.0
DPPH assay	78.07±1.73	62.25±1.65**	53.15±1.04**	47.97±1.07**	33.07±1.38**	48.17 ±1.30**
Reducing power assay	2.20±0.24	3.13±0.27*	3.35±0.28**	3.37±0.28**	2.61±0.28	3.56±0.28**
Total phenolics Content	0.12±0.01	0.19±0.01*	0.24±0.01**	0.30±0.01**	0.12±0.01	0.22±0.01**

Data are expressed as Mean ± SD (n=4), Control: Processing temperature 32±2°C and pH 7±0.4

* $p<0.05$ and ** $p<0.01$ compared to control.

Comparison of capsicums of different ripening stages

It was revealed from the present study that red capsicum showed better DPPH radical scavenging activity among the three in control conditions (Figure 1). However, after thermal and pH-dependent treatments, all the variants showed decreased DPPH radical scavenging profiles. The observation suggested that red capsicum contains greater amounts of hydrogen atom transferring antioxidant biomolecules. It is also implied that thermal or pH-dependent treatments decreased activities of less polar antioxidant biomolecules, which usually responds in DPPH radical scavenging assays[15]. Among the three variants, green capsicum had the maximum reducing power (Figure. 2). Reducing power increased significantly on treatment at pH 9.0 in case of green capsicum, but in other variants, there were tendencies of improvement, albeit non-significantly. The study indicated that green capsicum contains greater amounts of antioxidants which function through electron donation. pH dependent extraction at pH 9 probably facilitated their extraction in water significantly. In case of total phenolic contents, red and yellow capsicum showed almost similar results, whereas green capsicum scored less (Figure. 3).

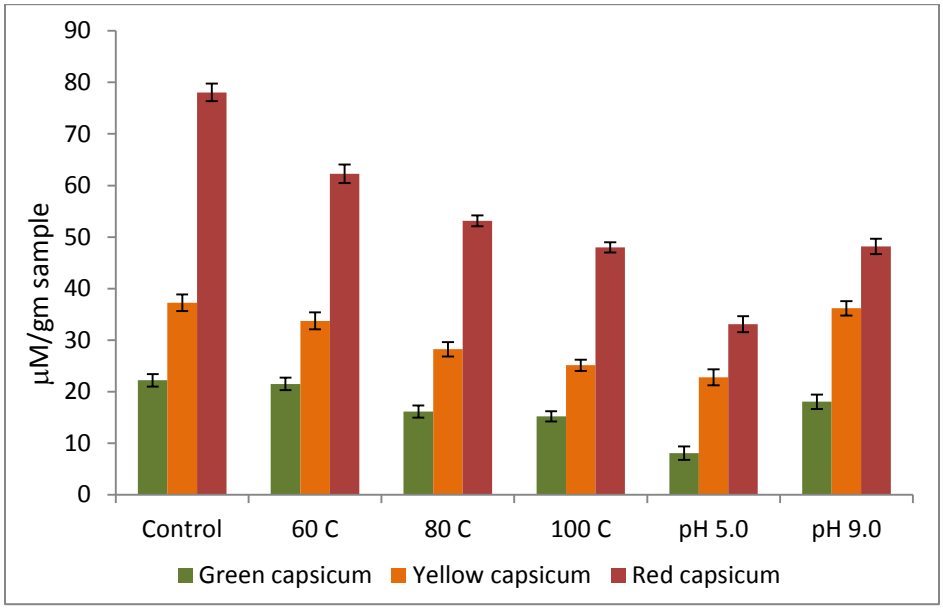

Figure 1: Comparative DPPH assay of the three capsicum variants in fresh and at different extraction conditions. Results are expressed as ascorbic acid equivalents (µM/gm sample).

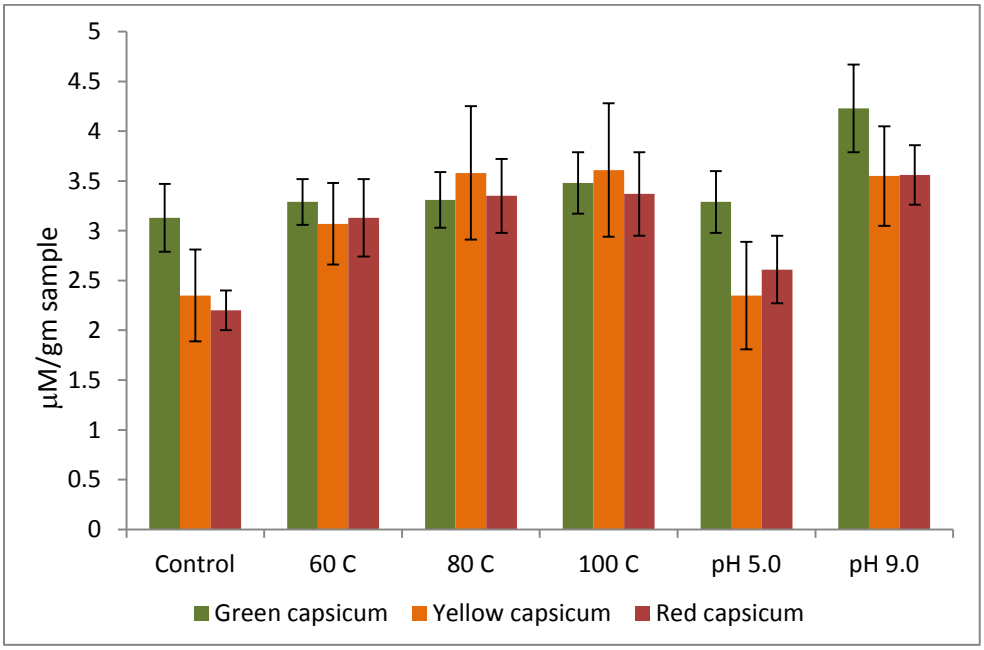

Figure 2: Comparative reducing power of the three capsicum variants in fresh and at different extraction conditions. Results are expressed as gallic acid equivalents (µM/gm sample).

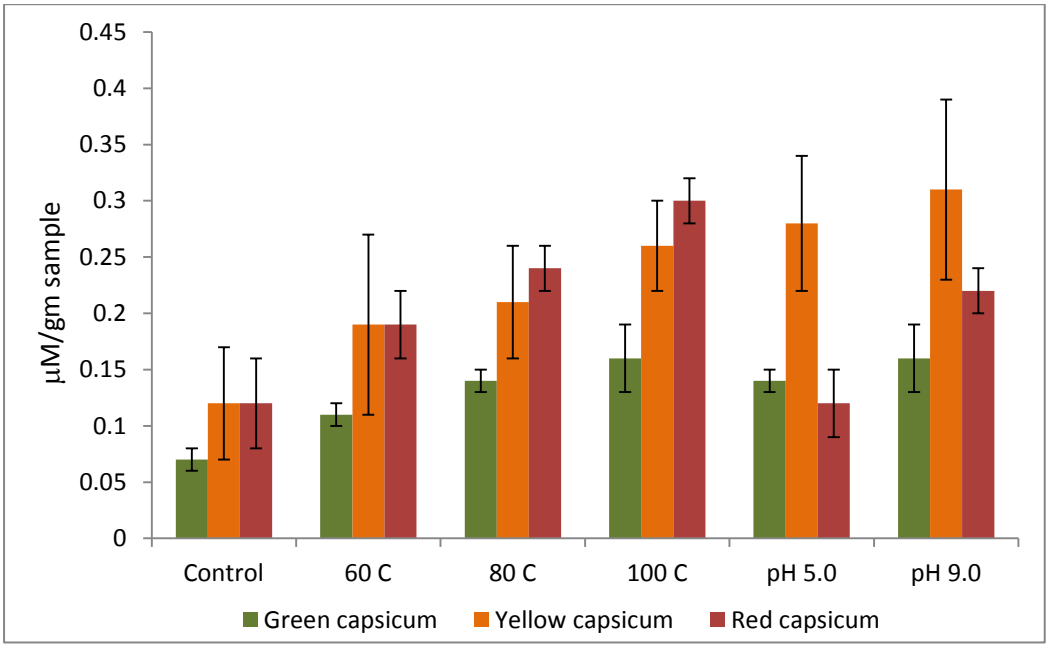

Figure 3: Comparative total phenolic contents of the three capsicum variants in fresh and at different extraction conditions. Results are expressed as gallic acid equivalents (µM/gm sample).

Thermal and pH-dependent treatments improved their phenolic contents, probably due to better solublization of the polyphenolics. It was also observed that in case of pH-dependent treatments, red capsicum lost its phenolics, probably due to rearrangement of the phenolic moieties, often observed in case of antioxidant biomolecules like anthocyanins and flavonoids. 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[19, 20]. The results from the present study indicated that there might be some transformation in the hydrogen atom transferring molecules of the capsicums upon thermal and pH treatment which could be unfavorable, as obvious from 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 three variants shown after thermal processing in water. 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[15]. 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. In this context, the present study indicated some positive effects of

thermal and pH treatment upon antioxidative potential of the three capsicum variants of different ripening stages 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 capsicum samples of three ripening stages 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 capsicums were mainly due to the polyphenolics extracted in the water by thermal processing as well as pH dependent extraction. However, DPPH radical scavenging activities were deteriorated significantly on treatments. This might indicate that less polar molecules were not extracted properly in such conditions. The improvements in the antioxidative potential of the three capsicums of different ripening stages on heat or pH-dependent treatment with water implied their role as functional foods, even after cooking.

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