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Thermal processing effects on in vitro Antioxidant activities of five common Indian Pulses

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ABSTRACT

Five different types of pulses commonly used in daily cuisine in India, viz. Lentil (*Lens culinaris*), Green gram (*Vigna radiate*), Bengal gram (*Cicer arietinum*), Red kidney beans (*Phaseolus vulgaris*) and Soybean (*Glycine max*), were analyzed for their *in vitro* antioxidant profile before and after thermal processing in water. The thermal processing included methods commonly employed in India for cooking, viz. pressure cooking, microwave treatment, boiling in water and boiling after overnight soaking. The assays performed included ABTS radical decolorization assay, DPPH radical decolorization assay, hydrogen peroxide scavenging assay and hydroxyl radical scavenging assay. The antioxidant profile of the five pulses improved significantly after microwave treatment as evident from all assays except DPPH assay. In case of Bengal gram, improvement was also observed after pressure cooking. In case of soybean, however, apart from microwave treatment, no significant improvement was observed. Enhanced activity of the pulses shown after thermal processing in water might be due to enhanced extraction of polyphenols like gallo-ellagitannins, phenolic acids or flavonoid glycosides, which have less solubility in normal water but enhanced solubility in hot water. The study indicated a systematic approach to ascertain antioxidant quality of pulses after thermal treatment with water.

INTRODUCTION

Legumes and pulses have long been a part of traditional diets in Asia, Africa and South America, which include most of the world's developing countries. They have been consumed as a rich source of protein in tandem with the cereal or tuber-based diets and have an important role in human nutrition. They are usually consumed as whole gram or as split pulses *(dhals).* In recent years, pulses and legumes have also been recognized as part of functional foods because pulses are rich sources of phenolic compounds and have significant antioxidant potential (Sreeramulu *et al.,* 2009). Pulses as rich protein sources are popular because animal proteins are scant and expensive or are not consumed especially in India due to religious and/or cultural reasons (Jain *et al.,* 2009).

In India, from time immemorial, many legumes and pulses have been consumed as part of a primarily cereal-based diet (Rao, 2002). Besides proteins, pulses are also good sources of vitamins, minerals, ω-3 fatty acids and dietary fiber or non-starch polysaccharides (NSP). However, recent studies revealed that pulses are also good sources of antioxidants. For example, Soybean is rich in isoflavones like genistein and daidzein and has a high antioxidant capacity (Ponnusha *et al.,* 2011). Lentils are good sources of the antioxidants – tartaric esters, flavonols and anthocyanins, which could also contribute to their marked antioxidant activity (Oomah *et al.,* 2011; Gharachorloo *et al.,* 2012). Kidney beans (i.e. rajmas) are replete with flavonoids and anthocyanins (Sreeramulu *et al.,* 2009). Recent researches showed that Green gram and Bengal gram are also good sources of antioxidants and have pharmacological activities (Jukanti *et al.,* 2012; Kim *et al.,* 2012). That is why, food industry is concentrated on the phenolic antioxidants of pulses as they retard oxidative degeneration of biomolecules like proteins, DNA and lipid, to prevent onset of a large number of diseases. In India, pulses are treated and cooked in a variety of methods based on tradition and taste preferences. Various treatment and cooking processes could affect the levels of nutritional and antioxidant factors of legume grains (Shah *et al.,* 2011; Hernandez-Salazar *et al.,* 2010). It have been seen that most of the research reports emphasized on determination of antioxidant potential of the pulses after cooking by classical methods (Filipiak-Florkiewicz *et al.,* 2012; Marka *et al.,* 2013; Karthiga and Jaganathan, 2013).

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Extraction with solvents like methanol, aqueous methanol and acetone were a common practice for the determination of bioactives as well as radical scavenging abilities. However, no studies were found where water extractives of the pulses were used directly for adjudication of antioxidant profile although water is the universal solvent and the only medium of cooking world-wide. The present study deals with five different types of pulses commonly used in daily cuisine in India, viz. Lentil (*Lens culinaris*), Green gram (*Vigna radiate*), Bengal gram (*Cicer arietinum*), Red kidney beans (*Phaseolus vulgaris*) and Soybean (*Glycine max*), for their *in vitro* antioxidant profile before and after thermal processing with water. The design resembled closely with common cooking procedures used in India. 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 the subject pulses. In this way, we would be able to know the appropriate cooking methods, which would retain the most effectiveness of the pulses 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′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS, were obtained from Sigma, USA. 2,2′-Diphenyl-1-picryl hydrazyl (DPPH) were obtained from Himedia, India. Ascorbic acid, hydrogen peroxide and ferrous sulphate heptahydrate 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

Five commonly used pulses, namely, Lentil (*Lens culinaris*), Green gram (*Vigna radiate*), Bengal gram (*Cicer arietinum*), Red kidney beans (*Phaseolus vulgaris*) and Soybean (*Glycine max*) were obtained from local markets in Ichapur, West Bengal. The samples were sorted, cleaned and stored in darkness in polyethylene containers at 4°C.

Thermal Processing of the samples

The pulses were washed well before applying different thermal stresses. The extractions were done using deionised distilled water with a solid-to-solvent ratio of 1:10 (w/v). About 10 gms of the samples were extracted without pulverization in view of their use in daily cooking. The following are the different methods of extraction –

I Raw sample – The samples were macerated in a mechanical blender. Then the mixtures were centrifuged at 5000 rpm for 10 minutes to get a clear supernatant. The supernatant were abbreviated as raw samples (RS) and used for further studies.

II Pressure cooking – The samples were subjected to a commercial pressure cooker for 10 minutes. Then the mixtures were centrifuged as above to get a clear supernatant. The supernatant were abbreviated as pressure cooked samples (PC) and used for further studies.

III Microwave cooking – The samples were subjected to a microwave oven for 1 minute. Then the mixtures were centrifuged as above to get a clear supernatant. The supernatant were abbreviated as microwave cooked samples (MO) and used for further studies.

IV Boiling – The samples were boiled for 15 minutes. Then the mixtures were centrifuged as above to get a clear supernatant. The supernatant were abbreviated as boiled samples (BS) and used for further studies.

V Over-night soaking – The samples were kept soaking overnight. The next day, the supernatant were centrifuged as above. The supernatant were discarded and the residues were dried in a hot-air oven at 70°C to a constant weight. The dried samples were then extracted with water in a boiling water bath for 15 minutes. It was then centrifuged as above and the supernatant were analyzed (abbreviated as overnight samples, ON).

Antioxidant assays

ABTS radical decolorization assay

The ABTS assay was performed using a previously described procedure (Baskar *et al.*, 2007). $ABTS^{-\bullet}$, the oxidant, was generated by persulfate 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 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 (µM/gm pulses).

DPPH radical decolorization assay

The DPPH assay was performed using a previously described procedure (Baskar *et al.*, 2007). 1 ml DPPH solution (3 mg 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 (µM/gm pulses).

H_2O_2 decomposition assay

The H_2O_2 assay was performed using a previously described procedure (Gulcin *et al.,* 2003). 0.4 ml of sample solutions were added to a 0.6 ml solution of 40mM of hydrogen peroxide in phosphate buffer (pH 7.4) and kept at 37°C for 10 minutes. Changes in absorbances were measured at 230 nm against a blank solution containing only phosphate buffer and sample in a Systronics spectrophotometer (model – 2202). The percentage scavenging of H_2O_2 by sample and standard compounds was determined as follows:

$$\text{Percentage scavenging of } H_2O_2 = \frac{Ao - A1}{Ao} \times 100$$

Where, A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the sample or standard (ascorbic acid). The results were expressed as ascorbic acid equivalents (µM/gm pulses).

Hydroxyl radical scavenging activity

The hydroxyl radical assay was performed using a previously described procedure (Sefatie *et al.,* 2013). 0.5 ml sample or buffer (in case of control) was added to 0.5 ml 1, 10-phenanthroline (5 mM) and 0.5 ml of $FeSO_4.7H_2O$ (0.75 mM) and mixed thoroughly. The samples were allowed to stand for 5 minutes and 0.5 ml of 0.01% (v/v) of H_2O_2 was then added. The mixtures were incubated at 37°C for 60 min. Absorbances were read at 536 nm in a Systronics spectrophotometer (model – 2202). The percentage scavenging effect was calculated as:

$$\text{Percentage scavenging} = \frac{A1 - Ao}{A2 - Ao} \times 100$$

where,

A_0 is the absorbance of the control (samples were replaced by buffer) A_1 is the absorbance in the presence of the samples or standard (ascorbic acid)

A_2 is the absorbance without H_2O_2

The results were expressed as ascorbic acid equivalents (µM/gm pulses).

Statistical Analyses

All the reactions were performed in quadruplicate and data are presented as mean ± standard deviation. Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons of the means. The value of $p < 0.05$ was taken as the criterion for statistically significant difference. The analyses were done with the software 'Prism 4.0' (GraphPad Inc., USA).

RESULTS

ABTS radical decolorization assay

The results of this assay indicated that antioxidant properties of all five pulses were increased after microwave irradiation and in cases of lentil, red kidney bean and soybean, the improvement were significant (Tables 1, 4 and 5). Almost 4-times increase in antioxidant potential was observed for soybean (from 6.33±1.35 µM/gm fresh sample to 23.59±1.42 µM/gm fresh sample, Table 5).

DPPH radical decolorization assay

The results of this assay indicated mixed responses. Lentil and Bengal gram showed significant improvement after overnight percolation treatment, red kidney bean and green gram showed improvement after boiling and soybean showed improvement after pressure cooking (Tables 1 – 5). Bengal gram showed most prominent improvement as there was 3.5-fold increment in the antioxidant potential (from 103.28±4.25 µM/gm fresh sample to 351.70±8.78 µM/gm fresh sample, Table 3).

H_2O_2 decomposition assay

The results of this assay indicated that antioxidant profile of all the pulses were increased albeit not significantly in all cases. All the pulses showed significant improvement after microwave treatment and the increment in antioxidant potential were 2 to 3-fold. Maximum improvement occurred in soybean (from 90.01±6.93 µM/gm fresh sample to 311.45±57.38 µM/gm fresh sample, Table 5).

Lentil (from 137.41±6.38 µM/gm fresh sample to 331.08±19.39 µM/gm fresh sample, Table 1) and Bengal gram (from 81.94±5.48 µM/gm fresh sample to 147.41±6.58 µM/gm fresh sample, Table 3) also showed significant improvements. In case of red kidney bean, significant improvement occurred in three thermal processing methods (viz. pressure cooking, microwave extraction and overnight soaking, Table 4).

Hydroxyl radical scavenging activity

The results of this assay also indicated that antioxidant profile of all the pulses were increased albeit not significantly in all cases. Green gram showed most prominent improvement upon microwave treatment (from 109.78±3.80 µM/gm fresh sample to 260.28±50.92 µM/gm fresh sample, Table 2).

Lentil (from 66.39±4.94 µM/gm fresh sample to 118.91±5.53 µM/gm fresh sample, Table 1) and Bengal gram (from 54.13±3.57 µM/gm fresh sample to 135.97±31.93 µM/gm fresh sample, Table 3) also showed significant improvement upon microwave extraction. Pressure cooking also showed improvement in the title assay. Lentil (from 66.39±4.94 µM/gm fresh sample to 164.49±18.31 µM/gm fresh sample, Table 1), green gram (from 109.78±3.80 µM/gm fresh sample to 181.21±23.50 µM/gm fresh sample, Table 2) and red kidney beans (from 88.54±3.58 µM/gm fresh sample to 179.24±19.52 µM/gm fresh sample, Table 4) showed significant improvement, whereas the improvement for Bengal gram and soybean were non-significant.

Table. 1: Antioxidant potential of Lentil (*Lens culinaris*) after processing with different thermal conditions. Results are expressed as ascorbic acid equivalents (µM/gm fresh sample).

Processing method	ABTS assay	DPPH assay	H_2O_2 assay	Hydroxyl radical assay
RS	17.50±1.74	471.44±21.73	137.41±6.38	66.39±4.94
PC	13.50±0.94**	165.11±13.00**	171.32±15.01*	164.49±18.31**
MO	21.20±1.50*	360.83±25.46**	331.08±19.39**	118.91±5.53**
BS	11.63±1.31**	99.53±15.32**	132.73±12.22	82.86±4.52
ON	17.10±1.49	590.89±14.93**	103.95±4.22*	98.24±10.83**

Data are Mean ± SD (n=4), RS: Raw sample, PC: Pressure cooked sample, MO: Microwave cooked sample, BS: water boiled sample, ON: over-night soaked sample. * $p<0.05$ and ** $p<0.01$ in comparison with RS.

Table. 2: Antioxidant potential of Green gram (*Vigna radiate*) after processing with different thermal conditions. Results are expressed as ascorbic acid equivalents (µM/gm fresh sample).

Processing method	ABTS assay	DPPH assay	H_2O_2 assay	Hydroxyl radical assay
RS	31.07±3.11	415.77±21.09	169.65±7.08	109.78±3.80
PC	33.61±2.91	440.57±33.61	144.46±8.56	181.21±23.50**
MO	34.80±1.94	109.36±6.98**	334.11±53.78**	260.28±50.92**
BS	30.36±2.08	610.04±21.40**	126.79±7.17	103.42±4.47
ON	16.26±1.56**	90.13±6.39**	136.06±8.61	123.04±4.17

Data are Mean ± SD (n=4), RS: Raw sample, PC: Pressure cooked sample, MO: Microwave cooked sample, BS: water boiled sample, ON: over-night soaked sample. * $p<0.05$ and ** $p<0.01$ in comparison with RS.

Table. 3: Antioxidant potential of Bengal gram (*Cicer arietinum*) after processing with different thermal conditions. Results are expressed as ascorbic acid equivalents (µM/gm fresh sample).

Processing method	ABTS assay	DPPH assay	H_2O_2 assay	Hydroxyl radical assay
RS	14.63±1.12	103.28±4.25	81.94±5.48	54.13±3.57
PC	9.11±0.92**	160.78±7.39**	104.84±6.45**	63.10±2.53
MO	16.23±0.89	60.21±3.29**	147.41±6.58**	135.97±31.93**
BS	11.12±1.96*	50.77±7.57**	75.98±5.23	65.15±2.61
ON	17.28±1.55	351.70±8.78**	93.42±5.33	79.34±1.73

Data are Mean ± SD (n=4), RS: Raw sample, PC: Pressure cooked sample, MO: Microwave cooked sample, BS: water boiled sample, ON: over-night soaked sample. * $p<0.05$ and ** $p<0.01$ in comparison with RS.

Table. 4: Antioxidant potential of Red kidney beans (*Phaseolus vulgaris*) after processing with different thermal conditions. Results are expressed as ascorbic acid equivalents (µM/gm fresh sample).

Processing method	ABTS assay	DPPH assay	H_2O_2 assay	Hydroxyl radical assay
RS	30.89±2.76	617.29±10.58	107.40±5.10	88.54±3.58
PC	35.12±2.78	529.26±14.09**	133.62±10.82**	179.24±19.52**
MO	37.10±2.29*	493.75±13.48**	289.15±8.78**	114.63±19.58
BS	30.12±3.75	893.03±29.54**	113.67±4.96	91.37±2.50
ON	16.33±1.89**	418.59±14.64**	138.93±5.89**	97.21±3.13

Data are Mean ± SD (n=4), RS: Raw sample, PC: Pressure cooked sample, MO: Microwave cooked sample, BS: water boiled sample, ON: over-night soaked sample. * $p<0.05$ and ** $p<0.01$ in comparison with RS.

Table. 5: Antioxidant potential of Soybean (*Glycine max*) after processing with different thermal conditions. Results are expressed as ascorbic acid equivalents (µM/gm fresh sample).

Processing method	ABTS assay	DPPH assay	H_2O_2 assay	Hydroxyl radical assay
RS	6.33±1.35	44.25±4.54	90.01±6.93	63.22±4.41
PC	7.21±0.76	91.31±5.53**	94.13±5.61	90.47±14.97
MO	23.59±1.42**	79.92±2.92**	311.45±57.38**	121.57±25.71**
BS	12.03±1.39**	47.96±4.05	113.15±9.36	77.84±2.47
ON	6.95±0.89	44.06±5.97	127.33±13.48	84.89±3.00

Data are Mean ± SD (n=4), RS: Raw sample, PC: Pressure cooked sample, MO: Microwave cooked sample, BS: water boiled sample, ON: over-night soaked sample. * $p<0.05$ and ** $p<0.01$ in comparison with RS.

DISCUSSION

Reactive oxygen species (ROS) have been implicated in various types of adverse pathophysiological conditions. The main ROS to be considered is superoxide anion ($O_2^{\bullet-}$), which is predominantly generated in mitochondrial electron transport chain. This deleterious species then produces more potent noxious species – hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD). H_2O_2 in turn produces another harmful species, hydroxyl radical ($OH^{\bullet}$), by a Fenton-type reaction in presence of systemic trace metal cations. Substances which could effectively scavenge these ROS would be useful in maintaining well being of humans.

In the present study, the four methods for adjudication of the antioxidant potential of the pulses were chosen specifically to answer the above objective. 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 (Sreeramulu *et al.,* 2009). The radical scavenging activities like ABTS assay and DPPH assay is generally used to indicate antioxidant potential of plant extracts. However, ABTS assay is performed in aqueous medium, whereas DPPH assay is based on non-aqueous less polar medium (i.e. alcohol). The extracts prepared in the present study might contain both polar and non-polar antioxidant biomolecules as aqueous extraction often results in mixture of polar (e.g. gallo-ellagitannins, ascorbic acid) and non-polar (e.g. flavonoids) antioxidant compounds. In the present study, more of the polar substances of these pulses might be extracted by microwave treatment, which gave positive result with ABTS assay and gave non-significant result with the other assays. Indeed, a previous study reported that this type of lack of correlation could be due to different responses of different phenolic compounds in different assay systems (Sreeramulu *et al.,* 2009).

Hydrogen peroxide decomposition assay indicated the probability of the extracts to protect physiological system from another potent threat, the hydroxyl radical. Hydroxyl radical, generated from hydrogen peroxide by Fenton reaction, is the starting point for lipid peroxidation in the membranes. Microwave treatment of the pulses indicated superior H_2O_2 decomposition, probably due to greater extraction of biomolecules capable of scavenging the deleterious substance. The same explanation could be issued for the hydroxyl radical scavenging abilities of the pulses where microwave extraction and pressure cooking improved their radical scavenging potentials.

The bioactives commonly present in pulses were reported to be effective against various types of toxic oxidants although they have received much less attention (Sreeramulu *et al.,* 2009). However, the effectiveness of the subject pulses against the most harmful ROS after thermal processing that closely resemble cooking methods employed in India was not studied previously. In this context, the study indicated effect of thermal stress upon antioxidative potential of five pulses, which would reflect their potential as functional food during human consumption.

A strong correlation was not observed among the radical scavenging abilities of the pulses with the extraction procedures. This result is in agreement with earlier reports where no correlations between antioxidant activities of 92 different plant extracts were observed (Mustafa *et al.,* 2006). Since, the responses of the polyphenolics towards different radicals might vary, the antioxidant activities of the extracts might not relate directly with the amount of different polyphenolics (Marja *et al.,* 1999). Thus, it would be better to adjudicate the antioxidant activities of the pulses as judicious consortium of different groups of bioactives that were present in the aqueous extracts. This was achieved by the study design of the present work, probably for the first time.

CONCLUSION

The major conclusion arising out of this research was that the antioxidant capacities of some of the pulses could be improved by thermal processing methods that resemble cooking. Enhanced activity of the pulses shown after thermal processing in water might be due to enhanced extraction of polar polyphenols like gallo-ellagitannins, and less polar polyphenols like phenolic acids or flavonoid glycosides, which have less solubility in normal water but enhanced solubility in hot water. Since a strong correlation was not observed between the antioxidant profiles of the pulses, it would be better to adjudicate the antioxidant activities of the pulses as judicious consortium of different groups of bioactives that were present in the aqueous extracts. It was proposed that there were some improvements in the antioxidative potential of the pulses on heat treatment with water, which implies their role as functional foods, even after cooking.

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