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Effect of Thermal Treatments Resembling Cooking on In Vitro Anti-Diabetic and Antioxidant Activities of Five Common Indian Pulses

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ABSTRACT

Pulses are consumed in India after cooking for their high contents of proteins and other beneficial bioactive as part of traditional diets. The present study dealt with five different types of pulses, 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* α - amylase and α-glucosidase inhibitory activities after thermal processing in water that resembled cooking methods. The results indicated that red kidney beans or rajma showed improvement in α - amylase inhibitory activity after microwave treatment, when the IC_{50} value became 1.85 µg /ml from the optimal value of 2.08 µg /ml. The efficiencies of all other pulses after thermal treatment deteriorated. Instead, IC_{50} values for α-glucosidase inhibitory activities were almost halved in cases of Bengal gram (33.39 to 18.57 µg/ml) and soybean (17.53 to 7.90 µg/ml), clearly indicating improvement of the enzyme inhibitory potential after microwave treatment. The FRAP assay results indicated that reductive power of all the pulses improved after microwave irradiation and pressure cooking, although the phenolic contents were diminished. These lent credence to the postulate that loss of polyphenolics could be compensated with formation of strong antioxidants like maillard products during thermal treatment, which helped in inhibiting the subject enzymes. The study also indicated that lipid peroxidation could be restrained by the pulse extracts after thermal treatments, which broadened the scope for the utilization of the subject pulses in controlling diabetic complications.

INTRODUCTION

Legumes and pulses have long been reported to be the traditional diets of the developing countries of Asia, Africa and South America. In India, many legumes and pulses have been consumed as part of a primarily cereal-based diet for their contents of high protein and other nutritional components[1]. Besides proteins, pulses are also good sources of vitamins, minerals, ω-3 fatty acids, phenolic antioxidants and dietary fiber or non-starch polysaccharides (NSP). In India, pulses are treated and cooked in a variety of methods based on tradition and taste preferences, which might affect the levels of nutritional and antioxidant factors of legume grains[2,3]. 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*), as they have good *in vitro* antioxidant profile before and after thermal processing with water that resembled cooking processes[4]. Moreover, all these five pulses are known to control elevated blood sugar levels, promote cardiovascular health, boost immunity systems and improve digestion due to their fiber and other nutrient contents. Soybean, in addition, has cholesterol-lowering effects that can reduce the risk of heart ailments all the more[5]. All these information lent credence to the fact that these five pulses might help in the management of blood glucose even after undergoing cooking processes practiced in the Indian households.

Diabetes mellitus is a metabolic syndrome characterized by increase in blood glucose level. Several strategies have been employed by the researchers worldwide to combat this deadly disease. One of the strategies is to inhibit the activities of polysaccharide hydrolyzing enzymes like α - *amylase* and α-glucosidase[6]. α - amylase is a prominent polysaccharide hydrolyzing enzyme found in the pancreatic juice and saliva, whereas α-glucosidase is present in the mucosal brush border of the small intestine which catalyzes the end step of digestion of starch and disaccharides. Inhibitors α - amylase and α-glucosidase thwart the breaking down of carbohydrates in the small intestine to reduce the postprandial blood glucose level[7]. Recent researches are emphasizing on the fact that α - amylase and α-glucosidase inhibitors are good targets for development of new drugs that can treat not only diabetes, but also obesity and hyperlipaemia[8]. Recently, it has been shown that phenolic-rich substances like turmeric extract and its' oleoresin might play a role in mediating α - amylase inhibition and therefore have potential to contribute to the management of type 2 diabetes[9].

The present study aimed to decipher the effect of the subject pulses on the *in vitro* α - amylase and α-glucosidase inhibitory activities after thermal processing in water that resembled cooking methods commonly practiced in Indian households. It should be noted that both the water extractive and the solid remains are consumed as '*Dahl*' in India, which means the entire antioxidative principles of the pulses are consumed as food. Given the important role of pulses play in daily nutrition, special emphasis was given on the antioxidant profile of the pulses *in vitro* before and after undergoing thermal treatment to understand the role of the polyphenolics in the inhibition of the key enzymes linked with type II diabetes. To our knowledge, it was one of the very few studies that dealt with human consumable water extractives of foodstuffs for their antidiabetic potential, and probably the first with the subject pulses. In this way, we would be able to get a preliminary idea about the appropriate cooking methods that retain the most effectiveness of the pulses for human consumption.

MATERIALS AND METHODS

Chemicals

Analytical grades of thiobarbituric acid (TBA), ascorbic acid, gallic acid, anhydrous aluminium chloride, sodium hydroxide and sodium carbonate were obtained from Merck, India. 2-Deoxy-D-ribose, 2,4,6-Tris-(2-pyridyl)-*s*-triazine (TPTZ), 3,5-Dinitrosalicylic acid (DNS) and Folin-Ciocalteau's solution were purchased from Loba Chemie, India. The enzymes α - amylase and α-glucosidase and their substrates were purchased from SRL, India. All other reagents and chemicals used were of analytical grade procured from local sources. Deionized distilled water was used in the entire study.

Preparation of samples

The pulses used in the present study were Lentil (*Lens culinaris*), Green gram (*Vigna radiate*), Bengal gram (*Cicer arietinum*), Red kidney beans (*Phaseolus vulgaris*) and Soybean (*Glycine max*), and coded as LC, VR, CA, PV and GM, respectively. The samples were procured from local markets of Barasat, Kolkata. They were checked for dirt or any visible damages prior to the study and discarded if found. 5 gms each of the samples were suspended in 50 ml double distilled water, separately. The samples were extracted by the following procedures –

(i) Heating at 80^0C for 10 minutes (coded as – HT),

(ii) Treating in a microwave oven at high power for 5 minutes (coded as – MW),

(iii) Putting in a pressure cooker for 10 minutes (coded as – PC).

To estimate the optimum activities of each sample, the samples were warmed separately at 60^0C for 10 minutes after suspended in 60% methanol in water. After extraction, the samples were centrifuged at 8000 rpm for 5 mins. The clear supernatants were used for *in vitro* antidiabetic and antioxidant assays and coded as OS.

In vitro α-Amylase inhibition assay

The inhibition assay was performed according to an established method with minor modifications[9]. 10-100 µl of sample in water and 20 µl of 0.02M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) containing *α-amylase* solution (0.5 mg/ml) were incubated at 37 ± 2^0C for 10 min. After pre-incubation, 25 µl of 0.5% starch solution in 0.02M sodium phosphate buffer was added to each tube at timed intervals and the volume was made up to 450 µl. The reaction mixtures were then incubated at 37 ± 2^0C for 10 min. The reaction was stopped with 50 µl of 3,5-dinitrosaliylic acid (DNS) reagent. The tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted with 500 µl water and absorbance was measured at 540 nm using a UV-Vis spectrophotometer (model – Systronics 2202). The amount of sample required to inhibit 50% of α-glucosidase under the assay conditions was defined as the IC_{50} value.

In vitro α-Glucosidase inhibition assay

This inhibition assay was performed according to an established method of with minor modifications[9]. *α*-glucosidase type I (20 µl, 1 U/ml in 50 mM phosphate buffer) was premixed with sample and made up to 500 µl with 50 mM phosphate buffer at pH 6.8. Then it was incubated for 5 min at 37 ± 2^0C. 1 mM *p*NPG (200 µl) in 50 mM of phosphate buffer was added to initiate the reaction and the mixture was further incubated for 20 min at 37 ± 2^0C. The reaction was terminated by the addition of 500 µl of 1M sodium carbonate and the final volume was made up to 1.5 ml with water. *α-glucosidase* activity of the mixtures was determined by measuring the quantity of nitrophenol released from *p*NPG. The absorbance of the mixtures at 405 nm was measured using a UV-Vis spectrophotometer

(model – Systronics 2202). The amount of sample required to inhibit 50% of *α-glucosidase* under the assay conditions was defined as the IC_{50} value.

Assay of glucose uptake in Yeast cells in vitro

Yeast cells were prepared according to an established method[10]. Briefly, commercial baker's yeast was washed by repeated centrifugation (3000×g; 5 min) in distilled water until the supernatant fluids were clear and a 10% (v/v) suspension was prepared in distilled water. Various concentrations of extracts (1–5 mg) were added to 1 mL of glucose solution (5, 10 and 25 mM) and incubated together for 10 min at 37 ^{0}C. Reaction was started by adding 100 µl of yeast suspension, vortex and further incubated at 37^{0}C for 60 min. After 60 min, the tubes were centrifuged (2500 × g, 5 min) and glucose was estimated in the supernatant. The amount of sample required to take up 50% of the glucose content of the experimental solution under the assay conditions was defined as the EC_{50} value.

Estimation of total phenolics content

Total phenolic compound contents were determined by the Folin-Ciocalteau method[11]. The samples (0.5 ml) were mixed with Folin-Ciocalteau reagent (5 ml, of 1:10 diluted sample with distilled water) for 5 min and aqueous sodium carbonate (4 ml, 1 M) was then added. The absorbance of the reaction mixture was then measured at 765 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid was used as standard. The results were expressed in terms of gallic acid equivalents (µg/gm sample).

FRAP: Ferric reducing antioxidant power

The experiment was done with a previously described procedure[12]. Briefly, a maximum of 100 µl of extract solution or standard was mixed with 1.9 mL of FRAP reagent and incubated at 37^{0}C for 30 mins. FRAP reagent was prepared by mixing 50 mL of 0.1 M acetate buffer (pH 3.6), 5 mL of 10 mM TPTZ solution and 5 mL of 20 mM $FeCl_3$ solution. After the stipulated time period, absorbance was measured at 593 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid is used as standard. Results are expressed as Gallic acid equivalents (µg/gm sample).

Inhibition of lipid peroxidation in vitro

Inhibition of lipid peroxidation *in vitro* by the samples was estimated with a previously described procedure with minor modifications[13]. A 10% (w/v) liver homogenate was prepared using ice-cold KCl (0.15M) with fresh goat liver and protein content was adjusted to 500µg/ml. In the control system, the lipid peroxidation was initiated by the addition of 0.1ml of $FeSO_4$(25 µM), 0.1ml of ascorbate (100 µM) and 0.1ml of KH_2PO_4(10 mM) and the volume was made up to 3 ml with distilled water and incubated at 37^0C for one hour. Then 1 ml of 10% TCA and 1 ml of 0.67% TBA in 50% acetic acid was added to this reaction mixture and the tubes were boiled for 30 mins in a boiling water bath. This was centrifuged at 3500 rpm for 10 min. The extent of inhibition of lipid peroxidation was evaluated by the estimation of Thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance of the clear supernatant at 532 nm. In the test, system homogenate was incubated with various concentrations of extracts (1-1000 µg/ml). Results were expressed in terms of gallic acid equivalents (µg/gm sample)

Statistical analysis

All experiments were done in quadruplicate. Statistical tests were carried out using the software SPSS Statistics 17.0 (IBM Corporation).

RESULTS

Inhibitory activities of the pulses against the two enzymes, viz. *α-amylase and α-glucosidase*, related to diabetes complications, were adjudicated by the IC_{50} values of the subject pulses, before and after thermal treatments. It was observed that IC_{50} values for *α-amylase* inhibition of the four pulses, except rajma, were increased after microwave treatment when compared to the ideal sample (Fig. 1). This indicated deterioration of the enzyme inhibitory potential of the samples. However, rajma showed better result after microwave treatment, when the IC_{50} value has become 1.85 µg/ml in comparison to 2.08 µg/ml of the ideal sample. Improvement of the inhibitory activity was also found after normal heat treatment in case of lentil (IC_{50} values diminished from 1.47 µg/ml to 1.21 µg/ml).

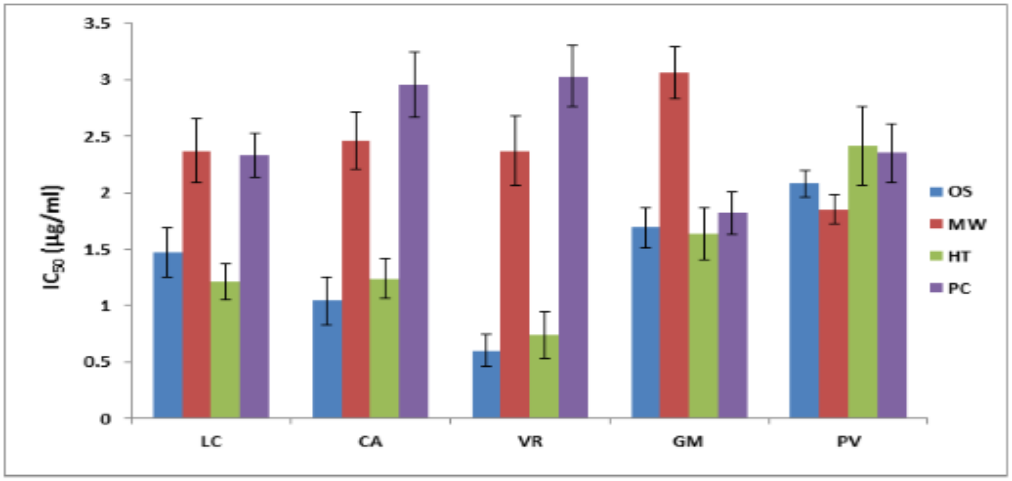

Fig 1. Comparative *α*-amylase activities of the pulses after thermal treatments, with respect to the standard sample (i.e. extracted with 60% aqueous methanol). OS – standard sample, MW – microwave treated sample, HT – water-boiled sample and PC – pressure cooked sample; LC – lentil, CA – Bengal gram, VR – green gram, GM – soybean and PV – rajma.

On the contrary, IC_{50} values for *α*-glucosidase inhibitory activities were notably lowered in cases of the two grams and soybean, clearly indicating an improvement of the enzyme inhibitory potential after microwave treatment (Fig. 2). In case of the other two pulses, there were improvements after microwave treatment, albeit within standard deviation limit. It was interesting to note that there were tendencies of improvement of enzyme activities in case of all heat treatment protocols in case of Bengal and green grams, although the lowering of IC_{50} values were within standard deviation limit.

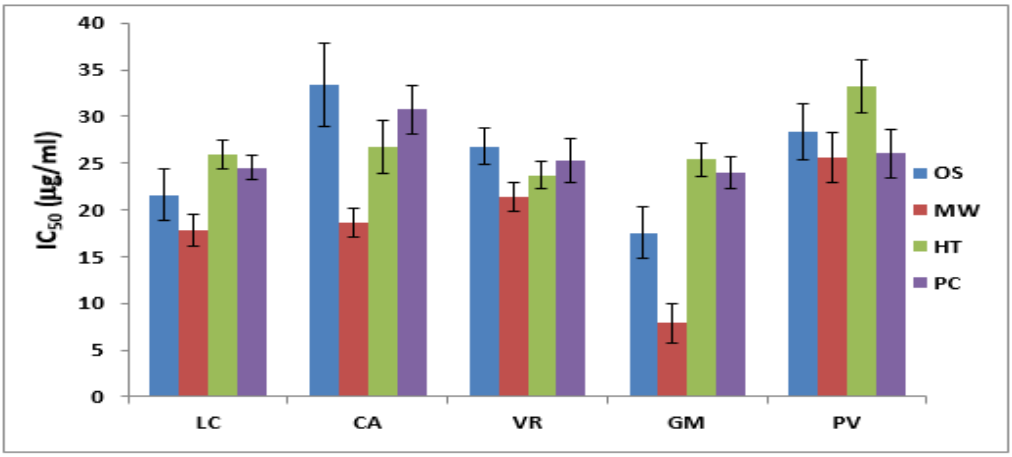

Fig 2. Comparative *α*-glucosidase activities of the pulses after thermal treatments, with respect to the standard sample (i.e. extracted with 60% aqueous methanol). OS – standard sample, MW – microwave treated sample, HT – water-boiled sample and PC – pressure cooked sample; LC – lentil, CA – Bengal gram, VR – green gram, GM – soybean and PV – rajma.

It was also observed that, apart from rajma, the four pulses were very effective to inhibit glucose uptake by live yeast cells after microwave treatment, as depicted in Fig. 3. The most effective of them were Bengal gram, where the EC_{50} value changed from 1.47 µg/ml to 0.33 µg/ml. No major change was observed in rajma after following the treatment regimens, except a serene indication of betterment after pressure cooking.

As we look into the total phenolic contents of the samples (Fig. 4), it was clear from the experiment that phenolic contents increased with heat treatment of the pulses. There were lowering of phenolic contents after microwave treatment or pressure cooking.

The FRAP assay results in Fig. 5 depicted that reductive power of all the pulses improved after microwave irradiation and pressure cooking. The gallic acid equivalent values increased appreciably after microwave treatment in case of the pulses except for soybean. However, in case of soybean, considerable improvement was observed after heat treatment and pressure cooking.

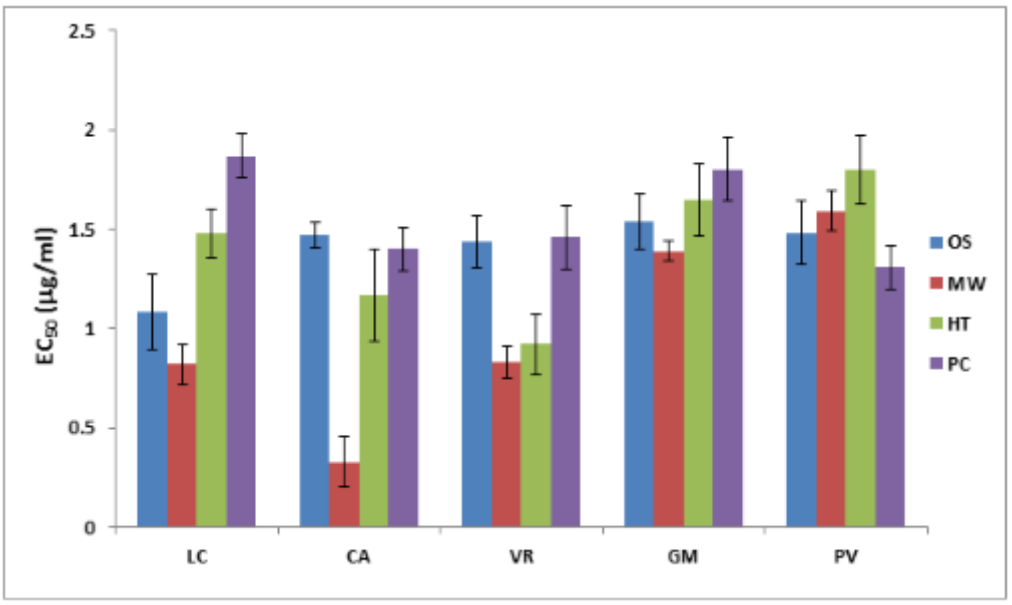

Fig 3. Comparative inhibitory capacities of the pulses after thermal treatments against glucose uptake by yeasts, compared to the standard sample (i.e. extracted with 60% aqueous methanol). OS – standard sample, MW – microwave treated sample, HT – water-boiled sample and PC – pressure cooked sample; LC – lentil, CA – Bengal gram, VR – green gram, GM – soybean and PV – rajma.

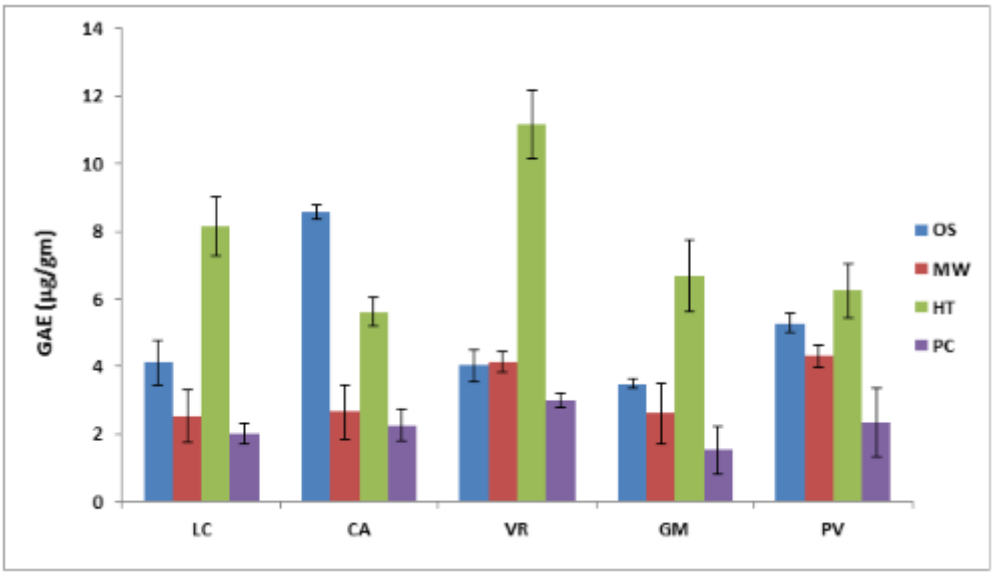

Fig 4. Comparative total phenolic contents of the pulses after thermal treatments, compared to the standard sample (i.e. extracted with 60% aqueous methanol). OS – standard sample, MW – microwave treated sample, HT – water-boiled sample and PC – pressure cooked sample; LC – lentil, CA – Bengal gram, VR – green gram, GM – soybean and PV – rajma.

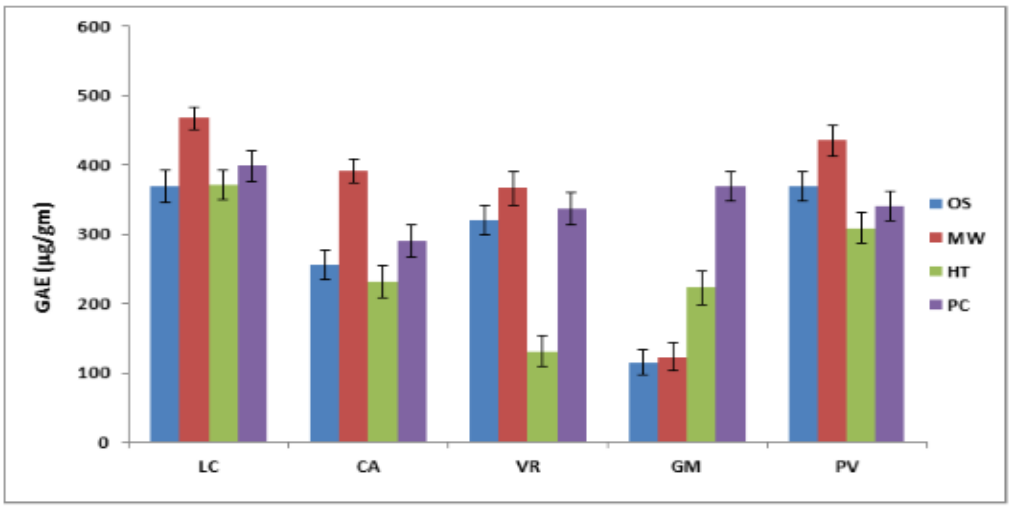

Fig 5. Comparative ferric reducing antioxidant potential (FRAP) of the pulses after thermal treatments, compared to the standard sample (i.e. extracted with 60% aqueous methanol). OS – standard sample, MW – microwave treated sample, HT – water-boiled sample and PC – pressure cooked sample; LC – lentil, CA – Bengal gram, VR – green gram, GM – soybean and PV – rajma.

The extracts, after thermal treatments, showed considerable improvement in their lipid peroxidation inhibitory activities in *in-vitro* model (Fig. 6). The gallic acid equivalent values improved two- to two-and-half-fold in case of lentil and Bengal gram in all treatment

protocols. Amongst the treatment protocols, notable improvement was observed after heat treatment and pressure cooking in case of all the pulses.

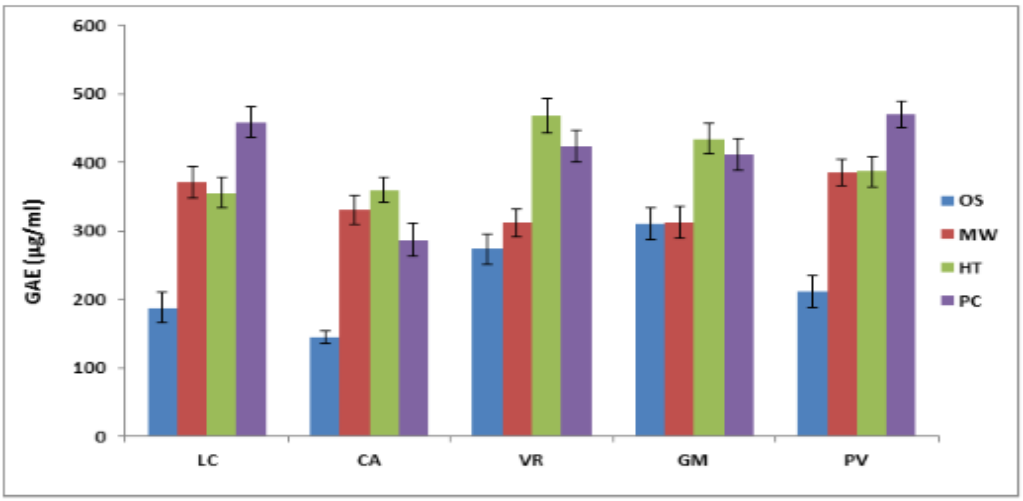

Fig 6. Comparative lipid peroxidation inhibitory activities of the pulses after thermal treatments, compared to the standard sample (i.e. extracted with 60% aqueous methanol). OS – standard sample, MW – microwave treated sample, HT – water-boiled sample and PC – pressure cooked sample; LC – lentil, CA – Bengal gram, VR – green gram, GM – soybean and PV – rajma.

DISCUSSION

In the present study, thermally treated pulses were primarily evaluated for their inhibitory effect on *α-amylase and α-glucosidase* enzymes by *in vitro* method to adjudicate their possible role in controlling diabetes mellitus. Thermal treatment protocols resembled with the cooking procedures followed in the households of India. However, pulses were also extracted with 60% aqueous methanol, which is the standard extraction procedure for estimation of pharmacognostic potentials of natural products. Those extracts were used as comparators having ideal pharmacognostic properties[12].

The results of enzyme inhibitory studies indicated that extracts of pulses, after thermal treatment, behave differently towards the two subject enzymes. They showed positive effects against *α-glucosidase,* although the effects were not prominent against *α*-amylase. The results clearly indicated the fact that similar types of bioactives were not responsible for the inhibition of the two enzymes. One another important aspect was also indicated by this experiment that standard extraction procedure with aqueous alcohols may not be the ideal for the adjudication of the pharmacognostic potential of the natural products.

The experiment for inhibition of glucose uptake by yeast cells was designed to check the extracts' abilities to act at the receptor level. It was observed that after microwave treatment, the pulses showed better efficiencies for inhibition of glucose uptake, as were observed by notable lowering of EC_{50} values. Extraction with hot water also improved the abilities of the two grams to some extent. In general, the study indicated that the bioactives of the pulses were not effective at the receptor level. Only after microwave extraction, the extractives might act at the receptor level, as well as could bind with the saccharide-digesting enzymes to prevent their activities.

In general, there was a tendency of lowering of the total phenolics contents after treatments, probably due to degradation and/or transformation of the otherwise susceptible phenolic biomolecules. However, after hot water treatment, phenolics contents increased in case of all the pulses. Degradation of polyphenolics has been reported previously after strong treatments like microwave irradiation or use of high pressure in a pressure cooker[14,15]. But it was observed that antioxidative power of the pulse extractives improved after microwave or pressure treatments, as was evident from the FRAP assay. These lent credence to the postulate that some type of transformation of the phenolics occurred which could be beneficial in inhibiting the subject enzymes. Loss of susceptible polyphenolics during heat treatments could be compensated by formation of non-nutritional bioactives like Maillard products, which have potential antioxidant capacities[16]. This substantiated the fact that the enzymes related to diabetes could be inhibited by some transformed metabolites of the polyphenolics present in the five pulses.

Recently, it has been established that lipid peroxidation can be considered as a marker of diabetes complications[17]. Altered insulin levels in blood creates a false immune response in the body thereby evoking respiratory burst of reactive oxygen species (ROS) from the immune cells, which ultimately leads to formation of lipid hydroperoxides. It has been observed in the present study that lipid peroxidation was restrained by the pulse extracts, probably by scavenging of the ROS by the antioxidants present in them. These positive effects provided by the water extractives of the five subject pulses broadened the scope for the utilization of the food stuffs as natural antioxidants that could also be efficiently utilized in controlling diabetic's complications.

CONCLUSION

One of the various strategies, used by the researchers worldwide nowadays, is to combat diabetes mellitus by inhibiting the activities of polysaccharide hydrolyzing enzymes like *α-amylase and α-glucosidase.* The present study indicated that thermal treatment of the five common Indian pulses improved their inhibitory capacities towards enzymes like *α-amylase and α-glucosidase* in general. The effects were more astounding in case of microwave treatment. Red kidney beans or rajma showed improvement in its' *α - amylase* inhibitory activity after microwave treatment, when the IC_{50} value became 1.85 μg/ml from the optimal value of 2.08 μg/ml. The efficiencies of all other pulses after thermal treatment deteriorated. However, IC_{50} values for *α*-glucosidase inhibitory activities were almost halved in cases of Bengal gram (33.39 to 18.57 μg/ml) and soybean (17.53 to 7.90 μg/ml), clearly indicating improvement of the enzyme inhibitory potential after microwave treatment. The antioxidant activities corroborate with the reducing potential of the extracts, although the polyphenolic levels were not very high after thermal treatments. Loss of susceptible polyphenolics during heat treatments was indicated to be compensated by formation of non-nutritional bioactives like Maillard products, which have potential antioxidant capacities. As a whole, it was indicated that thermal processes, that resemble cooking, might improve the pulses' abilities to inhibit enzymes like *α-amylase and α-glucosidase*, thereby improving their antidiabetic potential, if any.

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