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Impact of bioprocessing on phenolic content and antioxidant activity of soy seed to improve hypoglycemic functionality

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ABSTRACT

Hyperglycemia, a condition characterized by an abnormal excess of sugar in the blood, has been linked to the onset of type 2 diabetes mellitus and associated to oxidation-linked vascular complications. Medicinal plants and legumes have been shown to exert their hypoglycemic effect by several mechanisms which include the inhibition of the key enzymes, pancreatic α-amylase and intestinal α-glucosidase. The aim of the present study was to investigate the enrichment of soy seed (Glycine max) substrates with phenolic antioxidants via fungal-based solid-state bioconversion (SSB) system by dietary fungus (Rhizopus oligosporus) and its effect on the inhibition of above enzymes. Total phenol content (Lowry's method) and antioxidant activity (DPPH) were determined. Inhibition of α-amylase and α-glucosidase activities were measured using starch and p-nitrophenyl-α-D-glucopyranoside as substrates respectively. Protein and total phenol content of the seed extracts increased with fungal incubation time. The extract indicated high antioxidant activity (85±0.6%), during early growth stage (days 4-6) followed by reduced activity during later growth stage (days 8-10). A direct association between higher phenolic contents during early growth stage (days 4-6) and antioxidant activity suggested a link to mobilization of polymeric and hydrophobic phenolic forms. SSB process substantially improved in vitro α-amylase inhibition activity in soy seed extracts to 75% on day 4 which is compared to higher levels of antioxidant activity. It is concluded that SSB is a good strategy to improve the phenolic content of above seed for enhanced functionality with improved antioxidant activity that contributes to α-amylase inhibition relevant to potential diabetes management.

Key words: *In vitro* study, *Glycine max*, SSB, α-amylase and α-glucosidase inhibition, DPPH assay.

INTRODUCTION

The prevalence of diabetes mellitus (DM) is rapidly rising worldwide at an alarming rate. It is emerging as a major health problem in India [1] which may affect 366 million people in 2030. Two types of DM are currently known [2], one being insulin-dependent diabetes mellitus, IDDM and the other being type 2 non-insulin-dependent diabetes mellitus, NIDDM [3]. The most common acute complications of type 2 due to the hyperglycemia-induced pathogenesis are metabolic problems and infection [4]. Hyperglycemia, a condition characterized by an abnormal postprandial increase of blood glucose level, has been associated to oxidation-linked vascular complications [5]. It is possible to reduce the risks of chronic diseases and prevent progression by either enhancing the body's natural antioxidant defenses or by supplementing with dietary antioxidants [6]. Synthetic antioxidants like BHT and BHA[7] commonly used in processed foods have side effects and are carcinogenic [8]. *In vitro* studies suggest that food like vegetables, grains, seeds[2]and legumes[9] with antioxidants have protective effects against many diseases such as cancer, diabetes and cardiovascular diseases [10].

α-amylase and α-glucosidase are key enzymes involved in starch breakdown and intestinal absorption, respectively. Inhibition of these enzymes[11] can significantly decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet and therefore can be an important strategy in the management of hyperglycemia linked to type 2diabetes [12]. A main drawback of currently used synthetic α-glucosidase and α-amylase inhibitors such as acarbose is side effects such as abdominal distention, flatulence, meteorism and possibly diarrhea [13]. Therefore, focus has been shifted to natural enzyme inhibitors from dietary plants which have inhibitory effect against α-amylase and α-glucosidase activities and can be used as effective therapy for postprandial hyperglycemia with minimal side effects [14].

Solid State Bioconversion (SSB) is microbial bioprocessing of a solid food substrate that acts as a physical support and source of nutrients in the presence of low free liquid [15]. It is a simple alternative technology with a long history in Asia to improve the nutritional quality and palatable characteristics of cereals and legumes. *Rhizopus oligosporus* is a food-grade fungus that has been widely used in solid-substrate bioconversion systems to produce value-added food products [16, 17]. It is chosen because being food-grade fungus *Rhizopus oligosporus* is demonstrated to be effective in other substrates such as fava bean, cranberry pomace, and pineapple [18]. SSB would increase the phenolic content and antioxidant activity which will enhance the potential health-relevant functionality of fungal processed seed and legumes. It is used in the biotransformation of raw cassava for protein enrichment [19] and the production of lactic acid and fumaric acid [20]. *Bacillus subtilis* is also used for solid state bioconversion. *B. subtilis* BCRC14715-fermented black soybean possessed enhanced contents of aglycone and vitaminK2 as well as superoxide dismutase activity [21].

Soybean (*Glycine max*), a phenolic-rich legume consumed worldwide, received considerable attention of late decade for their potential role in reducing the formation and progression of certain types of cancers and some chronic diseases such as cardiovascular disease, Alzheimer's disease and osteoporosis[22]. Several anti-carcinogens, including phenolics such as phenolic acids, flavonoids and isoflavonoids have been identified in soybeans among which genistein, have been shown to inhibit α-glucosidase activity *in vitro* [23]. Therefore in this study, the potential of SSB using food-grade fungus was investigated to mobilize the conjugate forms of phenolic precursors naturally found in soyseed to improve its health-linked functionality.

MATERIALS AND METHODS

Collection of seeds and microorganism:
Plant seed (Soy-*Glycine max)* were procured from Namdhari seeds, Bangalore, Karnataka and were identified by Prof. S. B Sullia, taxonomist and microbiologist, Jain University, India. Specimen samples were deposited at Jain University herbarium (voucher no. 3412). *Rhizopus oligosporus* was procured from IMTECH, Chandigarh, India and was maintained on potato dextrose agar plates and sub-cultured monthly. The fungal culture at active sporulating stage (which is approximately three weeks of culture at room temperature) was used in this study [24]

Solid state bioconversion (SSB)
For SSB studies, 10 g of finely ground dry seed powder along with 25 ml distilled water were taken in 250 ml Erlenmeyer flasks and autoclaved. The fungal spores and mycelia were inoculated and incubated at room temperature for 10 days. The samples were extracted at two-day intervals for 10 days. The culture was homogenized and centrifuged at 15,000 g at 4 °C for 20 min. The supernatant was then filtered through a Whatman No. 1 filter paper[25].

Determination of total protein content
Total protein content of the bioprocessed seed extracts were determined by Lowry's method using BSA as standard and absorbance was measured at 660 nm[26]. All determinations were carried out in triplicates.

Total phenols content
Total phenol content of the bioprocessed seed extracts was determined using the method of McDonald *et al*.,2001 with slight modifications [27]. Gallic acid was used as the standard for the preparation of calibration graph. (concentration range: 0.025-0.4 mg/ml) Absorbance was measured at 765 nm. Phenol concentration in the extracts was calculated in terms of gallic acid equivalent (GAE).

Antioxidant activity

Antioxidant activity of the bioprocessed seed extracts was determined on the basis of the scavenging effect on the stable DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical activity[28]. DPPH solution was freshly prepared and kept in the dark at 4°C. To 3 ml of 60 µM DPPH, 100 µl of seed extracts were added and the absorbance was monitored at 517 nm. The radical scavenging activity of the SSB seed extracts were compared with the activity of equivalent concentration of ascorbic acid and expressed as DPPH radical inhibition percent. All determinations were performed in triplicates. The radical scavenging activities of the tested samples, expressed as percentage of inhibition were calculated according to the following equation [29].

Percentage inhibition of DPPH activity = [(AB- AA) / AB] × 100

Where AA and AB are the absorbance values of the test and of the blank sample respectively.

Determination of α-amylase activity

α-amylase activity was determined in bioprocessed seed extracts in a colorimetric reaction using 3,5-dinitrosalicylic acid[30]. A standard curve was generated using D-(+)-maltose monohydrate. Activity was calculated as units/ mg protein, where 1 unit was defined as the amount of enzyme required to liberate 1µmol of maltose under assay conditions. Data was reported as amylase inhibition (AI) index values, defined herein as the ratio of the amylase activity of the control (enzyme alone) to that of the enzyme/clonal extract mixture[31]. Values greater than 1 indicate amylase inhibition. The α-amylase activity is determined for unprocessed and bioprocessed seed extracts by same method.

Determination of α-glucosidase inhibition

The inhibitory activity of bioprocessed seed extracts against yeast α-glucosidase was determined by measuring the formation of *p*-nitrophenol by α-glucosidase after reaction with *p*-nitrophenyl-α-D-glucopyranoside (PNP) as substrate in the presence and absence of seed extracts. 0.2mL of 0.2 mM PNP substrate (dH_2O for control) and 0.2mL of the enzyme (α-glucosidase) was added to seed extract mixture. This reaction tube was incubated 30min in a 30°C water bath. The reaction was stopped by addition of 0.6mL of 1M sodium carbonate and absorbance was determined by spectrophotometer at 405 nm[31]. Data was reported as α- glucosidase inhibition (aGI) index values, defined as the ratio of the α-glucosidase activity of the control (enzyme alone) to that of the enzyme/ seed extract mixture. Values greater than 1 indicate α-glucosidase inhibition.

Statistical analysis

Experiments were performed in triplicates. The average values of the experiments for each day with standard deviations are reported in the figures. Statistical analysis was performed using MS- Excel software.

RESULTS AND DISCUSSION

During the SSB of seed substrates, the growth of the fungus was rapid from day 6 of inoculation and efficiently colonized the entire substrate by day 10.The fungal growth was monitored by the changes in the protein content of the seed extracts. On 6^{th} day, the protein content has increased in soy by 97% (Figure 1). The fungal growth was monitored by the changes in the protein content of the seed extracts. Earlier studies in cranberry, fava bean and mung bean extracts indicate that there was a linear increase in protein content with fungal growth [25,27].

The above studies indicated an efficient colonization of the fungus on the seed substrates, hence phenolic mobilization by the fungus was investigated. In the case of fava bean this enzymatic hydrolysis of conjugated phenolics resulted in improved free phenolic content which enhanced the health-relevant functionality of the substrate[30]. Free phenolics usually exist in conjugate forms with one or more sugar residues bound to hydroxyl groups or groups of compounds such as carboxylic and organic acids, amines and lipids[33]. The changes in the total phenolic content due to mobilization by *R. oligosporus* on the seed substrates are shown in Figure 2. The phenolic content doubled by day 6 when an increase in protein content was detected, indicating an active phenolic mobilization by the fungal enzymes[25]. Total phenol content has increased in soy on 6th day by 99%. In the case of soy bean substrate phenolic content has increased on 6^{th} day to 9.8 mg/g DW from 2.5 mg/g DW on 0^{th} day (Figure 2).

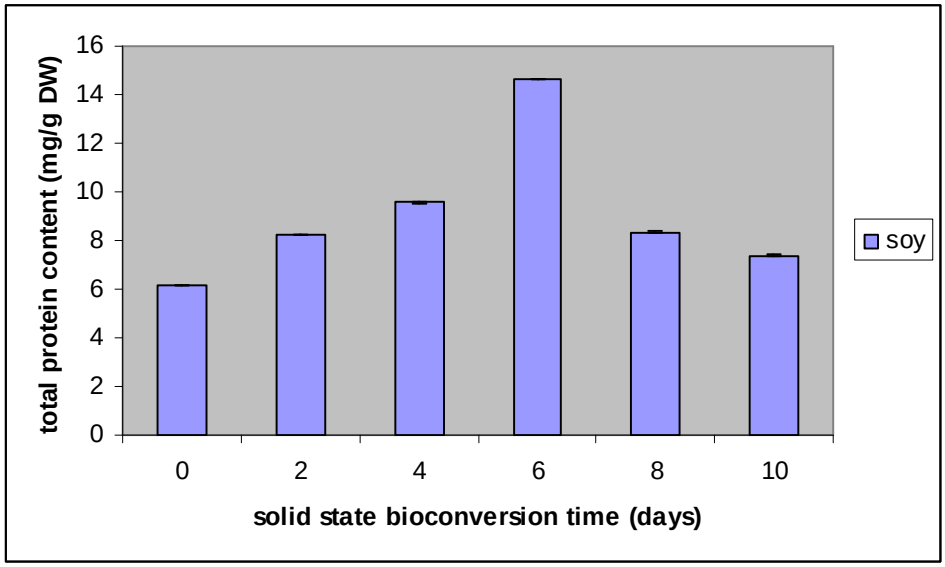

Figure 1: Effect of SSB on total protein content in soy extracts

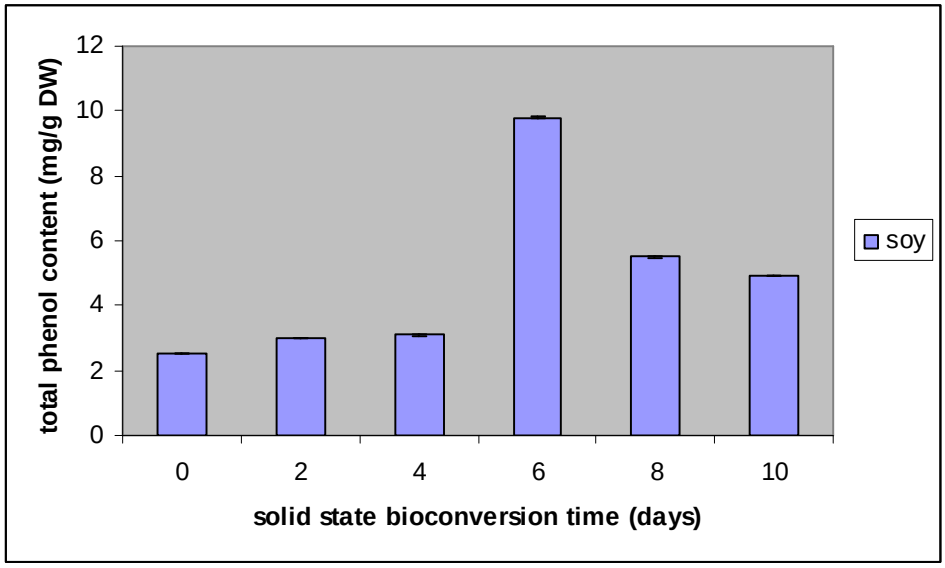

Figure 2: Effect of SSB on total phenol content of soy substrates

Higher phenolic content during late stages may also indicate a stress response due to nutrient depletion as the bioprocessing progressed as also reflected in higher antioxidant activity[25]. In the present study, antioxidant activity of the bioprocessed seed extracts was measured by the DPPH free radical scavenging method. The DPPH method estimates the ability of the extract to quench the DPPH free radical. Antioxidant activity has increased in bioprocessed soy on 4th day by 57.4% (Figure 3). The antioxidant activity of the bioprocessed seed extracts correlated to the phenolic content only during certain stages of growth (Figure 2 and Figure 3). This suggests that the antioxidant function of the seed extracts depend on the qualitative characteristics of phenolic profile and not just the total phenolic content.

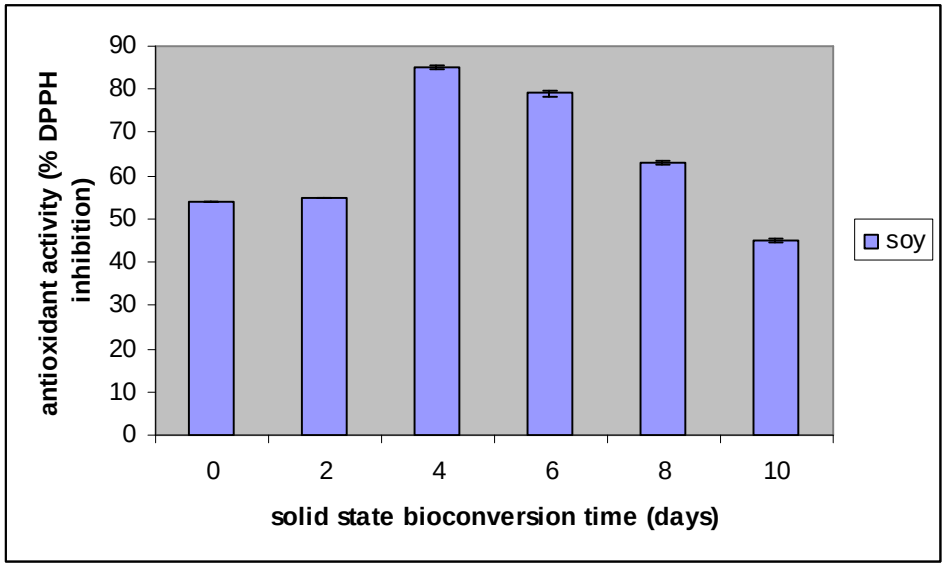

Figure 3: Antioxidant activity of soy substrates during SSB by *R. oligosporus* as measured by DPPH radical inhibition method.

α-Amylase is responsible for cleaving starch during digestive process, which is important to manage postprandial blood glucose levels. A bi-functional inhibitor from seed extracts which exhibit inhibitory activity towards trypsin-like and α-chymotrypsinlike serine proteinases as well as against α-amylases has been reported [34]. The α-amylase inhibitory activity of the bioprocessed seed extracts was moderately high during early incubation (days 0–2) (Figure 4). Extracts of *R. oligosporus*-bioprocessed soy had the strongest anti-amylase activity, specifically on 6th day of culture time (AI index value of soy = 3.6± 0.005) (Figure 4).

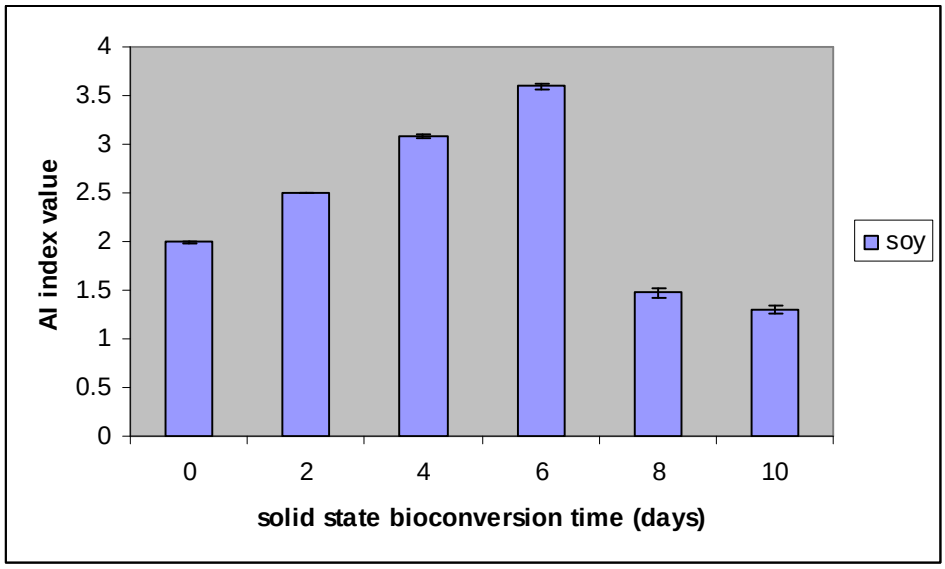

Figure 4: AI index in bioprocessed soy for alpha amylase inhibition

Higher inhibition was observed during days 4–10 which correlate with higher antioxidant activity (Figure 4 and Figure 6). Lower inhibition was observed during later stages of SSB, which might be due to the breakdown of phenolic compounds with limitation of nutrients for fungal subsistence and acute oxidative stress as indicated by high antioxidant activity. Extracts of *R. oligosporus*-bioprocessed soy extracts had the strongest anti-glucosidase activity, specifically on 6th day of culture time (soy, aGI index value = 2± 0.02) (Figure 5).

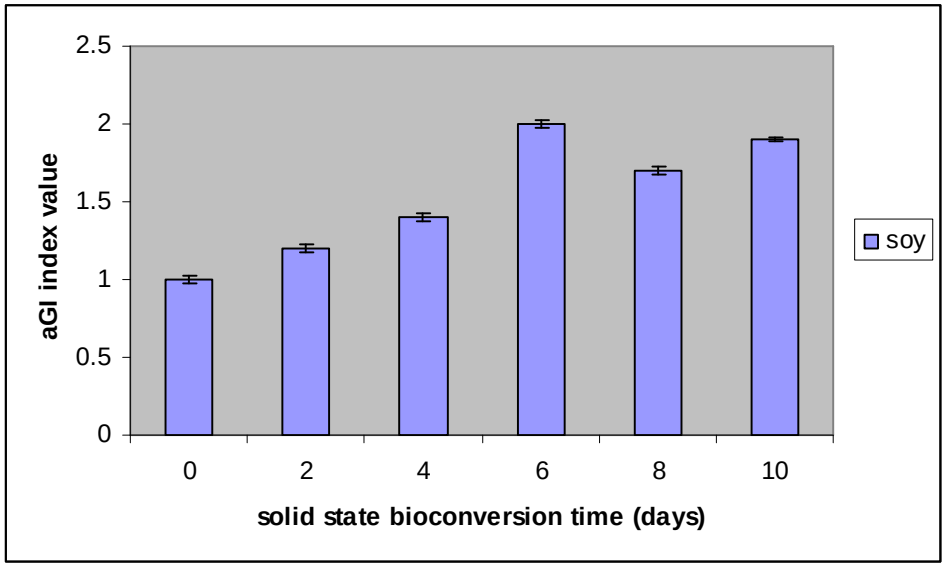

Figure 5: α-Glucosidase inhibition (aGI) by extracts of bioprocessed soy

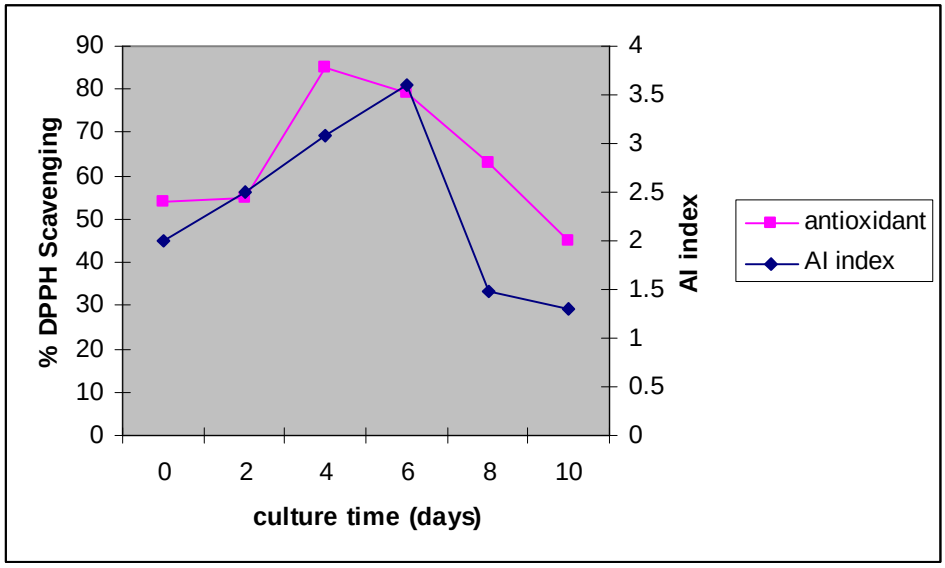

Figure 6: AI index versus DPPH scavenging activity of bioprocessed soy.

Results from the present study indicate that during the early stages of bioprocessing there is enhancement of total phenolics and antioxidant activity in the soy bean extract. This increase also correlates to higher α-amylase and α-glucosidase inhibition [25]. We propose that the improvement in functionality is due to the fungal enzymatic hydrolysis of the seed extracts.

Relationship of extract antioxidant activity to antidiabetic activity

As soybean extract anti-amylase activity was more pronounced than anti-α-glucosidase activity, we focused on the anti-amylase activity as representative of the soybean extract anti-diabetic activity for further comparison studies. For bioprocessed soybean extract anti-amylase activity was compared to antioxidant activity. These results suggest that antioxidant activity may play a role in the anti-amylase (e.g. antidiabetic) activity of bioprocessed soy bean extracts (Figure 6).

CONCLUSION

Present study revealed that during the early stages of bioprocessing, there is enhancement of total phenolics and antioxidant activity in the above seed extract. This increase in antioxidant activity also correlates to higher α-amylase inhibition which can control or prevent the onset of long-term complications of postprandial hyperglycemia and diabetes mellitus. Natural α-amylase and α-glucosidase inhibitors from food-grade plant sources offer an attractive therapeutic approach to the treatment of post-prandial hyperglycemia by decreasing glucose release from starch and delaying carbohydrate absorption in the small intestine and may have potential for use in the treatment of diabetes mellitus and obesity. Food-grade phenolic α-amylase inhibitors from dietary plant extracts are potentially safer, and therefore may be a preferred alternative for modulation of carbohydrate digestion and control of glycemic index of food products. The major implication of this research is that SSB can be an efficient strategy to improve the phenolic content of these seed extracts with associated enhancement of health-linked functionality.

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