ORIGINAL ARTICLE

Pigeon pea seed husks as potent natural resource of anti-oxidant and anti-hyperglycaemic activity

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Background: Pigeon pea (*Cajanus cajan* L. Millspaugh) is an important food legume. Its seed, root and leaves have been explored for various medicinal properties. Pigeon pea seed husks constitute the major by-product of milling industries and are mainly used as cattle feed. **Aim:** To explore anti-oxidant and anti-hyperglycaemic potential in pigeon pea seed husks. **Materials and Methods:** Methanolic extracts of pigeon pea seed husks were evaluated for various phytochemical constituents, free radicals scavenging and anti-oxidant potentials *in vitro*. Crude methanol extract was further evaluated for its anti-hyperglycaemic activity in starch-induced postprandial hyperglycaemic rats. **Results:** Methanolic extracts of seed husks were found to be the rich source of polyphenols and protein and therefore possess potent free radicals scavenging, anti-oxidant activities *in vitro*. Furthermore, crude methanol extract of pigeon pea seed husks significantly ($P < 0.05$) mitigated starch-induced postprandial glycaemic excursions and reduced glycaemic load in rats similar to the standard drug acarbose. **Conclusion:** Observations made in this study indicate that husks of pigeon pea seeds may serve as potential natural resource for the development of anti-oxidant rich anti-hyperglycaemic functional food and/or nutraceuticals.

Key words: Anti-hyperglycaemic activity, anti-oxidant activity, *Cajanus cajan*, pigeon pea seed husks

INTRODUCTION

Pigeon pea (*Cajanus cajan* L. Millspaugh) is an important food legume. It is a rich source of starch, protein, calcium, manganese, crude fibre, fat, trace elements and minerals.[1] Pigeon pea seeds are made up of 85% cotyledons, 14% seed coat, about 1% embryo and contain a variety of dietary nutrients.[2] The cotyledons are rich in carbohydrates (66.7%) while a major proportion (about 50%) of seed protein is located in the embryo.[1] According to an estimate it is cultivated on 4.79 M ha in 22 countries.[3]

Besides its high nutritional value, pigeon pea is also used as traditional folk medicine in India, China, Philippines and some other nations. Pigeon pea floral decoctions are traditionally used for treating ailments such as bronchitis, cough and pneumonia. Scorched seeds, when added to coffee alleviate headache and vertigo.[1] Fresh seeds are believed to help incontinence of urine in males, while immature seeds are recommended for treatment of kidney ailments.[4] Dried roots of pigeon pea are used as an alexeritic, anti-helminthic, expectorant, sedative and vulnerary.[1] In India, pigeon pea leaves are used for curing sores, wounds, abdominal tumours and diabetes.[5,6] The young leaves of pigeon pea are also chewed for treating cough, diarrhoea,[7] traumatism, burnt infection, bedsore, toothache, mouthwash, sore gums, child-delivery and dysentery.[8,9] Leaves have been well explored for their anti-oxidant properties[10] and are reported to be the rich source of flavonoids, isoflavonoids and stilbenes.[11]

Although, the husk of pigeon pea is utilised mainly as cattle feed, recent reports find it to be an excellent material for treating waste waters containing low concentration of metal ions,[12] in manufacture of biscuits[13] and also as an anti-microbial agent.[14] Despite the fact that husk of leguminous plant seeds constitute major by-product of milling industries, little attention have been paid to explore their medicinal properties. Recently, we have explored and identified anti-oxidant and anti-hyperglycaemic activities in number of Indian food materials.[15-19] In the present study, we report analysis of phytochemical components, anti-oxidant properties and anti-hyperglycaemic activity in pigeon pea seed husk.

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MATERIALS AND METHODS

Chemicals

Chemicals used in this study were purchased from Sigma-Aldrich chemicals (St Louis, MO USA),

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Merck (India) Ltd. (Mumbai, India) and S.D. Fine Chemicals Ltd. (Mumbai, India).

Preparation of Extracts

C. cajan seed husks (CCHs) were obtained from a pulse processing plant in Kurnool, Andhra Pradesh (India). Maceration was employed for their extraction. Four kilograms of CCH was soaked in 8 L of methanol (labelled CCHME) and acidified methanol (labelled CCHacME) for 7 days and filtered. The marc was soaked again for 3 days. Hence obtained extracts were concentrated in a rotary evaporator (Heidolph LABOROTA Collegiate, Germany) and stored at room temperature for the study.

Analysis of Chemical Components

Total polyphenolic content

Total phenolic content in the extracts was measured using Folin-Ciocalteu reagent.[20] Briefly, 25 µL of extract (10 mg/mL in DMSO) was diluted with 2.5 mL of distilled de-ionised water followed by addition of 250 µL of Folin-Ciocalteu reagent (1 M) and 250 µL of Na_2CO_3 (20% w/v). The mixture was incubated at room temperature for 60 min and absorbance was measured spectrophotometrically at 765 nm. Results were expressed in terms of micrograms Gallic acid equivalent (GAE).

Total anthocyanins

Presence of anthocyanins in the extract was determined applying the method described earlier.[21] Briefly, to 25 mM potassium chloride solution (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5), equal volumes of extract (10 mg/mL in DMSO) was added and absorbance was measured at 510 and 700 nm, respectively. Data was expressed using molecular extinction co-efficient, molecular weight of anthocyanins and an absorbance of A = [(A_{510}-A_{700} pH 1.0- (A_{510}-A_{700}) pH 4.5] as milligrams of anthocyanins per 100 g of extract.

Total protein content

Total protein content in the extract was determined using Bradford's reagent.[22] Briefly, 10 µL of extract (10 mg/mL in DMSO) was incubated with 240 µL of Bradford reagent and absorbance was read at 595 nm spectrophometrically. Protein concentration was expressed applying a BSA regression curve.

Determination of Free Radicals Scavenging Anti-Oxidant Potentials

$ABTS^+$cation scavenging

End point decolourisation of 2, 2'-Azinobis (3-ethyl benzthiazoline-6-sulphonic acid) cation ($ABTS^+$) was measured following the method reported earlier.[23] Determination of $ABTS^+$ scavenging concentration 50% (SC_{50}) in reaction medium was performed with several serial dilutions of the extract. Percentage scavenging of $ABTS^+$ was calculated by applying formula: [(Absorbance control-Absorbance test)/Absorbance control] × 100. The SC_{50} values were calculated applying suitable regression analysis.

DPPH radical scavenging

Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined as reported earlier.[16] Briefly, in a 96-well microplate reaction mixture was prepared with 25 µL extract (5 mg/mL DMSO), 100 µL of 0.1 M Tris–HCl buffer (pH 7.4), 125 µL DPPH solutions (0.5 mM in methanol) and incubated in dark for 30 min. DPPH decolourisation was recorded at 517 nm. Percentage DPPH scavenging and SC_{50} values were calculated as above.

ABTS [2, 2'-Azino Bis (3-Ethyl Benzothiazoline-6-Sulphonic acid)] oxidation assay

Mixture of ABTS liquid substrate and ABTS enhancer solution in the ratio of 10:1 was prepared[16] and stored in dark. To 160 µL of this mixture, 20 µL of test extract (5 mg/mL in phosphate buffer saline) was added in 96-well microplate. Oxidation of ABTS mixture was initiated by addition of 20 µL of potassium persulphate (6.9 mM, pH 8.0). Individual blanks for test samples were prepared to correct the background absorbance where the ABTS solution was replaced with 160 µL of PBS. Absorbance was read at 734 nm. The percentage of oxidation inhibition by the test materials was determined as explained earlier.

Reduction of nitro blue tetrazolium

Nitro blue tetrazolium (NBT) reducing activity in extract was determined following the method reported earlier.[16] Briefly, in a 96-well plate 100 µL phosphate buffer (50 mM, pH 10), equal quantity of NBT (1 mM, prepared in same buffer) and 50 µL of extract (5 mg/ml DMSO) was mixed and incubated for 15 min. A blank with extract in absence of NBT was run to correct background absorbance. Reduction of NBT was measured at 560 nm using a BioTek synergy4 multimode microplate reader (BioTek Instruments Inc, Winooski, VT, USA). The percentage of NBT reduction by extract was calculated applying the formula mentioned earlier. Reducing power 50% (RC_{50}) was determined as procedures applied for calculation of SC_{50} values.

Ferric chloride reducing activity

$FeCl_3$ reducing activity[15] was modified to suit 96 well plates reading. In Eppendorf tubes, 100 µL of various dilutions of sample extract were mixed with 100 µL of phosphate buffer and incubated with 100 µL of 1% potassium ferricyanide at 50°C for 20 min. The reaction was terminated by the addition of 10% trichloroacetic acid (TCA) and centrifuged (Heraeus, Biofuge stratos) at 3000 rpm for 10 min. One hundred microlitre of supernatant was transferred to 96

well micro plates. Further, 100 µL of distilled water and 20 µL of 0.1% $FeCl_3$ was added and mixed well. Absorbance was measured at 700 nm and percentage of reducing power was determined as NBT reducing method. Reducing power 50% (RC_{50}) was determined as mentioned above.

Hydroxyl radical mediated 2-deoxy-D-ribose oxidation

The method is based on spectrophotometric measurement of thiobarbituric acid (TBA)-reactive carbonyl compounds formed during oxidation of 2-deoxy-D-ribose by hydroxyl radicals.[19] Hydroxyl radicals were generated by Fenton reaction (Fe^{3+}-ascorbate-EDTA-H_2O_2). The reaction mixture contained $FeCl_3$ (25 mM) pre-mixed with EDTA (10 mM) in KH_2PO_4 buffer (pH 7.4), 2-deoxy-D-ribose (10 mM), H_2O_2 (10 mM), ascorbic acid (1 mM) and 35 µL sample. Tubes containing samples were placed in a water bath at 37°C for 1 h. Thereafter, 1 mL each of 1% thiobarbituric acid and 2.8% TCA were added. Reaction mixture was heated in a water bath at 80°C for 20 min. After reaching room temperature absorbance of the mixture was measured at 532 nm. The percentage protection of 2-deoxy-D-ribose by test material was determined applying the formula given earlier. The inhibitory concentration 50% (IC_{50}) was calculated applying methods above.

Prevention of glucose induced haemoglobin glycation

Blood was collected from adult male Wistar rats in tubes containing EDTA and centrifuged at 1000 rpm for 20 min. Pellet was suspended in PBS (pH 7.4). Red blood cells (RBCs) were lysed with two volumes of lysis buffer. Centrifugation was carried out to remove debris and supernatant containing haemoglobin was collected and diluted with PBS so as to get 5 g/dL concentration. Next, 500 µl of above solution was transferred to Eppendorf tube and incubated with 200 µL extract (10 mg/mL DMSO) for 10 min. Further, 500 µL solution containing glucose (2 g/100 mL) and gentamycin (20 mg/mL) was added and again incubated for 72 h. Amount of glycated haemoglobin in the reaction mixture was calculated by measuring absorbance at 443 nm.[24]

Intestinal α-glucosidase inhibition

Inhibition of rat intestinal α-glucosidase enzyme was determined as reported earlier.[19] Twenty microlitre of extract (10 mg/mL DMSO) was incubated with 50 µL of crude intestinal α-glucosidase for 5 min and then with 50 µL of substrate 5 mM p-nitrophenyl-α-D-glucopyranos ide. Absorbance was measured at 405 nm. Percent enzyme inhibition was calculated applying formula mentioned earlier.

Animal experiments

Animal experiments were performed using adult male Wistar rats (180-220 g body weight). Institutional Animal Ethical Committee (CPCSEA Reg. No. 97/1999, Government of India) approval for experimental protocol was obtained. All experiments with live animals were performed in compliance with relevant laws and institutional guidelines. Experiments were performed as reported earlier.[16] All animals were fasted overnight. Next day forenoon, blood was collected from retro-orbital plexus in EDTA-containing tubes. Plasma glucose levels for basal ('0' h) value were measured by glucose-oxidase test method using auto-blood analyser instrument (Seimens Dimension Xplus analyser, New York, USA).

Rats were divided into test, control and standard groups (six rats in each group). Samples were triturated with normal saline to form a suspension and administered at a dose of 500 mg/kg body weight to respective groups of animals orally through gastric intubation. Control group of animals were administered normal saline. Thirty minutes after sample administration, soluble-starch dissolved in normal saline at a dose of 2 g/kg body weight was administered. Thereafter, blood was collected at the intervals of 30, 60, 90 and 120 min post-starch feeding. Plasma was separated out for glucose measurement as described earlier. Two-hour postprandial glycaemic load ($AUC_{0\text{-}120\ min}$ mg/dL) was calculated following trapezoidal rules.

Statistical Analysis

One way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was applied to compare differences in animal study groups. The criterion for statistical significance was $P < 0.05$. Statistical analyses were performed by using GraphPad PRISM Version 5.01 (GraphPad Software, Inc. California, USA).

RESULTS

Phytochemical Components

The analysis of major phytochemicals in *C. cajan* husk is presented in Table 1. The yields of methanolic and acidified methanol were similar. Total anthocyanins could not be detected in acidified methanol extract. However, the total polyphenol content was 21% and total protein was 42% more in acidified methanol extract of the husk [Table 1].

Free Radicals Scavenging Anti-Oxidant Activity

Various free radical scavenging activities and anti-oxidant potential of CCH are presented in Table 2 and in Figure 1.

Table 1: Chemical components analysed in husk of *C. cajan* seeds

	Yield (%w/w)	TPP (µg GAE)	TA (%w/w)	TP (µg BSAE)
CCHME	5.6	150±2	0.12±0.01	500±20
CCHacME	6.4	190±1	ND	860±20

CCHME – *C. cajan* husks' methanol extract, CCHacME – *C. cajan* husks' acidified methanol extract; TPP – Total poly phenols; TA – Total anthocyanins; TP – Total protein; GAE – Gallic acid equivalent; BSAE – Bovine serum equivalent; ND – Not detected. Values represent mean±SD, *n*=3

ABTS radical scavenging potential was observed more in methanolic extract than acidified methanol extract of CCH. However, DPPH radical scavenging and preventive potential of hydroxyl radicals induced damage to 2-deoxy-D-ribose sugar was similar in both the extracts. Interestingly, $FeCl_3$ and NBT reducing property was observed more in acidified methanol extract than methanol extract of CCH [Table 2]. Preventive potential of methanolic extract for ABTS oxidation was twice than that of acidified methanol extract. However, reverse was the case observed in high glucose induced glycation of haemoglobin [Figure 1].

Inhibition of Rat Intestinal α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity was found to be more than three times higher in methanolic extract of CCH than the acidified methanolic extract of CCH [Figure 1].

Starch Tolerance Test

Methanolic extract of CCH was evaluated to test its activity on starch-induced postprandial glycaemia in rats. Figure 2a presents shape of starch tolerance test in rats. Prior administration of methanolic extract reduced postprandial excursion of glucose following starch tolerance test. Anti-hyperglycaemic potential of methanolic extract was found similar to standard drug acarbose. Figure 2b presents 2 h glycaemic load following starch tolerance test. It is observed that methanolic extract of CCH significantly ($P < 0.05$) reduced starch-induced postprandial glycaemic load [Figure 2b].

Table 2: Free radicals scavenging (SC_{50}), reducing power (RC_{50}) and anti-oxidant (IC_{50}) values of different extracts of *C. cajan*

	ABTS (µg/mL)	DPPH (µg/mL)	$FeCl_3$ (µg/mL)	NBT (µg/mL)	AOX (µg/mL)
CCHME	9.0±0.4	233±45	60.6±5.3	4.2±0.5	8.2±0.4
CCHacME	14.2±5.1	240±12	45.2±4	2.5±0.6	8.9±0.6
ASC	7±0.6	103±0.4	NM	5.9±0.9	3.0±0.5

CCHME – *C. cajan* husks' methanol extract; CCHacME – *C. cajan* husks' acidified methanol extract; ASC – Ascorbic acid; DPPH – 2, 2'-diphenyl-1-picryl hydrazyl; $FeCl_3$ – Ferric chloride; NBT – Nitro blue tetrazolium; AOX – Anti-oxidant activity (2-deoxy-D-ribose oxidation assay); NM – Not measured. Values represent mean±SD, *n*=3

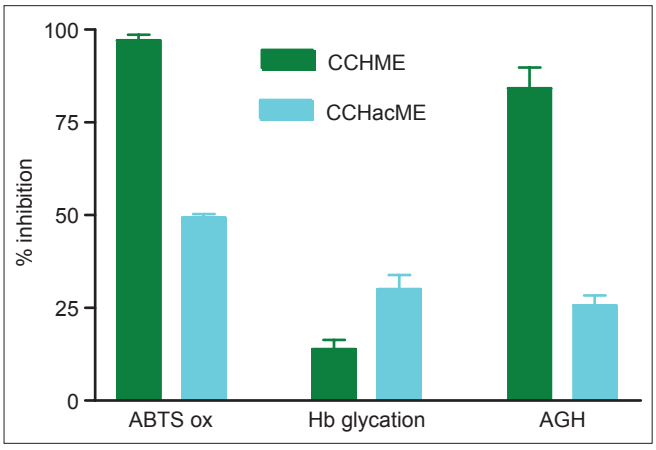

Figure 1: Capacity of *C. Cajan* husks' in preventing ABTS oxidation (ABTS ox), Haemoglobin glycation (Hb glycation) and inhibition of rat intestinal α-glucosidase enzyme (AGH). CCHME – *C. cajan* husks' methanol extract; CCHacME – *C. cajan* husks' acidified methanol extract. ABTS oxidation was measured with 100 µg concentration, Hb glycation was measured with 1000 µg concentration and AGH inhibitory activity was determined with 100 µg concentration of respective extracts in the reaction medium. Values represent mean ± SEM, *n* = 3

DISCUSSION

Modern epidemic of lifestyle-related diseases like type-2 diabetes mellitus (T2DM), cardiovascular disorders (CVDs) and obesity are thought to be the product of industrialisation, progressive modernisation and globalisation.[25] Lifestyle changes include adverse behavioural patterns like increased stress and decreased physical activity. Increasing epidemic of these disorders among south Asians is attributed to migration and nutritional transition from traditional dietary habits to those possessing greater amount of carbohydrates, saturated and *trans*-fats, and lower amounts of dietary fibre.[26] Now processed and calorie rich food is conveniently cheap and easily available making it the main menu for

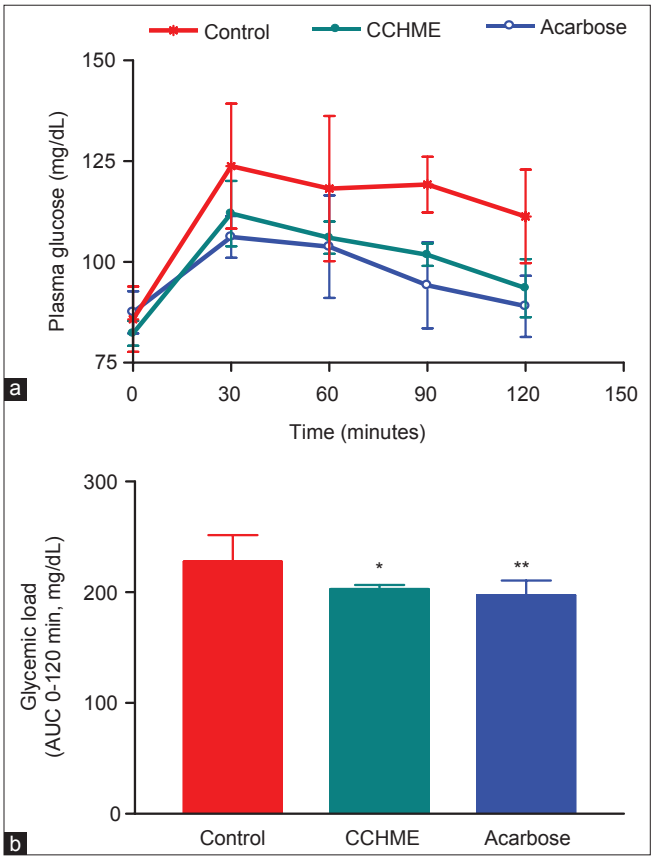

Figure 2: (a) Shape of starch tolerance test; (b) two hour glycaemia load following oral administration of *C. cajan* husks' methanol extract (CCHME) and standard drug acarbose. Values represent mean ± SEM, *n* = 6 rats in each group. ANOVA followed by Dunnett's multiple comparison tests was applied to find differences between the groups. *$P < 0.05$, ** $P < 0.01$ when compared with control

common man.[27] A typical south Asian meal usually comprises of a greater percentage of carbohydrate than that of standard European meals[28] and especially in southern India, this habit (high intake of polished white rice) has been linked to the development of T2DM.[29]

Highly processed, calorie dense, nutrient depleted diet leads to exaggerated postprandial spikes in blood glucose and induces immediate oxidative stress.[15,17] Postprandial hyperglycaemia is a prominent and early defect ensuing T2DM,[30] an independent predictor of development of future cardiovascular events,[31] atherosclerosis and development of oxidative stress.[32] Postprandial state is also referred to as pro-oxidative state, which involves active oxidative metabolism and generation of free radicals.[18]

Taken together, these developments highlights the need for the development and use of appropriate diets and/or nutraceuticals that can reduce the burden of postprandial hyperglycaemia and oxidative stress along with possessing potent free radicals scavenging properties, which consequently will prevent development of lifestyle-related diseases.

Analysis of our research finds that husk of *C. cajan* seed is an economic and readily available rich natural source of polyphenols and protein. It has the potential of scavenging a variety of free radicals like $ABTS^{+}$, DPPH and possesses potent reducing power ($FeCl_3$ and NBT). Furthermore, it displays anti-oxidant activity (ABTS oxidation) and the ability to prevent free radicals induced damage to biomolecules like 2-deoxy-D-ribose and haemoglobin.

We selected methanolic extract of seed husks for study of anti-hyperglycaemic activity for; it displayed more α-glucosidase inhibitory activity than the acidified methanol extract. Inhibitors of intestinal α-glucosidase enzyme like acarbose have been successfully utilised in clinics to reduce burden of starch-induced postprandial hyperglycaemia. Methanolic extract of seed husk potentially mitigated development of postprandial hyperglycaemic spikes and glycaemic load close to clinically used drug acarbose. These observations present exciting opportunity for its development as economic functional food and/or nutraceutical possessing both potent anti-oxidant and anti-hyperglycaemic activity. This is the first report identifying potent anti-oxidant and anti-hyperglycaemic in pigeon pea seed husk.

CONCLUSION

Pigeon pea seed husks possess potent anti-oxidant and anti-hyperglycaemic activity. It may become an economical natural organic resource for development of functional food/nutraceuticals meant for hyperglycaemic individuals.

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