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Composition and enzyme inhibitory properties of finger millet (*Eleusine coracana* L.) seed coat phenolics: Mode of inhibition of α-glucosidase and pancreatic amylase

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ABSTRACT

Inhibitors of alpha glucosidase and pancreatic amylase play a vital role in the clinical management of postprandial hyperglycemia. Although, powerful synthetic inhibitors are available, natural inhibitors are potentially safer. Phenolic compounds from the millet seed coat were extracted with acidified methanol and characterised by HPLC and ESI-MS. These phenolics showed strong inhibition towards α-glucosidase and pancreatic amylase and the IC_{50} values were 16.9 and 23.5 μg of phenolics, respectively. The enzyme kinetic studies, using Michaelis–Menton and Lineweaver–Burk equations, indicated that, in the presence of millet phenolics, the Michaelis–Menton constant (K_m) remained constant but the maximal velocity (V_{max}) decreased, revealing a non-competitive type of inhibition. The study indicated the therapeutic potentiality of millet phenolics in the management of postprandial hyperglycemia.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterised by hyperglycemia, resulting from insufficient or inefficient insulin secretion, with alterations in carbohydrate, protein and lipid metabolism. Recent reports indicate that hyperglycemia could induce non-enzymatic glycosylation of various proteins, resulting in the development of chronic complications in diabetes (Lebovitz, 1998, 2001). Therefore control of postprandial blood glucose surges is critical for treatment of diabetes and for reducing chronic vascular complications (Baron, 1998; Lebovitz, 2001). One of the therapeutic approaches for decreasing postprandial hyperglycemia is to prevent absorption of carbohydrates after food uptake. Complex polysaccharides are hydrolyzed by amylases to dextrins or oligosaccharides that are further hydrolyzed to glucose by intestinal α-glucosidase before being absorbed into the intestinal epithelium and entering blood circulation. Therefore, amylase and α-glucosidase inhibitors may help to reduce postprandial hyperglycemia by partially inhibiting the enzymatic hydrolysis of complex carbohydrates, and hence may delay the absorption of glucose. Acarbose, voglibose and miglitol are widely used, either alone or in combination with insulin secretogogues, for patients with type II diabetes (Saito, Sakai, Sekihara, & Yajima, 1998). However these inhibitors are reported to cause several side effects, such as liver disorders, flatulence and abdominal cramping. In addition, some of them may increase the incidence of renal tumours, hepatic injury and acute hepatitis. Several other safer natural amylase and α-glucosidase inhibitors have been reported from plant sources (Hiroyuki, Tomohide, & Kazunori, 2001; Matsui et al., 2001).

Finger millet (*Eleusine coracana* L.) or ragi is one of the minor millets cultivated in India and many of the African countries. The millet diet is known for high sustaining power and is usually recommended for diabetics. Epidemiologically lower incidence of diabetes has been reported in the millet-consuming population. Lower glycemic responses to the millet diets have been reported elsewhere (Ramanathan & Gopalan, 1957). However, there are no specific scientific reports on the health benefits of millet constituents, with specific reference to the influence of its phenolic compounds, on the carbohydrate-digesting enzymes, and particularly pancreatic amylase and intestinal α-glucosidase – the two key enzymes involved in the regulation of glucose homeostasis.

Most of the phenolics of millet are concentrated in the seed coat and hence, in the present study, determination of the composition of the finger millet seed coat phenolic compounds, and evaluation of these phenolics in the inhibition of pancreatic amylase and intestinal α-glucosidase, were carried out. Enzyme kinetic studies were also performed to understand the possible mode of inhibition of these phenolic compounds.

2. Materials and methods

2.1. Materials

Finger millet (GPU 28 variety) was procured from the University of Agricultural Sciences, Bangalore, India. The grains were cleaned

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and kept in a cool (8 ± 0.5 °C) and dry place (relative humidity 55–60%) prior to use. The phenolic standards, namely, gallic, protocatechuic, gentisic, vanillic, caffeic, syringic, para-coumaric, ferulic and trans-cinnamic acids, catechin, *p*-nitrophenyl-alpha-D-glucopyranoside (PNP-glycoside), rat intestinal acetone powder and porcine pancreatic amylase, were purchased from Sigma–Aldrich chemical company (St. Louis, USA). All the reagents used were of analytical grade, and glass (double)-distilled water was used for the preparation of the reagents.

2.2. Preparation of millet seed coat matter

The millet was sprayed with 7% (w/v) water and equilibrated for 10 min and pulverized in a horizontal carborundum disc mill (SABKO, Rajkot, India). The meal was sifted through 85 mesh (180 μm BSS sieve) and the tailings were again pulverized and sifted. The process of pulverizing and sieving was twice repeated. The fractions that passed through the Ist, 2nd and 3rd stage were pooled and designated as refined ragi/millet flour. Tailings from the third stage grinding (particle size 60 mesh, 250 μm BSS Sieve) were collected and designated as seed coat-rich fraction (SCRF) and were used for the extraction of phenolic compounds. Proximate composition of SCRF was determined by the AACC methods (AACC, 2000). Total dietary fibre was determined by rapid enzymatic assay, according to the method of Asp, Claves, Johnson, Halmer and Siljestrom (1983). The results were expressed as means of three independent determinations for each sample.

2.3. Extraction of polyphenols

The seed coat matter (1 g) was extracted by refluxing in 1% HCl-methanol at 60 °C for 10 min (100 ml × 4) and the extracts were pooled and concentrated under vacuum in a rotary flash evaporator according to the method of Chethan and Malleshi (2007).

2.4. Assay of total phenolics content

Total phenolics content of the millet seed coat extract was assayed according to the spectrophotometric method of Singleton, Orthofer, and Lamuela-Raentos (1995). Briefly, 0.1 ml of the acidified methanolic extract was mixed with 5 ml of distilled water in a 50 ml volumetric flask and treated with 2.5 ml of Folin-Ciocalteau reagent (diluted 1:2 with water) and 7.5 ml of 15% sodium carbonate solution, mixed thoroughly and made up to 50 ml. The blue colour developed was read against a reagent blank at 760 nm after 30 min. Gallic acid served as the reference standard and total phenolics content was expressed as gallic acid equivalents.

2.5. Separation of phenolics

The phenolic constituents of the millet seed coat extract were separated by reverse phase HPLC, on a Shimpak C-18 column (model LC-10A, Shimadzu Corporation, Japan), using a diode array detector operating at 280 and 320 nm. An isocratic solvent system, consisting of water:acetic acid:methanol (80:5:15), was used as a mobile phase at a flow rate of 1 ml/min. Standards, of caffeic, coumaric, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids, were used for identification of phenolics.

2.6. Direct infusion electrospray insertion mass spectrometry (ESI-MS)

The ESI-MS fingerprints of the millet seed coat phenolics were obtained with an Alliance, Waters 2695 mass spectrometer (Waters corporation, Micromass Ltd, UK) operating at ESI (-ve mode) according to the method of Markowicz et al. (2007). The capillary voltage was 3.0 kV; source and desolvation temperatures were 120 °C and 300 °C, respectively; cone gas (argon) and desolvation gas (nitrogen) flow rates were 50 l h^{-1} and 500 l h^{-1}, respectively. The *m*/*z* values obtained for the different standard phenolics and the *m*/*z* values of the different compounds, available in the literature, were used to match the *m*/*z* values in the spectra obtained for the millet seed coat phenolics.

2.7. Inhibition of α-glucosidase

α-Glucosidase was prepared from rat intestinal acetone powder according to the method of Oki, Matsui, and Osajima (1999). In brief, rat intestinal acetone powder (100 mg) was homogenised in 3 ml of 0.9% NaCl solution. After centrifugation at 12,000×*g* for 30 min, the clear supernatant was used for the assay. The inhibitory effect of the millet seed coat phenolic extract on rat intestinal α-glucosidase was assayed according to the procedure described previously by Matsui et al. (2001), with minor modifications. The enzyme (0.02 units; one unit of enzyme activity is defined as the amount of enzyme required to release one micromole of para nitro phenol from PNP-glycoside per minute under assay conditions) and suitable aliquots of millet seed coat phenolic compounds (to obtain ≈30–80% inhibition) were pre-incubated for 10 min at 37 °C in 0.1 M PIPES [piperazine-1,4-bis (2-ethane sulphonic acid)] buffer, pH 6.8. At the end of pre-incubation, 0.5 ml of 2.0 mM PNP-glycoside (*p*-nitrophenyl-alpha-D-glucopyranoside) was added to the enzyme-phenolic extract mixture and incubated at 37 °C for 30 min. The reaction was terminated by adding 1 ml of 0.64% N-(1-naphthyl) ethylenediamine solution (pH 10.7). Enzyme activity was determined by measuring the *p*-nitrophenol released from PNP-glycoside at 400 nm. Enzyme inhibitory reactions, for all the phenolic extract concentrations, were carried out in three replications. Suitable solvent control for particular phenolic extract concentrations was maintained.

2.8. Inhibition of pancreatic amylase

The inhibition properties of the millet seed coat phenolics extract against pancreatic amylase were assayed according to the procedure described previously by Bernfeld (1955) with minor modifications. The amylase activity was determined, using soluble starch (1%) as a substrate in 0.1 M sodium phosphate buffer containing 1 mM $CaCl_2$, pH 7.4 (final volume 2.0 ml). The enzyme solution (0.33 units; one unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per minute under assay conditions) and suitable aliquots of millet seed coat phenolic compounds (to obtain 30–80% inhibition) in 1 ml were pre-incubated for 10 min at 37 °C. The residual amylase activity was measured by adding 1 ml of starch substrate and incubating at 37 °C for 30 min. The reaction was terminated by adding 2.0 ml of dinitrosalicylic acid solution (DNS) and boiled for 5 min in a boiling water bath, and the total volume was made up to 20 ml with water. Enzyme activity was quantified by measuring the maltose equivalents released from starch at 540 nm. Enzyme inhibitory reactions for all the phenolic extract concentrations were carried out in three replications. Appropriate solvent controls were maintained.

2.9. Determination of IC_{50}

In order to determine the IC_{50} values, the enzyme activities of α-glucosidase and pancreatic amylases were determined in the presence of the millet seed coat extract at various concentrations (4–34 μg of phenolics for α-glucosidase and 9–85 μg of phenolics for pancreatic amylase). IC_{50} is defined as the concentration of phenolics required to inhibit 50% of the enzyme activity.

2.10. Kinetics of enzyme inhibition

The mode of inhibition of α-glucosidase and pancreatic amylase by millet seed coat phenolic extract was determined by using Michaelis–Menton and Lineweaver–Burk equations. PNP-glycoside, in the concentration range 0.5–4.0 mM, and soluble starch, in the range 0.25–1.5%, were used as substrates for α-glucosidase and pancreatic amylase, respectively. Enzyme activities were determined in the absence or presence of different concentrations of millet seed coat phenolic extract. The concentrations of phenolic compounds used for the inhibitory kinetics of pancreatic amylase were 25.6, 34.2 and 42.7 μg/ml, whereas, 6.4, 8.5 and 10.7 μg/ml were used for α-glucosidase inhibitory kinetics. Dixon plots (Dixon, 1953) were used to determine the inhibitory constants (K_i), whereas secondary plots of $[S]/V$ Vs. [I] (Bowden, 1974) were used to determine the dissociation constants (K_i') of phenolic compounds.

3. Results and discussion

The millet seed coat contained 17.8% of protein and about 64% of carbohydrates, including 41% of dietary fibre. The millet seed coat matter is a rich source of phenolic compounds (10% w/w) compared to about 2.3% (w/w) phenolics in whole meal. Histochemical localisation of phenolic compounds in the millet kernel indicated that the phenolics are mainly concentrated in the testa or seed coat layers (data not presented). Reverse phase HPLC analysis indicated the presence of various types of phenolic compounds, including polyphenols and phenolic acids, such as gallic, protocatechuic, gentisic, caffeic, vanillic, syringic, ferulic, *p*-coumaric and transcinnamic acids, along with some unidentified peaks in millet seed coat phenolics extract (Fig. 1).

Since identifications of all the peaks were not accomplished by RP-HPLC, direct infusion electrospray ionisation mass spectrometry (ESI-MS) was used to identify the phenolic compounds of seed coat extract. The ESI-MS fingerprint of the millet seed coat extract is shown in Fig. 2 (full spectra), which indicates the presence of several phenolic compounds with different molecular weights ranging from 100–2000 Da. Phenolic compounds were identified by their *m*/*z* values, from ESI-MS fingerprints (zoomed spectra) and are listed in Table 1. Mass spectra of the finger millet extract showed the presence of naringenin, kaempferol, luteolin glycoside, phloroglucinol, apigenin, (+)-catechin/(−)-epicatechin, trans-feruloyl-malic acid, dimer of prodelphinidin (epi/gallocatechins; 2GC), diadzein, catechin gallates, trimers and tetramers of catechin. Some of these phenolic compounds are also present in the methanolic extracts of peas (Duenas, Estrella, & Hernandez, 2004) and green tea. ESI-MS has been extensively used for the identification of phenolic compounds from cereals, legumes, yerba

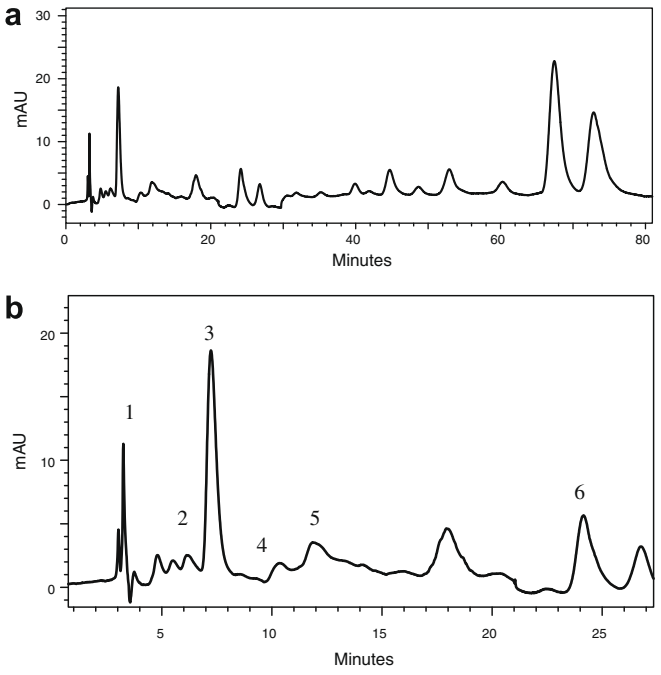

Fig. 1. Component phenolics of millet seed coat extract (HPLC chromatograms at 320 nm; (a) full chromatogram, (b) zoomed chromatogram, peak no. 1-gallic acid, 2-protocatechuic acid, 3-gentisic acid, 4-caffeic acid, 5-vanillic acid, 6-ferulic acid).

Table 1
Phenolic compounds identified from the ESI-MS fingerprint of millet seed coat extract.

Phenolic compound	$[M–H]^-$ (*m*/*z*)*
Gallic acid	169.13
Gentisic acid	153.12
Protocatechuic acid	153.12
Syringic acid	197.64
Transcinnamic acid	147.09
Caffeic acid	179.20
Ferulic acid	193.34
p-Hydroxy benzoic acid	137.14
4-*O*-Methyl gallic acid	183.16
Kaempferol	285.24
Naringenin	271.02
Phloroglucinol	125.13
Apigenin	269.01
(+)-Catechin/(−)-epicatechin	289.30
Luteolin glycoside	447.03
Trans feruloyl-malic acid	308.23
Dimer of prodelphinidin (2GC)	609.65
Diadzein	253.38
Catechin gallates	440.97
Trimers of catechin	865.64
Tetramers of catechin	1153.88

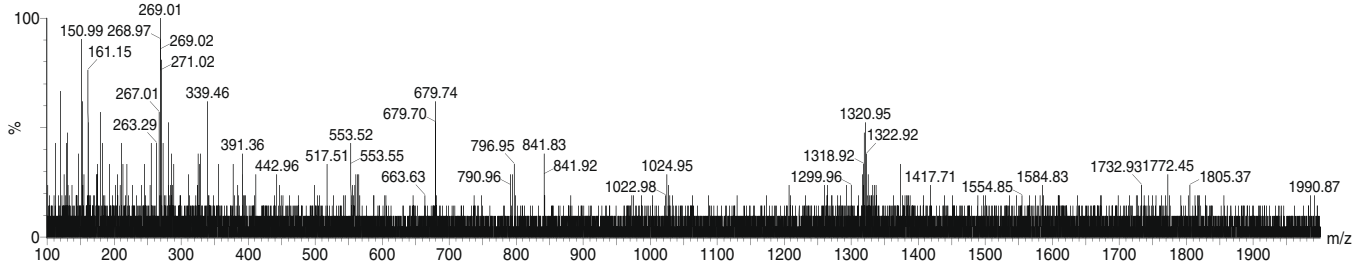

Fig. 2. ESI-MS fingerprints of millet seed coat extract (full spectra).

mate, green tea and wine extracts (Ǵomez-Ariza, Garćıa-Barrera, & Lorenzo, 2006; Markowicz et al., 2007; Møller, Catharino, & Eberlin, 2005).

Alpha-amylases are endoglucanases, which hydrolyze the internal alpha 1,4 glucosidic linkages in starch. α-Glucosidase is one of the glucosidases located in the brush border surface membrane of intestinal cells, and is a key enzyme for carbohydrate digestion. These enzymes have been recognized as therapeutic targets for modulation of postprandial hyperglycemia. Postprandial hyperglycemia is the earliest metabolic abnormality to occur in type 2 *diabetes mellitus* (Lebovitz, 1998). Postprandial blood glucose levels may be elevated in the presence of normal levels of fasting plasma glucose, constituting an early stage in type 2 diabetes, referred as 'postprandial diabetes' (Baron, 1998). This state, not only initiates the development of micro- and macro-vascular complications, but can also contribute to a more rapid progression to symptomatic diabetes by causing glucose toxicity in muscle and pancreatic cells. Early identification of postprandial hyperglycemia and its effective control will aid in early intervention and prevention of diabetic complications (Ratner, 2001). The millet seed coat phenolics inhibited both pancreatic amylase and α-glucosidase in a dose-dependent manner (Fig. 3A and B). The IC_{50} values for α-glucosidase and amylase were found to be 16.9 and 23.5 μg millet seed coat phenolics, respectively (Table 2). There are reports of established α-glucosidase inhibitors and their effects on blood glucose levels after food uptake (Chen, Nakashima, Kimura, & Kimura, 1995). Plant phenolic compounds modulate the enzymatic breakdown of carbohydrates by inhibiting amylases and glucosidases (McDougall et al., 2005). Various biological and health-beneficial effects have been demonstrated by phenolic compounds in plants (Middleton, 1996).

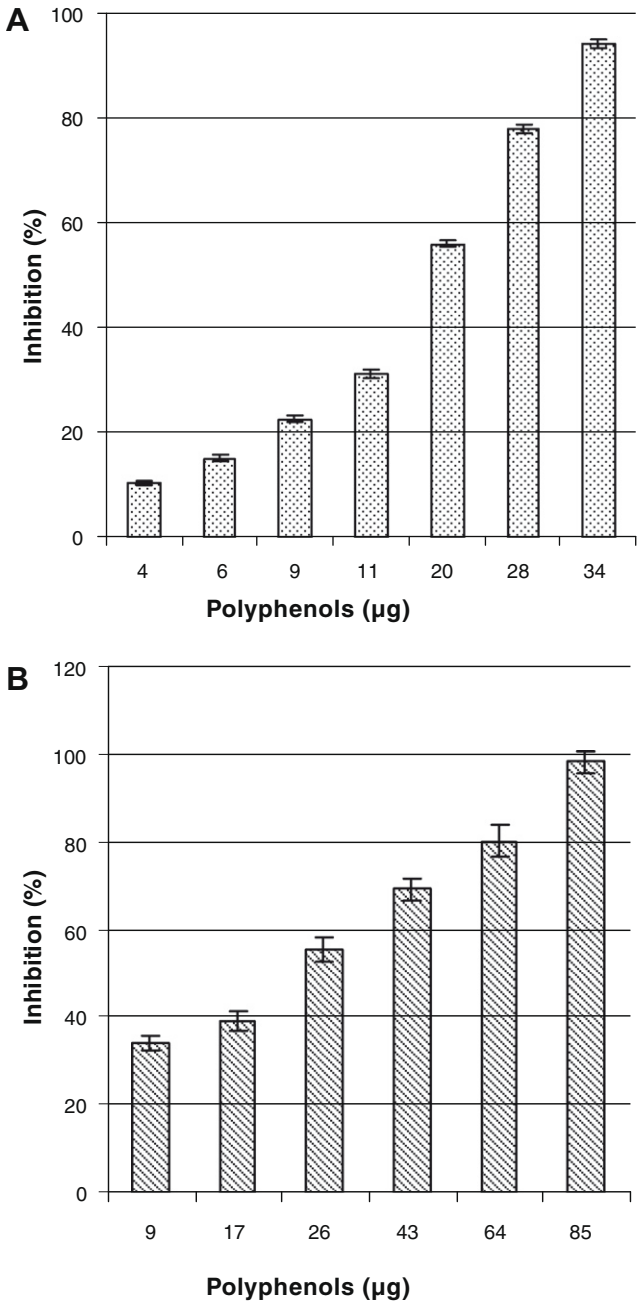

Fig. 3. Inhibitory effect of different levels of millet seed coat extract phenolics on A. α-glucosidase (0.02 units, 30 min incubation) and B. pancreatic amylase (0.33 units, 30 min incubation).

In the present study, the initial velocity '*v*' of the hydrolysis reactions catalysed by α-glucosidase and pancreatic amylase was measured at various substrate concentrations [*S*] in the presence and absence of a fixed millet seed coat phenolic compound [*I*], as indicated in Fig. 4. The double reciprocal plots show that straight lines were obtained with PNP-glycoside and starch substrates for α-glucosidase (Fig. 4A) and amylase (Fig. 4B), respectively. Both the slope '*s*' and vertical axis intercept '*i*' increase with increasing phenolic compound concentration. These results indicate that the binding of the phenolic compounds affected the velocity of the reaction catalysed by α-glucosidase and amylase, proportionately to the concentration of the phenolic compounds in the reaction mixture, without affecting the K_m. Non-competitive inhibition of millet seed coat phenolics on the α-glucosidase-catalysed hydrolysis of PNP-glycoside and amylase-catalysed hydrolysis of starch can account for these results.

The inhibitory kinetics of millet seed coat phenolics on the α-glucosidase-catalysed hydrolysis of PNP-glycoside (a short-chain substrate) and amylase-catalysed hydrolysis of starch (a long-chain substrate) were evaluated using Lineweaver–Burk and Dixon plots. The effect of either inhibitor or substrate concentration on the slope and vertical axis intercept of the corresponding plots was analysed. Kinetic constants for the inhibition of α-glucosidase and pancreatic amylase are listed in Table 2. α-Glucosidase has a Michaelis–Menton constant (K_m) of 8×10^{-3} M for PNP-glycoside and V_{max} value of 83.3×10^3 μmoles. Apparent V_{max} values in the presence of 6.4, 8.5 and 10.7 μg of millet seed coat phenolics were found to be 76.9×10^3, 71.4×10^3 and 62.5×10^3 μmoles, respectively. The K_m for pancreatic amylase under the reaction conditions was found to be 1% starch and V_{max} value of 66.0×10^{-2} μmoles of

Table 2
Kinetic properties of millet seed coat extract on α-glucosidase and pancreatic α-amylase.

S.No	Parameter	α-glucosidase	Pancreatic α-amylase
1.	K_m	[a]8.0×10^{-3} M	[b]1%
2.	V_{max}	[c]83.3×10^{3}	[d]66.0×10^{-2}
3.	$V_{max'}$	[c]76.9×10^{3} (6.4μg)[e]	[d]31.3×10^{-2} (25.6μg)[e]
4.	$V_{max'}$	[c]71.4×10^{3} (8.5μg)[e]	[d]27.0×10^{-2} (34.2μg)[e]
5.	$V_{max'}$	[c]62.5×10^{3} (10.7μg)[e]	[d]21.7×10^{-2} (42.7μg)[e]
6.	IC_{50} (μg)	16.9	23.5
7.	K_i (μg)	5.0	10.0
8.	K_i'(μg)	2.5	7
9.	Mode of inhibition	Non-competitive	Non-competitive

[a] PNP-glycoside.
[b] Starch.
[c] μmoles of para-nitrophenol released/min.
[d] μmoles of maltose eq released/min.
[e] Concentration of millet seed coat phenolic compounds.

maltose (Table 2). The apparent V_{max} values in the presence of 25.6, 34.2 and 42.7 μg of millet seed coat phenolics were found to be 31.3×10^{-2}, 27.0×10^{-2} and 21.7×10^{-2} μmoles, respectively. The inhibitory constants (K_i), determined from Dixon plots for α-glucosidase and pancreatic amylase, were 5.0 and 10 μg of millet seed coat extract phenolics, respectively (Table 2). The dissociation constant (K_i') for α-glucosidase was found to be 2.5 μg, whereas pancreatic amylase had a K_i' of 7 μg of phenolic compounds, as determined from secondary plots of $[S]/V$ Vs. $[I]$ (Bowden, 1974).

Inhibition of α-glucosidase and pancreatic amylase by different classes of phenolic compounds is described in the literature (Kim, Jeong, Wang, Lee, & Rhee, 2005; Shim et al., 2003; Tadera, Minami, Takamatsu, & Matsuoka, 2006). α-Glucosidase and pancreatic amylase were effectively inhibited by naringenin, kaempferol, luteolin, apigenin, (+)-catechin/(−)-epicatechin, diadzein and epigallocatechin gallate (Tadera et al., 2006). These flavonoids exhibited a mixed and close to non-competitive type of inhibition on yeast α-glucosidase. ESI-MS analysis indicated the presence of these flavonoid compounds in the millet seed coat extract (Table 1). Pine bark extract (PBE) non-competitively inhibited human salivary and porcine pancreatic amylases. However, a combination of non-competitive and uncompetitive inhibition was observed in the study of α-glucosidase inhibition of PBE against yeast *S. cerevisae* α-glucosidase (Kim et al., 2005). The non-competitive nature of the inhibition of α-glucosidase and porcine pancreatic amylase was reported for *Rhus chinensis* extract, a Korean herb traditionally used in the treatment of type 2 diabetes in Korea (Shim et al., 2003). The millet seed coat extract exhibited an inhibitory activity similar to that of acarbose against α-amylase (Alkazaz et al., 1996).

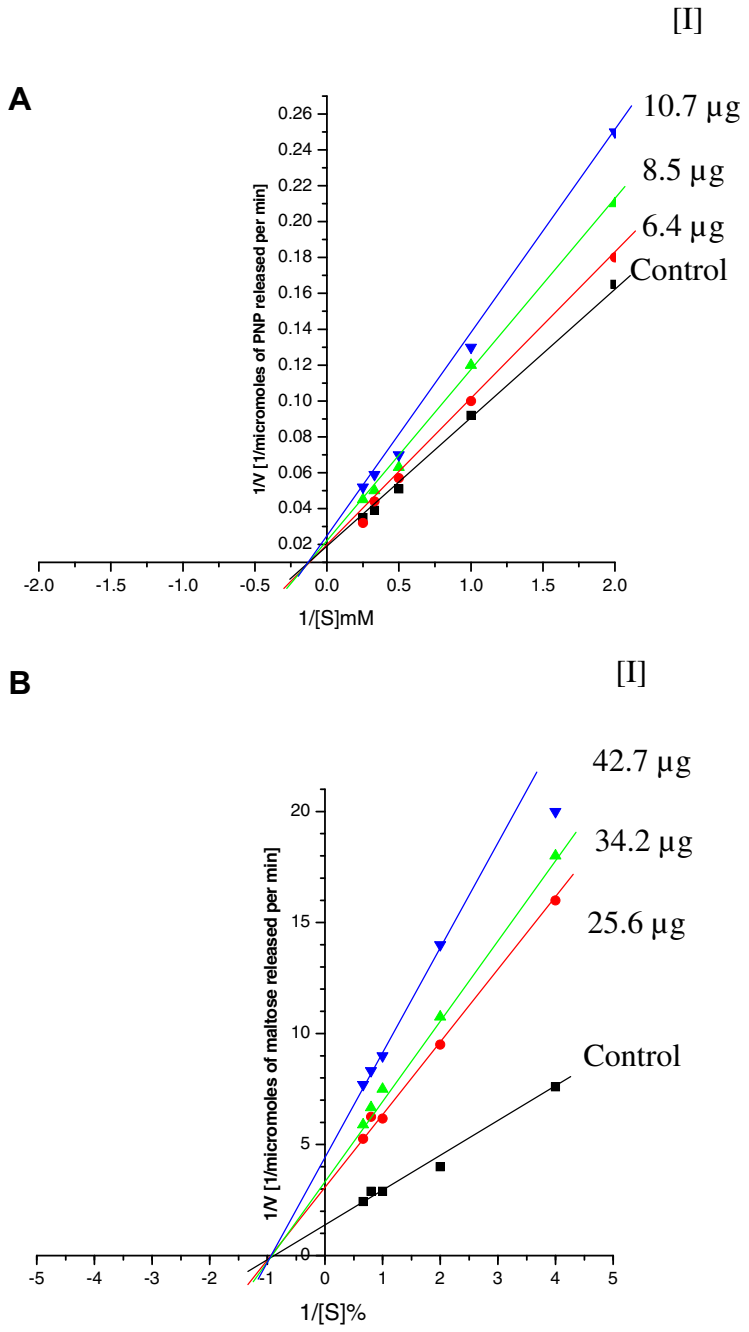

Fig. 4. L–B plot for determining the kinetic constants for α-glucosidase (A) and pancreatic amylase (B), Inhibitor concentrations [*I*] are indicated.

Finger millet diets have been recommended for diabetics and epidemiological reports indicate a lower incidence of diabetes amongst millet consumers. However, the mechanisms of action were not clearly reported. The results of this investigation suggest that, the phenolic compounds present in the millet seed coat may regulate the glucose uptake from the intestinal lumen by inhibiting carbohydrate digestion and absorption, leading to normal glucose homeostasis. Further, there are extensive studies on the role of phenolic compounds in the regulation of glucose homeostasis. Phenolics from tea extract (epigallocatechin gallate, epicatechin gallate, tannins and theaflavins) have been reported to possess insulin-enhancing properties (Anderson & Polansky, 2002), and to regulate hepatic glucose output. Dietary phenolic acids, such as chlorogenic acid, caffeic, catechin, ferulic, tannic and gallic acids, have been reported to reduce glucose uptake by favouring dissipation of the Na^+ electrochemical gradient, which provides the driving force for active glucose accumulation and hence glucose transport (Welsch, Lachance, & Wasserman, 1989) and are known to decrease glucose absorption and regulate postprandial hyperglycemia. It is also possible that, phenolic compounds from millet seed coat extract may regulate the glucose absorption by mechanisms other than enzyme inhibition.

4. Conclusions

The study indicates that, the millet seed coat contains a complex mixture of several phenolic compounds, belonging to different classes, ranging from lower molecular weight simple phenols to higher molecular weight polyphenols, such as tannins and anthocyanins. These seed coat phenolics are effective non-competitive inhibitors of carbohydrate-hydrolyzing enzymes. Further, millet seed coat extract may interfere with or delay absorption of dietary carbohydrates in the small intestine, leading to suppression of postprandial blood glucose surges. Food-grade phenolic inhibitors from millet seed coat extracts are potentially safer, and therefore may be preferred alternatives for inhibition of carbohydrate breakdown and control of glycemic index of food products.

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