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Inhibition of aldose reductase from cataracted eye lenses by finger millet (*Eleusine coracana*) polyphenols

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ABSTRACT

Retinopathy is a major cause of blindness in the Western world, while cataract is one of the three major causes of blindness worldwide. Diabetes is one of the major risk factor in retinopathy and cataract. The prevalence of blindness in India is 15 per 1000 while cataract alone accounts for 80% of this blindness. Diabetes induced cataract is characterized by an accumulation of sorbitol which is mediated by the action of a key enzyme aldose reductase (AR). Non-enzymatic glycation (binding of glucose to protein molecule) induced during diabetes appear to be the key factor for AR mediated sugar-induced cataract. Finger millet polyphenols (FMP) being a major anti-diabetic and antioxidant component, we have evaluated them for AR inhibiting activity. Phenolic constituents in FMP such as gallic, protocatechuic, *p*-hydroxy benzoic, *p*-coumaric, vanillic, syringic, ferulic, *trans*-cinnamic acids and the quercetin inhibited cataract eye lens effectively, the latter was more potent with an IC_{50} of 14.8 nM. Structure function analysis revealed that phenolics with OH group at 4th position was important for aldose reductase inhibitory property. Also the presence of neighboring *O*-methyl group in phenolics denatured the AR activity. Finger millet seed coat polyphenols (SCP) has been found to inhibit AR reversibly by non-competitive inhibition. Results thus, provide a stronger evidence for the potentials of FMP in inhibiting cataractogenesis in humans.

1. Introduction

Aldose reductase (AR, alditol: $NADP^+$ 1-oxidoreductase, EC 1.1.1.21) is a monomeric reduced NADPH dependent enzyme and a member of aldo–keto reductase super family.[1] It has been implicated in the etiology of complications in diabetes such as neuropathy, nephropathy, retinopathy and cataractogenesis.[2] This enzyme reduces glucose to sorbitol in the presence of NADPH (Scheme 1A–C). Although limited levels of aldose reductase (AR) and high levels of polyol dehydrogense (PD) relative to the animal lens has been depicted in human lens; AR is confined primarily to the lens epithelium. Two- to threefolds higher in juvenile lenses than in adult lenses which is sufficient to cause significant osmotic pressure leading to cataractogenesis.[3] Increased levels of sorbitol in the tissues of diabetic subjects and also in diabetic animals have indeed been reported.[4] A vast literature survey showed that the cataract progression could be slowed or prevented by the use of natural therapies, particularly with plants having high flavonoid contents. Also considerable in vivo AR inhibitory effect and hypoglycemic activity of flavonoids observed in the presence of higher levels of varieties of phenolics in finger millet (*Eleusine coracana*)[5] lead us to explore the AR inhibitory potentials of the millet phenolics.[6]

Aldose reductase from the eye lens is capable of binding to NADPH and glyceraldehyde via histidine-110 and tyrosine-48 residues, located at or near nucleotide-binding site of the enzyme and have been shown to be important in the binding or orientation of the reduced nucleotide.[7] Aldehydes that contained an aromatic ring were found to induce oxidation of nicotinamide adenine dinucleotide phosphate reduced (NADPH), consistent with crystallographic data that aldose reductase possesses a large hydrophobic binding site. AR is known to exhibit (β/α) triosephosphate isomerase (TIM) barrel fold, a novel NADPH binding domain[8] and either of the two amino acids in the active site that is Histidine-110 and Tyrosine-48 could be the proton donor.[9]

Finger millet contains about 5–8% protein, 1–2% ether extractives, 65–75% carbohydrates, 15–20% dietary fibre and 2.5–3.5% minerals.[10] It is also good source of polyphenols among cereals. The millet polyphenols, an important phytochemicals with one or more aromatic rings, with hydroxyl groups in different pattern/positions have been shown to exhibit significant antioxidant activity including free radical scavenging by the abstraction of protons.[11] Further, several health beneficial properties, such as anti-inflammatory, antiviral, anticancer and platelet aggregation inhibitory activity have also been documented.[5] Regular consumption of finger millet is known to reduce the risk of *diabetes mellitus*[12] and

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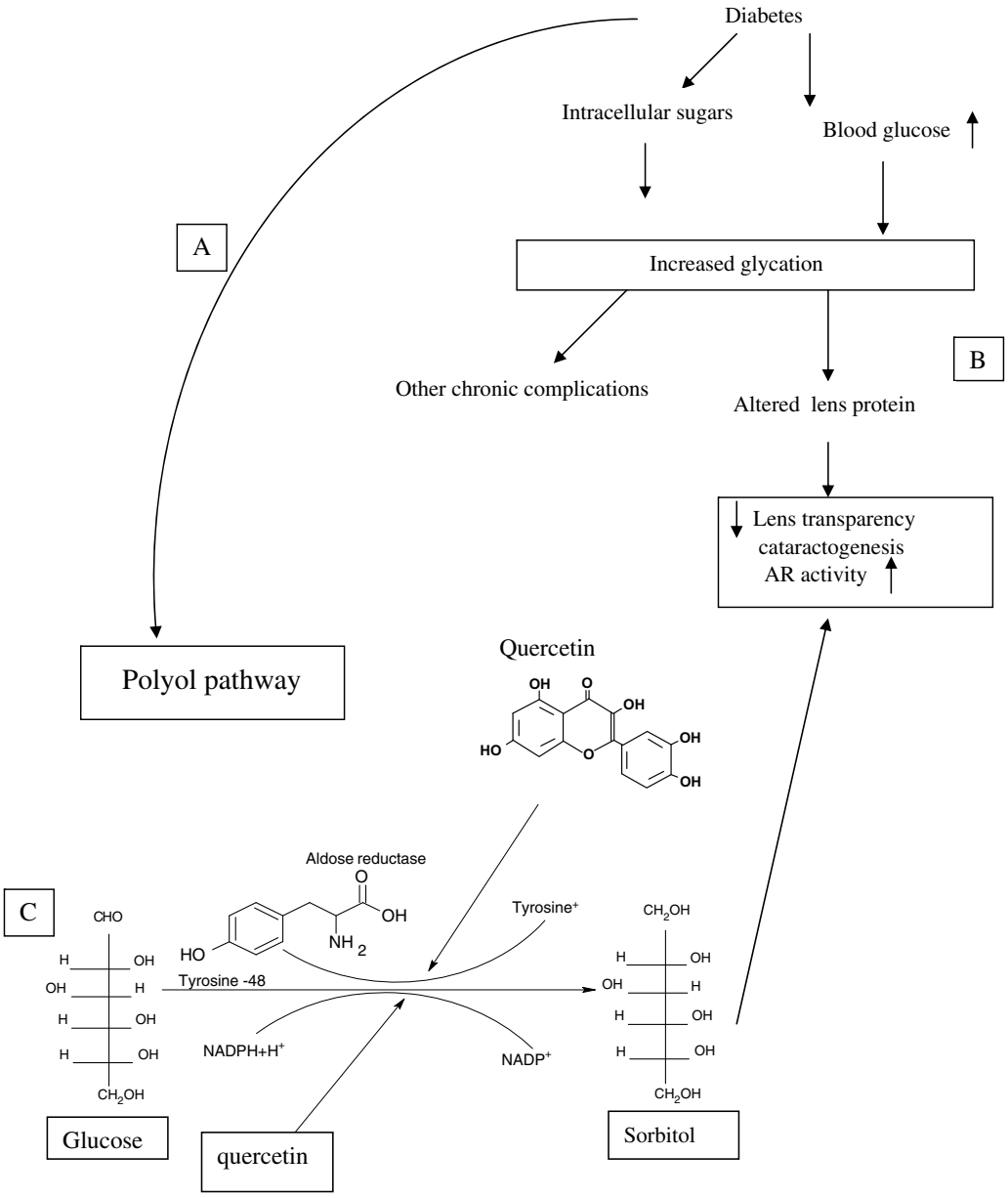


Scheme 1. Etiology of complications of diabetes, (A) complications leading to polyol pathway, (B) complications leading to decrease in lens transparency and formation of cataract and (C) action of quercetin inhibiting polyol pathway in two modes.

gastrointestinal tract disorders[13] and these properties were attributed to its polyphenols content. The utilization of whole meal cereals with bran, including the seed coat matter in food formulations is increasing world wide since they are a rich source of dietary fibre, which provides several health benefits to the consumer. Finger millet seed coat is edible and now-a-days it is readily available as a by-product in the millet malting[14] and milling industries.[15] Finger millet polyphenols are concentrated in the seed coat.[10] There have been numerous reports on inhibitors from extracts of some plants on NADPH oxidation in the presence of glyceraldehydes, or inhibitors of sorbitol production in tissues exhibiting non-competitive inhibition against both DL-glyceraldehyde and NADPH.[16,17] Inhibition of malt amylases by finger millet phenolics showed a mixed non-competitive mode of inhibition.[18]

In the present study, therefore, the emphasis has been on the role of finger millet seed coat polyphenols against one of the common diabetic complications, namely, cataractogenesis. AR enzyme being crucial in cataractogenesis via a polyol pathway, AR inhibitory effect of finger millet polyphenols and the constituent phenolics responsible for the inhibition along with the nature and kinetics of inhibition is addressed.

2. Results and discussion

Finger millet kernel contains 15–17% (w/w) seed coat. The polyphenol contents of the seed coat (FMSC-12.2%), was several fold higher compared to the refined flour (0.8%), consisting mainly endosperm matter.[10] In view of this the SCP was evaluated for the AR inhibition. The AR activity in cataracted eye lenses was 0.66 unit/mg protein (Fig. 1). The K_m (app) of the substrate gylceraldehyde and NADPH was 1.8 mM (Fig. 2A) and 67.56 μM (Fig. 2B), respectively. The results matched with the K_m reported for glyceraldehyde as 1.1 mM.[19] The optimized conditions were 1.8 mM, 68 μM for glyceraldehyde and NADPH respectively, used for fur-

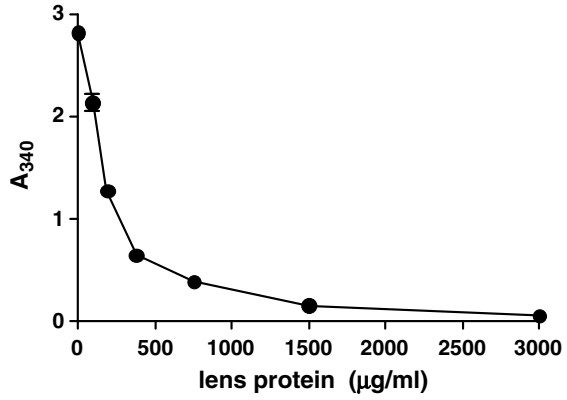


Figure 1. Aldose reductase activity at different concentrations of lens protein measured by oxidation of NADPH at 340 nm.

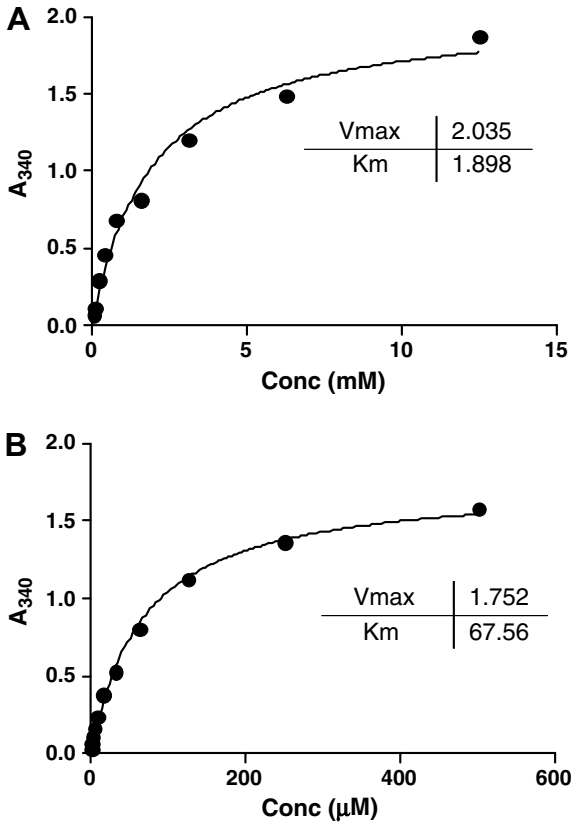


Figure 2. Optimization conditions for aldose reductase activity. (A) Michealis–Menten constant at varying concentration of D-glyceraldehyde and at saturation concentration of NADPH (0.1 mM) and optimized AR enzyme. (B) Michealis–Menten constant at varying concentration of NADPH at saturation concentration D-glyceraldehyde (10 mM) and optimized AR enzyme.

ther inhibitory studies. Dose dependent inhibitory effects of polyphenols extracted from native millet on the activity of the AR isolated from cataracted eye lenses were observed and showed an IC_{50} of 60.12 µg/ml (Fig. 3). The aldose reductase inhibition by polyphenols could be by preventing either the enzymatic conversion of (a) glyceraldehyde to glycerol and (b) glucose to sorbitol, thereby replenishing the depletion of NADPH levels. It is important to notify here that NADPH is used for several critical reductive metabolic steps, such as the detoxification of reactive oxygen species and hydroperoxides etc. Indeed a large supply of NADPH pool

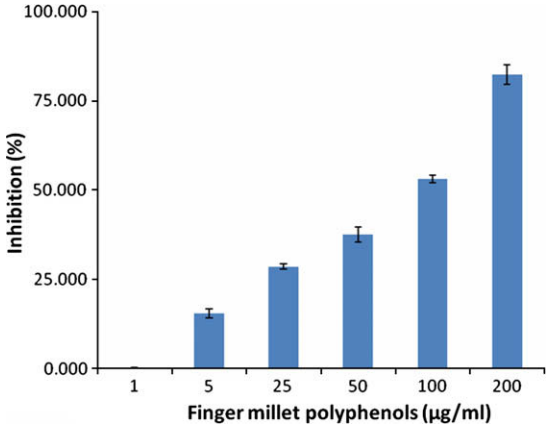


Figure 3. Inhibitory effects of crude finger millet polyphenols extract at varying concentrations on aldose reductase activity using optimized glyceraldehyde, NADPH and enzyme.

has been shown to envisage cytoprotection against oxidative stress.[8,20,21,22]

The crude polyphenols extracted using HCl-methanol were fractionated into component phenolic acids by HPLC (Fig. 4). It mainly constituted by ferulic (32.8%), *p*-hydroxy benzoic (17.9%), protocatechuic (15.3%), gallic (12.6%), *p*-coumaric (4.4%), syringic (4.0%), vanillic (3.8%), *trans*-cinnamic (3.6%) acids and quercetin (5.6%). Among these phenolic acids, quercetin, protocatechuic and *trans*-cinnamic acids showed 4 - 5 fold higher activity with an IC_{50} of 25.2, 42.7 & 68.1 µg/ml than synringic (172.1 µg/ml) and *p*-coumaric (162.3 µg/ml) acids. Gallic acid also showed an IC_{50} at 97.3 µg/ml (Table 1). *p*-Hydroxy benzoic, vanillic and ferulic acids showed negligible or no AR inhibitory activity. Data thus indicated that, quercetin is the most potent AR inhibitory component among finger millet polyphenolic constituents. The activity was correlated with antioxidant potency with the correlation coefficient ($r = 0.99$, $p \leqslant 0.1$) between antioxidant and AR inhibitory effect of phenolic constituents suggesting proton abstracting ability is responsible for AR inhibitory effect. Significant reduction in the activity from *trans*-cinnamic acid (*trans*-3-phenylacrylic acid) to

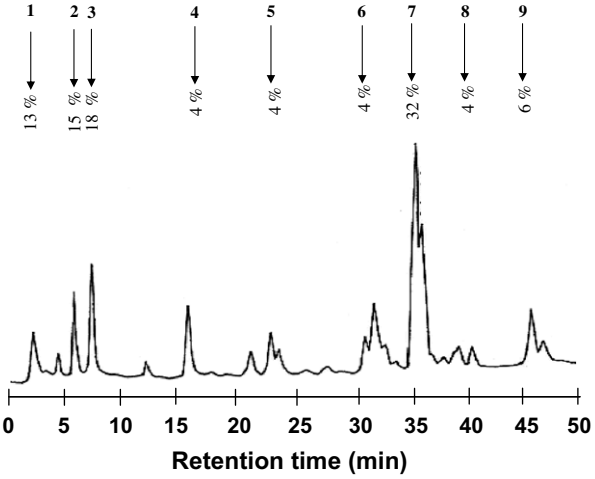
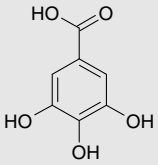
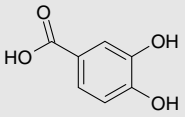
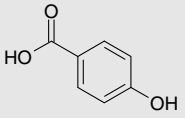
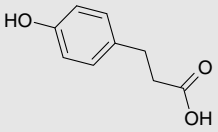
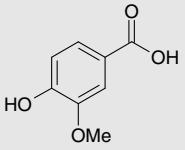
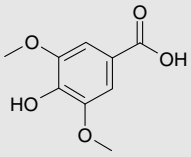
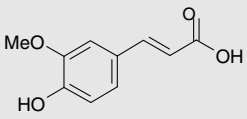
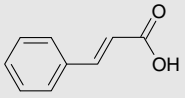
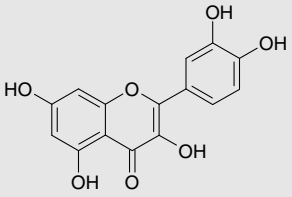


Figure 4. HPLC chromatogram of native millet polyphenols. Polyphenols were fractionated on ODS-2, C-18 column, using 0.1% tri-fluoroacetic acid: methanol at a flow rate of 1.0 ml/min. Peaks, (1) gallic acid; (2) protocatechuic acid; (3) *p*-hydroxy benzoicacid; (4) vanillic acid; (5) *p*-coumaric acid; (6) syringic acid; (7) ferullic acid; (8) *trans*-cinnamic acid; (9) Quercetin. Relative percent of phenolic acids are depicted on respective peaks.

Table 1
The inhibitory activity of finger millet seed coat phenolics and its constituents on antioxidant and aldose reducatse activity.

Phenolics	Structure	AR IC_{50} (μg/ml)	DPPH scavenging effect IC_{50} (μg/ml)
Gallic acid 3,4,5-Trihydroxybenzoic acid		97.3 ± 2.5	26.9 ± 10.1
Protocatechuic acid 3, 4-Dihydroxybenzoic acid		42.7 ± 3.2	77.63 ± 6.2
p-Hydroxy benzoic acid 4-Hydroxybenzoic acid		—	183.7 ± 4.2
p-Coumaric acid *Trans*-4-hydroxycinnamic acid		162.31 ± 12.6	112.01 ± 7.2
Vanillic acid 4-Hydroxy-3-methoxybenzoic acid		—	176.5 ± 11.3
Syringic acid 3,5-Dimethoxy-4-hydroxybenzoic acid		172.1 ± 8.2	155.6 ± 7.6
Ferulic acid 4-Hydroxy-3-methoxycinnamic acid		—	189.1 ± 8.1
Trans-cinnamic acid 3-Phenylacrylic acid		68.1 ± 4.8	96.7 ± 4.4
*Quercetin 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-Benzopyran-4-one		25.23 ± 2.2	56.8 ± 1.8
Crude phenolic extract		60.12 ± 3.4	90.12 ± 2.8

Finger millet seed coat phenolics and its constituents were identified by HPLC and fractionated by preparative HPLC. Fractions were examined for antioxidant and aldose reductase inhibitory property.
* Quercetin showed potent activity.

ferulic acid (4-hydroxy-3-methoxycinnamic acid), which showed no inhibition and gallic acid (3,4,5-trihydroxy benzoic acid) to syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) indicates that, the presence of methoxy group may be abolishing the AR inhibition, which could be due to interruption in the red–ox flow between phenolic acids and the enzyme. Results were substantiated by previous observation[23,24] that the hydroxylation in the 4th position is crucial for AR inhibitory effect. Besides, the lack of

activity in ferulic and syringic acids although having 4-hydroxyl group enunciates the importance of the presence of neighboring hydroxyl groups as seen in 3,4,5-dihydroxy benzoic (gallic) and 3,4-dihydroxybenzoic (protocatechuic) acids. Compounds similar in structure such as flavonoids, benzopyrans, spirohydantoins, alkaloids, non-steroidal anti-inflammatory drugs (NSAID), and quinones have also been shown to inhibit the enzyme with varied degrees of efficacy and specificity.[25,26] Systematic designing of related molecules and examination of AR inhibitory activity may further throw some light on the structure–activity relationship.

Quercetin therefore is a potent AR inhibitor of sorbitol accumulation in polyol pathway at step C in Scheme 1, in finger millet polyphenols. It was observed previously in colored rice by Morimitsu et al.[27] Results were further substantiated by examining the potency of pure quercetin on AR activity. Since, crude extract of finger millet seed coat polyphenols showed twofold lesser activity than pure compound quercetin; suggests the synergistic or combinational effect of phenolics in the crude extract. The mode of action of quercetin was investigated by determining Michaelis–Menten constant and Lineweaver–Burk plot equation. Data indicated a noncompetitive inhibition of AR by quercetin, since it did not alter the K_m but the V_{max} (Fig. 5). On the other hand, the IC_{50} at a very low range 14.79 nM by quercetin similar to that of a regulatory endogenous inhibitor may suggest the possibility of efficient inhibition of AR in vivo also.[28]

Noncompetitive inhibitors in general are those substances that form strong non-covalent bonds with enzymes and consequently are not displaced by the addition of excess of substrate thereby, rendering this type of noncompetitive inhibition as reversible reactions. Quercetin, a non-competitive inhibitor may render reversible inhibition of AR by successfully blocking the polyol pathway leading to cataractogenesis. Quercetin, which acts as a noncompetitive inhibitor. It is presumed that, it binds to the free enzyme at or near the active site but not to the enzyme–substrate (ES) complex. Thus, the strong hydrogen abstracting ability of quercetin (Table 1) may replace the proton donation from AR-Histidine-110/Tyrosine-48, which is a key step in the NADPH regenerating potential substantiating the effective AR blockade activity. AR inhibition potentially results in no or only traces accumulation of sorbitol in vivo which is beneficial to overcome osmotic pressure that may also affect eye lens.

Thus the present study emphasizes on phytochemicals particularly quercetin, present in the finger millet seed coat polyphenols showing noncompetitive inhibitions as potential alternatives to synthetic inhibitors against aldose reductase as also suggested by Kim et al., and Lee et al.[29,30] This approach may circumvent the toxic effects of clinically tested AR inhibitors such as sorbinil, statil, epalrestat, tolrestat and alrestatin.

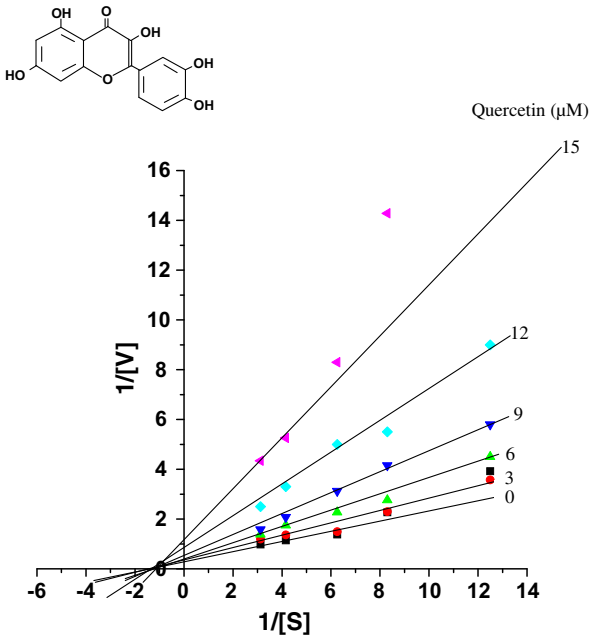


Figure 5. LB plot of the aldose reductase hydrolysis reaction with variable substrate concentrations (0.05–2.5 mM) and at fixed concentration of quercetin (0–15 μM) as indicated on the graph.

3. Experimental

3.1. Chemicals

DL-glyceraldehyde, phenylmethane sulphonyl fluoride (PMSF), NADPH, phenolic standards: gallic, vanillic, ferulic, *p*-coumaric, *p*-hydroxy benzoic, syringic, *trans*-cinnamic, *p*-catechuic, triflouroacetic acids and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol (HPLC grade), lithium sulphate (Li_2SO_4) was purchased from Merck (India). Cataractous eye lenses were collected from a local eye hospital. Other reagents and solvents were of analytical grade.

3.2. Milling

A popular high yielding finger millet cultivar (GPU 28) procured from the University of Agriculture Sciences, Bangalore, India. The millet was cleaned for extraneous matter and sprayed with 5% additional water, tempered for about 10 min and pulverized in a comminuting mill (Apex comminuting mill, Apex Constructions Ltd, England). Soon after pulverizing, the meal was sieved through '180 μ' openings and the tailings ('+180 μ' fraction) was again pulverized immediately and sieved through the same sieve, and the process was repeated for the third time. The flour ('−180 μ' fraction) from 1, 2 and 3 passes were pooled and designated as refined flour fraction (RFF), whereas, the tailings was termed as seed coat fraction (SCF).[10]

3.3. Polyphenol extraction

An aliquot (1 g) of finger millet seed coat fraction (SCF) was extracted with ≅50 ml of 1% HCl–methanol by refluxing in a boiling water bath for 30 min.[10] The refluxed material was filtered using Whatman No. 1, filter paper. The residue was again refluxed with fresh solvent. The process was repeated till the extract tested (with F/C reagent) negative to polyphenols. The filtrates pooled together and their polyphenol content was estimated[31], using gallic acid as reference standard and presented as gallic acid equivalent (GAE). Further it was fractionated into component phenolics by HPLC according to Chethan and Malleshi.[10] The polyphenol were fractionated into the component phenolics by reverse-phase analytical HPLC [Shimadzu LC-10A liquid chromatograph fitted with 250 mm × 4.6 mm ODS-2 C_{18} column and equipped with CBM-10A system controller, SPD-M10 AVP photo diode array detector and a software class 10A]. The mobile phase consisted of a binary solvent system using water acidified with 0.1% trifluoroacetic acid (solvent A) and 100% methanol (solvent B), kept at a flow rate of 1.0 ml/min. The gradient program initiated with 80% eluent A and 20% eluent B, ramped linearly to 60% solvent A and 40% solvent B within 40 min. This proportion (60:40) was maintained for next 10 min and subsequently, the solvent gradient was reverted to the initial conditions (80:20::A:B) within next 5 min (total run time was 55 min). Detection and quantification of the eluted phenolics was performed at 295 nm.

Briefly, different phenolic standards (Sigma Chemical Co., USA) were prepared separately (1 mg/ml). Concentration and retention time in min for each was established. Phenolic acids in samples were identified based on the retention time relative percentage of each constituent was then calculated.

3.4. Isolation of enzyme from cataracted human eye lens

Cataracted human eye lenses were washed with saline and their fresh weights were recorded. The lenses were pooled and homogenized in (1:2, w/v) sodium phosphate buffer (0.135 M, pH 7.0) containing 0.5 mM PMSF and 10 mM β-mercaptoethanol, centrifuged at 8000*g* for 30 min at 4 °C. The supernatant was used for determination of AR activity and estimation of protein content.[32] The activity was calculated as;

$$\text{Activity (U/ml)} = \frac{(\Delta A\ \text{test/min} - \Delta A\ \text{control/min})}{(6.2 \times \text{vol taken for analysis}) \times \text{total volume (ml)}}$$

where 6.2 = micromolar extinction coefficient of NADPH at 340 nm

$$\text{Specific activity (U/mg protein)} = \frac{\text{Activity (U/ml)}}{\text{Protein conc. (mg/ml)}}$$

3.5. AR inhibitory activity

The inhibitory activity of the millet seed coat polyphenols (SCP) on aldose reductase was carried out using all the optimized data in a 1 ml cuvette optimized amount of enzyme (750 μg/ml protein), varying concentration of polyphenol extract (1–200 μg/ml) in 50 mM sodium potassium phosphate buffer (pH 6.0) containing 5 mM β-mercaptoethanol, 0.7 M NADPH, 0.4 M Li_2SO_4 and 2.5 mM of glyceraldehyde (substrate) were taken. The reaction was initiated by addition of glyceraldehyde and the decrease in the optical density at 340 nm was recorded. IC_{50} of the polyphenol extract was calculated using Graphpad software (ver 5.0) USA.

The same conditions were used for fractionated phenolics ranging (1–200 μg/ml) for their inhibitory effects. Guided by this the phenolic compound which showed higher inhibitory activity was taken further for kinetic studies.

3.6. Kinetics of AR inhibition by polyphenols

The inhibition was measured in the presence of commercially available quercetin at different concentrations (0–15 μM) and increasing concentration of glyceraldehydes (substrate, 0.05–2.5 mM). Mode of inhibition was determined by Lineweaver–Burk plot analysis of the data, which is calculated from the results according to Michaelis–Menten kinetics in order to understand the probable mode of action.

3.7. Scavenging of 1,1-diphenyl-2-picrylhydrazyl radical

The effect of SCP extract on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was studied, employing the modified method described earlier by Yamaguchi et al.[33] 1.5 ml of DPPH solution (0.1 mM, in 95% ethanol) was incubated with varying concentrations of the fractionated phenolic compound (1–200 μg/ml). The reaction mixture was shaken well and incubated for 20 min at room temperature and the absorbance of the resulting solution was read at 517 nm against the blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation;

$$\text{Inhibition}(\%) = (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$$

3.8. Statistical analysis

All analysis were performed in triplicate and the data were calculated as means ± standard deviations. Correlation coefficient was analyzed using Graphpad software (Ver 5.0) USA.

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