

Evaluation of Antioxidant and Antidiabetic properties of selected small millets

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ABSTRACT

Multi-target, multi-channel and synergistic properties are the common features in the action of herbal medicines and nutraceuticals, due to variety of constituents within a single natural product. Owing to these properties, herbal medicines and nutraceuticals may be beneficial in dealing with diabetes itself as well as its complications. As diet plays an important role in the overall well-being of an individual and the utilization of wholegrain cereals in food formulations is increasing worldwide, since they are rich in phytochemicals and other nutrients that are useful in treating several health issues. The dietary polyphenols are known to reduce carbohydrate digestibility and regulate postprandial glycemic response. Moreover, polyphenols are known to inhibit glucose absorption and prevent advanced glycation end product (AGE) formation. It is believed that antioxidant activity might be correlated with antidiabetic activity and therefore, it is interesting to see the potential of extracted active ingredients for treating diabetes. Hence the present study has been designed to investigate the efficiency of whole millet extracts on metabolic alterations in glucose metabolism and their antioxidant status.

Keywords: Barnyard, Foxtail, Proso, Little, Finger, Pearl, Khodo, Antioxidant, Antidiabetic

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder of the endocrine system. The incidence of diabetes is escalating globally. The prevalence of diabetes amongst all age-group's worldwide is estimated to increase from 2.8% in 2000 to 4.4% in 2030. This correlates to the total number of people with diabetes rising from 171 million in 2000 to a staggering 366 million in 2030[1]. Comparing the different types of diabetes, type 2 diabetes, the most prevalent form both in global and Indian scenario is the non-insulin dependent diabetes mellitus (NIDDM type-2) which is associated with elevated post prandial hyperglycemia, is responsible for 90-95% of diabetes cases which are a direct result of increased urbanization, high rates of obesity, sedentary lifestyles and stress. Not only does diabetes negatively impact the health and social wellbeing of sufferers but also brings forth devastating economic impact. Glucose is the main energy source for the body, and in the case of DM, management of glucose becomes irregular. Keeping blood glucose levels close to normal and preventing diabetic complications are the major goals in the treatment of DM. In addition, the metabolic deregulation associated with diabetes mellitus also causes secondary pathophysiological changes in multiple organ systems that are associated with oxidative stress and tissue damage [2]. Various experimental and clinical investigative reports indicated that elevated blood glucose levels in diabetic individuals lead to oxidative stress with subsequent formation of advanced glycation end products (AGE) [3,4]. Oxidative stress

due to increased ROS generation and an imbalance in oxidative/antioxidative equilibrium in hyperglycemia plays a major role in diabetic complications [5]. The medicinal value of these plants lies in some chemical substances that produce a definite physiological effect on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, phenolic compounds and Flavonoids. Among these, Flavonoids are the ubiquitous group of plant secondary metabolites demonstrating a wide range of biochemical and pharmacological effects, including anti-diabetic, antioxidant, anti-inflammatory, antibacterial, antifungal [6, 7].

For instance, the use of whole grain based products or the extracts of *Echinochloa* spp., *Pennisetum typhoideum*, *Panicum miliaceum*, *Panicum sumatrense*, *Eleusine coracana*, *Paspalum scrobiculatum* and *Setaria italica*, have been well documented for their dietary polyphenols[8], antioxidant, hypocholesterolaemic, hypolipidemic, insulinemic activities and lower the plasma glucose levels in diabetic subjects.[9]

In the present study, we focused on four different types of assays that are antioxidant and two different types of antidiabetic assays using six millets were carried out and compared with standards[10,11].

MATERIALS AND METHODS

Collection of plant materials – The plant materials for this present study consists of seeds of seven different samples of millets viz *Echinochloa* spp., *Pennisetum typhoideum*, *Panicum miliaceum*, *Panicum sumatrense*, *Eleusine coracana*, *Paspalum scrobiculatum* and *Setaria italica*, were collected. The samples were collected, cleaned, crushed to coarse powder using grinder and stored in air tight bags.

Extraction – Weighed amount of samples were boiled different solvents like absolute ethanol and methanol using Soxhlet apparatus for 2hrs not exceeding its boiling point to prepare the respective extracts. The obtained extracts were filtered using Whatmann filter paper No.1 and concentrated by flash evaporation. The extracts were stored in desiccators and used for further study.

Isolation of Enzymes:

Pancreatic Amylase- Pancreatic amylase was isolated based on the method described by Chougaleet *al.*, (2009), rat was sacrificed, quickly dissected and pancreas was immediately washed with cold saline 3-4 times. The pancreatic tissue was finely cut into small pieces and homogenized in cold saline. The mixture was then centrifuged with protease inhibitor and supernatant was used as crude enzyme. Procedure was carried out at 4⁰ c.

Salivary amylase- Salivary amylase was prepared by diluting the human saliva with saline and stored in cold condition at 4⁰c.

α -Glucosidase- α -glucosidase was isolated from rat small intestine by following the method of Sunil *et al* (2009). briefly 20 hrs fasted animal was sacrificed and immediately the intestine was cut between the part below duodenum and above cecum, then rinsed with ice

cold saline and homogenized with maleate buffer (100mM, pH 6.0). Centrifuged and the homogenate was used as α -glucosidase solution.

Positive control: -Acarbose a known drug for inhibiting carbohydrate hydrolyzing enzyme was used as a positive control.

Antioxidant assays

DPPH radical scavenging assay

In this assay, free radical scavenging activity of crude extract was determined by measuring the bleaching of purple colored methanol solution of DPPH. The radical scavenging activity was determined as described elsewhere. One millilitre from a 0.5 mM methanol solution of the DPPH radical was mixed to 2.0 mL of different concentrations (10 to 50 μ g/mL) of 95% ethanol and methanol extracts; were added 2.0 ml of 0.1 M sodium acetate buffer (pH 5.5). The mixtures were well shaken and kept at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a UV Spectrophotometer. BHT was used as positive control, whereas methanol was used as negative one. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the given equation.

$$\text{DPPH radical scavenging activity (\%)} = [(Abs\ control - Abs\ sample) / (Abs\ control)] \times 100$$

H₂O₂ Assay

The ability of all seeds extracts to scavenge hydrogen peroxide was determined by preparing a solution of hydrogen peroxide (2 mM) in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm. Extracts samples (10-50 μ g/mL) in ethanol were added to a hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of both extracts and standard compounds are calculated by using following equation.

$$\text{H}_2\text{O}_2\text{ Scavenged activity (\%)} = [(Abs\ control - Abs\ sample) / (Abs\ control)] \times 100$$

β -Carotene belching

Antioxidant activity was determined using β -carotene bleaching test. 1 mL of β -carotene solution (0.2 mg/mL in chloroform) was added to 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20. (shon et al , 2003). The mixture was evaporated at 40 °C for 10 min using rotary evaporator to remove chloroform. The resultant mixture was immediately diluted with 100 mL of distilled water to form emulsion. 5 mL of the emulsion was transferred into different test tubes containing 0.2 mL of samples in 70% ethanol at different concentrations (10- 50 μ g/mL). 0.2 mL of 70% ethanol in 5mL of the above emulsion was used as control. Standard (propyl gallate) at the same concentration as samples was used for comparison. The tubes were gently shaken and placed at 45 °C in a water bath for 60 min. The absorbance of the samples, standard and control was measured at 470 nm using a UV Spectrophotometer

against a blank, consisting of an emulsion without β -carotene. The measurement was carried out at initial time ($t = 0$) and successively at 30 and 60 min[12]. All samples were assayed in triplicate and averaged. The antioxidant activity (AA) was measured in terms of successful bleaching of β -carotene using following equation.

$$\%Inhibition = [1 - (AS(0) - AS(60)) / (Ac(0) - Ac(60))] \times 100$$

Where AS(0) the initial absorbance of the sample at AS(60) the absorbance of the sample at 60 min, Ac(0) the absorbance of the negative control at 60min. The extract concentration providing 50% antioxidant activity (IC₅₀) was calculated from the graph of antioxidant activity percentage against extract concentration.

Determination of FRAP

The antioxidant capacity of each sample was estimated according to adapted procedure of Benzie and Strain (1996) with some modifications. FRAP reagent was prepared as using 300 mM acetate buffer, pH 3.6 (3.1 g sodium acetate trihydrate, plus 16-mL glacial acetic acid made up to 1L with distilled water); 10 mM TPTZ (2,4,6-tri(2-pyridyl)-striaizine), in 40 mM HCl; and 20 mM FeCl₃ 6H₂O in the ratio of 10:1:1 to give the working reagent. FRAP reagent prepared freshly and warmed at 37°C, was mixed with 100L test sample in 80 % methanol, standards, or extraction solvent as reagent blank. After 30 min, the absorbance was measured at 595 nm wavelength. The result was expressed as milligrams of equivalents per 100 g of fresh sample (mg TE/g of FW).

Antidiabetic assays

In-vitro α - Glucosidase Inhibition Assay: α -glucosidase will be done based on the method described by Sunil et al (2009). Rat intestinal α -glucosidase was isolated and premixed with the extract at various concentrations (0.2-1 μ g/ml) and 3mM p-nitrophenyl α -D-glucopyranoside as a substrate in phosphate buffer was added to the mixture. The mixture was incubated at 37°C for 30 min and stopped by adding 2ml of 0.1M Na₂CO₃. α -glucosidase activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl α -D-glucopyranoside at 400nm in a spectrophotometer.

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

In Vitro α -Amylase Inhibition Assay: The α -amylase (Salivary and Pancreatic) inhibitory activity will be determined based on spectrophotometer assay using acarbose as the reference drug (Ali et al 2006). The amylase inhibition was performed by using 40 μ l of plant extract (0.2-1 μ g/ml in DMSO), 160 μ l of buffer (pH 6.8) and 400 μ l of starch in phosphate buffer (pH 6.9 with 0.006 M sodium chloride). The reaction was started by the addition of 200 μ l of the enzyme solution. The tubes were incubated at 37°C for 30 min. The final concentration of the each mixture ranges from 0.2- 1mg/ml, 0.25 % (w/v) starch and 1 unit/ml enzyme. 100 μ l of DNS (3, 5 Dinitrosalicylic acid) was added and placed in 85°C water bath for 15 min, cooled and diluted with 900 μ l of distilled water. The activity was determined by measuring the absorbance at 540nm. For control plant extract was replaced

with 40µl of DMSO representing 100% activity, simultaneously for blank, the enzyme was replaced with distilled water and the same procedure was carried out as above. Percentage of inhibition was found out by using the formula as given by (Pavana *et al.* , 2010).

RESULT AND DISCUSSION

Antioxidant assays

A direct relationship has been reported between the levels of phenolic compounds and antioxidant potential of plants. Phenolic compounds exhibit their protective action through various mechanisms like preventing the generation of carcinogens from precursors by acting as blocking agents. The compound which possess large amount of Flavonoids has found to have inherent ability to modify the body reactions to allergens, viruses and carcinogens.

DPPH radical scavenging assay

The degree of discoloration indicates the scavenging capacity of the extract. The effect of antioxidants on the DPPH radical scavenging was thought to result from their hydrogen donating ability. DPPH scavenging activities of different millets were compared with standard (ascorbic acid) by evaluating antioxidant efficiencies, known as IC₅₀. The lower the IC₅₀ number, the greater the overall effectiveness of the antioxidant in millet samples. IC₅₀ value for DPPH scavenging for methanol extract of different millet extracts varied from 645.2-99.5 µg/ml. The khodo millet showed lowest IC₅₀ value (99.5) which was found to be much closer to the standard butylated hydroxy toluene (BHT) followed by little millet (200.9). In addition, there was a statistically significant correlation between the amount of phenolic compounds and DPPH scavenging activity in all the extracts. IC₅₀ values of different extracts are summarized in Table 1.

H₂O₂ Scavenging Activity

The results of H₂O₂ scavenging activity indicated that all the millet extracts showed a considerable amount of H₂O₂ scavenging activities at a concentration range from 10-50 µg/ml. there was a steady and gradual increase in scavenging action with rise in concentration. The ethanolic extracts of Khodo millet showed IC₅₀ value of 102.3 µg/ml which was very close to IC₅₀ value of std. ascorbic acid i.e., 110.5 ± 2.306 µg /mL and the highest value was showed by proso millet 600.8 µg/ml. IC₅₀ values of different extracts are summarized in Table 1.

β-carotene bleaching

The bleaching effect was measured by the peroxidation of β-carotene. Antioxidants can reduce the extent of β-carotene destruction by neutralizing the lipoate-free radical and other free radicals formed in the system. Accordingly, the absorbance decreased rapidly in reaction mixtures without extracts, whereas in the presence of extracts the reaction mixtures retained their colour and thus absorbance for a longer time.

The IC of different extracts in inhibiting the bleaching of β-carotene suggests that bleaching can be moderately inhibited by all seven extracts which attributes to the fact that the

presence of different antioxidant molecules in extracts might be responsible for inhibition of β -Carotene by neutralizing the formation of free radicals. IC_{50} values of different extracts are summarized in Table 1.

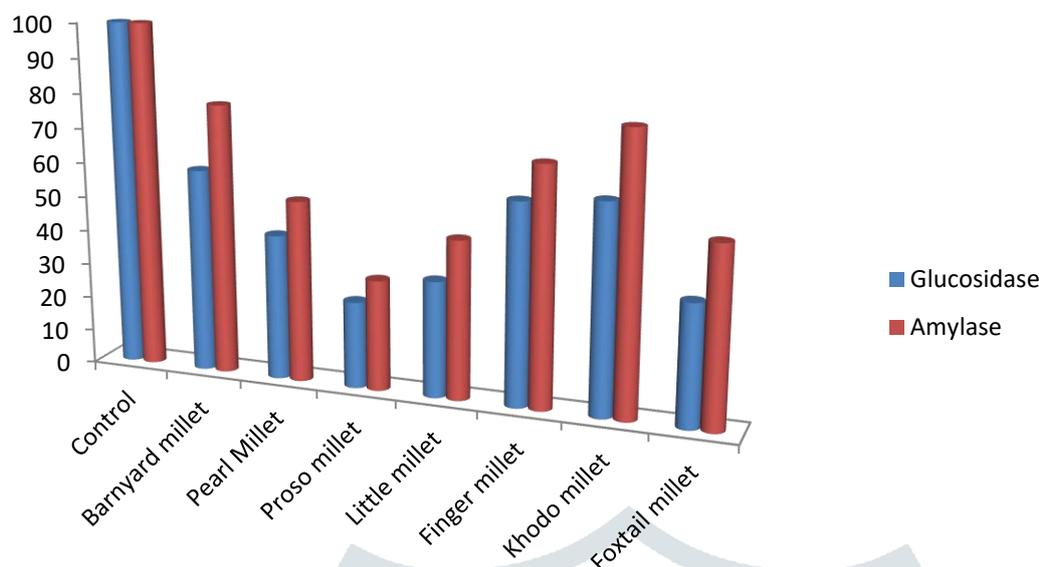
Table: 1 represents IC_{50} values of different antioxidant assays in selected millet extracts

Sl.No	Millet samples	DPPH Scavenging activity (IC_{50} in μ g/ml)	H_2O_2 Assay (IC_{50} in μ g/ml)	β -Carotene Assay (IC_{50} in μ g/ml)	FRAP Assay (IC_{50} in μ g/ml)
1.	Barnyard millet	310.6 \pm 7.403	300.5 \pm 9.632	350.2 \pm 9.845	250.1 \pm 7.238
2.	Pearl Millet	315.9 \pm 6.833	310.5 \pm 5.373	290.2 \pm 5.321	200.3 \pm 4.213
3.	Proso millet	645.2 \pm 1.233	600.8 \pm 2.335	595.8 \pm 1.002	500.1 \pm 5.277
4.	Little millet	200.9 \pm 7.302	209.9 \pm 5.023	215.5 \pm 1.023	195.7 \pm 9.232
5.	Finger millet	508.3 \pm 8.322	415.7 \pm 3.002	450.5 \pm 9.054	401.2 \pm 4.377
6.	Khodo millet	99.5 \pm 8.309	102.3 \pm 1.022	110.7 \pm 7.055	150.3 \pm 5.235
7.	Foxtail millet	509.1 \pm 1.523	510.9 \pm 9.885	597.1 \pm 1.023	500.1 \pm 6.237
8.	Control	100.5 \pm 1.203	110.5 \pm 2.306	110.2 \pm 4.200	150.2 \pm 3.026

Antidiabetic assays

Invitro α - Glucosidase Inhibition Assay: α - Glucosidase is the most important enzyme in carbohydrate digestion. Among the seven millet extracts, other than Proso Millet, all the other millet extracts showed significant relative inhibition for α - glucosidase activity as shown in Graph 1. Particularly Khodo Millet showed good inhibition (60.25%) followed by Barnyard Millet (59.01%) & Finger Millet (58.2%).

In Vitro α -Amylase Inhibition Assay: All the extracts were analysed for the presence of inhibitor of α -Amylase as bioactive constituent. Only Khodo (80.3%), Barnyard (78.2%), little (69.2%) and Finger Millet (68.9%) showed measurable inhibition to α -Amylase as shown in the Graph 1.



Graph 1: Represents comparative effect of Millet extracts on various enzymes

CONCLUSION

All the millet grains used in this study consists of rich source of bioactive compounds that may help to regain immune power and maintain various metabolic reactions inside the body to dominate over a wide range of stress generated due to free radicals.

Results obtained from the above study clearly indicate the presence of various types of phytochemicals in Khodo, Little, Finger, Barnyard and Pearl millet extracts. The Khodo, Little and Barnyard, Finger Millet showed excellent antioxidant properties in four different antioxidant assays compared to Foxtail and Proso Millet. Among the various extracts, the well known millets for said to have loads of phytochemicals are not uniformly effective against selected enzymes of carbohydrate metabolism. Only Khodo, Little, Barnyard and Finger Millet showed effective inhibition against α -Glucosidase and Amylase enzymes.

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