



Comparative analysis of in vitro antidiabetic and antioxidant activity of different supernatant liquids (millets soaked water) of different millets

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Abstract



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This study assessed four distinct millet grains' possible antidiabetic and antioxidant qualities in vitro. The highest levels of flavonoids (115.8 mg CE/100 g) and total phenolics (136.4 mg FAE/100 g) were found in finger Italian millet. The maximum concentrations of ABTS radical scavenging activity (IC₅₀ = 362.40 µg/mL and 381.65 µg/mL, respectively) and DPPH (IC₅₀ = 359.6 µg/mL and 436.25 µg/mL, respectively) were found in barnyard and finger Italian millet. The percentage inhibition of α-glucosidase (18.07 µg/mL) and α-amylase (10.56 µg/mL) was also significantly lower in finger Italian millet compared to acarbose (IC₅₀ = 59.34 µg/mL and 27.73 µg/mL, respectively), and the formation of AGEs (33.68 µg/mL) compared to aminoguanidine (AG) (52.30 µg/mL). Flavonoids comprised all eight phenolic chemicals found in finger Italian millet, with flavanols being the most common subclass. Finger Italian millet has the potential to be developed as a functional food since its flavonoids, when combined, have significant roles in the prevention and control of type 2 diabetes.

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INTRODUCTION

Type 2 diabetes (T2D) is the most prevalent form of the disease, developing around 90% of all cases globally. Affected by insulin resistance and malfunctioning pancreatic β-cells, type 2 diabetes is typified by hyperglycemia and abnormal carbohydrate metabolism. Reactive oxygen species (ROS) have been linked to prolonged exposure to elevated blood glucose levels. The development of diabetes is primarily caused by excessive ROS-induced oxidative stress, which damages cells and causes dysregulations in several gene expressions that impair insulin production and insulin transmission. It has been

discovered that postprandial blood glucose levels are crucial for the initiation and progression of T2D problems. Advanced glycation endproducts (AGEs) and excessive non-enzymatic glycation of proteins are further consequences of hyperglycemia [1]. By causing nephropathy, cataracts, vasculopathy, and atherosclerosis, the glycation changes can worsen the diabetic pathology. Necessary enzymes for the digestion and release of glucose from food include intestinal α -glucosidase and pancreatic α -amylase. One of the most successful treatment approaches for postprandial hyperglycemia is inhibiting these enzymes to delay glucose absorption. The prevalence of diabetes has made it necessary to look more closely for safe, natural treatments and dietary changes that can be used to manage the condition instead of synthetic drugs.

Polyphenols from cereal grains and other medicinal plants are examples of phytochemicals that may have therapeutic benefits, including reducing the problems of diabetes and obesity and inhibiting α -amylase and α -glucosidase, according to mounting evidence. The word "millet" refers to a group of small-seeded, edible grasses grown in arid and semi-arid areas of the world and are members of the Poaceae (formerly Gramineae) family. As the sixth most important cereal in the world, millets are naturally resistant to most biotic and abiotic stressors [2]. For many people in Asia and Africa, they are a staple diet. Millets are nutrient-dense in multigrain and gluten-free cereal products and offer significant health advantages. Besides their nutritional benefits, millets are rich in phytochemicals, mainly phenolic compounds, which may help treat metabolic conditions like diabetes, cancer, and heart disease. Identifying the many chemicals in grains is crucial since they may have additive and synergistic actions that contribute to these positive health outcomes. However, several variables influence cereals' nutritional and phytochemical contents, including genotype, soil, and climatic and environmental factors. It is important to remember that both the phenolic concentration and the specific phenolic type affect the inhibition of digestive enzymes [3].

Research on millet phenolics is currently limited despite growing efforts to identify natural, potent inhibitors of advanced glycation production to lessen its detrimental effects. To investigate the

possible applications of millet types as natural antioxidants and therapies for creating functional meals, it is required to assess their phenolic composition and bioactivities. Although there are several research on medicinal plants, fruits, and vegetables, this study is the first to disclose possible antiglycation capabilities of millet grains as far as we are aware. This study aimed to evaluate various millet cultivars for their antidiabetic potential *in vitro* [4].

MATERIALS AND METHODS

MATERIALS

Different kinds of whole millet grains used in this investigation were given. An electric mill was used to clean and grind the millet grains into a fine powder, sieved through mesh 40. Before being extracted, the samples were kept at -20°C [5].



Figure 1 Images of Millet Grains

Preparation of Ethanolic Solution:

With a few changes, soluble phenolic compounds were extracted. Using a Soxhlet system, samples were defatted with hexane to eliminate lipids. An orbital shaker extracted soluble phenolics from 5 g of defatted samples using 70% ethanol (1:20 w/v) for one hour at 50°C . Following 10 minutes of centrifugation at $4000\times g$, the supernatant was gathered, and the residue was twice extracted using the same setup. After being concentrated under vacuum at 40°C , the mixed supernatants

were freeze-dried. The lyophilized solids were reconstituted in ethanol and kept at -20°C for further use [6].

TPC (Total Phenolic Content):

Using a 24-well microplate and ferulic acid as the reference, total phenolic content (TPC) was determined. In summary, 200 µL of Folin-Ciocalteu reagent was mixed with 100 µL of sample extracts, standard, or a 95% (v/v) methanol blank, and the mixture was carefully agitated. After adding 800 µL of 700 mM sodium carbonate, the mixture was left to rest for two hours at room temperature.

Molecular Devices' SpectraMax i3 plate reader was utilized to measure the absorbance at 765 nm. The ferulic acid standard curve calculated the total phenolic content of milligrams of ferulic acid equivalent per 100 grams of sample (mg FAE/100 g) [7].

TFC (Total Flavonoid Content):

A 24-well microplate was used to measure the total flavonoid content (TFC) of ethanol extracts using the modified AlCl₃ method. In brief, 75 µL of NaNO₂ (50 g L⁻¹) and 1 mL of distilled water were mixed with 250 µL of sample extracts and standard. 75 µL of AlCl₃ (100 g L⁻¹) was added to the reaction mixture after 5 minutes.

600 µL of distilled water and 500 µL of 1 M NaOH were added after 6 minutes. After 30 seconds of shaking in a SpectraMax i3 plate reader (Molecular Devices), the absorbance at 510 nm was measured. Results were reported as milligram catechin equivalents per 100 g of sample (mg CE/100 g), with catechin as a standard.

CTC (Total Condensed Tannin Content):

The vanillin assay was modified to assess the ethanol extract's total condensed tannin content (TCT). In a 96-well microplate, 40 µL of the sample solution and 200 µL of the 4% vanillin reagent were combined.

After 20 minutes at 500 nm, the absorbance of the combination kept at 30 °C was measured against a blank solution made using the same method as before, with 4% hydrochloric acid used in place of the 4% vanillin reagent. Results were reported as milligrams of catechin equivalents per gram of

sample (mg CE/100 g) on a dry weight basis, using catechin as a standard [8].

Antioxidant Activities Assays :

Radical Scavenging Activities:

DPPH:

The DPPH assay used a 24-well microplate reader based on the modified method. In brief, 2 mL of newly prepared 100 µM DPPH radical solution (4 mg DPPH in 100 mL 95% v/v methanol) was mixed with 200 µL sample extracts of different concentrations. After 30 minutes of room temperature incubation, the absorbance at 517 nm was measured. The IC₅₀ value, which indicates the concentration of test extracts needed to scavenge the DPPH free radical by 50%, was used to express the results [9] , [10].

ABTS:

A modest adjustment was made to this procedure. Equal volumes of 2.45 mM potassium persulfate solution and seven mM ABTS solution were reacted for 16 hours at room temperature in the dark to create the ABTS+ stock solution. An absorbance of about 0.70 at 734 nm was obtained by diluting the ABTS+ stock solution with ethanol. 1 mL of newly prepared ABTS+ radical solution was mixed with 80 µL of sample extracts in various concentrations. The absorbance at 734 nm was measured appropriately. Results were presented as IC₅₀, the concentration of test extracts needed to scavenge 50% of the ABTS radical. Enzymes for the Digestion of Carbohydrates and Glycation Inhibitions

α-Amylase Inhibitory Assay:

Sekhon-Loodu and Rupasinghe's α-amylase inhibitory test was modified and used. Briefly, 20 mM sodium phosphate buffer with six mM NaCl (pH 6.9) was used to dissolve the test extracts, enzyme, and soluble starch. The buffer solution was used to dissolve the test extracts at varying concentrations. 100 µL of test extract at several concentrations and 250 µL of pancreatic pig α-amylase (1 U/mL) diluted in buffer (pH 6.9) were added to a test tube. 250 µL of 0.5% starch was added to the mixture after it had been pre-incubated for 15 minutes at 37 °C. The mixture was then vortexed and incubated for 20 minutes at 37 °C. One milliliter of dinitrosalicylic acid color reagent was then used to terminate the reaction.

After five minutes in a heating block, the tubes were allowed to cool to ambient temperature before being diluted with ultrapure water. A Spectra Max i3 plate reader was used to measure the absorbance at 540 nm after 200 microlitres of the reaction mixture were transferred into a 96-well transparent plate. 100% enzyme activity was represented by the control, which was α -amylase without any inhibitor. Suitable test extract blanks that included the reaction mixture—aside from the enzyme—were utilized. For comparison tests, acarbose, a well-known α -amylase inhibitor, accounted for the color interference. The test sample's % inhibition of α -amylase was calculated as follows:

$$\text{Inhibition (\%)} = 100 \times \frac{(AC - AS)}{AC}$$

α -Glucosidase Inhibitory Assay:

With few modifications, The test for α -glucosidase inhibition was altered. A SpectraMax i3 plate reader recorded the absorbance at 405 nm. As a positive control, the prescription diabetes medication acarbose was utilized. The enzyme and substrate mixture without inhibitors—instead, buffer—served as the control. Except α -glucosidase, the sample blanks were combinations of test sample, substrate, and buffer. Like the α -amylase assay, the test sample's α -glucosidase inhibition (%) was determined.

AGE Formation Inhibition :

With equal amounts of D-glucose (36 mg/mL) and bovine serum albumin as well as the test samples or negative control, or aminoguanidine at levels of 0.01, 0.02, 0.04, 0.05, 0.1, 0.2, 0.5, and 1.0 mg/mL, the incubation mixes had a final volume of 1.0 mL. A well-known inhibitor of AGE production, aminoguanidine, served as a positive control. Excitation and emission wavelengths of 340 and

420 nm were used to observe fluorescent AGEs on a microplate reader. The experiments were carried out in triplicate. The following formula was used to determine the percentage of AGE inhibition [11]:

$$\text{Inhibition (\%)} = \left[1 - \left(\frac{\text{Fluorescent of the Test}}{\text{Fluorescent of the Control}} \right) \right] \times 100$$

The IC50, the concentration of test extracts needed to produce a 50% inhibition of AGE formation, was used to express the results.

Statistical Analysis:

GraphPad Prism 8.0 was used to analyze the data. One-way analysis of variance (ANOVA) and Tukey's test were used to compare the mean values of the various millet phenolic fractions at a significance threshold of $p < 0.05$. The mean \pm standard deviation (SD) was used to illustrate the results [12].

RESULTS AND DISCUSSION

TPC, TFC, and CTC of Ethanol Extracts:

The primary antioxidants that directly increase cereals' antioxidant capacity are polyphenols. **Table 1** displays the soluble TPCs for the four types of millet: finger Italian millet (FIM), barnyard millet (BM), Italian millet (IM), and Buck Wheat Millet (BWM) 107.8–136.4 mg ferulic acid equivalent/100g, DW. TPC of BWM, BM, and FIM did not significantly differ. However, FIM soluble TPC was the highest. The soluble TPC in Italian millet was substantially lower ($p < 0.05$). Important antioxidants called flavonoids help lower the chance of developing chronic diseases. **Table 2** and **Table 3** show that the free phenolic fraction consisted of various flavonoids; no phenolic acids were found. This is consistent with research that shows flavonoids make up the

Table 1 Total condensed tannin content (CTC), total flavonoid content (TFC), and total phenolic content (TPC) of millet grain types

Millet	TPC (mg Equivalent of Ferulic Acid/100 g, DW)	TFC (mg Equivalent of Catechin/100 g, DW)	CTC (mg Equivalent of Catechin/100 g, DW)
M	124.2 \pm 6.35 a	102.8 \pm 9.1 a	51.51 \pm 4.82 a
I'M	106.7 \pm 6.03 b	106.3 \pm 9.1 a	35.36 \pm 3.57 c
BM	128.4 \pm 4.96 a	102.2 \pm 10.4 a	58.53 \pm 3.64 a
FIM	135.5 \pm 7.06 a	116.7 \pm 9.1 a	16.64 \pm 4.96 b

The mean \pm SD is used to express the results. BM, barnyard millet; FIM, finger Italian millet; DW, dry weight sample; M, millet; IM, Italian millet.

largest class of free phenolic fractions and that phenolic acids are primarily found in bound fractions. The range of TFC in millet cultivars' free fractions was 101.3–115.8 mg CE/100g. Among these four types, there was no significant difference ($p < 0.05$), although the highest TFC was found in finger Italian millet. In comparison to IM (36.37 mg CE/100g, DW) and FIM (17.65 mg CE/100g, DW), the BM (59.54 mg CE/100g, DW) and BWM (50.50 mg CE/100g, DW) types in this investigation exhibited considerably greater ($p < 0.05$) CTC. Finger millet has a substantially higher CTC than pearl, proso, foxtail, kodo, and tiny millets. The climate, agricultural environment, and genotype may be responsible for this.

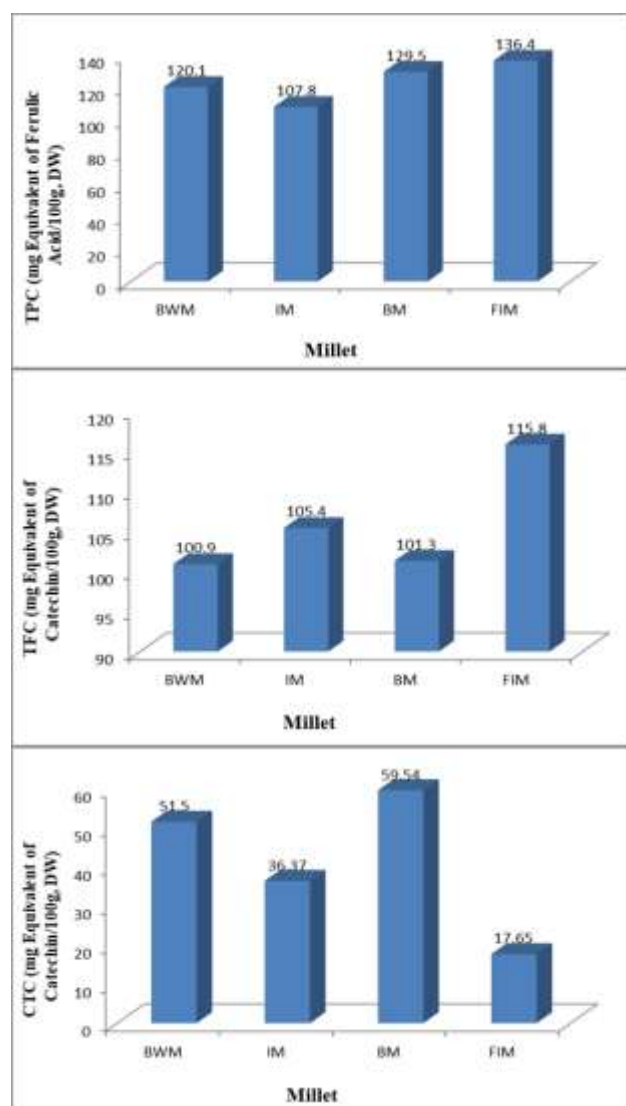


Figure 2 Total condensed tannin content (CTC), total flavonoid content (TFC), and total phenolic content (TPC) of millet grain types

Antioxidant Activity of Ethanolic Solution:

Phenolic chemicals found in plants help to protect the body from oxidative stress, diabetes, cancer, and cardiovascular disease. Phenolic substances found in various meals have been shown to contribute significantly to their antioxidant properties. As a result of their safety and nutritional benefits, plant-based therapies are promising options to consider. Similarly, finger Italian millet (381.65 $\mu\text{g/mL}$) and barnyard millet (362.40 $\mu\text{g/mL}$) showed lower IC_{50} values and the most excellent ABTS radical scavenging activity. Similar to their DPPH radical scavenging activity, millet and Italian millet showed significantly lower ABTS radical scavenging activity than Trolox (76.60 $\mu\text{g/mL}$, respectively, with IC_{50} values of 410.35 and 422.35 $\mu\text{g/mL}$). Our research supports earlier investigations that discovered vigorous antioxidant activity in vitro in millet cultivars' soluble phenolic components.

Antidiabetic Activity:

In vitro, Postprandial blood glucose levels are the primary factor to be controlled in managing type 2 diabetes. Dietary polyphenols have been shown to have antihyperglycemic effects in addition to their antioxidant qualities via binding to glucose transporters and blocking digestive enzymes. Dietary starch and oligosaccharides are hydrolyzed into glucose by the enzymes α -amylase and α -glucosidase, increasing postprandial glucose levels. Inhibiting α -amylase and α -glucosidase activity is a key preventive strategy for managing type 2 diabetes. This study examined how ethanol extracts of millet types inhibited α -amylase, α -glucosidase activity, and AGE formation (IC_{50} values). Ethanol extracts from barnyard millet and finger Italian millet inhibited α -glucosidase, with respective IC_{50} values of $20.90 \pm 7.46 \mu\text{g/mL}$ and $18.07 \pm 3.27 \mu\text{g/mL}$. This was noticeably more effective than the often prescribed standard medication, acarbose (IC_{50} value: $59.34 \pm 3.07 \mu\text{g/mL}$). The α -glucosidase inhibition was significantly lower in millet and Italian millet, with IC_{50} values of $193.85 \pm 3.63 \mu\text{g/mL}$ and $499.76 \pm 5.46 \mu\text{g/mL}$, respectively. Polyphenols have been shown to inhibit enzymes via nonspecific binding. Polyphenols with higher molecular weights and polymerization levels block α -glucosidase more effectively. Millet extracts inhibited α -glucosidase

more than α -amylase. Alternatively, our results suggest that genotypes or environmental conditions influence the content and concentration of individual phenolic compounds. Finger The highest α -amylase inhibition was seen in Italian millet ethanol extracts, with IC₅₀ values of $10.56 \pm 1.43 \mu\text{g/mL}$. A significant difference ($p < 0.05$) was not found between Buck Wheat millet ($32.59 \pm 4.61 \mu\text{g/mL}$) and Italian millet ($18.89 \pm 2.57 \mu\text{g/mL}$). The IC₅₀ value of barnyard millet was $81.32 \pm 3.54 \mu\text{g/mL}$, which was the lowest inhibition of α -amylase when compared to acarbose ($27.73 \pm 7.34 \mu\text{g/mL}$).

The soluble and bound phenolics of foxtail and tiny millet cultivars (albeit the soluble fractions showed more potent inhibition) were found to have lower IC₅₀ values than the standard medication, acarbose. Flavonoids inhibit α -glucosidase and α -amylase through the hydroxyl group at C3 and the double bond between C2 and C3 on their C-rings. Recent research has also found that phenolic compounds' inhibitory activities on digestive enzymes can be mediated by their hydrogen bonding with residues of amino acids in the digestive enzymes' active sites, which prevents the enzymes' catalytic activity with carbs. Soluble millet phenolics, primarily flavonoids, inhibit α -glucosidase and α -amylase, potentially reducing glucose release and absorption in the small intestine.

Significantly, more efforts are being made to develop natural chemicals or items that can mitigate the harmful effects of AGEs. Recent research has shown that polyphenols may have antiglycation properties. Finger Italian millet ethanol extract inhibited AGE formation with the highest IC₅₀ value ($33.68 \pm 5.98 \mu\text{g/mL}$) compared to aminoguanidine, a recognized antiglycation drug (IC₅₀ = $52.30 \pm 2.31 \mu\text{g/mL}$). Growing research demonstrates that polyphenols' ability to suppress protein glycation is highly related to their antioxidant activity and phenolic concentration. Phenolic components of medicinal plants demonstrated more antiglycation activity than aminoguanidine. Polyphenols mitigate the adverse effects of advanced glycation by inhibiting (1) ROS formation, (2) Schiff base, (3) Amadori products and dicarbonyl formation, (4) activating the detoxification enzyme system, and (5) blocking the interaction between AGEs and receptors.

Phenolic Compound Analyses Using UHPLC-Q-TOF-MS2:

Dietary phenolic compounds are the main bioactive ingredients in various fruits, vegetables, drinks, and whole grains that help prevent chronic diseases and enhance existing ones. In the current study, UHPLC-Q-TOF-MS2 in a negative mode was used to positively or tentatively identify the phenolic contents of soluble ethanol extracts from barnyard millet and finger Italian millet. **Table 2** and **Table 3** summarize the mass and UV spectral data. Using UV spectra data, retention time (RT) comparison with known authentic standards, and UHPLC-Q-TOF-MS2 confirmation, phenolic identification and characterization were accomplished. Phenol-Explorer, an online comprehensive dietary polyphenol database, was used to identify tentative compounds.

As indicated in **Table 2** and **Table 3**, Eight (8) and seven (7) phenolic compounds were discovered tentatively from finger Italian and barnyard millet soluble extracts, respectively. All phenolic chemicals found in finger Italian millet were flavonoids, with the most common subclass being flavanols. Flavones, flavonols, isoflavonoids, dihydroflavonols, and their glycosides were among the other phenolics that belonged to various flavonoid subclasses. This result is in line with what other investigations have shown. The soluble ethanol extracts of barnyard millet and finger Italian millet contained no phenolic acids.

Table 2 shows that Compound 2 ([M-H]⁻ m/z = 289.0721) was positively recognized as catechin compared with a catechin standard. Additionally, at m/z 289.0721, Compounds 2 and 4 displayed the identical [M-H]⁻, indicating that they might be an isomeric pair. Consequently, Compounds 3 and 4 were tentatively identified as epicatechin and catechin, respectively. There have been prior reports of epicatechin and catechin in finger millet. Phenol-Explorer, an online polyphenol database, provided the preliminary identifications for the remaining seven phenolics (**Table 2**). Similarly, **Table 3** shows that Compound 1 ([M-H]⁻ m/z = 289.0721) was positively recognized as catechin by comparison with the catechin standard. At m/z 285.0408, compounds 3 and 4 displayed the same [M-H]⁻, suggesting they might be an isomeric pair.

Since catechins have been demonstrated to help reduce hyperglycemia and improve diabetes, this

Table 2 Using quadrupole time-of-flight mass spectrometry in ultra-high performance liquid chromatography (UHPLC-Q-TOF-MS2), phenolic components were detected

RT (min)	Molecular Formula	[M-H] ⁻ (m/z)	Compound Identified
8.73	C ₂₁ H ₂₄ O ₁₁	451.1249	(+)-Catechin 3-O-glucose
9.54	C ₁₅ H ₁₄ O ₆	289.0721	(+)-Catechin
9.67	C ₂₁ H ₂₂ O ₁₂	465.1041	Dihydromyricetin 3-O-rhamnoside
11.06	C ₁₅ H ₁₄ O ₆	289.0722	(-)-Epicatechin
12.42	C ₁₅ H ₁₂ O ₇	303.0514	Dihydroquercetin
12.67	C ₁₆ H ₁₂ O ₄	267.0664	Formononetin
15.63	C ₁₅ H ₁₀ O ₆	285.0408	Kaempferol
16.42	C ₁₅ H ₁₀ O ₅	269.0457	Apigenin

Table 3 UHPLC-Q-TOF-MS2 was used to identify phenolic chemicals in barnyard millet ethanol extracts.

RT (min)	Molecular Formula	[M-H] ⁻ (m/z)	Compound Identified
9.54	C ₁₅ H ₁₄ O ₆	289.0721	(+)-Catechin
12.68	C ₁₆ H ₁₂ O ₄	267.0664	Formononetin
15.63	C ₁₅ H ₁₀ O ₆	285.0408	Kaempferol
16.38	C ₁₅ H ₁₀ O ₆	285.0408	Luteolin
16.43	C ₁₅ H ₁₀ O ₅	269.0457	Apigenin
16.44	C ₁₆ H ₁₂ O ₇	315.0513	Isorhamnetin
16.50	C ₁₇ H ₁₄ O ₇	329.067	3,7-Dimethylquercetin

Table 4 Polyphenolic component quantification using a high-performance liquid chromatography photodiode array (HPLC-PDA) in finger Italian and barnyard millet extracts

Compound	RT (min)	Finger Italian Millet (µg/100g)	Barnyard Millet (µg/100g)	Polyphenolic Class
Catechin	9.54	577.49	146.1	Flavonoid
Quercetin	16.2	Not Defined	Not Defined	
Caffeic acid	11.13	Not Defined	Not Defined	Phenolic acid
p-Coumaric acid	12.94	Not Defined	Not Defined	
Ferulic acid	13.57	Not Defined	Not Defined	
Gallic acid	2.17	Not Defined	Not Defined	

may explain the increased inhibitory activities of digestive enzymes linked to type 2 diabetes. However, other flavonoids present may also work in concert to amplify this impact. In earlier research, catechin was identified as the main phenolic compound in finger millet; however, the amount of catechin in this study was lower than in those earlier findings. The soluble fractions of barnyard and finger Italian millets did not include the other five criteria. Most cereal phenolic acids are thought to be found in bound form rather than free fraction. According to reports, phenolic acids are the most common phenolics in bound fractions.

CONCLUSION:

The current study presents new information on some millet grains' potential as antioxidants and antidiabetic agents. The extracts of barnyard millet and finger Italian millet demonstrated high antioxidant capacity. Comparing these millet types' soluble phenolics—mostly flavonoids—to the widely used medication acarbose, they showed strong suppression of α -glucosidase and α -amylase activities, suggesting that they may be able to lower postprandial hyperglycemia by delaying the digestion of carbohydrates. Additionally, phenolic fractions, primarily flavonoids, displayed vigorous antiglycation

activities, suggesting that they may be able to lessen the harmful effects of AGEs. Therefore, additional animal model research is required to validate these millet grains' antidiabetic properties. The results of this study can be applied to the creation of functional foods and ingredients for the management and prevention of diabetes and other chronic diseases.

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Author Contribution

All authors made substantial contributions to the conception, design, acquisition, analysis, or interpretation of data for the work. They were involved in drafting the manuscript or revising it critically for important intellectual content. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work, ensuring its accuracy and integrity.

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