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Optimization of Germination Enhances Nutritional Composition and *In vitro* Enzyme Inhibitory Properties of Selected Millets

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ABSTRACT: Germination is an effective bioprocessing technique that improves the nutritional quality and functional characteristics of cereal grains by activating endogenous enzymes and modifying the biochemical composition of seeds. Millets are recognised as nutrient-dense cereals with low glycaemic potential; however, comparative information on how germination affects their nutrient composition and enzyme inhibitory properties remains limited. This study evaluated the effects of germination on the nutritional composition of *Pennisetum glaucum* (pearl millet), *Panicum miliaceum* (proso millet), *Panicum sumatrense* (little millet), and *Echinochloa frumentacea* (barnyard millet), as well as their *in vitro* inhibitory activities against α-amylase, α-glucosidase, and acetylcholinesterase. The mode of enzyme inhibition was characterised through kinetic analysis, and germination conditions were optimised using response surface methodology.

Germinated millet flours were analysed for carbohydrate, reducing sugar, protein, and dietary fibre content using standard analytical procedures. Enzyme inhibitory activity against α-amylase, α-glucosidase, and acetylcholinesterase was evaluated using established *in vitro* assays, and inhibitory potency was estimated by IC_{50} determination. Lineweaver–Burk plots were used to determine the mode of enzyme inhibition. Central Composite Design coupled with Response Surface Methodology was applied to optimise soaking and germination conditions for maximum enzyme inhibitory activity. Germination enhanced the nutritional attributes of the selected millets: pearl millet showed the highest protein and reducing sugar content, while little millet showed the highest dietary fibre content. All germinated samples showed concentration-dependent inhibitory activity against the three enzymes evaluated, with little millet the most potent α-amylase inhibitor (IC_{50} = 4.95 ± 0.20 µg mL^{-1}). Kinetic analysis indicated competitive inhibition of α-amylase and α-glucosidase, and non-competitive inhibition of acetylcholinesterase. Response surface optimisation identified 12 h of soaking followed by 6 h of germination as the optimum processing conditions for maximising enzyme inhibitory activity. Germination improved the nutritional quality and *in vitro* enzyme inhibitory properties of the millets investigated,

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highlighting their potential as ingredients for functional cereal-based foods. The biological activities reported are, however, based exclusively on *in vitro* assays; further phytochemical characterisation together with animal and clinical studies are needed to establish their physiological relevance and health benefits.

1. INTRODUCTION

The rising global burden of non-communicable diseases (NCDs), together with mounting concerns over food security and climate change, has intensified the search for foods that combine strong nutritional value with genuine health-promoting potential. Dietary intervention is now widely regarded as a practical strategy for reducing the burden of chronic metabolic disorders, particularly type 2 diabetes mellitus, obesity, cardiovascular disease, and age-related neurodegeneration. This has fuelled growing scientific and industrial interest in functional foods that go beyond basic nutrition to deliver bioactive compounds capable of supporting long-term health. Among the cereal grains studied for this purpose, millets have emerged as one of the most promising candidates, owing to their favourable nutrient composition, resilience under harsh agro-climatic conditions, and compatibility with sustainable farming practices (Krishnan, 2024).

Millets comprise a diverse group of small-seeded cereal crops, including pearl millet (Pennisetum glaucum), proso millet (Panicum miliaceum), little millet (Panicum sumatrense), and barnyard millet (Echinochloa frumentacea), which have served as staple foods across Asia and Africa for centuries. Unlike major cereals such as rice and wheat, millets tolerate drought, poor soil fertility, and high temperatures, allowing cultivation under resource-limited conditions with comparatively low water and input requirements (Harish et al., 2024). In recognition of their contribution to sustainable agriculture and nutritional security, the United Nations declared 2023 the International Year of Millets, drawing renewed global attention to millet cultivation, processing, and utilisation, and encouraging researchers to view millets not only as climate-resilient crops but also as functional ingredients capable of addressing both nutritional deficiencies and lifestyle-related disorders.

Interest in millets stems largely from their nutritional profile. They are rich in complex carbohydrates, dietary fibre, plant protein, essential amino acids, and minerals such as iron, calcium, magnesium, phosphorus, and zinc, alongside phytochemicals including phenolic acids, flavonoids, tannins, lignans, anthocyanins, phytosterols, and bioactive peptides that together contribute to their antioxidant and functional properties. Compared with refined cereals, millets generally contain more slowly digestible and resistant starch, giving a lower glycaemic response after consumption. Recent work in cereal science indicates that these phytochemicals modulate oxidative stress, inflammation, and metabolic pathways linked to chronic disease, although the prevailing view is that such effects arise from the collective action of multiple bioactive constituents rather than any single compound. Whole-grain millet products are therefore increasingly explored as functional ingredients that can deliver synergistic nutritional benefit while retaining their traditional dietary role.

Despite these advantages, the health-promoting potential of raw millet grains is often limited by naturally occurring antinutritional factors such as phytates, tannins, and endogenous enzyme inhibitors, which can reduce mineral bioavailability, impair protein digestibility, and restrict nutrient absorption. Considerable research has therefore focused on processing technologies that improve nutrient accessibility while preserving or enhancing bioactive composition. Germination is one of the most effective, low-cost, and environmentally sustainable options, since it triggers natural biochemical transformation without sophisticated equipment or chemical treatment. During germination, water uptake activates endogenous hydrolytic enzymes such as amylases, proteases, and phytases, which break down storage macromolecules into simpler, more bioavailable forms. These changes increase the availability of free amino acids, soluble sugars, vitamins, and minerals while reducing phytate and other antinutritional compounds; germination has also been shown to improve dietary fibre accessibility and promote the release of phenolics, flavonoids, γ-aminobutyric acid (GABA), and other antioxidant metabolites. As a result, germinated cereal grains typically show improved nutritional quality, digestibility, and functional potential relative to their ungerminated counterparts.

Beyond nutrient composition, germination alters the concentration and bioavailability of compounds capable of interacting with biological systems, which has generated interest in evaluating germinated millets as functional ingredients for the dietary management of metabolic disorders. The extent of these changes, however, varies considerably among millet species and depends on processing variables such as soaking duration, germination time, temperature, and moisture. Optimising

germination conditions and systematically characterising the resulting nutritional and functional properties are therefore essential steps toward standardised millet-based products with reproducible quality and biological activity.

Functional foods are intended to support health through regular dietary consumption rather than pharmacological action, and cereal-based functional foods are of particular interest because they combine nutritional adequacy with phytochemical diversity. Germinated cereals are promising in this regard, since germination increases the concentration, bioaccessibility, and biological activity of several health-relevant constituents (Jacob et al., 2024; Sadh et al., 2024). One of the most widely studied mechanisms by which such foods may support metabolic health is modulation of the carbohydrate-digesting enzymes α-amylase and α-glucosidase. Salivary and pancreatic α-amylase initiates starch hydrolysis by cleaving internal α-1,4-glycosidic bonds to yield maltose, maltotriose, and dextrins, while intestinal α-glucosidase completes digestion by releasing free glucose for absorption. Rapid digestion and absorption of carbohydrate contribute to postprandial hyperglycaemia, a key risk factor for insulin resistance, type 2 diabetes, and cardiovascular complications, and partial inhibition of these enzymes is an established strategy for improving glycaemic control (Mwakalukwa et al., 2020; Ahmad et al., 2020).

Synthetic α-glucosidase inhibitors such as acarbose, miglitol, and voglibose remain widely prescribed for controlling postprandial glucose, but prolonged use is often associated with gastrointestinal side effects, including abdominal discomfort, flatulence, and diarrhoea, which reduce patient compliance. This has driven interest in naturally occurring enzyme inhibitors from edible plants as milder, potentially safer alternatives. Phenolic compounds, flavonoids, condensed tannins, anthocyanins, and bioactive peptides in cereal grains can interact with digestive enzymes through hydrogen bonding, hydrophobic interactions, and conformational change, lowering catalytic efficiency; rather than fully suppressing enzyme activity, many food-derived inhibitors act moderately, which is considered advantageous because it slows glucose release without severely disrupting normal digestion (Jacob et al., 2024; Sharma et al., 2024).

Millets are of particular interest here because of their distinctive phytochemical composition and comparatively low glycaemic index. Germination appears to further increase the abundance and accessibility of phenolic acids, flavonoids, GABA, and other antioxidant metabolites, contributing to greater antioxidant capacity and enzyme inhibitory activity. By activating endogenous hydrolytic enzymes, germination reshapes both the nutritional profile of millet grains and the composition of bioactive molecules that interact with carbohydrate-digesting enzymes, positioning germinated millets as candidate ingredients for functional foods aimed at supporting glycaemic regulation (Jacob et al., 2024; Sadh et al., 2024).

Beyond metabolic disease, dietary bioactive compounds may also influence neurological health through modulation of cholinergic neurotransmission. Alzheimer's disease, the most common form of dementia, is marked by progressive cognitive decline accompanied by degeneration of cholinergic neurons and depletion of acetylcholine. Acetylcholinesterase (AChE), which hydrolyses acetylcholine, is an important therapeutic target because excessive enzymatic activity accelerates neurotransmitter breakdown and contributes to cognitive dysfunction. While clinically approved AChE inhibitors remain the mainstay of symptomatic treatment, a growing body of evidence suggests that naturally occurring phytochemicals — polyphenols, flavonoids, alkaloids, and other plant metabolites — can also interact with the catalytic and peripheral binding sites of AChE and reduce its activity under experimental conditions (Kumar et al., 2023).

Interpreting AChE inhibition by food extracts nonetheless calls for caution. Unlike purified pharmaceutical inhibitors, crude plant extracts are complex mixtures whose composition varies with species, genotype, cultivation conditions, and processing method. Moreover, *in vitro* enzyme inhibition does not automatically translate into physiological efficacy, since digestion, intestinal absorption, hepatic metabolism, blood–brain barrier permeability, and systemic bioavailability all shape biological activity in vivo. *In vitro* inhibition assays are therefore best regarded as an initial screening step that identifies promising food matrices for further mechanistic and preclinical study, rather than as direct evidence of therapeutic efficacy (Jacob et al., 2024).

Inhibition assays alone do not explain how a bioactive compound affects enzyme function, which is why kinetic analysis is an important complement to functional food research. Determining the Michaelis constant (Km) and maximum reaction velocity (Vmax) allows competitive, non-competitive, uncompetitive, and mixed modes of inhibition to be distinguished: competitive inhibitors typically raise the apparent Km without changing Vmax by competing with substrate for the active site, whereas non-competitive inhibitors lower Vmax without materially affecting substrate affinity by binding elsewhere on the enzyme. Such mechanistic detail strengthens the interpretation of inhibitory activity and allows more meaningful comparison between food-derived extracts and established pharmaceutical inhibitors (Moghimi et al., 2021; Mwakalukwa et al., 2020). Integrating kinetic

analysis with nutritional profiling, rather than treating these as separate lines of evidence, gives a more complete picture of the functional properties of germinated cereals and strengthens the scientific basis for developing evidence-based functional foods (Jacob et al., 2024; Sadh et al., 2024).

Despite this progress, the millet literature remains fragmented. Most published work has examined either the nutritional changes associated with germination or the biological activity of individual millet species in isolation, and comparative studies that jointly evaluate nutritional enhancement, digestive enzyme inhibition, AChE inhibition, enzyme kinetics, and germination optimisation across multiple millet species within one experimental framework remain scarce (Jacob et al., 2024; Sadh et al., 2024). Much of the existing work also emphasises antioxidant activity and total phenolic content over the mechanistic interaction between germinated extracts and therapeutically relevant enzymes, so the mode of inhibition of α-amylase, α-glucosidase, and AChE by germinated millet extracts remains poorly characterised, limiting both scientific interpretation and comparison with established dietary enzyme inhibitors (Mwakalukwa et al., 2020; Moghimi et al., 2021). A further gap concerns process optimisation: germination-induced biochemical change depends heavily on variables such as soaking duration, germination time, moisture, and temperature, yet relatively few studies have used statistical approaches such as Response Surface Methodology to optimise these variables for both nutritional quality and biological activity, and the resulting lack of standardised processing conditions limits reproducibility and industrial application (Sharma et al., 2024). Addressing these gaps calls for integrated investigations that combine compositional analysis, bioactivity assessment, kinetic evaluation, and process optimisation within a single experimental framework — an approach that provides stronger scientific evidence than studies examining these parameters individually.

The present study addresses these gaps through a comparative evaluation of four nutritionally important millet species — pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), little millet (*Panicum sumatrense*), and barnyard millet (*Echinochloa frumentacea*) — that integrates proximate nutritional analysis, *in vitro* inhibition of α-amylase, α-glucosidase, and acetylcholinesterase, enzyme kinetic characterisation, and statistical optimisation of germination conditions within a single experimental design. A particular strength of the approach is the use of kinetic analysis to resolve the mechanism of inhibition through changes in Km and Vmax, which offers greater biological insight than percentage inhibition or IC_{50} values alone. The application of Central Composite Design (CCD) coupled with Response Surface Methodology further allows systematic optimisation of soaking and germination conditions to maximise functional properties. Throughout, enzyme inhibitory activity is interpreted strictly within the scope of *in vitro* experimentation, consistent with current recommendations for functional food research, providing a foundation for future phytochemical, preclinical, and clinical work.

Based on this rationale, the central hypothesis of the study is that controlled germination enhances the nutritional quality and modifies the biochemical composition of the selected millet species, thereby increasing their capacity to inhibit the carbohydrate-digesting enzymes α-amylase and α-glucosidase, as well as acetylcholinesterase, *in vitro*. These biological activities are further expected to depend on germination conditions and to correspond to specific, kinetically identifiable modes of enzyme inhibition.

To test this hypothesis, the study was designed with the following objectives:

- To evaluate the effect of germination on the proximate nutritional composition of pearl millet, proso millet, little millet, and barnyard millet.
- To investigate the *in vitro* inhibitory activity of germinated millet extracts against α-amylase, α-glucosidase, and acetylcholinesterase using established enzyme inhibition assays.
- To determine the inhibitory potency (IC_{50}) of each germinated millet extract and compare enzyme inhibitory responses among the selected species.
- To characterise the mechanism of enzyme inhibition through Lineweaver–Burk kinetic analysis, determining Km, Vmax, and the mode of inhibition.
- To optimise soaking and germination conditions using Central Composite Design and Response Surface Methodology to maximise enzyme inhibitory activity and nutritional quality.

● To formulate an optimised germinated millet blend based on the resulting nutritional and functional characteristics for potential use in functional cereal-based foods.

By providing an integrated evaluation of nutritional enhancement, enzyme inhibitory activity, mechanistic kinetics, and process optimisation in germinated millets, this study contributes to the growing field of functional food research. Its findings clarify how controlled germination shapes both nutritional composition and biological functionality, offering a scientific basis for developing standardised millet-based functional foods, and the kinetic evidence generated here provides a foundation for future work involving phytochemical characterisation, molecular docking, bio - accessibility studies, and animal and clinical validation.

2. MATERIALS AND METHODS

2.1 Study Material

All chemicals and reagents used in this study were of analytical grade. α-Amylase (EC 3.2.1.1), α-glucosidase (EC 3.2.1.20), acetylcholinesterase (AChE; EC 3.1.1.7), soluble starch, p-nitrophenyl-α-D-glucopyranoside (pNPG), 3,5-dinitrosalicylic acid (DNS), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, acarbose, phosphate buffer salts, Tris-HCl, sodium carbonate, and other analytical-grade reagents were purchased from recognised suppliers. All aqueous solutions were prepared using deionised water.

2.2 Procurement and Germination of Millet Grains

Pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), little millet (*Panicum sumatrense*), and barnyard millet (*Echinochloa frumentacea*) were procured from a certified local supplier in Tamil Nadu, India. Foreign material and damaged grains were removed manually before washing thoroughly with distilled water.

The cleaned grains were soaked overnight in distilled water at room temperature using a grain-to-water ratio of 1:3. After soaking, excess water was drained and the hydrated grains were spread uniformly on sterile trays lined with moist muslin cloth. Germination was carried out under controlled laboratory conditions for predetermined durations according to the experimental design, with moisture maintained by periodic spraying of sterile distilled water. Following germination, the sprouts were dried in a hot-air oven at 60°C to constant weight, milled using a laboratory grinder, sieved through a 60-mesh sieve, and stored in airtight polyethylene containers at 4°C until further analysis. Similar germination procedures have been reported for cereal grains by Hung et al. (2020) and Wu et al. (2023).

2.3 Proximate Nutritional Analysis

The proximate composition of germinated millet flour was determined according to the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC, 2019). Total carbohydrate content was estimated using the standard AOAC procedure. Reducing sugars were quantified by the dinitrosalicylic acid (DNS) colorimetric method of Miller (1959). Protein content was determined using the Bradford dye-binding assay with bovine serum albumin as the reference standard (Bradford, 1976). Total dietary fibre was estimated using the enzymatic-gravimetric method (AOAC Method 991.43; AOAC, 2019). All analyses were performed in triplicate, and results are expressed as grams per 100 g dry weight (mean ± standard deviation).

2.4 *Invitro* α-Amylase Inhibitory Assay

Inhibitory activity of germinated millet extracts against α-amylase was determined using the dinitrosalicylic acid (DNS) colorimetric assay originally described by Bernfeld (1955), with minor modifications based on the procedure of Nair et al. (2013). Briefly, different concentrations of millet extract (100–500 µg mL^{-1}) were incubated with α-amylase solution (1 mg mL^{-1}) prepared in phosphate buffer (pH 7.4) at 37 °C for 10 min. The reaction was initiated by adding 1% soluble starch solution and incubated for a further 10 min under identical conditions. DNS reagent was then added to terminate the reaction, and the mixture was heated before cooling to room temperature. Absorbance was measured at 540 nm using a microplate reader [Make: "BIO-RAD" & Model: IMARK]. Acarbose served as the positive control, and reaction mixtures lacking enzyme were used as blanks. Percentage inhibition was calculated using Equation (1), and IC_{50} values were determined by nonlinear regression analysis.

2.5 *Invitro* α-Glucosidase Inhibitory Assay

α-Glucosidase inhibitory activity was determined following the method of Kim et al. (2005), with slight modification. Millet extracts (100–500 µg mL^{-1}) were incubated with yeast α-glucosidase in 50 mM phosphate buffer (pH 6.8) at 37 °C for 5 min, and the reaction was initiated by adding pNPG as substrate. Following incubation, 50 mM sodium carbonate was added to terminate the reaction, and the liberated p-nitrophenol was quantified at 410 nm. Acarbose was used as the positive control, and reagent blanks without enzyme were included to correct for background absorbance. Percentage inhibition and IC_{50} values were calculated as described previously (Kim et al., 2005; Nair et al., 2013).

2.6 Acetylcholinesterase Inhibitory Assay

Acetylcholinesterase inhibitory activity was evaluated using the Ellman colorimetric method (Ellman et al., 1961), with modifications described by Pohanka et al. (2011). Millet extracts (100–500 µg mL^{-1}) were mixed with acetylcholinesterase solution (0.5 mg mL^{-1}), DTNB (3 mM), and Tris-HCl buffer (pH 8.0). Following incubation at 37 °C for 10 min, acetylthiocholine iodide (15 mM) was added to initiate the reaction, and formation of 5-thio-2-nitrobenzoate was monitored at 412 nm using a microplate spectrophotometer. Control reactions lacking enzyme were included to account for non-enzymatic hydrolysis. Percentage inhibition and IC_{50} values were calculated according to Ellman et al. (1961).

2.7 Determination of IC_{50} Values

Dose–response curves were generated by plotting percentage inhibition against the logarithm of inhibitor concentration. IC_{50} values were estimated using nonlinear regression (four-parameter logistic model) in GraphPad Prism version, following the recommendations of Motulsky and Christopoulos (2004); this approach provides greater statistical reliability than linear interpolation for estimating half-maximal inhibitory concentrations.

2.8 Enzyme Kinetic Analysis

The inhibition kinetics of α-amylase, α-glucosidase, and acetylcholinesterase were investigated according to Michaelis–Menten enzyme kinetics (Michaelis & Menten, 1913). Initial reaction velocities were determined at varying substrate concentrations in the presence and absence of millet extracts. Lineweaver–Burk double-reciprocal plots (Lineweaver & Burk, 1934) were constructed by plotting 1/[S] against 1/V. The apparent Michaelis constant (Km) and maximum reaction velocity (Vmax) were calculated by linear regression, and the mode of inhibition (competitive or non-competitive) was interpreted from changes in these kinetic parameters following the criteria of Segel (1993).

2.9 Optimization of Germination Conditions

The effects of soaking time and germination duration on enzyme inhibitory activity were optimised using Response Surface Methodology (RSM) implemented in Design-Expert® version 13 (Stat-Ease Inc., Minneapolis, MN, USA). A Central Composite Design (CCD) was used to investigate the interactive effects of the independent variables. Experimental responses were fitted to a second-order polynomial model, and analysis of variance (ANOVA) was performed to evaluate model significance. Model adequacy was assessed using the coefficient of determination (R^2), adjusted R^2, predicted R^2, the lack-of-fit test, and the desirability function, following the methodology of Box and Wilson (1951) and Montgomery (2019).

2.10 Statistical Analysis

All experiments were performed using three independent biological replicates, with each analytical measurement conducted in triplicate. Results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics version 29.0. Differences among treatment groups were evaluated by one-way ANOVA followed by Tukey's post hoc test at $p < 0.05$ (Montgomery, 2019). Pearson's correlation analysis was used to determine relationships among nutritional parameters and enzyme inhibitory activities. IC_{50} values were estimated by nonlinear regression in GraphPad Prism (Motulsky & Christopoulos, 2004).

3. RESULTS AND DISCUSSION

3.1 Preliminary Analysis

The proximate composition of the germinated millet samples is presented in Table 1. Considerable variation was observed among the four species with respect to reducing sugars, carbohydrates, protein, and dietary fibre, reflecting species-specific responses to germination. Pearl millet showed the highest protein (12.5 ± 1.02 g/100 g) and reducing sugar content (5.3 ± 0.05 g/100 g), consistent with enhanced protein mobilisation and carbohydrate hydrolysis during germination, while proso millet had the highest carbohydrate content (65.2 ± 0.57 g/100 g) and little millet the highest dietary fibre content (7.6 ± 0.02 g/100 g); barnyard millet showed intermediate values across all parameters.

Table 1. Comparative proximate composition of germinated pearl, proso, little, and barnyard millets

Millet Type	Reducing sugar (g)	Carbohydrate (g)	Protein (g)	Fibre (g)
Pearl Millet	5.3 ± 0.05	57.6 ± 1.15	12.5 ± 1.02	2.1 ± 1.19
Proso Millet	2.8 ± 0.6	65.2 ± 0.57	5.6 ± 0.5	3.9 ± 0.08
Little Millet	4.6 ± 0.8	50.3 ± 0.9	9.4 ± 0.5	7.6 ± 0.02
Barnyard Millet	3.7 ± 0.1	61.2 ± 0.5	6.6 ± 0.01	6.5 ± 0.1

These results indicate that germination retained the underlying nutritional diversity among the species while producing distinct compositional profiles for each. Germination activates endogenous hydrolytic enzymes — amylases, proteases, phytases, and cellulases — that mobilise reserve macromolecules for embryo growth: starch is hydrolysed to soluble sugars, storage proteins are partially degraded to peptides and amino acids, phytate-bound minerals become more bioavailable, and cell-wall breakdown releases bound phenolics, together improving nutritional quality and functional potential (Balyatanda et al., 2024; Jacob et al., 2024; Sadh et al., 2024). The higher protein content in pearl millet likely reflects greater mobilisation of storage protein, whereas the higher fibre content in little millet suggests better retention of structural polysaccharides despite metabolic activation; comparable patterns have been reported for finger, foxtail, and proso millet, although the extent of enhancement depends on genotype, soaking conditions, germination duration, and drying method (Balyatanda et al., 2024; Rajkumar et al., 2024). γ-Aminobutyric acid, total phenolics, flavonoids, antioxidant activity, and mineral bioavailability are also known to increase with germination but were outside the scope of the present proximate analysis and warrant further study to link compositional change with functional outcome (Jacob et al., 2024; Sadh et al., 2024).

3.2 α-Amylase Inhibitory Activity

The α-amylase inhibitory activity of the germinated millet extracts is summarised in Table 2 and varied considerably among species. Little millet showed the lowest, and therefore most potent, IC_{50} (4.95 ± 0.20 μg mL^{-1}), followed by pearl millet (19.37 ± 0.50 μg mL^{-1}), barnyard millet (29.33 ± 0.40 μg mL^{-1}), and proso millet (50.26 ± 0.10 μg mL^{-1}); the reference inhibitor acarbose gave an IC_{50} of 70.72 ± 0.50 μg mL^{-1}. All four extracts therefore inhibited α-amylase more strongly than acarbose across the concentrations tested, and percentage inhibition likewise varied by species across the concentration range examined.

Table 2. α-Amylase inhibitory activity of germinated millet extracts at different concentrations

Millet Type	Concentration (μg/mL)	% Inhibition	IC_{50} Value
Pearl Millet	100	83.67	66.66 ± 0.3
	200	74.24	44.75 ± 0.2
	300	60.23	28.6 ± 0.6
	400	55.48	26.93 ± 0.9
	500	35.86	19.37 ± 0.5
Proso Millet	100	85.63	68.47 ± 1.1
	200	80.45	64.97 ± 0.2

	300	76.58	60.11 ± 0.6
	400	70.24	55.23 ± 1.3
	500	65.32	50.26 ± 0.1
Little Millet	100	74.26	44.53 ± 0.8
	200	55.62	25.75 ± 1.2
	300	36.89	20.72 ± 0.5
	400	20.16	6.741 ± 0.4
	500	8.25	4.95 ± 0.2
Barnyard Millet	100	80.56	64.0 ± 1.2
	200	74.63	59.77 ± 0.5
	300	71.56	54.09 ± 1.1
	400	76.59	40.13 ± 1.3
	500	56.23	29.33 ± 0.4
Standard	100	93.64	90.21 ± 0.8
	200	90.63	83.32 ± 0.7
	300	88.54	81.75 ± 0.1
	400	70.56	75.75 ± 0.2
	500	68.42	70.72 ± 0.5

Differences in inhibitory response among species probably reflect differences in phytochemical composition and the extent of biochemical change during germination. Germination is known to increase the concentration and bioaccessibility of phenolic acids, flavonoids, and other antioxidant metabolites through enzymatic breakdown of cell-wall complexes and conversion of bound phenolics to free forms; these compounds can inhibit α-amylase by interacting with residues around the catalytic site through hydrogen bonding, hydrophobic interactions, and π–π stacking (Bhujle et al., 2025; Jacob et al., 2024). Because total phenolic content, flavonoid concentration, and individual phytochemicals were not quantified in this study, the specific compounds responsible for inhibition cannot be identified, and the inhibitory activity observed should be interpreted as a cumulative extract effect rather than evidence for any single bioactive molecule; HPLC, LC–MS/MS, and molecular docking studies would help link phytochemical composition to enzyme inhibition more directly in future work (Bhujle et al., 2025; Jenipher et al., 2024).

3.3 α-Glucosidase Inhibitory Activity

Inhibitory activity of the germinated millet extracts against α-glucosidase is presented in Table 3, with IC_{50} values ranging from 18.37 to 64.72 μg mL^{-1} across species and percentage inhibition also varying across the concentration range tested.

Table 3. α-Glucosidase inhibitory activity of germinated millet extracts

Millet Type	Concentration (μg/mL)	% Inhibition	IC_{50} Value
Pearl Millet	100	73.87	76.66 ± 0.03
	200	64.64	54.75 ± 0.2
	300	58.83	38.6 ± 0.06
	400	55.98	26.93 ± 0.09
	500	45.36	18.37 ± 0.57

Proso Millet	100	75.93	78.47 ± 1.1
	200	70.54	74.97 ± 1.02
	300	66.54	50.11 ± 0.06
	400	60.64	45.23 ± 0.3
	500	55.21	40.26 ± 1.1
Little Millet	100	74.06	74.23 ± 1.08
	200	65.20	65.75 ± 0.2
	300	56.89	50.24 ± 0.05
	400	40.65	48.41 ± 0.04
	500	38.05	44.75 ± 0.9
Barnyard Millet	100	70.96	64.0 ± 0.28
	200	64.37	54.77 ± 1.5
	300	61.60	49.09 ± 0.15
	400	56.91	40.65 ± 0.3
	500	50.03	37.33 ± 0.9
Standard	100	83.04	91.81 ± 1.08
	200	80.93	87.32 ± 1.7
	300	78.55	80.65 ± 0.01
	400	70.05	70.75 ± 0.12
	500	68.28	64.72 ± 1.05

Since α-glucosidase catalyses the terminal step of carbohydrate digestion, its modulation is a widely explored nutritional strategy for reducing postprandial glucose excursions. Germination is reported to increase the release of hydroxycinnamic acids, hydroxybenzoic acids, flavonoids, and related phenolics through enzymatic breakdown of the grain matrix, and compounds such as ferulic, p-coumaric, caffeic, and vanillic acid, together with quercetin and kaempferol derivatives, have all been implicated in interactions with digestive enzymes (Sadh et al., 2024; Zhang et al., 2022). The present findings suggest that germinated millet extracts contain constituents capable of interacting with α-glucosidase, but this remains limited to *in vitro* evidence; biological efficacy after dietary consumption depends on digestion, intestinal absorption, metabolism, gut microbiota interactions, and systemic bioavailability, none of which were assessed here, so these results should be regarded as preliminary support for further mechanistic and preclinical study rather than direct evidence of antidiabetic efficacy (Bhujle et al., 2025; Jacob et al., 2024).

3.4 Acetylcholinesterase Inhibitory Activity

Acetylcholinesterase (AChE) inhibitory activity is summarised in Table 4. All germinated millet extracts showed measurable inhibition, with variation among species in both percentage inhibition and IC_{50}.

Table 4. Acetylcholinesterase inhibitory activity of germinated millet extracts.

Millet Type	Concentration (µg/mL)	% Inhibition	IC_{50} Value
Pearl Millet	100	63.70	61.66 ± 0.35
	200	54.42	52.75 ± 0.02
	300	48.03	48.06 ± 1.16
	400	45.88	36.30 ± 0.1
	500	30.36	28.97 ± 1.70
Proso Millet	100	66.10	68.70 ± 0.1

	200	61.03	64.63 ± 0.26
	300	66.24	50.01 ± 1.36
	400	52.68	40.38 ± 04
	500	47.12	30.98 ± 1.01
Little Millet	100	64.98	78.25 ± 0.05
	200	55.89	64.85 ± 1.02
	300	53.78	60.94 ± 1.85
	400	47.64	56.41 ± 0.5
	500	36.89	45.05 ± 0.19
Barnyard Millet	100	60.60	74.0 ± 1.2
	200	54.07	62.87 ± 0.6
	300	50.98	57.98 ± 1.05
	400	47.27	50.58 ± 1.39
	500	40.85	42.26 ± 0.79
Standard	100	73.40	81.01 ± 0.8
	200	70.63	77.92 ± 0.07
	300	68.05	80.65 ± 1.18
	400	60.59	72.58 ± 1.02
	500	58.28	63.02 ± 0.05

Natural dietary polyphenols have drawn increasing attention because several plant-derived compound classes interact with AChE through multiple binding mechanisms while also showing antioxidant and anti-inflammatory activity. Millets contain diverse phenolic acids, flavonoids, lignans, and phytosterols that may contribute collectively to this inhibition, and germination further increases the availability of these compounds by activating metabolic pathways and releasing bound phenolics from the cell wall (Jacob et al., 2024; Sadh et al., 2024). This activity should nonetheless be interpreted cautiously: the present design did not assess blood–brain barrier permeability, neuronal uptake, pharmacokinetics, toxicity, or cognitive performance, so the findings indicate only that germinated millet extracts contain constituents capable of interacting with AChE *in vitro* and should not be read as evidence of neuroprotective efficacy.

3.5 Enzyme Kinetic Analysis

Kinetic parameters derived from Lineweaver–Burk plots are presented in Tables 5–7. For α-amylase and α-glucosidase, kinetic analysis indicated competitive inhibition, reflected in an increased apparent Km with little change in Vmax, whereas AChE inhibition was consistent with a non-competitive mechanism, showing reduced Vmax and comparatively smaller change in Km.

Table 5. Michaelis–Menten plots for α-amylase inhibition

Sample	Vmax	Km	R^2 Value	Type of Inhibition
Pearl Millet	1.3271	1.2433	0.9711	Competitive Inhibition
Proso Millet	1.6620	2.6620	0.9964	Competitive Inhibition
Little Millet	1.6595	2.6595	0.9763	Competitive Inhibition

Barnyard Millet	1.7890	3.0658	0.9464	Competitive Inhibition

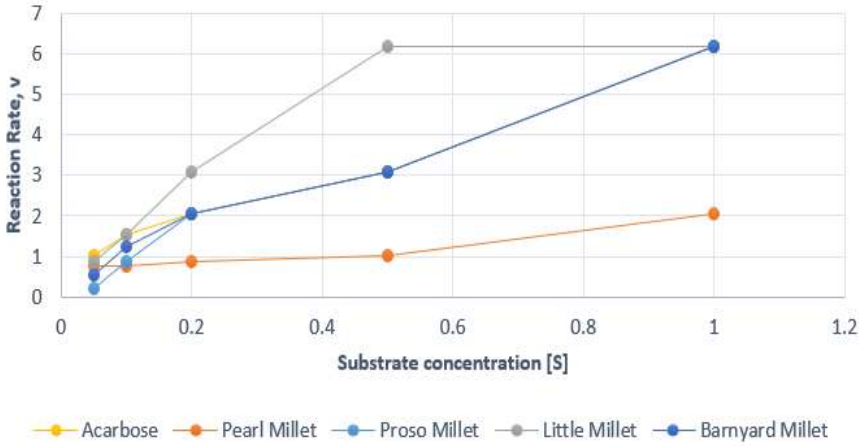


Figure 1. Lineweaver–Burk plots illustrating α-amylase inhibition

Table 6. Kinetic studies of the millets for α-glucosidase inhibition

Sample	Vmax	Km	R^2 Value	Type of Inhibition
Pearl Millet	1.6452	2.1938	0.9764	Competitive Inhibition
Proso Millet	1.6728	3.7528	0.9932	Competitive Inhibition
Little Millet	1.9587	9.099	0.9789	Competitive Inhibition
Barnyard Millet	2.9730	19.5470	0.9647	Competitive Inhibition

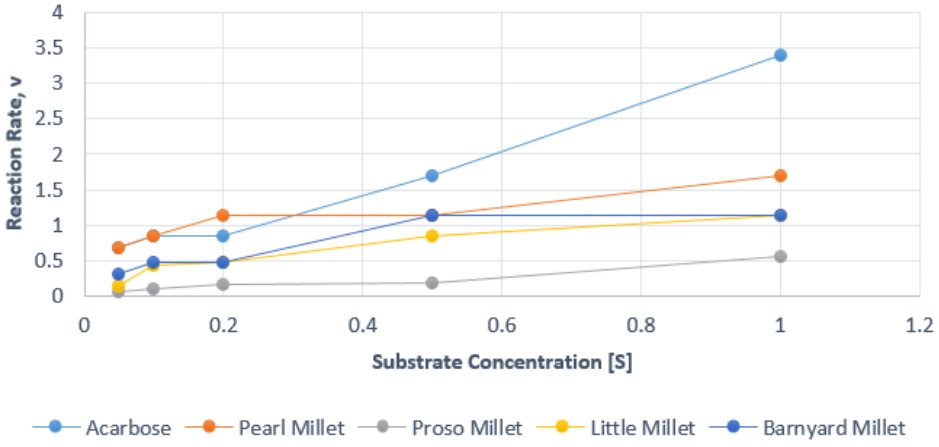


Figure 2. Lineweaver–Burk plots illustrating α-glucosidase inhibition

Table 7. Kinetic studies of the millets for α-glucosidase inhibition

Sample	Vmax	Km	R^2 Value	Type of Inhibition
Pearl Millet	9.6487	2.8796	0.9856	Non-Competitive Inhibition
Proso Millet	5.6970	2.2358	0.9920	Non-Competitive Inhibition
Little Millet	3.5874	2.0872	0.9745	Non-Competitive Inhibition

Barnyard Millet	2.7982	2.7057	0.9808	Non-Competitive Inhibition

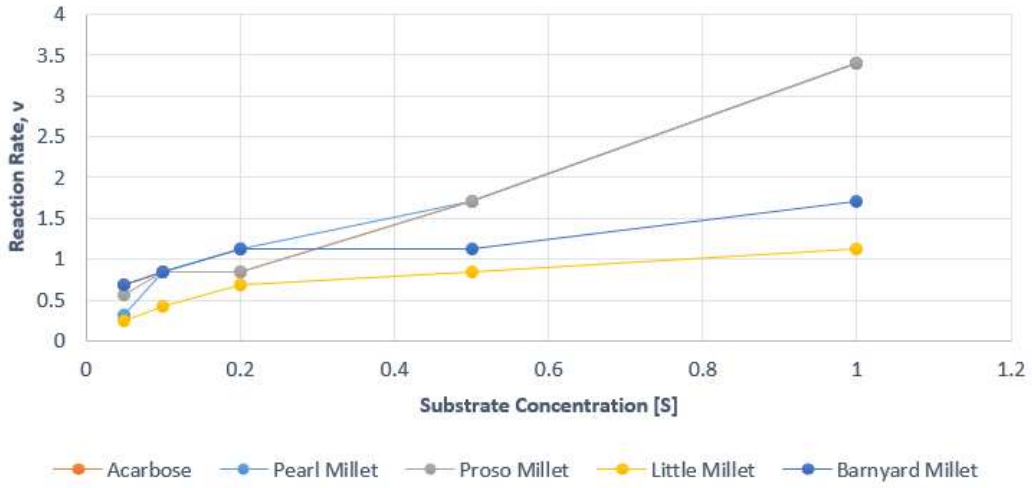
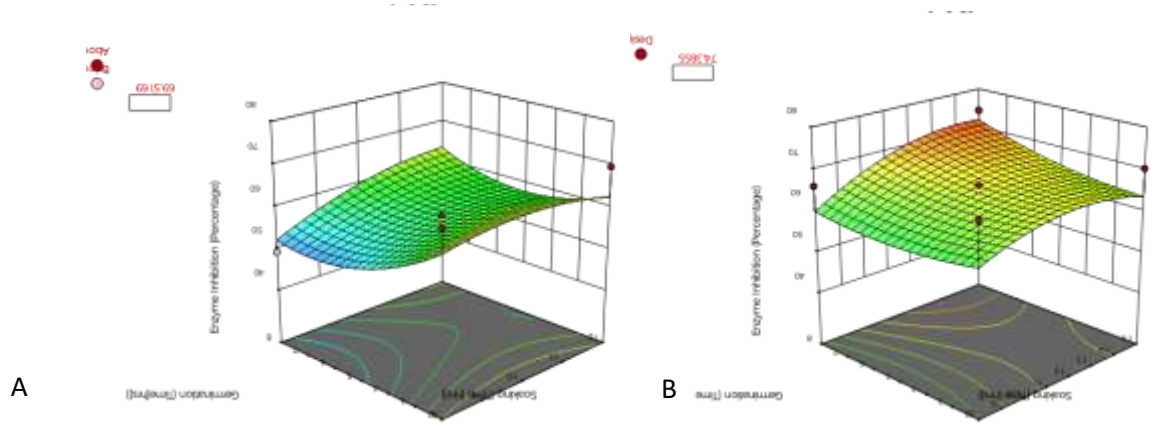


Figure 3. Lineweaver–Burk plots illustrating acetylcholinesterase inhibition.

Competitive inhibition indicates that inhibitory molecules compete with substrate for the enzyme active site, raising the apparent Km while leaving Vmax largely unaffected, whereas non-competitive inhibition reflects binding at an alternative regulatory site, lowering catalytic efficiency without substantially altering substrate affinity; similar behaviour has been reported for several plant-derived phenolics and flavonoids acting as natural enzyme inhibitors (Bhujle et al., 2025). Because Lineweaver–Burk transformations can amplify experimental error by weighting low-substrate-concentration measurements more heavily, future work should complement reciprocal-plot analysis with nonlinear Michaelis–Menten regression, molecular docking, and molecular dynamics simulation to strengthen the mechanistic evidence.

3.6 Optimization of Germination Conditions

Response Surface Methodology was used to optimise soaking and germination conditions, with the resulting three-dimensional response surfaces shown in Figure 10. The optimisation model identified 12 h of soaking followed by 6 h of germination as the conditions that maximised enzyme inhibitory activity while maintaining favourable nutritional characteristics.



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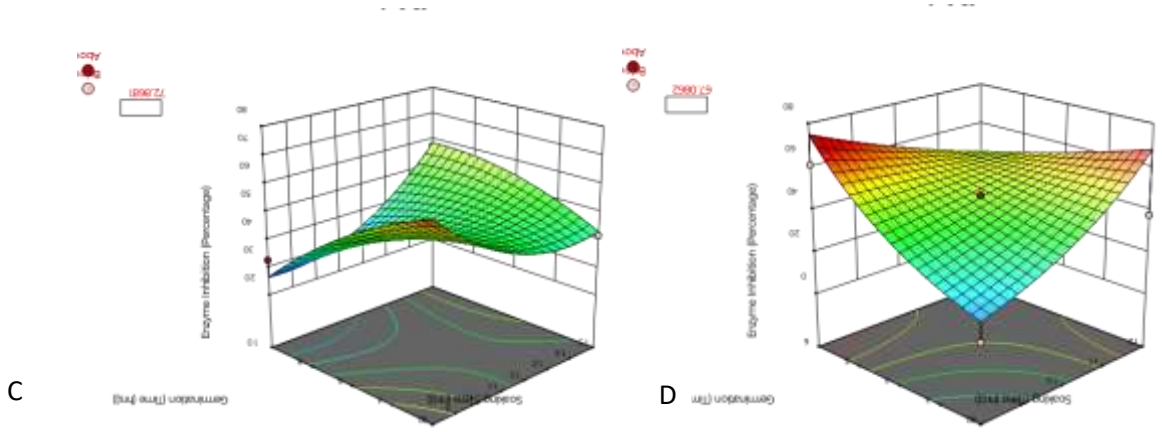

Figure 4. Three-dimensional response surface plots showing the effect of soaking and germination time on enzyme inhibitory activity - Barnyard (A), Little (B), Pearl (C), Proso (D)

Germination conditions strongly influence moisture uptake, enzymatic activation, metabolic flux, and the synthesis or release of bioactive compounds, and optimisation is important because excessive germination can degrade phenolic compounds and deplete storage nutrients, while insufficient germination may fail to activate the enzymatic pathways needed for nutritional enhancement (Balyatanda et al., 2024; Bhujle et al., 2025). The present model offers a useful framework for standardising germination conditions, but it evaluated only soaking time and germination duration; variables such as temperature, relative humidity, drying conditions, extraction solvent, and particle size should be incorporated into future optimisation work aimed at industrial-scale production.

3.7 Correlation Analysis

Pearson correlation analysis showed positive relationships among α-amylase, α-glucosidase, and acetylcholinesterase inhibitory activities.

Table 8. Pearson correlation heatmap showing relationships among enzyme inhibitory activities

Pearson coefficient	α - amylase	α - glucosidase	Acetylcholinesterase
α - amylase	1	0.90004	0.97349
α - glucosidase	0.90004	1	0.92803
Acetylcholinesterase	0.97349	0.92803	1

The positive correlations among the three inhibition assays suggest that common classes of bioactive constituents may contribute to more than one biological activity; correlation does not, however, establish causation, and further phytochemical characterisation is needed to identify the compounds responsible for these relationships.

4. CONCLUSION

Germination improved the nutritional quality and *in vitro* enzyme inhibitory properties of the four millet species examined here, with clear species-specific differences: pearl millet showed the highest protein and reducing sugar content, little millet the highest dietary fibre content and the strongest α-amylase inhibition, and all species showed measurable α-glucosidase and acetylcholinesterase inhibitory activity. Kinetic analysis indicated competitive inhibition of α-amylase and α-glucosidase and non-competitive inhibition of acetylcholinesterase, and Response Surface Methodology identified 12 h of soaking followed by 6 h of germination as the optimum processing condition for maximising enzyme inhibitory activity. Together, these findings support the potential of germinated millets as ingredients for functional cereal-based foods targeting glycaemic regulation. The biological activities reported here are, however, based exclusively on *in vitro* assays; further phytochemical characterisation together with animal and clinical studies will be needed to establish their physiological relevance and any health benefit before therapeutic or nutraceutical claims can be made.

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AUTHOR CONTRIBUTIONS

Sathviga S: Methodology, Laboratory Analysis.

Anbarasi P: Conceptualization, Investigation.

Shyamala Gowri S: Data Curation, Formal Analysis.

Vaidhyanathan B: Conceptualization, Investigation.

Shanmuga Priya B: Data Curation, Formal Analysis.

Pavithra M K S: Supervision, Writing—Review & Editing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

Ethical approval was not required for this study.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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