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ORA- Experimental Study

Phytochemical Characterization and *In vitro* Evaluation of the Antioxidant, Anti-inflammatory, and Antidiabetic Activities of *Pennisetum glaucum* (Pearl Millet) Ethanolic Seed Extract

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ABSTRACT:

Background: *Pennisetum glaucum* is a rich source of nutrients that is valued due to the presence of various phytochemicals with health promoting effects. Nevertheless, a complete study involving phytochemical characterization together with several *in vitro* biological activities remains scarce. **Objective:** In this study, we conducted a characterization of phytochemical constituents in the ethanolic seed extract of *Pennisetum glaucum* as well as its antioxidant, anti-inflammatory and antidiabetic enzyme inhibitory activities. Methods: Ethanolic seed extract was extracted via Soxhlet extraction method. Phytochemical analysis of the plant extract was carried out by Fourier Transform Infrared (FTIR) spectroscopy and Gas Chromatography Mass Spectrometry (GC-MS). Anti-inflammatory activity was studied in terms of bovine serum albumin denaturation assay, egg albumin denaturation and human red blood cells membrane stabilization assay. Antioxidant activity was studied using DPPH, hydrogen peroxide, FRAP, ABTS and nitric oxide scavenging assays. Antidiabetic activity was measured in terms of α-amylase and α-glucosidase inhibitions. One-way ANOVA was used for data analysis and differences were considered to be statistically significant at $P < 0.05$. **Results:** Along with several bioactive phytoconstituents discovered by GC-MS analysis, the FTIR study identified particular functional groups including hydroxyl, carbonyl, aromatic, and aliphatic that are connected to phenolic and flavonoid compounds. 16.2 µg/mL (BSA), 10.0 µg/mL (egg albumin), 7.5 µg/mL (membrane stabilization), 5.5 µg/mL (DPPH), 10.8 µg/mL ($H2O_2$), 5.8 µg/mL (ABTS), 6.2 µg/mL (nitric oxide), 11.7 µg/mL (α-amylase) inhibitory activity. **Conclusion:** The results indicated that the ethanolic extract of P. glaucum seeds possesses concentration-dependent antioxidant, anti-inflammatory, and antidiabetic enzyme inhibitory activity *in vitro* experiments. These results may serve as the basis for further quantitative phytochemical and in vivo studies.

KEYWORDS: Antidiabetic activity, Anti-inflammatory activity, Antioxidant activity, FTIR, GC-MS, Pearl millet, *Pennisetum glaucum*.

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1. INTRODUCTION

Functional foods have become popular in the last few years because of the benefits that they offer to consumers beyond those offered by regular foods. Prolonged disorders such as diabetes mellitus, obesity, heart diseases, and inflammation have made people seek alternatives in the natural diet so that they are able to maintain optimal health status and prevent disease. [1] Cereal grains have been selected to be rich sources of healthy foods since they are packed with nutrients, low glycemic index, and bioactive plant compounds. One type of millet is pearl millet, which is also referred to as bajra. [2]

There is much to say about pearl millet because it is widely known that this plant has the ability to tolerate extreme growing conditions and it possesses extraordinary nutritive properties. The seed of pearl millet contains carbohydrates, proteins, fibers, amino acids, vitamins, and various minerals such as iron, magnesium, zinc, and phosphorus. [3] One needs to pay attention to the fact that pearl millet contains more minerals and fibers than any other common cereal. Pearl millet is the nourishing food which can become an indispensable element of your daily ration. [4]

Apart from the nutrients, pearl millet contains a lot of phytochemicals contributing to its therapeutic qualities. Some of the phytochemicals found in pearl millet include tannins, phenolic acids, phytosterols, flavonoids, alkaloids, terpenoids, and other types of antioxidants. [5,6] All these phytochemicals have been found to show such therapeutic effects as being antioxidants, anti-inflammatory, antimicrobial, anti-cancerous, and antidiabetic. [7] Phenolic compounds and flavonoids, in particular, are known for their ability to remove ROS and lower oxidative stress, thus preventing harm to cellular structures. It is clear that oxidative stress is among the main reasons for the development of various diseases; therefore, pearl millet is beneficial. [8]

Another key factor related with the pathogenesis of metabolic and degenerative diseases includes inflammation. It may be stated that the bioactive compounds present in pearl millet may exert a possible impact on inflammation through the decrease in pro-inflammatory agents as well as the prevention of oxidative destruction. [9] Also, it should be noted according to the results obtained recently, phytochemicals contained in food can influence carbohydrate metabolism by reducing the potential of such enzymes as α-glucosidase and α-amylase. [10]

Phytochemical detection and identification are central aspects of the assessment of their medicinal value. Modern techniques such as GC-MS and FTIR have been found to be very useful in phytochemical analysis. The GC-MS technique aids in detecting volatile and semi-volatile compounds, whereas FTIR provides data regarding the functional groups present in phytochemicals. This renders the techniques relevant in carrying out phytochemical studies. [11] Whereas some studies have focused on the nutritional composition and some biological activities of *Pennisetum glaucum*, very few studies incorporating phytochemical analysis along with biological activity have been conducted on this plant. It may be further added that very few studies have employed FTIR and GC-MS analysis in conjunction with antioxidant, anti-inflammatory, and carbohydrate hydrolyzing enzyme inhibitory activity of the ethanolic seed extract. The present investigation is therefore an attempt to conduct an integrated phytochemical and biological analysis of this plant species.

The above reasoning led us to the hypothesis that the ethanolic seed extract of *Pennisetum glaucum* has bioactive phytochemicals that can be determined through FTIR and GC–MS analysis, which could have antioxidant, anti-inflammatory, and α-amylase and α-glucosidase inhibition properties when tested under *in vitro* conditions.

In light of the above, the current study was designed to determine the phytochemical composition of the ethanolic seed extract of *Pennisetum glaucum* by FTIR and GC–MS analysis and to determine its *in vitro* antioxidant, anti-inflammatory, and enzyme inhibition activities against diabetes.

2. MATERIALS AND METHODS

Collection and Extraction of Pearl Millet Extract: *Pennisetum glaucum* seeds were collected from local millet stores in Chennai, Tamil Nadu, India. Taxonomic identification of the plant material was done by the Department of Plant Biology and Biotechnology, SDNB Vaishnav College for Women, Chromepet, Chennai, India (Identification Number: SDNB/PBPB/AP/2026/0015). Taxonomic classification of the plant was determined to be *Pennisetum glaucum* (L.) R.Br., Prodr. Fl. Nov. Holland.1:159 (1810). The seeds were washed, dried in shade at room temperature, and ground to make a fine powder using laboratory grinding machine. About 100 grams of the powdered seeds were extracted with 95% ethanol in a Soxhlet apparatus for 8 hours.

The choice of ethanol as the solvent is based on its intermediate polarity that makes possible the extraction of different bioactive plant-derived compounds such as phenolic acids, flavonoids, terpenoids, phytosterols and fatty acids. Moreover, the solvent in question is considered safe, environmentally friendly and can be regarded as food grade, which makes it convenient for the extraction of *Pennisetum glaucum* with further analysis by FTIR and GC-MS. The extract was passed through Whatman No. 1 filter paper and concentrated under reduced pressure at 40°C using rotary vacuum evaporator. The concentrate was dried to constant weight resulting in 12.4 g of dried extract with extraction yield of 12.4% (w/w). The dried extract was kept in sterile airtight amber-colored glass bottles at 4°C until the phytochemical studies and *in vitro* biological tests were performed. All experiments were done with the same extraction batch for the sake of experimental consistency. Batch-to-batch reproducibility was not tested and is one of the drawbacks of the current study.

Qualitative Phytochemical Analysis: Preliminary qualitative phytochemical analysis of the ethanolic seed extract of *Pennisetum glaucum* was done using standard phytochemical analysis technique to determine the presence of different types of phytochemical constituents. For qualitative determination, the extract was tested for the presence of alkaloids (by Mayer's test & Wagner's test), flavonoids (alkaline reagent test), phenolic compounds and tannins (Ferric chloride test), saponins (Foam test), terpenoids (Salkowski test), steroids/phytosteroids (Liebermann-Burchard test), glycosides (Keller-Killiani test) and carbohydrates (Molisch's test). Appearance of characteristic color or precipitates indicated the presence of different phytochemical constituents. [12]

FTIR Spectroscopic Analysis: Analysis of the functional groups of the ethanolic extract was performed using the Fourier Transform Infrared (FTIR) Spectrometry technique. Dried samples of the ethanolic extracts were combined with spectroscopic grade potassium bromide (KBr), after which pellets were made out of them. The spectral data was obtained using the FTIR spectrophotometer at wavenumber ranging from 4000 – 400 cm^{-1}. [13]

Identification of Phytochemical Composites by GC-MS Technique: The phytochemicals in the ethanolic seed extract of *Pennisetum glaucum* were analyzed by PerkinElmer GC 2400 Gas Chromatograph together with PerkinElmer MS 2400 QT Mass Spectrometer. The chemical compounds were separated through the 5-MS capillary column (5% phenyl–95% dimethylpolysiloxane; 30 m × 0.25 mm i.e., 0.25 µm film thickness). High-grade helium (99.999%) served as the carrier gas at a constant flow of 1.0 mL/min. An injection volume of 1 µL was applied through split injection method (10:1) at an injection temperature of 250°C.

The temperature of the oven was set to be between 50°C and 250°C at the rate of 10°C/min. The mass spectrometry analyses were conducted in EI mode at 70 eV. The ion source temperature, quadrupole temperature, and transfer line temperature were controlled at 250°C, 150°C, and 280°C, respectively. The mass spectrum was measured in full scan mode in m/z range of 40–500 with a solvent delay of 4 min. The compounds detected in the oil were tentatively identified using comparison of their mass spectra with those from the NIST Mass Spectral Library. The

identification of the compounds can be regarded as tentative since retention indices and authentic standards were not utilized in this study. [14]

In Vitro Anti-inflammatory Screening

Controls Used in the Assays: Appropriate controls were included in all experiments. Ascorbic acid was used as the positive control in the antioxidant assay, diclofenac sodium served as the positive control in the anti-inflammatory assays, and acarbose was used as the positive control in the α-amylase and α-glucosidase inhibitory assays. Negative controls consisted of reaction mixtures containing all assay components except the test extract or standard drug. Reagent blanks containing the respective reagents without extract or standard were included to correct for background absorbance. Where applicable, the solvent used for extract preparation served as the vehicle control and was tested under identical experimental conditions.

BSA Denaturation Test: The anti-inflammatory effect of the crude extract was studied by means of the denaturation assay of bovine serum albumin. In brief, 0.5 ml of 1% BSA solution and 0.5 ml of extracts at different concentrations (20-100 µg/ml) were placed in a 5ml test tube. The solution was buffered to a pH value of 6.3 by 1N HCl. This reaction mixture was then maintained (for 20 min) at 37ºC and then heated to 57ºC for 3 min. Absorbance values were determined with a UV-Vis Spectrophotometer after allowing the reaction mixture to cool at 660 nm wavelength. Diclofenac Sodium was employed as the control. The percentage denaturation of protein inhibition was determined via:

Inhibition (%)=$A_c - A_t / A_c \times 100$

A_c (Absorbance of the control)

A_t (Absorbance of the test/sample)

Egg Albumin Denaturation Test: Egg albumin denaturation assay was done by mixing 0.2 ml of fresh egg albumin, 2.8 ml of phosphate buffer solution (pH 6.4), and 2 ml of solution containing different concentrations of ethanoic extract was prepared and maintained at 37°C (for 15 min). Further, this test solution was incubated at 70°C for 5 min. Finally, the OD values were taken after cooling at 660 nm. The denaturation inhibition percentage of protein was estimated. The positive control was diclofenac sodium. Appropriate reagent blanks were prepared for absorbance correction. Absorbance was determined at 660 nm, and percentage inhibition was determined with respect to the control.

Membrane Stabilization Assay

Ethical Considerations: Human red blood cells (HRBCs) used in the membrane stabilization assay were obtained from residual blood samples remaining after completion of an Institutional Ethics Committee-approved study (IEC Ref. No. 01804/2024/IECSMCH). No additional blood samples were collected specifically for the present investigation. The residual samples were anonymized prior to laboratory use, and no participant-identifiable information was accessed.

HRBC Membrane Stabilization test was employed to test the anti-inflammatory effect. HRBCs were collected by drawing blood in heparinized tubes and subjected to centrifugation for ten minutes at 3000 rpm. Isotonic saline was utilized for washing the packed cells which were then re-suspended to make 10% v/v suspension. The test was performed using 1 mL phosphate buffer, 2 mL hypo tonic solution of saline, 0.5 mL of HRBC and extracts of different concentrations. After incubation for 30 minutes at 37°C. The test solutions were centrifuged at 3000rpm. Sodium diclofenac is chosen as the standard drug. [15] The standard anti-inflammatory drug was diclofenac sodium. Reagent blank was prepared with phosphate buffered saline without extract. The hemoglobin release was taken at 560 nm, and percentage inhibition was determined with respect to the control.

Antioxidant Activity In vitro

DPPH Assay: The in vitro antioxidant activity was assessed by DPPH radical scavenging method. Various concentrations of extract were mixed with 0.1 mM DPPH solution and incubated for 30 min in the dark at room temperature. Measurement of absorbance was done at 517 nm. Ascorbic acid was taken as the standard antioxidant. The positive

control was taken as ascorbic acid, while reaction mixture devoid of the extract was taken as negative control. Ethanol along with the reagent DPPH without sample was used as a blank. Absorbance was measured at 517 nm with the help of a UV-Visible spectrophotometer, and percentage of radical scavenging activity was calculated in comparison with the control.

H_2O_2 Scavenging Test: H_2O_2 (Hydrogen Peroxide) scavenging test was performed by combining solutions of extracts with 40 mM H_2O_2 which was dissolved in PBS with pH 7.4. Measurement of absorbance was done after 10 minutes at a wavelength of 230 nm compared with blank solution. Ascorbic acid acted as the control. . Ascorbic acid was used as the positive control, and reagent blank devoid of extract was used for background correction. Absorbance was read at 230 nm, and percentage scavenging activity was calculated in comparison with the control.

Ferric Reducing Antioxidant Power (FRAP) Test: Acetate buffer, TPTZ solution, and ferric chloride were mixed to prepare the FRAP solution. Extracts were combined with fresh FRAP solution then kept for thirty minutes at 37°C. OD was determined at 593 nm wavelength. Higher absorbance meant stronger reducing power of the test substance. Ascorbic acid was used as the reference standard, and a reagent blank was used before measuring absorbance at 593 nm.

ABTS Radical Scavenging Activity Assay: The ABTS radicals were formed by addition ABTS stock solution to potassium persulfate and kept in dark for overnight. To determine ABTS radical scavenging effect, the stock was diluted to OD of 0.70 ± 0.02 at 734 nm. Then, the extracts at different concentration were mixed with test solution and OD was noted after 5 minutes at 734 nm. The results were compared using Ascorbic acid as positive control. The reference standard was taken as ascorbic acid, and appropriate reagent blank was included for background correction. Absorbance was measured at 734 nm, and percentage inhibition was calculated in comparison with the control.

Nitric Oxide Radical Inhibitory Activity Assay: Nitric oxide scavenging activity was measured through the addition of sodium nitroprusside. The mixture of sodium nitroprusside with the extract was incubated at 28°C (for 150 minutes). The Griess reagent was then added to the mixture followed by the measurement of absorbance at 546 nm. [16] The positive control was taken as ascorbic acid, and appropriate blanks were used to eliminate background absorbance. Absorbance was read at 546 nm, and percentage inhibition was calculated in comparison with the control.

Determination of In vitro Antidiabetic effects

α-Amylase Inhibition Test: Evaluation of antidiabetic property in vitro was performed using the inhibitory activity on α-amylase enzymes. An aqueous pearl millet extract was added to varying concentrations of α-amylase enzyme solutions and maintained at 37°C for 10 min. Further, a 1% starch solution was mixed and incubated at the same temperature for 15 more minutes. The enzymatic activity was quenched with DNSA reagent and kept in a water bath at 100°C for 5 min.

The positive control was acarbose. Reagent blank was prepared with all reagents except enzyme. The absorbance was determined at 540 nm, and percentage inhibition was determined with respect to the control.

Inhibition Percentage was calculated as below.

Inhibition (%)=$A_c - A_t / A_c \times 100$

A_c (Absorbance of the control)

A_t (Absorbance of the test/sample)

α-Glucosidase Inhibition Assay: The inhibitory effects of ethanolic extract on α-glucosidase enzymes was investigated. The substrate used in this test was p-nitrophenyl-α-D-glucopyranoside (pNPG). Pearl millet extract in different concentrations was mixed with α-glucosidase enzyme solution and kept at 37°C (for 15 minutes). Further, the substrate pNPG was mixed and the solution was incubated for another 20 minutes at 37°C. The reaction was quenched using sodium carbonate and OD was taken at 405 nm. Acarbose was used as Positive control. [17-19]

Inhibition proportion was calculated by the formula:

Inhibition (%)=$A_c - A_t / A_c \times 100$

A_c (Absorbance of the control)

A_t (Absorbance of the test/sample)

Statistical Analyses: All experiments were performed in triplicate, and the results are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism software (Version 9.0; GraphPad Software, San Diego, CA, USA). Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post hoc test. A p-value < 0.05 was considered statistically significant. IC_{50} values were determined by plotting concentration-response data and fitting the data using a nonlinear regression (dose-response) model in GraphPad Prism. The concentration required to achieve 50% inhibition (IC_{50}) was calculated from the fitted curve.

3. RESULTS

Phytochemical Characterization of *Pennisetum glaucum* Ethanolic Seed Extract: The qualitative phytochemical screening of ethanolic extracts from seeds of *Pennisetum glaucum* showed the presence of various active phytochemical components such as alkaloids, flavonoids, phenols, tannins, terpenoids, steroids/phytosterols, saponins, glycosides, and carbohydrates (Table 1). The above observations show the chemical diversity of the extract and suggest their biological activities.

Table 1: Preliminary qualitative phytochemical screening of ethanolic seed extract of *Pennisetum glaucum*.

Phytochemical Constituent	Result
Alkaloids	Present (+)
Flavonoids	Present (+)
Phenolic compounds	Present (+)
Tannins	Present (+)
Terpenoids	Present (+)
Steroids/Phytosterols	Present (+)
Saponins	Present (+)
Glycosides	Present (+)
Carbohydrates	Present (+)

FTIR-Based Functional Group Characterization: The FTIR spectroscopy of the ethanolic extract of *Pennisetum glaucum* showed that several bioactive functional groups related to phytochemicals with therapeutic significance were present (Figure 1). The wide band at 3200 and 3400 cm^{-1} is due to the O-H bond vibration, which means the presence of phenols and alcohols. The vibration of aliphatic C-H bond is seen in the region between 2920 and 2850 cm^{-1}, and this could be a result of the fatty acids and their by-products. The carbonyl bond of esters, ketones, and aldehydes is seen in the region between 1700 and 1740 cm^{-1}. The aromatic vibration at 1600 cm^{-1} is due to the aromatic C-C bond, but the vibrations between 1200 and 1000 cm^{-1} are C-O and C-N bonds in carbohydrates, alcohols, and amines.

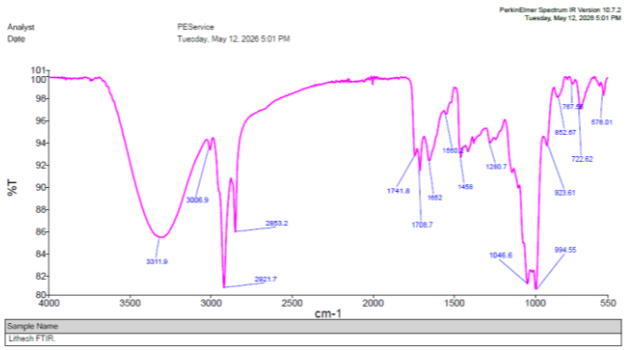

Figure 1: FTIR spectrum of ethanolic extract of *Pennisetum glaucum* showing characteristic functional groups of bioactive phytoconstituents.

GC–MS Identification of Bioactive Phytoconstituents: Phytochemicals were observed in the pearl millet extracts through GC-MS, and these revealed the chemical diversity of the extracts (Table 2). There were major peaks within the range of retention times 9.73 and 28.01 minutes. Isopropyl myristate was found to be the predominant constituent in the extract at a retention time of 17.97 minutes, while β-sitosterol was second at a retention time of 28.01 minutes. Other compounds present included Neophytadiene at a retention time of 18.12 minutes; tert-Hexadecanethiol at a retention time of 24.97 minutes; 1,2-benzenedicarboxylic acid diundecyl ester at a retention time of 19.34 minutes; and 2-hexadecen-1-ol at a retention time of 18.57 minutes.

Additional chemicals include 1,2-Propanediamine, 1-Iodo-2-methylnonane, and 2-Formyl-9-[α-D-ribofuranosyl] hypoxanthine (Figure 2). The identification of phytosterols, terpenoids, fatty acid esters, and nitrogen compounds indicates that pearl millet may have great pharmacological potential, especially concerning antioxidant, anti-inflammatory, and antidiabetic uses. The chemicals characterized in this study through GC-MS were tentatively characterized through a comparison of their mass spectra to those contained in the National Institute of Standards and Technology (NIST) mass spectral library. The chemicals thus identified should therefore be viewed as tentative since no analysis of retention indices and authentic standards was undertaken in the current study.

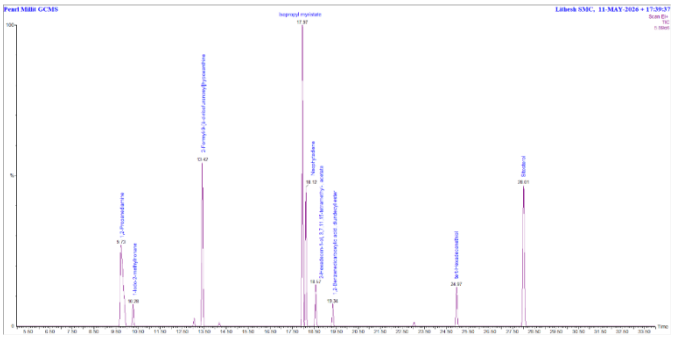

Figure 2: GC-MS Chromatogram of ethanolic extract of *Pennisetum glaucum* depicting the phytochemical constituents identified.

Table 2: Phytoconstituents that have been bioactively identified in *Pennisetum glaucum* extract via GC-MS analysis.

S. No.	Retention Time (min)	Identified Compound	Probable Chemical Class	Reported Biological Activities
1	9.73	1,2-Propanediamine	Diamine compound	Antioxidant, antimicrobial activity
2	10.28	1-Iodo-2-methylnonane	Halogenated hydrocarbon	Bioactive hydrocarbon derivative
3	13.42	2-Formyl-9-(β-D-ribofuranosyl) hypoxanthine	Nucleoside derivative	Antioxidant and metabolic regulatory activity
4	17.97	Isopropyl myristate	Fatty acid ester	Anti-inflammatory, skin permeation enhancer, antioxidant potential
5	18.12	Neophytadiene	Diterpene hydrocarbon	Anti-inflammatory, antioxidant, antimicrobial activity
6	18.57	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, acetate	Terpenoid ester	Antioxidant and anti-inflammatory activity
7	19.34	1,2-Benzenedicarboxylic acid, diundecyl ester	Phthalate ester derivative	Antimicrobial and antioxidant properties
8	24.97	tert-Hexadecanethiol	Sulfur-containing compound	Antioxidant and protective biological activities
9	28.01	Phytosterol	Plant sterol	Antidiabetic, anti-inflammatory, antioxidant, hypocholesterolemic activity

Anti-inflammatory Activity

Bovine Serum Albumin (BSA) Denaturation Test

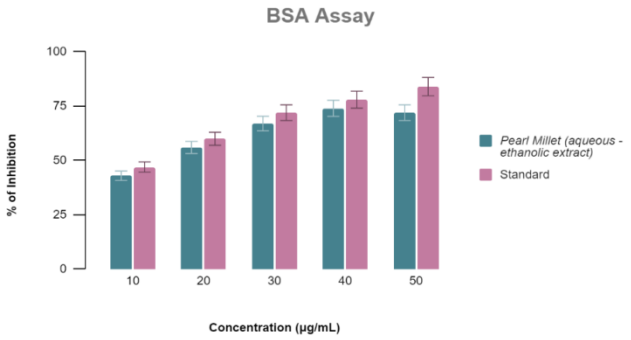

Figure 3. Inhibition of bovine serum albumin (BSA) denaturation by the ethanolic extract of *Pennisetum glaucum* compared with diclofenac sodium. Values are expressed as mean ± SD (n = 3). The extract exhibited concentration-dependent inhibition of protein denaturation, whereas diclofenac sodium showed comparatively greater inhibitory activity. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

The test extract was observed with protein denaturation inhibition in a concentration basis in the BSA test system

(Figure 3). The percentage of inhibition increased from 10 to 50 µg/mL, which proves that an rise in concentration led to an higher inhibitory activity towards inflammation. The highest inhibitory activity was at 50 µg/mL, which proved the efficiency of the extract in protein denaturation inhibition. Although the inhibition rate was slightly lower compared to diclofenac sodium – standard anti-inflammatory drug – it still proved good anti-inflammatory activity. The estimated IC_{50} values of the ethanolic extract and diclofenac sodium were 16.2 µg/mL and 12.9 µg/mL, respectively.

Egg Albumin Denaturation Test

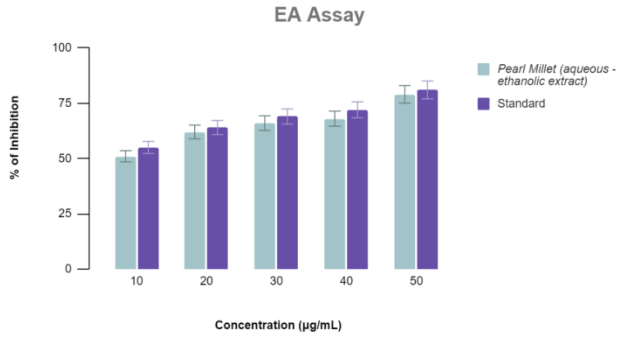

Figure 4: Inhibition of egg albumin denaturation by the ethanolic extract of *Pennisetum glaucum* compared with diclofenac sodium. Values are expressed as mean ± SD (n = 3). Both the extract and diclofenac sodium demonstrated *in vitro* concentration-dependent inhibition of protein denaturation, with diclofenac sodium exhibiting comparatively greater activity. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (P < 0.05).

It was found out that pearl millet extract inhibits egg albumin protein denaturation in a measure dependent way (Figure 4). The higher amount of pearl millet extract caused a greater inhibition of egg albumin denaturation. As the highest inhibitory effect was observed at the dose of 50 µg/mL, it could be said that there were bioactive substances in pearl millet extract, which inhibited inflammatory reactions. The estimated IC_{50} values of the ethanolic extract and diclofenac sodium were 10.0 µg/mL and 9.0 µg/mL, respectively.

Membrane Stabilization Assay

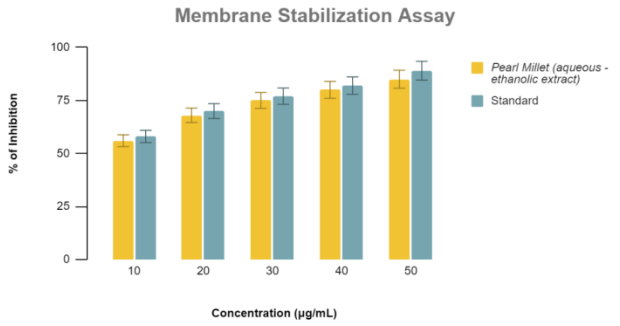

Figure 5: Human red blood cell (HRBC) membrane stabilization activity of the ethanolic extract of *Pennisetum glaucum* compared with diclofenac sodium. Values are expressed as mean ± SD (n = 3). Membrane stabilization activity increased with increasing concentration of the extract and was comparable to the standard at higher concentrations. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (P < 0.05).

In the assay for membrane stabilization, it was seen that pearl millet extract had a high protective effect on human erythrocytes against hemolysis (Figure 5). The percentage inhibition of lysis increased in proportion to the concentration of the extract applied. The sample demonstrated a good *in vitro* membrane stabilizing activity due to the existence of phytosteroids and derivatives of fatty acid.

Antioxidant Activity

DPPH Radical Scavenging Assay

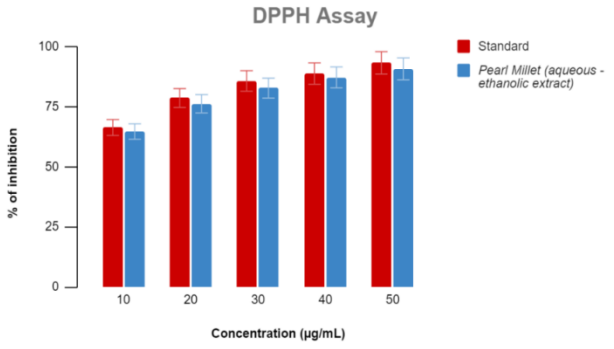

Figure 6: DPPH radical scavenging activity of the ethanolic extract of *Pennisetum glaucum* compared with ascorbic acid. Values are expressed as mean ± SD (n = 3). The radical scavenging activity increased in a concentration-dependent manner, with ascorbic acid exhibiting comparatively higher activity than the extract at the tested concentrations. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison

test, and differences were considered statistically significant at P < 0.05.

The pearl millet extract demonstrated *in vitro* scavenging properties of DPPH free radicals, based on the concentration of extract used. The scavenging effect was increase with increasing concentration, with the peak scavenging effect being noted at the highest concentration used. This indicated the ability of the phytoconstituents in the pearl millet to donate hydrogen atoms (Figure 6). The estimated IC_{50} values of the ethanolic extract and ascorbic acid were 5.5 µg/mL and 5.0 µg/mL, respectively.

H_2O_2 Scavenging Assay

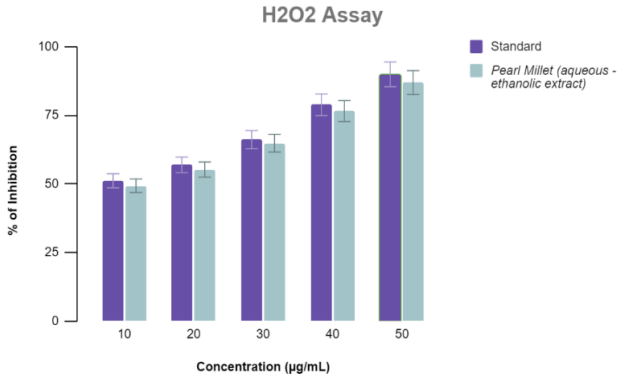

Figure 7: Hydrogen peroxide scavenging activity of the ethanolic extract of *Pennisetum glaucum* compared with ascorbic acid. Values are expressed as mean ± SD (n = 3). Both the extract and the standard exhibited concentration-dependent scavenging activity, with ascorbic acid showing comparatively greater activity. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (P < 0.05).

There was indication of efficient scavenging effects of H_2O_2 free radicals by the extract. Scavenging was concentration-dependent, and thus the pearl millet phytochemicals possess the potential to counteract reactive oxygen species (ROS), which in turn lowers oxidative stress (Figure 7). The maximal scavenging effect was witnessed at 50 µg/mL . The estimated IC_{50} values of the ethanolic extract and ascorbic acid were 10.8 µg/mL and 9.0 µg/mL, respectively.

Ferric Reducing Antioxidant Power (FRAP) Assay

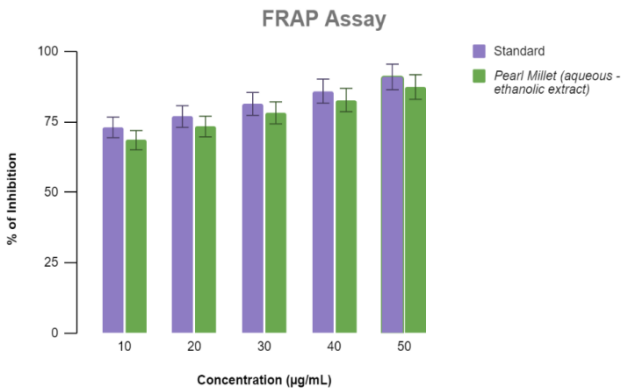

Figure 8: Ferric reducing antioxidant power (FRAP) of the ethanolic extract of *Pennisetum glaucum* compared with ascorbic acid. Values are expressed as mean ± SD (n = 3). The reducing power increased with increasing concentration, while the standard exhibited comparatively higher reducing activity than the extract. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (P < 0.05).

The Pearl Millet Extract demonstrated a significant *in vitro* ferric ion reduction capability. It was perceived that there was a increase in the OD values in a concentration dependent manner (Figure 8). This implies that there is the presence of electron donors that can reduce the ferric ions into ferrous ions thereby acting as antioxidants. The estimated IC_{50} values of the ethanolic extract and ascorbic acid in the FRAP assay were 6.8 µg/mL and 5.8 µg/mL, respectively.

ABTS Radical Scavenging Assay

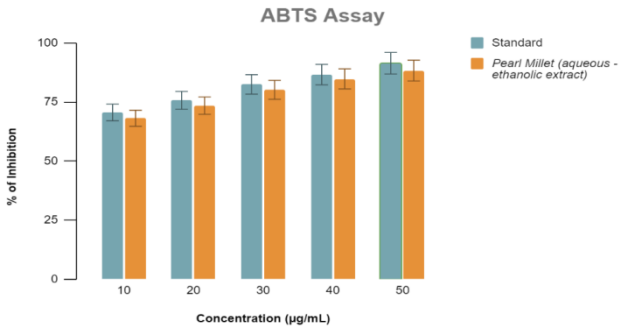

Figure 9: ABTS radical scavenging activity of the ethanolic extract of *Pennisetum glaucum* compared with ascorbic acid. Values are expressed as mean ± SD (n = 3). The extract demonstrated in vitro concentration-dependent ABTS radical scavenging activity, whereas ascorbic acid exhibited comparatively greater scavenging activity across the tested concentrations. Statistical

analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (P < 0.05).

This assay showed that Pearl Millet Extract exhibited high radical scavenging efficiency. Percentage inhibition increased dose-dependently. Highest radical scavenging efficiency was achieved at 50 µg/mL concentration (Figure 9). The estimated IC_{50} values of the ethanolic extract and ascorbic acid in the FRAP assay were 6.8 µg/mL and 5.8 µg/mL, respectively.

Nitric Oxide Radical Inhibition test

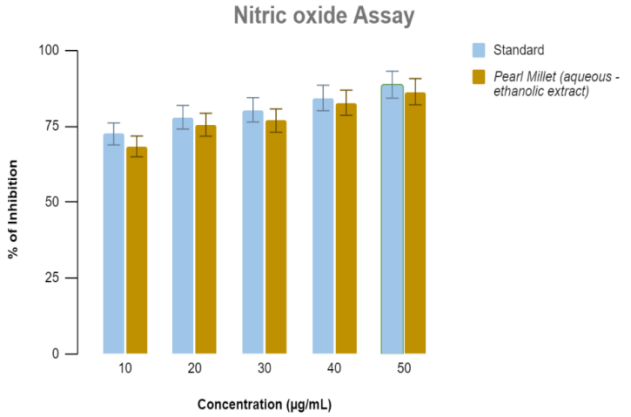

Figure 10: Nitric oxide scavenging activity of the ethanolic extract of *Pennisetum glaucum* compared with ascorbic acid. Values are expressed as mean ± SD (n = 3).

Nitric oxide scavenging activity increased with increasing concentration of the extract, while the standard showed comparatively higher activity. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (P < 0.05).It was evident that Pearl Millet Extract has good inhibitory efficiency on nitric oxide radicals. Percentage inhibition was dose-dependent. It implied that Pearl Millet Phytochemicals possess the ability to inhibit reactive nitrogen species and inflammation oxidative pathway activities (Figure 10). The estimated IC_{50} values of the ethanolic extract and ascorbic acid were 6.2 µg/mL and 5.0 µg/mL, respectively

Antidiabetic Effectiveness

Alpha Amylase Inhibitory effect

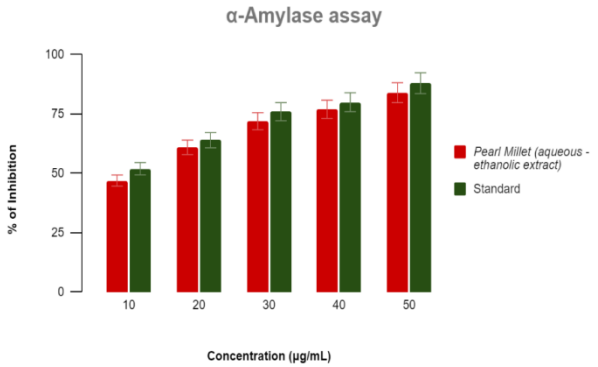

Figure 11: α-Amylase inhibitory activity of the ethanolic extract of *Pennisetum glaucum* compared with acarbose. Values are expressed as mean ± SD (n = 3). The extract exhibited concentration-dependent enzyme inhibition, while acarbose demonstrated comparatively greater inhibitory activity across the tested concentrations in this in vitro assay. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (P < 0.05).

Pearl millet extract had significant alpha amylase inhibitory value, based on concentration. The percentage inhibition kept increasing with an increase in the concentration, meaning that there was successful inhibition of starch digestion (Figure 11). Maximum inhibition concentration was attained at 50 µg/mL concentration, but lower than the efficacy of the drug acarbose used as a standard. This shows the invitro activity of pearl millet in controlling postprandial blood glucose levels. The estimated IC_{50} values of the ethanolic extract and acarbose were 11.7 µg/mL and 8.5 µg/mL, respectively.

Alpha Glucosidase Inhibitory effect

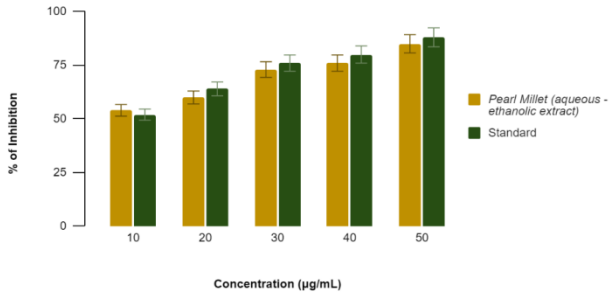

Figure 12: α-Glucosidase inhibitory activity of the ethanolic extract of *Pennisetum glaucum* compared with acarbose. Values are expressed as mean ± SD (n = 3). Both the extract and acarbose demonstrated concentration-dependent inhibition, with the extract exhibiting comparable inhibitory activity under

the experimental conditions in the current *invitro* assay. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

There was significant alpha glucosidase inhibitory activity. An increase in enzyme inhibition based on concentration was observed, with maximum inhibition achieved at 50 µg/mL concentration. Inhibition of alpha glucosidase enzyme will delay metabolism and absorption of glucose (Figure 12). The estimated IC_{50} values of the ethanolic extract and acarbose were 7.0 µg/mL and 9.5 µg/mL, respectively.

4. DISCUSSION

This work investigates the chemical composition and biological effects of *Pennisetum glaucum* (Pearl Millet) using FTIR, GC-MS, anti-inflammatory, antioxidant, and antidiabetic assays. It is determined that pearl millet contains a wide array of biologically active components that have the capacity to generate a range of positive biological effects via a complicated molecular mechanism. [19]

The FTIR results indicate the occurrence of hydroxyl, carbonyl, aromatic, and aliphatic functional groups, meaning that the existence of phenolic acids, flavonoids, fatty acid derivatives, alcohols, and esters is possible. These above functional groups play a vital role in free radical elimination, enzyme inhibition, and cellular protection. [20] Hydroxyl groups of phenolics have the capacity to donate hydrogen or an electron to ROS and destroy them. In such a way, they prevent the cells from oxidative stress. Carbonyl and aromatic functional groups are the indication of phytochemicals with redox-regulatory activities that are essential for the conservation of cellular homeostasis. [21] The extensive absorption peak obtained at 3300–3400 cm^{-1} is indicative of O–H stretching vibrations from phenols and alcohol molecules, while the absorption peak obtained at 2920 cm^{-1} is indicative of C–H stretching vibrations from aliphatic hydrocarbon molecules. The absorption peak at 1700 cm^{-1} is indicative of C=O stretching vibrations from carbonyls, while the absorption peaks obtained at 1000 to 1200 cm^{-1} are indicative of C–O stretching vibrations from carbohydrates and phenolic molecules. The spectrum features noted are consistent with previous findings on FTIR spectra of *Pennisetum glaucum* and other cereal grains, indicating the presence of various phytochemical molecules in the ethanolic seed extract. [2,3]A number of bioactive substances such as β-sitosterol, neophytadiene, isopropyl myristate, tert-hexadecanethiol, and other fatty acid ester derivatives have been detected by GC-MS method. Out of the above-mentioned substances, β-sitosterol is the most extensively investigated phytosterol characterized by different biological processes including antioxidative, anti-inflammatory, hypoglycemic, and lipolytic effects. [22-24] Previous studies suggest that β-sitosterol may suppress NF-κB-mediated inflammatory signaling; however, this mechanism was not investigated in the present study. [25-27] Moreover, β-sitosterol enhances the effect of insulin due to the instigation of the PI3K/Akt pathway that ensures glucose absorption. Therefore, the presence of β-sitosterol may partially contribute to the observed biological activities; however, this association remains hypothetical because the present study evaluated a crude extract and did not isolate or experimentally validate the activity of individual compounds [28] In the current study, however, chemical characterization was done primarily through comparison to those contained in the NIST mass spectral library. Retention index analysis and authentic standard analysis were not within the scope of the current study. The chemicals thus identified can only be considered as tentative and need validation in future studies.

The presence of phytosterols, terpenoids, fatty acid esters, and other plant compounds might be responsible for their antioxidant, anti-inflammatory, and enzyme-inhibitory properties to some extent, since these groups of substances were earlier shown to possess such actions. However, these associations still need further confirmation through experiments. Because this particular *invitro* study tested only an ethanolic extract of the plant, the biological properties demonstrated are most probably due to the combined action of various phytochemicals present in the

extract. Individual compounds like β-sitosterol and neophytadiene cannot be credited for any specific biological activity unless they are separated and tested individually.

From the anti-inflammatory assay results, pearl millet extract demonstrated the capability to hinder protein denaturation and membrane stabilization of red blood cells. It is important to note that protein denaturation is one of the common conditions that arise because of inflammation due to alteration of proteins that initiate an immune response resulting in tissue damage. [19] The inhibition of protein denaturation by pearl millet extract is thought to be as a consequence of phytoconstituent properties that are responsible for the prevention of protein oxidation. [29] Another property of pearl millet extract is its membrane stability, implying its ability to protect membranes of lysosomes. The function of lysosomes in the production of hydrolytic enzymes increases inflammation; therefore, the protection of cellular membranes is possible. [30]

The generation of oxidative stress is an essential factor to the emergence of chronic inflammatory conditions, diabetes, neurodegeneration and cardiovascular diseases. [31] All the antioxidant tests performed in this experiment indicated a similar pattern that had a concentration-based effect on radical scavenging. When it comes to the DPPH and ABTS assays, such methods evaluate the capacity of substances to reduce free radicals by donating an electron or transferring hydrogen atoms. In the FRAP test, however, the reducing power is assessed in terms of ferric ion reduction. The substantial influence exhibited by the tests suggests that there are various antioxidants in pearl millet with distinct mechanisms of action. Neophytadiene and β-sitosterol might boost the effect of the antioxidant enzyme defense system by upregulating SOD, CAT, and GPx levels. At the same time, phenolic compounds could help neutralize ROS and inhibit oxidation. [32]

Pearl millet anti-diabetic activity was reached due to the preventing action of enzymes responsible for carbohydrate breakdown. The activity of these enzymes is connected with the transformation of carbohydrate macromolecules into glucose molecules that can easily be absorbed in the intestine. This leads to the retardation of carbohydrate metabolism and their transformation into glucose, which results in influencing the level of glycemia after meals. [33] It is possible to stop the function of enzymes due to the ability of phenolic compounds to attach to the functional site of the enzyme by means of hydrophobic interaction and hydrogen bonds with the amino acids of the catalytic site. In addition, it is known that β-sitosterol is able to enhance insulin sensitivity through glucose transporter regulation.

One interesting observation from the ongoing experiment includes the relationship among antioxidants, anti-inflammatories, and antidiabetics. This is because chronic hyperglycemia results in the generation of increased levels of ROS, which in turn trigger inflammatory responses that cause insulin resistance. Pearl millet's bioactives act through reducing inflammation by lowering oxidative stress, hence breaking the process. This infers that the biological effects of pearl millet may not result from individual bioactives, but from their synergy.

On the whole, the current study showed that *Pennisetum glaucum* extract had different phytochemical components determined by FTIR and GC-MS techniques and exhibited concentration-dependent antioxidant, anti-inflammatory, and α-amylase/α-glucosidase inhibitory activities through in vitro experimentations. The above result is the initial scientific support for the biological significance of *Pennisetum glaucum*. Nonetheless, since the present study has been confined to the in vitro experiments performed on a crude extract, the biological activities noted in the current study should be treated cautiously. Future studies must concentrate on quantitative phytochemical composition, isolating compounds, fractionation guided by bioactivities, and other aspects. The observed activities are based on in vitro experimental models and should not be interpreted as evidence of therapeutic efficacy. Further cellular, animal, and clinical studies are required to validate the biological effects of the extract. Furthermore, although GC-MS

analysis identified several phytochemical constituents, their individual contributions to the observed biological activities were not experimentally evaluated, and therefore any association should be considered preliminary.

Limitations: The present study should be considered a preliminary investigation of the phytochemical profile and biological potential of *Pennisetum glaucum*. Although taxonomic identification of the plant was performed, a herbarium voucher specimen was not prepared and deposited. The phytochemical assessment was primarily qualitative and quantitative estimation of total phenolic content, total flavonoid content, and other major phytoconstituents was not undertaken. Similarly, GC–MS analysis provided tentative identification of phytochemical constituents based on comparison with the NIST mass spectral library; retention indices and authentic reference standards were not used for further confirmation. Detailed GC–MS chromatographic data, including peak area percentages and compound abundance, were also not presented. In addition, comprehensive quality-control assessment of the plant material for heavy metals, pesticides, aflatoxins, and microbial contamination was not performed.

The biological evaluation was conducted as an initial in vitro assessment, and the observed antioxidant, anti-inflammatory, and enzyme-inhibitory activities should not be interpreted as evidence of therapeutic efficacy under physiological conditions. The study did not include cellular or in vivo investigations to confirm the biological effects observed in the experimental models. Furthermore, factors such as bioavailability, metabolism, pharmacokinetic behavior, toxicity, safety profile, and clinical effectiveness were not evaluated. Formal evaluation of normality and homogeneity of variance, together with comprehensive reporting of statistical parameters, can be incorporated in future investigations. Furthermore, the relationship between individual phytochemical constituents and the observed biological activities was not evaluated in the present preliminary study. Although GC–MS analysis identified several phytochemical constituents, their individual contributions to the observed biological activities were not experimentally evaluated, and therefore any association should be considered preliminary. Furthermore, only a single batch of the ethanolic extract was evaluated in the present study; therefore, batch-to-batch phytochemical and biological variability could not be assessed. In addition, comprehensive physicochemical quality-control parameters and extract standardization measures required to establish identity, purity, and reproducibility were not evaluated in the present study.

Future studies will therefore focus on quantitative phytochemical characterization, preparation and deposition of authenticated voucher specimens, retention-index- and/or authentic-standard-based confirmation of GC–MS compounds, comprehensive chromatographic data reporting, rigorous quality-control assessment, independent biological replication, detailed statistical analysis, phytochemical–activity correlation studies, and validation using cellular, animal, and clinical models. These investigations will provide a more comprehensive understanding of the biological potential and possible therapeutic relevance of *P. glaucum*.

5. CONCLUSION

The ethanolic seed extract of *Pennisetum glaucum* exhibited notable in vitro antioxidant, anti-inflammatory, and enzyme-inhibitory activities. The extract demonstrated concentration-dependent α-amylase and α-glucosidase inhibitory activities, along with antioxidant activity and anti-inflammatory effects in protein denaturation and HRBC membrane stabilization assays. GC–MS and FTIR analyses indicated the presence of diverse phytochemical constituents that may contribute to the observed biological activities. However, these findings are based solely on in vitro experimental models and should not be interpreted as evidence of therapeutic efficacy. Further studies involving detailed phytochemical characterization, cellular investigations, animal experiments, and clinical validation

are required to confirm the biological potential of the extract.

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Reviewing & editing: AP, SR, MP

Approval of final manuscript: All authors

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Ethical Statement: This study did not involve human participants or experimental animals; therefore, Institutional Ethics Committee approval and informed consent were not applicable.

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