



Effect of lipids and phytosterols from *Pennisetum glaucum* grains on anti-inflammation: An *in Silico*, *In Vitro* and *In Vivo* Studies

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Abstract: The current research is engrossed on screening anti-inflammatory activity of methanolic grain extract of *Pennisetum glaucum* using *in silico*, *in vitro* and *in vivo* methods. *In silico* studies were carried out using docking (CB-Dock2), Ramchandran plot (procheck). *In vitro* studies were carried out using inhibition of protein denaturation, membrane stabilization activity while *in vivo* studies using carrageen induced paw edema in rodents. Docking studies were performed for natural compounds against PDB ID: **1FT4**, PDB ID: **3NS7**, PDB ID: **1KQU** and PDB ID: **3E7G**. The docking results revealed that all of the selected proteins had the greatest vina scores with all of the phytoconstituents from the extract and the common medication indomethacin, indicating a better receptor-ligand binding affinity. In *in vitro* results MEPG showed higher inhibition at 500µg/ml (46.4%) compared to aspirin 100µg/ml (60.7%) for protein denaturation. The extract demonstrated 56.6% suppression of heat-induced hemolysis at 500 g/ml, while aspirin demonstrated 73.3% inhibition at 100 g/ml. The extract inhibited hypotonicity-induced hemolysis by 59.3% at 500 g/ml and Diclofenac sodium 100 g/ml with 75% inhibition at 100 g/ml. The extract at doses of 200 mg/kg, 400 mg/kg, and the common medicine indomethacin at 3 mg/kg bd.wt significantly reduced paw edoema in an animal model.

It is clear from the above that *Pennisetum glaucum* methanolic grain extract had strong anti-inflammatory activity.

Keywords: *Pennisetum glaucum*, docking studies (CB-Dock2), Ramchandran plot (procheck), anti-inflammatory.



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INTRODUCTION

Inflammation is a local response (reaction) to foreign and endogenous stimuli in a living, vascularized tissue. The Latin form of the word is "inflammare," which means "to burn." Fundamentally, inflammation serves two purposes: to contain tissue damage and to localise and eradicate the cause. The disease can be classified as acute or chronic, depending on the host's immune capacity and response time (Mohan H, 2015). The cytotoxic actions of carrageenan cause the inflammation to progress while stimulating phospholipase A2. The cyclooxygenase pathway is activated by this model. The biphasic curve of the carrageenan-induced edema (Shelar PA et al., 2020). Carrageenan-induced paw edema is a well-known acute form of inflammation that is widely used for screening potential anti-inflammatory medications. A biphasic edema was brought on by injecting carrageenan into the subplantar surface of rat paws. The early phase, which begins around 1 hour, is associated with the release of histamine, serotonin, bradykinin, and to a lesser extent

prostaglandins produced by cyclooxygenase enzymes (COX), whereas the delayed phase (after 1 hour) is associated with neutrophil infiltration and the continuation of prostaglandin production (Mansouri MT et al., 2015).

Many prevalent illnesses and diseases are caused by inflammation. Fatty acids and phytosterols influence inflammation through a variety of mechanisms, such as cell surface and intracellular receptors/sensors that control inflammatory cell signalling and gene expression patterns. A mediator of some of the actions of fatty acids on inflammatory cells, or at least one that is connected to them, appears to be responsible for modifications to the fatty acid composition of cell membranes (Calder PC, 2011). Changes to these elements may have an impact on various processes, including the structure of fatty acids and polypeptide facilitator generation, the fluidity of the barrier, the formation of rafts of lipids, cellular communication leading to altered expression of genes, and even more.

Pennisetum glaucum commonly known as Pearl Millet (Bajra) is the poor men's staple food in India and Africa in the family *Poaceae*. The plant is found to possess several properties like antibacterial, anti-sickling, immunosuppressant, fungistat, analgesic, antiallergic, anti-inflammatory, hepatoprotective, antiallergic, arteriodilator, anti-cancer, antiseptic, antioxidant, antimalarial, hepatoprotective, anti-HIV and antihistaminic. *Pennisetum glaucum* contains fatty acids like linoleic Acid, oleic Acid, linolenic acid, arachidonic acid, stearic acid, palmitic acid and phytosterols like ergosterol, β -sitosterol, stigmasterol, and campesterol (Yuan L, 2019). Employing *in silico*, *in vitro*, and *in vivo* methodologies, and the current work sought to assess the anti-inflammatory effects of the methanolic grain extract of *Pennisetum glaucum*.

Materials and methods

Procurement of grains of *Pennisetum glaucum*

The registered millet shop in Hyderabad is where the *Pennisetum glaucum* grains were purchased. Professor and Head of Department of Botany, Government Degree College, Kukatpally, identified and verified this specimen. After being cleansed, the grains were ground into a coarse powder. The material was ground into a powder and either stored or transported for extraction.

Preparation of methanolic grain extract of *Pennisetum glaucum*

Soxhlet extraction is a persistent process of extraction that allows the extractor to repeatedly circulate the same solvent. Following extraction, the solvent is evaporated in this process. The liquid that has been condensed from the solvent's vapours is then reintroduced to the drug for ongoing extraction (Ganga RM et al., 2021).

Preliminary Phytochemical Analysis

In order to determine the different plant-based compounds found in the methanolic grain extract of *Pennisetum glaucum*, the extract was submitted to preliminary phytochemical research.

GC-MS analysis

GC-MS analysis of methanolic grain extract of *Pennisetum glaucum* was done at the Sophisticated Analytical Instrument Facility (SAIF) labs, Osmania University, Hyderabad using standard GC-MS model (Ganga RM et al., 2021).

Acute Toxicity Studies

Utilising OECD 425 recommendations, the acute toxicity studies were completed. The GRCP, Bachupally, Hyderabad, India (Reg. No. 1175/PO/ERe/S/08/CCSEA), has an animal facility that has been certified by CPCSEA for use in research.

Experimental Protocol

Wistar albino rats weighed between 200 and 250 g were purchased from Jeeva Life Sciences in Hyderabad. The animals were cared for and kept in good condition in accordance with the approved procedures of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Docking studies for molecules

Molecular docking tests were carried out to assess the intensity and mechanism of interactions between phytoconstituents and the targets acceptable to examine the link between the target proteins and ligands at the molecular level. CB-Dock (<http://cao.labshare.cn/cb-dock/>) was used to simulate the docking

process. It has the ability to recognise the binding sites of a given protein automatically, determine the centre position, alter the size of the docking box, and dock with the query ligands using the well-known docking programme (AutoDock Vina). The protein data bank (PDB) (<http://www.rcsb.org>) was used to download the 3D crystal structures of the target proteins, and Gaussian software was used to optimise the chosen phytoconstituents. After that, the phytoconstituents and the protein were submitted to CB-Dock for docking. To compare the theoretical binding affinities of phytoconstituents to the important targets, docking scores were used. According to Liu et al. (2020), the chosen phytochemical components of the extract were docked against PDB IDs 1FT4, 3NS7, 1KQU, and 3E7G.

The Ramachandran Plan

In order to assess the model's quality, a Ramachandran plot was created using the PROCHECK validation server (Ganga RM et al., 2021). This plot's allowed and prohibited regions were used to determine the model's quality.

Anti-inflammatory activity:

In vitro methods for evaluation of anti-inflammatory activity:

1. Protein denaturation inhibition:

Procedure:

The reaction mixture was made up of test extracts and a 1% bovine albumin fraction aqueous solution. To alter the pH of the mixture a slight volume of 1N hydrochloric acid was added. After 20 min at 37 °C and 20 min at 51°C, the turbidity was measured at 660 nm after the sample extracts were brought to room temperature (Ganga RM et al., 2020). The below formula was used to compute the percentage inhibition of protein denaturation:

$$\% \text{ inhibition} = (\text{Absorbance Control} - \text{Absorbance Sample}) \times 100 / \text{Absorbance control}$$

2. Membrane stabilisation:

a. Red Blood cells (RBCs) suspension:

Healthy hominid volunteers who had not taken any NSAIDs for two weeks before to the experiment's start had their blood drawn, and it was then transferred to centrifuge tubes. The tubes were cleaned three times with an equivalent volume of normal saline after being centrifuged at 3000 rpm for 10 minutes (Karim N et al., 2019). The blood's volume was determined, and it was then reconstituted in a 10% v/v solution with normal saline.

b. Heat Induced Haemolysis

The test sample for the reaction mixture (2 ml) was divided into 1 ml portions with varying concentrations (100 - 500 μ g/ml) and 1 ml portions with 10% RBC suspension. The control test tube just had saline added in place of the test sample. The medication of choice was aspirin. The reaction mixture in each centrifuge tube was incubated for 30 min at 56 °C in a water bath. After the development period was complete, the tubes were cooled under flowing water. After centrifuging the reaction mixture for five minutes at a speed of 2500 rpm, the supernatants' absorbance at 560 nm was measured (Karim N et al., 2019). All of the test samples were subjected to the experiment in triplicate.

c. Hypotonicity Induced Haemolysis

Different extract concentrations (ranging from 100 to 500 µg/ml), reference samples, and controls were each combined with 1 ml of phosphate buffer, 2 ml of hyposaline, and 0.5 ml of HRBC solution independently. The typical medication was diclofenac sodium (100µg/ml). All of the assay mixtures underwent a 30-minute incubation period at 37°C and 3000 rpm centrifugation. Using a spectrophotometer set at 560 nm, the supernatant liquid was decanted to determine how much haemoglobin was present (Karim N et al., 2019). The resulting formula was used to define the percent inhibition of hemolysis:

$$\% \text{ inhibition} = (\text{Absorbance control} - \text{Absorbance sample}) \times 100 / \text{Absorbance control}$$

$$\% \text{ protection} = 100 - (\text{OD sample} / \text{OD control}) \times 100$$

In vivo methods for evaluation of anti-inflammatory activity:

Carrageenan induced paw edema model:

Thirty healthy Albino rats of either sex weighing 200-250 gm were selected for the study. They were divided into 5 groups, each containing 6 animals (n=6). In this method, albino rats were fasted in individual cages for 24 hours. Group I received normal saline. Group II received Carrageenan, Group III received MEPG of 200 mg/kg, p.o. Group IV received MEPG of 400 mg/kg, p.o. Group V received Indomethacin (3 mg/kg, bd.wt, p.o.). Group III, IV and V received their respective drugs before 1h of carrageenan administration. Paw edema was induced by injecting 0.1 ml of 1% w/v carrageenan into sub-plantar tissues of the left hind paw of each rat. Paw thickness was measured immediately after carrageenan injection (time 0) and at 1, 2, 3, 4, 5 and 6h using vernier calliper to assess the degree of inflammation (Ganga RM et al., 2018).

% inhibition was intended by means of the resulting formula:

$$\% \text{inhibition} = \frac{(V_c - V_t) \times 100}{V_c}$$

RESULTS

The anti-inflammatory properties of *Pennisetum glaucum*'s methanolic grain extract were investigated. Below are listed all of the study's findings. The *Pennisetum glaucum* grain extract was made using the soxhlation method. The following formula was used to obtain the extract's % yield.

$$\text{Percentage yield of extract} = \frac{\text{Amount of extract}}{\text{Amount of powder}} \times 100$$

Amount of powder

$$= 30.0 / 250.0 \times 100$$

$$= 12.0\% \text{ w/w.}$$

Preliminary phytochemical analysis

Fatty acids, sterols, phenol, flavonoids, carbohydrates, proteins, tannins, alkaloids, and terpenoids were found in the *Pennisetum glaucum* methanolic grain extract, according to the preliminary phytochemical analysis.

Acute toxicity studies

Even at 2000 mg/kg body weight in methanolic grain extract of *Pennisetum glaucum*, no symptoms of toxicity or fatality were present. Even 14 days of observation revealed that all animals were secure. At 200 and 400 mg/kg bd.wt, the pharmacological evaluations were conducted.

GC-MS RESULTS

Table 1: GC-MS analysis of methanolic grain extract of *Pennisetum glaucum*

S. No.	Compound	Mol. Weight	Retention Time	Area Present	Base m/z
1	Glucose	180.56	23.56	7.12	131.2
2	Linoleic Acid	288.44	22.64	2.14	55.05
3	Linolenic Acid	278.430	24.230	0.150	42.06
4	Oleic Acid	282.470	22.88	0.190	74.10
5	Arachidonic Acid	304.47	25.12	0.18	43.05
6	Lupeol	426.72	32.71	0.16	191.0
7	Vanillic Acid	168.14	29.8	2.56	25.6
8	Palmitic Acid	256.4	21.26	0.05	43.05
9	Stearic Acid	284.48	26.16	0.17	44.31
10	Campesterol	400.68	31.37	0.82	43.05
11	Ferrulic Acid	194.18	27.5	1.98	32.4
12	Ergosterol	396.65	31.95	0.05	96.10
13	Sitosterol	414.71	32.05	14.04	396.35
14	Tocopherol	416.68	30.25	0.71	151.0

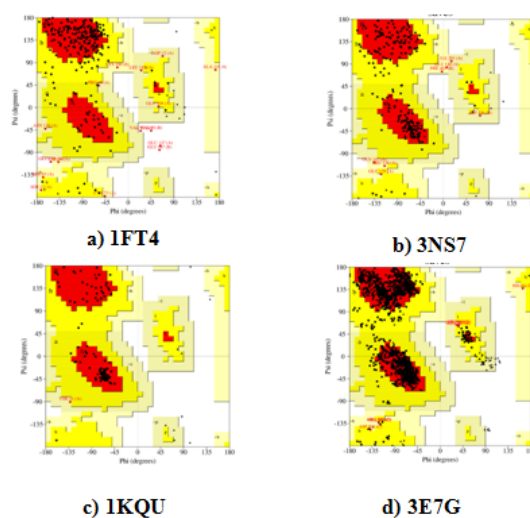
In silico analysis**Table 2: Molecular docking**

Sl no	Compounds	1FT4	3NS7	1KQU	3E7G
1	Glucose	-5.5	-5.3	-3.0	-5.9
2	Linoleic Acid	-4.7	-5.3	-6.4	-6.4
3	Oleic Acid	-4.9	-5.3	-4.5	-7.0
4	Linolenic Acid	-5.1	-4.3	-6.3	-6.8
5	Arachidonic Acid	-5.3	-5.6	-6.8	-7.6
6	Stearic Acid	-4.3	-4.5	-5.9	-6.6
7	Palmitic Acid	-4.4	-4.6	-5.8	-6.0
8	Lupeol	-7.4	-6.7	-5.8	-9.7
9	Vanillic Acid	-5.4	-5.5	-5.6	-6.8
10	Ferulic Acid	-5.7	-5.6	-6.4	-7.3
11	Campesterol	-7.7	-6.6	-8.6	-10.1
12	Sitosterol	-6.9	-6.6	-5.8	-9.9
13	Ergosterol	-7.8	-7.3	-5.8	-10.8
14	Tocopherol	-6.1	-6.5	-7.3	-8.9
15	Indomethacin	-6.7	-6.3	-8.8	-9.4

Vinascore, the more negative the vinascore, the more favorable the binding.

Table 3: Ramachandran plot status with protein with 1FT4, 3NS7, 1KQU and 3E7G

Residues	1FT4	3NS7	1KQU	3E7G
Most favorable region (%)	71.4	77.9	91.7	90.6
Additional allowed regions (%)	22.6	18.9	7.3	8.9
Generously allowed regions (%)	4.0	3.2	0.9	0.5
Disallowed regions (%)	2.0	0.0	0.0	0.0

**Figure 1: Ramchandran plot with selected proteins**

***In vitro* anti-inflammatory activity:**

Table 4: Inhibition of Protein Denaturation:

Treatment	Concentration	Percentage Inhibition
Control	-	-
MEPG	100 µg/ml	10.7
	200 µg/ml	17.8
	300 µg/ml	28.5
	400 µg/ml	35.7
	500 µg/ml	46.4
Aspirin	100 µg/ml	60.7

Table 5: Membrane Stabilisation:

A. Heat Induced Haemolysis:

Treatment	Concentration	Percentage Inhibition
Control	-	-
MEPG	100 µg/ml	13.3
	200 µg/ml	26.6
	300 µg/ml	33.3
	400 µg/ml	46.6
	500 µg/ml	56.6
Aspirin	100 µg/ml	73.3

Table 6: B. Hypotonicity Induced Haemolysis:

Treatment	Concentration	Percentage Inhibition
Control	-	-
MEPG	100 µg/ml	18.7
	200 µg/ml	28.1
	300 µg/ml	40.6
	400 µg/ml	46.8
	500 µg/ml	59.3
Diclofenac Sodium	100 µg/ml	75

Anti-inflammatory activity *in vivo*:

Table 7: Carrageenan induced paw edema:

Edema and a change in paw thickness result with the administration of carrageenan into the paw. The appropriate test extract and the reference medication were given to the animals. Following an hour, the animals received 0.1 ml of a 1% carrageenan solution in the subplantar area of the left hind paw. Paw thickness is assessed every hour between 0 and 6 hours.

Groups	0 th h	1h	2 h	3 h	4 h	5 h	6 h	% Inhibition (4hrs)
Control	0.960±0.02	0.960±0.02	0.960±0.02	0.960±0.02	0.960 ± 0.02	0.960 ± 0.02	0.960 ± 0.02	-
Disease Control	1.950±0.040*	2.060 ± 0.040*	1.850±0.040*	1.70 ±0.040*	1.460 ± 0.050*	1.230 ±0.020	0.960 ± 0.020	-
MEPG (200 mg/kg)	1.480±0.020 ^{*Ae}	1.610±0.010 ^{*Aa}	1.410±0.010 ^{*Aa}	1.300 ±0.020 ^{*Ad}	1.160 ±0.020 ^{**Aac}	1.070 ±0.020	0.960 ± 0.020	20.730
MEPG (400 mg/kg)	1.4600±0.040 ^{*Ad}	1.630 ±0.020 ^{*Ab}	1.450±0.020 ^{*Aa}	1.330 ±0.020 ^{*Ae}	1.230 ±0.020 ^{*Ac}	1.080 ± 0.010	0.960 ± 0.020	31.200
Indomethacin (3.0 mg/kg)	1.6100±0.030 ^{*A}	1.760±0.020 ^{*A}	1.600±0.020 ^{*A}	1.480 ±0.010 ^{*B}	1.350±0.020 ^{*C}	1.160±0.030	0.960 ± 0.020	46.830

Standards are conveyed as Mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were expressed as when compared to control (* = p<0.0001, ** = p<0.005), disease control (A = p<0.0001, B = p<0.001, C = p<0.05), standard (a=p<0.0001, b=p<0.0005, c=p<0.005, d=p<0.01, e=p<0.05)

DISCUSSION

Computer-based drug design techniques such as molecular docking, which evaluates tiny compounds by positioning and scoring them in a protein's binding site, continue to show considerable potential. Using CB-Dock2, isolated chemicals from *Pennisetum glaucum* methanolic grain extract and common medications were docked (Liu Y et al., 2020). The countless residents known in the extract are glucose, linoleic Acid, oleic Acid, linolenic acid, arachidonic acid, stearic acid, palmitic acid, lupeol, vanillic acid, ferulic acid, campesterol, sitosterol, ergosterol, tocopherol and indomethacin were subjected to docking against PDB ID: 1FT4, PDB ID: 3NS7, PDB ID: 1KQU and PDB ID: 3E7G. According to the docking results, campesterol, sitosterol, ergosterol, lupeol, linoleic, oleic, and linolenic acids, arachidonic, stearic, and palmitic acids, glucose, tocopherol, and indometacin all displayed the highest vina scores with all of the chosen proteins, indicating a higher affinity for receptor-ligand binding. Docking results made it abundantly evident that the chemical ingredients stated above may have demonstrated a comparable mechanism to those of common anti-inflammatory medications like indomethacin. The proteins were identified as PDB ID: 1FT4, PDB ID: 3NS7, PDB ID: 1KQU, and PDB ID: 3E7G. The proteins were modelled, and the 3D model's properties were assessed using the PROCHECK programme and the Ramachandran plot. It is clear from the Ramachandran plot that predicted models have most advantageous regions, additional allowed regions, generally allowed regions, and banned regions. A Ramachandran plot analysis of the protein residues yielded a percentage distribution that indicates whether or not the anticipated models are accurate.

In vitro Anti-inflammatory:

When an external stressor or compound, such as a potent acid or base, a concentrated inorganic salt, an organic solvent, or heat is used, proteins lose their tertiary and secondary structures, a process known as denaturation. Lupeol and beta sitosterol inhibited thermal induced protein denaturation (Leelaprakash G, 2011).

Membrane stabilisation:

Because the erythrocyte membrane and the lysosomal membrane are comparable (Gandhidasan, R et al., 1991, Shenoy S et al., 2010). The stability of the HRBC membrane has been employed to investigate the anti-inflammatory efficacy *in vitro*. Lysosomal stabilisation is critical in regulating the inflammatory response because it prevents the release of lysosomal components of activated neutrophils, such as bacterial enzymes and proteases, which aggravate tissue inflammation and damage upon extracellular release. These enzymes' extracellular activity is thought to be connected to either acute or chronic inflammation. Assays for membrane stabilisation demonstrate the anti-inflammation properties of fatty acids such as oleic acid, stearic acid, palmitic acid, and arachidonic acid.

Anti-inflammatory activity *in vivo*

A biphasic natural occurrence, carrageenan-induced paw edoema. Histamine, serotonin, and bradykinins, which are released from mast cells into the nearby injured tissues, are responsible for the initial phase (1–2 h after carrageenan injection). Prostaglandins, leukotrienes, and a number of cytokines, including IL-1, IL-6, IL-10, and TNF, are among the arachidonate metabolites that are released during the second phase of the inflammatory response (3–6 h after carrageenan injection) (Solanki HK et al., 2015). Linoleic acid, palmitic acid, and linolenic acid are fatty acids that reduce inflammation by decreasing the enzymes cyclooxygenase and 5-lipoxygenase, which are essential enzymes responsible for many types of inflammation. The limiting enzyme COX produces prostaglandin E2 (PGE2), a fatty acid with a 20-carbon chain that causes fever, malaise and swelling (Frederick A et al., 1980). Stearic acid yog saturated fatty acid nrog saw at 18 carbons. It mediates the inflammatory response by partially suppressing HIF1 (Miao H et al., 2015). In carrageenan-induced inflammation, HIF-1 stimulates the expression of proinflammatory cytokines and macrophage aggregation, invasion, and motility, all of which are inflammatory processes (Gupta MB et al., 1980). Lupeol is a triterpene that is pentacyclic. TNF and IL-1 secretion are also somewhat decreased by it. The monohydroxybenzoic acid vanillic acid is one of them. According to (Calixto-Campos C et al., 2015),

it has analgesic and anti-inflammatory actions via reducing the production of pro-inflammatory cytokines. The antioxidant ferrulic acid is employed because it prevents the manufacture of prostaglandins, which combats carrageenan-induced paw edema. Treatment reduced cytokine levels while increasing antioxidant enzyme activity (Yukihiro O, 1992). Campesterol is a phytosterol that resembles cholesterol in terms of its chemical composition. It's associated with the production and release of inflammatory cytokines and also contains anti-inflammatory effects. As a result, these ingredients could be made into medications to treat bodily inflammatory disorders (Shen X et al., 2017). As Akt activity is decreased, tocopherols have an anti-inflammatory impact. This will be accomplished by stopping Akt from stimulating the gene transcription nuclear factor NF-KB, which in turn causes the generation of inflammatory cytokines in carrageenan-induced edema (Reiter E et al., 2007). The edema produced by tPA and the activity of the enzyme mieloperoxidase are reduced by phytosterols known as beta-sitosterol. Numerous authors claimed that sitosterol has anti-inflammatory properties in rat models of Brewer's yeast- and cotton-pellet-induced pyrexia, carrageenan-induced edema, and cotton pellet implantation (Gupta MB et al., 1980). Ergosterol, a sterol present in fungal cell membranes and similar to mammalian cholesterol, preserves the structural integrity of the cell membrane. According to (Kobori M et al., 2007), ergosterol peroxide inhibits the expression of TNF- and IL-1 that is triggered by LPS and is a crucial step in all forms of inflammation. Anti-inflammatory properties of lipids such as linoleic acid, palmitic acid, linolenic acid, oleic acid, arachidonic acid, and stearic acid, flavonoids such as vanillic acid, terpenes such as lupeol, phenols such as ferulic acid, and sterols such as campesterol, ergosterol, and sitosterol.

CONCLUSION

According to *in silico*, *in vitro* and *in vivo* investigations methanolic grain extract of *Pennisetum glaucum* has anti-inflammatory activity. More research is required to identify the specific phytochemical components of the extract and determine the precise mechanism underlying its anti-inflammatory effect.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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