AJBAR Vol. 5(5), October 2026: 140-150, ISSN: 2811-2881

Arid Zone Journal of Basic and Applied Research
Faculty of Science, Kashim Ibrahim University
Maiduguri, Nigeria

Journal homepage: https://www.azjournalbar.com

Research Article

Phytochemical composition, mineral element profile, and *in vitro* anti-inflammatory activity of *Piliostigma reticulatum* leaf fractions

Mshelia, H.S.*, M. S. Sule, and Nasiru Abdullahi,
Department of Biochemistry, Bayero University, P.M.B. 3011, Kano, Nigeria
*Corresponding author's Email: msheliahamman@yahoo.com, doi.org/10.55639/607.020100155

ARTICLE INFO:
Keywords:
Piliostigma reticulatum, Phytochemical, Mineral elements, Anti-inflammatory, Albumin denaturation.

ABSTRACT

Piliostigma reticulatum (DC.) Hochst. is a Sahelian leguminous shrub used in traditional medicine for inflammatory conditions across West Africa. This study characterised the phytochemical composition, mineral element profile, and in vitro anti-inflammatory activity of six leaf fractions obtained by sequential solvent partitioning. Qualitative screening detected flavonoids, steroids, alkaloids, terpenoids, glycosides, and tannins across all fractions. Quantitative analysis revealed that fraction MEOHF6 contained the highest concentrations of flavonoids (16.84 g/100g), alkaloids (23.57 g/100g), glycosides (18.46 g/100g), and terpenoids (8.85 g/100g). Mineral element analysis by AAS identified pharmacologically relevant concentrations of Ca, P, K, Mg, Mn, Fe, Zn, Se, and Cu. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation inhibition, proteinase inhibition, and erythrocyte membrane stabilisation assays at 25–200 µg/mL. MEOHF6 recorded IC_{50} values of 131.9 µg/mL (albumin denaturation), 54.2 µg/mL (proteinase inhibition), and 171.3 µg/mL (membrane stabilisation)—the only fraction achieving membrane stabilisation IC_{50} within the tested range. Fraction HEOHF4 recorded the lowest proteinase IC_{50} (41.0 µg/mL) in the entire study. These findings identified MeOHF6 as the fraction with the most consistent activity across the three assays.

Corresponding author: Mshelia, H.S, **Email**: msheliahamman@yahoo.com
Department of Biochemistry, Bayero University, P.M.B. 3011, Kano, Nigeria

INTRODUCTION

Inflammation underlies cardiovascular disease, type 2 diabetes, and certain cancers. Mainous *et al.* (2024) demonstrated that poverty combined with chronic inflammation increases mortality risk more than two-fold for heart disease in adults aged 40 and above. Current anti-inflammatory agents—NSAIDs and corticosteroids—carry dose-limiting gastrointestinal, renal, and cardiovascular adverse effects (Adams *et al.*, 2011; Bindu *et al.*, 2020), creating an urgent need for safer alternatives in sub-Saharan Africa (Lopez-Candales *et al.*, 2017).

Piliostigma reticulatum (DC.) Hochst. (Caesalpiniaceae) is a dioecious leguminous shrub distributed across the Sahelo-Sudanian belt. Pharmacological evaluations substantiate several traditional therapeutic applications of the plant, revealing a complex phytochemical profile across its leaves, stem bark, and roots that includes polyphenols, flavonoids, tannins, alkaloids, glycosides, and terpenes (Adeyanju et al., 2019; Babajide et al., 2008). Specific

constituents, notably C-methylated flavonols such as 6-C-methylquercetin-3-methyl ether and 6,8-di-C-methylkaempferol-3-methyl ether, exhibit broad-spectrum antimicrobial efficacy against various bacterial and fungal strains, including *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (Adeyanju *et al*., 2019; Babajide *et al*., 2008). Furthermore, methanolic leaf extracts demonstrate substantial antioxidant and anti-inflammatory activities, displaying high total phenolic content alongside significant inhibition of heat-induced albumin denaturation (Boualam *et al*., 2021), while bark-derived condensed tannins markedly reduce carrageenan-induced paw edema in vivo (Diatta *et al*., 2019). Additional documented effects include prominent antidiarrheal properties mediated by reductions in defecation frequency, enteropooling, and gastrointestinal motility (Salawu *et al*., 2007), alongside demonstrated in vivo antiplasmodial activity and a favorable acute safety profile with an LD_{50} exceeding 5000 mg/kg (Bello et al., 2022). Despite extensive traditional use, systematic phytochemical characterisation and anti-inflammatory evaluation of the leaf fraction remain incomplete. This study determined the phytochemical constituents, mineral element composition, and in vitro anti-inflammatory activity of six leaf fractions to identify the most consistent pharmacologically active fraction against inflammation.

MATERIALS AND METHODS

Plant material collection and identification

Fresh leaves of *Piliostigma reticulatum* were harvested from naturally occurring populations in Konduga LGA, Borno State, north-eastern Nigeria (GPS coordinates: approximately 11.69586°N, 13.27555°E), during the late dry season (February–March) in 2024, when secondary metabolite concentrations in leaves are typically at their peak. Collection was conducted between 07:00 and 10:00 hours to minimise degradation of heat-labile phyto-constituents. Only mature, healthy, undamaged leaves without evidence of pathogen attack or mechanical injury were collected. Approximately 3 kg of fresh leaf material was obtained. Botanical identification was performed at the University of Maiduguri Herbarium (voucher: UMM/FPH/FAA/010). No collection permit was required as plant is found growing abundantly in the wild and Ethical clearance was obtained from ACUREC, Bayero University Kano (BUK/ACUREC/CAP/PG174).

Processing, Extraction and fractionation

Fresh leaves were rinsed under running tap water to remove surface debris, followed by a final rinse with distilled water. The cleaned leaves were spread in a single layer on clean stainless steel drying racks and air-dried at ambient temperature (28–32°C) under shade conditions to prevent photodegradation of secondary metabolites, for a period of 14–21 days or until a constant dry weight was attained. Dried leaf material was then pulverised to a coarse powder using a stainless-steel electric blender, passed through a 1.5 mm mesh sieve to achieve particle size uniformity, and stored in airtight amber glass containers at 4°C until use.

The two crude extracts were prepared by cold maceration. Five hundred grams each of the dried leaf powder was macerated in 2.5 litres of 80% ethanol (ethanol: distilled water, 4:1 v/v) and 99% methanol respectively in a clean glass vessel with intermittent stirring for 72 hours at room temperature. The macerates were filtered using Whatman No. 1 filter paper, and the marc was re-extracted twice under the same conditions for 48 hours each. The filtrates were concentrated under reduced pressure at 40°C using a rotary evaporator (Heidolph Laborota 4000, Germany) to yield the crude extracts. The crude extracts were further dried in a desiccator over silica gel, and the percentage yield calculated gravimetrically.

Partitioning of the crude extracts were independently carried out as described by Ibrahim et al. (2012). Here, 30g of silica gel will be dissolved in ethyl-acetate ($100cm^3$) and packed into a glass column with measurement of 1.5*50cm. The extract was eluted using running solvents system: ethyl-acetate methanol with gradual increase in polarity (10:1, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10) thereafter the extracts were subjected to the column packed with silica gel pre equilibrated with ethyl-acetate. Fractions eluted were collected in aliquots with 20ml

beaker. These eluted fractions were subjected to analytical TLC where fractions with similar RF values and relative mobility in solvent systems was pooled together to get active plant fractions which was used for the *in vitro* assays. The solvent fractionation yielded six fractions coded MeOHF1, MeOHF5, MeOHF6 (for methanol fraction) and HeOHF4, HeOHF11, HeOHF12 (for Hydro-Ethanol fraction). All fractions were stored at 4°C until analysis.

Qualitative and quantitative phytochemical analysis

Qualitative phytochemical screening of the solvent fractions was carried out using standard colourimetric and precipitation-based methods to detect the presence or absence of major classes of secondary metabolites, including alkaloids (Dragendorff's and Mayer's reagents), flavonoids (Shinoda test and lead acetate test), tannins (ferric chloride test and Stiasny reaction), terpenoids and steroids (Liebermann-Burchard test) and glycosides (Keller-Kiliani and Bornträger's tests), following the procedures of Harborne (1998). The Phytochemical compositional score was reported based on colour intensity of the test sample with +, ++ and +++ reported as slightly available, available and abundantly available

For the quantitative determination of Phytochemicals, about 2 – 5g of sample was weighed and dissolved with hexane in a 1.0ml vial. The prepared sample was then injected into a Buck scientific (USA) BLC10/11 High Performance Liquid Chromatography (HPLC) system with a fluorescence detector (excitation at 295 nm and emission at 325 nm) and an analytical silica column (25 cm x 4.6mm ID, stainless steel,5 μm) was used to analyse phytochemicals. The mobile phase used was hexane: tetrahydrofuran: isopropanol (1000:60:4 v/v/v) at a flowrate of 1.0ml/min. Standard samples were also prepared using similar method. Concentration of phytochemicals in samples was calibrated using authentic standards. Using the results obtained the concentration of phytochemicals in the sample was calculated, using the formula below;

[PHYTO] = [A SAMPLE x [STD] (ppm) x V HEX (ml)]/ [A STD x Wt SAMPLE (g)]

Where;

[PHYTO] = concentration of phytochemicals in ppm

[STD] = concentration of standard

A SAMPLE = area of sample

A STD = area of standard

V HEX = volume of hexane

Wt SAMPLE = weight of sample

Determination of Mineral Elements and Heavy Metals

To analyze mineral and heavy metal concentrations, 5.0-g samples were weighed into high-form porcelain crucibles and incinerated in a muffle furnace at 550 °C until complete organic oxidation yielded a white or grey ash. For mineral analysis, the resulting ash was dissolved in 2 mL of concentrated HNO_3, quantitatively transferred to a 100-mL volumetric flask, diluted to volume with distilled water, and homogenized. The concentrations of iron (Fe), magnesium (Mg), and calcium (Ca) were determined via flame atomic absorption spectrophotometry (FAAS) using a Model 210VGP atomic absorption spectrophotometer (Buck Scientific). The instrument was operated in integrated mode utilizing an air-acetylene flame as the atomization source. Element-specific hollow cathode lamps were employed as light sources at their respective resonance wavelengths: 248.3 nm for Fe, 285.2 nm for Mg, and 422.7 nm for Ca. To prevent refractory chemical interferences during Ca determination, lanthanum chloride (LaCl3) was added to the samples and standard solutions as a releasing agent.

Potassium (K) levels were analyzed via flame emission photometry using a Jenway PFP7 flame photometer (Cole-Parmer). The instrument operated using an integrated air-acetylene flame mixture to achieve thermal excitation of the target analyte. Quantitation was based on atomic emission intensity isolated through a specific optical filter at the characteristic potassium resonance line of 766.0 nm.

For both pathways, standard calibration curves ((R^2 > .999) were prepared using certified multi-element stock solutions. Samples were aspirated in triplicate, and blank corrections were applied to calculate the final elemental concentrations.

For heavy metal determination in plant fractions, the initial ashing and dissolution steps were replicated. However, the mixture was sequentially swirled and heated in a fume hood until frothing ceased and a clear, pale-yellow solution formed. After cooling, the solution was diluted to 100 mL with distilled water and filtered through Whatman No. 1 filter paper. Heavy metal concentrations in the filtrate were measured using an Atomic Absorption Spectrophotometer (Buck 230, Buck Scientific) configured with element-specific lamps and corresponding wavelengths under an air-acetylene integrated flame mode. Following standard guidelines (AOAC, 2000), concentration values for all minerals and heavy metals were quantified by extrapolation from the calibration curves of their respective standard solutions.

In vitro anti-inflammatory assays

The *in vitro* anti-inflammatory assay of the six fractions extracts of *Piliostigma reticulatum* was carried out by determining the inhibition of albumin Denaturation and anti-protinase action using Piroxicam as the positive control drug in all three assays with the IC50 values for piroxicam reported as 48.11 µg/mL (albumin denaturation), 38.15 µg/mL (proteinase inhibition) and 52.59 µg/mL (membrane stabilization) confirming the sensitivity of the assay models.

Inhibition of Albumin Denaturation.

Anti-inflammatory effects of the crude extracts of *Piliostigma reticulatum* was carried by assaying the inhibition of albumin denaturation as described by Mizushima *et al*. (1968), Sakat *et al*. (2010) and Leelaprakash *et al*. (2011). Test extracts and 1 % aqueous solution of bovine albumin fraction termed as reaction mixture was mixed and with the pH of the reaction mixture being adjusted with 1N HCl. The reaction mixture was incubated at 37ºC for 20 minutes before being heated to 51oC for same time as incubation period. Sample turbidity was measured using UV-Visible Spectrophotometer at 660nm after cooling. This experiment was done in triplicates and the %inhibition of protein denaturation was calculated as:

%Inhibition = (Abs Control-Abs Sample) ×100/Abs Control

Antiprotieinase Action

Antiproteinase action was carried out as described by Leelaprakash and Dass (2011). The reaction mixture comprised of 1ml 20mM Tris HCL Buffer with a Ph of 7.4, 0.06mg trypsin and 1ml test sample was incubated at 37ºC (5 minutes) before addition of 0.8% (w/v) casein. The mixture above was further incubated for 2 minutes and 2ml of 70% perchloric acid was added to stop the reaction. The suspension was centrifuged and absorbance read at 210nm against buffer as blank. The percentage inhibition of proteinase inhibitory activity was calculated thus:

% Inhibition= (Abs control- Abs sample)×100/ Abs control

Anti-Inflammatory (Membrane Stability) Activity Assay

The human red blood cell (HRBC) membrane stabilization method was used as described by Shinde et al. (1999). The principle is based on the analogy between HRBC membrane and lysosomal membrane; stabilization of HRBC membrane implies stabilization of lysosomal membrane.

Fresh whole blood (5 mL) was collected from a healthy volunteer who had not taken non-steroidal anti-inflammatory drugs (NSAIDs) for 2 weeks, into an EDTA tube. Ethical clearance was obtained and informed consent was given. The blood was centrifuged at 3000 rpm for 10 min, and the supernatant was discarded. The packed cells were washed three times with isosaline (0.9% NaCl) and a 10% (v/v) HRBC suspension was prepared with isosaline. The assay mixture (4.5 mL) contained 1.0 mL phosphate buffer (pH 7.4), 2.0 mL hyposaline (0.36% NaCl), 0.5 mL HRBC suspension, and 1.0 mL of test sample or standard drug (25–200 µg/mL). The control was prepared with 1.0 mL distilled water instead of test sample, representing 100% lysis. All mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm for 20 min. Hemoglobin content in the supernatant was estimated spectrophotometrically at 560 nm.

membrane stabilization was calculated as:

$$\text{Percent of hemolysis} = \frac{\text{OD of test}}{\text{OD of control}} \times 100. \quad (1)$$

The percentage of protection can be hence calculated from the equation as given below:

$$\text{Percent of protection} = 100 - \frac{\text{OD of test}}{\text{OD of control}} \times 100. \quad (2)$$

Here "OD of test" is optical density or the test sample's absorbance and "OD of control" is optical density or absorbance of the negative control.

Statistical analysis

Data were reported as the mean ± SEM for triplicate determinations of each sample. All data analyses were performed using the SPSS statistical package, Version 22 (SPSS Inc., Chicago, IL).

RESULTS

Qualitative phytochemical screening

All six phytochemical classes were detected across every fraction (Table 1). Fractions MeOHF6 and MeOHF1 showed the richest profiles, with flavonoids, alkaloids, glycosides, and tannins all rated highly present (+++). Steroids were uniformly slightly present (+).

Table 1: Qualitative Phytochemical Profile of *P. reticulatum* Leaf Fractions

Phytochemical	MeOHF5	MeOHF1	HeOHF4	HeOHF11	HeOHF12	MeOHF6
Flavonoids	++	+++	++	+	+	+++
Steroids	+	+	+	+	+	+
Alkaloids	+++	+++	+++	+++	++	+++
Terpenoids	+	++	+	+	+	++
Glycosides	++	+++	++	++	++	+++
Tannins	++	+++	+++	++	+++	+++

Note: + = slightly present; ++ = present; +++ = highly present. Methods: Harborne (1998).

Quantitative phytochemical composition

MeOHF6 recorded the highest flavonoids (16.84 ± 0.00 g/100g), alkaloids (23.57 ± 0.02 g/100g), glycosides (18.46 ± 0.03 g/100g), and terpenoids (8.85 ± 0.02 g/100g). MeOHF1 carried the highest tannins (22.73 ± 0.03 g/100g) (Table 2).

Table 2: Quantitative Phytochemical Content of P. reticulatum Leaf Fractions (g/100g)

Phytochemical	MeOHF5	MeOHF1	HeOHF4	HeOHF11	HeOHF12	MeOHF6
Flavonoids	7.66±0.04	11.73±0.03	8.63±0.03	3.87±0.04	4.63±0.03	16.84±0.00
Alkaloids	15.69±1.53	21.72±0.01	16.34±0.02	14.35±0.02	9.88±0.02	23.57±0.02
Tannins	13.86±0.41	22.73±0.03	14.53±0.02	8.55±0.03	18.24±0.03	19.65±0.02
Glycosides	11.21±1.36	17.03±0.04	7.15±0.04	4.70±0.74	7.69±0.03	18.46±0.03
Terpenoids	5.34±0.12	7.86±0.03	2.53±0.02	3.74±0.03	2.13±0.01	8.85±0.02
Steroids	0.81±0.09	1.30±0.04	0.86±0.03	0.65±0.02	0.53±0.03	1.43±0.02

Note: Key: Values are expressed as Mean ± S.E.M., n = 3. HEOH = hydro-ethanolic extract; MEOH = methanolic extract.

Mineral element composition

The mineral composition of crude extracts and fractions of P. reticulatum is presented in Table 3. Potassium was the most abundant element, ranging from 66.35 ± 0.02 mg/100g (HeOHF12) to 116.15 ± 0.02 mg/100g (HeOH crude), followed by phosphorus (32.15 ± 0.02 to 72.01 ± 0.01 mg/100g) and calcium (14.72 ± 0.01 to 42.92 ± 0.02 mg/100g). Crude MeOH extract was generally richer in minerals than its fractions, with the exception of MeOHF1 which retained high calcium (42.53 ± 0.02 mg/100g), magnesium (21.42 ± 0.02 mg/100g) and iron (2.53 ± 0.02 mg/100g). MeOHF6, which exhibited the highest anti-inflammatory activity, showed the highest sodium content (14.53 ± 0.02 mg/100g) among fractions. Trace elements zinc, iron, manganese and selenium were present in low concentrations (<7 mg/100g), with selenium being the least abundant (<0.5 mg/100g) across all samples.

Table 3: Mineral Element Composition of P. reticulatum Leaf Fractions (mg/100g)

Elements	HeOH	MeOH	Relative proportion of Fractions (mg/100g)					
			MeOHF5	MeOHF1	HeOHF4	HeOHF11	HeOHF12	MeOHF6
Calcium	33.79± 0.02	42.92±0.02	21.46±0.02	42.53±0.02	31.16±0.01	14.72±0.01	22.23±0.02	38.71±0.02
Phosphorus	65.22±0.02	72.01±0.01	32.15±0.02	44.52±0.02	37.18±0.02	41.67±0.02	49.63±0.02	51.55±0.02
Potassium	116.15±0.02	92.51±0.02	66.94±0.01	81.52±0.02	71.13±0.01	91.64±0.02	66.35±0.02	88.62±0.02
Sodium	8.65± 0.02	7.51±0.01	7.14±0.02	8.37±0.02	5.64±0.03	3.67±0.02	4.56±0.02	14.53±0.02
Magnesium	12.65±0.02	12.01±0.01	4.17±0.02	21.42±0.02	11.72±0.02	6.82±0.02	8.13±0.02	12.75±0.02
Manganese	4.77± 0.01	6.21±0.01	1.97±0.02	9.51±0.01	6.87±0.02	2.97±0.02	3.32±0.02	6.15±0.02
Iron	1.75±0.02	1.65±0.01	0.46±0.02	2.53±0.02	3.16±0.01	1.72±0.01	0.66±0.01	2.16±0.02
Zinc	0.84±0.01	1.00±0.01	0.36±0.02	0.52±0.02	1.18±0.02	0.67±0.02	0.40±0.02	0.73±0.02
Selenium	0.42±0.02	0.35±0.02	0.09±0.01	0.22±0.02	0.33±0.01	0.06±0.01	0.37±0.02	0.23±0.01
Copper	0.65±0.02	0.41±0.01	0.14±0.02	0.35±0.00	0.44±0.03	0.17±0.02	0.43±0.02	0.24±0.02

Key: Values are expressed as Mean ± S.E.M., n = 3. HEOH = hydro-ethanolic extract; MEOH = methanolic extract.

In Vitro Anti-Inflammatory Activities

Table 4: Percentage Inhibition of Albumin Denaturation by Piliostigma reticulatum Leaf Fractions Across Concentrations

Fraction	25	50	100	200	IC_{50} (µg/mL)
MeOH F5	21.33±0.02	26.43±0.01	32.33±0.03	54.63±0.01	>200
MeOH F1	26.62±0.01	31.35±0.03	42.25±0.02	61.14±0.02	141.1
HEOH F4	12.43±0.02	17.56±0.02	23.33±0.03	31.74±0.01	>200
HEOH F11	10.35±0.02	22.63±0.02	32.27±0.02	41.76±0.01	>200
HEOH F12	11.35±0.02	18.23±0.01	31.42±0.03	46.22±0.02	>200
MeOH F6	**31.41±0.00**	**38.34±0.01**	**44.26±0.02**	**62.24±0.03**	**131.9**

Note. Values are Mean ± SEM of triplicate readings, expressed as percentage inhibition relative to the unprotected heat-denaturation control. IC_{50} = concentration producing 50% inhibition, calculated by linear interpolation

between bracketing concentration points. >200 = IC_{50} not achieved within the 25–200 µg/mL concentration range. IC_{50} values ≤150 µg/mL shown in bold (indicating pharmacologically meaningful potency).

Results from Table 4 indicated differential potency across fractions. MeOH F6 demonstrated the greatest inhibitory activity, increasing from 31.41% ± 0.00% at 25 µg/mL to 62.24% ± 0.03% at 200 µg/mL, with an IC50 of 131.9 µg/mL. MeOH F1 also exhibited substantial activity, ranging from 26.62% ± 0.01% to 61.14% ± 0.02%, with an IC50 of 141.1 µg/mL. Both fractions met the criterion for pharmacologically meaningful potency (IC50 ≤ 150 µg/mL). In contrast, MeOHF5 (21.33% ± 0.02% to 54.63% ± 0.01%), HEOH F4 (12.43% ± 0.02% to 31.74% ± 0.01%), HEOH F11 (10.35% ± 0.02% to 41.76% ± 0.01%), and HEOH F12 (11.35% ± 0.02% to 46.22% ± 0.02%) failed to achieve 50% inhibition within the tested concentration range, yielding IC50 values > 200 µg/mL.

Table 5: Percentage Inhibition of Proteinase Activity by *Piliostigma reticulatum* Leaf Fractions Across Concentrations

Fraction	25	50	100	200	IC_{50} (µg/mL)
MeOH F5	12.25±0.04	18.43±0.03	32.45±0.02	44.86±0.03	>200
MeOH F1	27.12±0.03	37.56±0.02	55.26±0.01	71.37±0.01	85.2
HEOH F4	36.74±0.02	57.44±0.00	71.35±0.02	87.41±0.02	**41.0**
HEOH F11	18.33±0.01	31.14±0.02	40.48±0.02	61.28±0.02	145.8
HEOH F12	12.75±0.02	23.13±0.01	44.33±0.04	65.14±0.03	127.3
MeOH F6	**36.79±0.03**	**48.53±0.04**	**66.22±0.00**	**82.38±0.02**	**54.2**

Note. Values are Mean ± SEM of triplicte readings; percentage inhibition of trypsin-mediated proteolytic activity relative to an uninhibited enzyme control. IC_{50} computed by linear interpolation. >200 = IC_{50} not achieved. IC_{50} ≤60 µg/mL shown in bold. HeOHF4 achieved the lowest IC_{50} across the entire study (41.0 µg/mL); MeOHF6 returned the second-lowest IC_{50} at 54.2 µg/mL.

Proteinase inhibition assay results revealed a markedly different pattern from the denaturation data, with fraction HEOHF4 emerging as the most potent inhibitor. It achieved 57.44% inhibition at just 50 µg/mL and 87.41% at the maximum concentration, recording an IC_{50} of 41.0 µg/Ml, the lowest value obtained in any assay across the entire study. MeOHF6 ranked second, with inhibition climbing from 36.79% at 25 µg/mL to 82.38% at 200 µg/mL (IC_{50} = 54.2 µg/mL). other Fractions reported intermediate activity with IC_{50} values of 85.2, 127.3, and 145.8 µg/mL respectively. MeOHF5 was the weakest performer in this assay, failing to attain 50% inhibition within the tested range. The exceptional performance of HEOHF4 in the proteinase assay, despite its moderate albumin-denaturation inhibitory capacity, points to a specific concentration of protease-inhibitory constituents.

Table 6: Percentage Erythrocyte Membrane Stabilisation by *Piliostigma reticulatum* Leaf Fractions Across Concentrations

Fraction	25 (%)	50 (%)	100 (%)	200 (%)	IC_{50} (µg/mL)
MeOH F5	8.72±0.01	20.56±0.01	36.53±0.04	44.32±0.02	>200
MeOH F1	14.02±0.03	21.49±0.03	33.26±0.00	38.15±0.02	>200
HEOH F4	15.24±4.24	24.88±0.02	29.64±0.03	33.94±0.02	>200
HEOH F11	11.83±0.02	16.14±0.02	22.43±0.02	28.34±0.02	>200
HEOH F12	8.46±0.02	16.52±0.02	18.25±0.01	26.25±0.04	>200
MeOH F6	**20.54±0.02**	**31.23±0.02**	**43.26±0.02**	**52.71±0.01**	**171.3**

Note. Values are Mean ± SEM of triplicate readings; percentage protection of erythrocyte membranes from hypotonic lysis relative to an unstabilised haemolysis control. IC_{50} computed by linear interpolation. >200 = IC_{50} not achieved within 25–200 µg/mL range. RBC membrane stabilisation is a validated model of lysosomal membrane-stabilising anti-inflammatory activity analogous to the mechanism of NSAIDs.

The membrane stabilisation assay produced the most discriminatory results of the three *in vitro* screens: MeOHF6 was the sole fraction to breach the 50% protection threshold within the tested concentration window, attaining 52.71 ± 0.01% protection at 200 µg/mL (IC_{50} = 171.3 µg/mL). All other fractions plateaued below 50%, with maximum values spanning 26.25% (HEOHF12) to 44.32% (MeOHF5). The MeOH F6 fraction attenuated lysosomal enzyme-mediated oxidative tissue damage as reflected in the serum MDA and antioxidant enzyme profiles. That fraction U is the only fraction capable of lysosomal membrane stabilisation within the tested range reinforces its pharmacological uniqueness and validates its selection as the primary fraction for in vivo investigation.

DISCUSSION

The consistent presence of flavonoids, alkaloids, tannins, glycosides, terpenoids and steroids across the crude extracts and all six fractions of *P. reticulatum* leaf (Tables 1 and 2) is consistent with earlier phytochemical screenings of the same species. Boualam *et al*. (2021) similarly reported the presence of polyphenols, tannins, flavonoids and alkaloids in methanolic leaf extracts of *P. reticulatum* and the closely related *P. thonningii*, and observed a higher phenolic concentration in the methanolic fraction relative to other solvent systems, broadly mirroring the pattern observed here in which the methanolic extract and its derivative fractions, particularly MeOHF1 and MeOHF6, generally returned higher phytochemical yields than the hydroethanolic counterparts. The dominance of alkaloids by mass in every sample (9.88-23.57 g/100 g) is notable given that alkaloids, tannins and flavonoids have each been independently implicated in anti-inflammatory bioactivity through distinct mechanisms, including free-radical scavenging, inhibition of pro-inflammatory enzymes and stabilisation of cell membranes (Naz *et al*., 2017).

The in vitro anti-inflammatory evaluation of *Piliostigma reticulatum* leaf fractions revealed distinct, assay-specific potencies, with fraction MeOH F6 emerging as the most comprehensively active candidate. A key mechanism of tissue injury in inflammatory conditions involves the denaturation of tissue proteins and the subsequent release of lysosomal enzymes, which propagate cellular damage and lipid peroxidation (Onaolapo & Onaolapo, 2021). The capacity of these leaf fractions to prevent protein denaturation, inhibit proteolytic enzyme activity, and stabilize cellular membranes underscores their therapeutic potential as source materials for novel anti-inflammatory agents.

Protein denaturation is a well-documented cause of inflammatory disorders, frequently resulting in the loss of biological function and the

autoantigenic activation of tissue proteins. In the present study, all evaluated fractions demonstrated a concentration-dependent rise in percentage albumin denaturation inhibition, confirming dose-related protein-protective activity across the panel (see Table 4). Fraction MeOHF6 achieved the highest inhibition at every concentration tested, ranging from 31.41% at 25 µg/mL to 62.24% at 200 µg/mL. Crucially, it recorded the lowest half-maximal inhibitory concentration (IC_{50}) of 131.9 µg/mL, indicating the greatest potency among all six fractions. Fraction MeOH F1 was the next most potent, achieving an IC_{50} of 141.1 µg/mL and reaching 61.14% inhibition at the maximum concentration. Conversely, fractions MeOHF5, HeOHF4, HeOHF11, and HeOHF12 demonstrated comparatively weak protein-denaturation inhibitory capacity, with IC_{50} values exceeding the 200 µg/mL threshold. The near-identical, high-concentration performance of fractions MeOHF6 and MeOHF1 suggests a shared phytochemical basis. It is highly plausible that their abundant alkaloid and glycoside content contributes synergistically to this protein-stabilizing effect, preventing the hydrophobic exposure that leads to thermal denaturation (Sakat et al., 2010).

Leukocyte proteinases, such as trypsin and chymotrypsin, are heavily implicated in tissue destruction during inflammatory responses, and their inhibition serves as a critical checkpoint in modulating chronic tissue injury. The proteinase inhibition assay results revealed a markedly different pattern from the denaturation data, shifting the profile of structural potency.

In this screen, fraction HEOHF4 emerged as the most potent inhibitor, achieving a remarkable 57.44% inhibition at a low concentration of 50 µg/mL and 87.41% at 200 µg/mL. This performance translated to an IC_{50} of 41.0 µg/mL, the lowest value obtained in any assay across the entire study. Fraction MeOHF6 ranked as the second most active inhibitor, exhibiting an IC_{50} of 54.2 µg/mL (see Table 5). The remaining fractions reported intermediate activity, whereas MeOHF5 was the weakest performer, failing to attain 50% inhibition within the tested range (IC_{50} > 200 µg/mL).

The erythrocyte membrane stabilization assay provided the most stringent and discriminatory results among the three in vitro screens. Because the erythrocyte membrane structurally resembles the lysosomal membrane, stabilization against hypotonic lysis serves as a reliable surrogate model for evaluating lysosomal membrane-stabilizing anti-inflammatory activity, a pathway analogous to the mechanism of non-steroidal anti-inflammatory drugs (NSAIDs; Shinde et al., 1999). As presented in Table 6, fraction MeOH F6 was the sole fraction to breach the 50% protection threshold within the tested concentration window, attaining 52.71% protection at 200 µg/mL with a calculated IC_{50} of 171.3 µg/mL. All other evaluated fractions plateaued prematurely below the 50% marker, with maximum protection values spanning a modest 26.25% (HEOH F12) to 44.32% (MeOH F5), resulting in IC_{50} values exceeding 200 µg/mL. By preventing hypotonic lysis, fraction MeOH F6 demonstrates a unique capacity to maintain membrane integrity. In a physiological context, this prevents the extracellular leakage of destructive lysosomal enzymes—such as proteases and acid hydrolases—thereby checking the escalation of localized tissue damage. The singular capability of MeOHF6 to achieve cell membrane stabilization within the tested range reinforces its unique pharmacological profile.

CONCLUSION

All six leaf fractions of *P. reticulatum* contain diverse secondary metabolites and pharmacologically relevant mineral. MeOHF6 exhibited the most consistently potent in vitro anti-inflammatory activity across three assays, establishing it as the primary candidate for *in vivo* evaluation with a moderate IC50 Value of 41-171 µg/Ml.

RECOMMENDATIONS

In vivo evaluation of MeOHF6 in the carrageenan-induced paw oedema model is recommended. GC-MS and LC-MS/MS compound identification should follow. Fraction HeOHF4 warrants dedicated sub-fractionation targeting triterpenoid protease inhibitors.

AUTHORS' CONTRIBUTIONS

Mshelia, H.S. designed, conducted the study, performed statistical analysis, and drafted the manuscript. M. S. Sule and Nasiru Abdullahi supervised the research and reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Adams, R. J., Piantadosi, C., Ettridge, K., Miller, C., Wilson, C., Tucker, G., & Hill, C. L. (2011). Functional limitations and the need for medicines among persons with chronic conditions. *Australian and New Zealand Journal of Public Health*, *35*(4), 392–396.

AOAC. (2020). *Official methods of analysis* (21st ed.). Association of Official Analytical Chemists.

Bello, I., Ojo, O. A., Ojo, A. B., & Oluwafemi, S. (2022). Elemental and medicinal evaluation of the leaf extract of Piliostigma reticulatum (DC) Hochst. International Journal of Research Granthaalayah,10(8),123–135. https://doi.org/10.29121/granthaalayah.v10.i8.2022.4718

Bindu, S., Mazumder, S., & Bandyopadhyay, U. (2020). Non-steroidal anti-inflammatory drugs (NSAIDs) and organ damage: A current perspective. *Biochemical Pharmacology*, *180*, Article 114147.

Boualam, K., Ndiaye, B., Harhar, H., Tabyaoui, M., Ayessou, N., Taghzouti, K., & Talbi, M. (2021). Study of the phytochemical composition, the antioxidant and the anti-inflammatory effects of two Sub-Saharan plants: *Piliostigma reticulatum* and *Piliostigma thonningii*. Evidence-Based Complementary and Alternative Medicine, 2021, 9925131. https://doi.org/10.1155/2021/9925131

Harborne, J. B. (1998). *Phytochemical methods: A guide to modern techniques of plant analysis* (3rd ed.). Chapman and Hall.

Koto-te-Nyiwa, N., Mbala, B. M., Gbolo, B. Z., Liyongo, C. I., Mpiana, P. T., & Ngbolua, K. N. (2015). A review on phytochemistry and pharmacology of Piliostigma species. *International Journal of Innovation and Applied Studies*, *11*(1), 150–158.

Leelaprakash, G., & Dass, S. M. (2011). In vitro anti-inflammatory activity of methanol extract of Enicostemma axillare. *International Journal of Drug Development and Research*, *3*(3), 189–196.

Lopez-Candales, A., Hernandez Burgos, P. M., Hernandez-Suarez, D. F., & Harris, D. (2017). Linking chronic inflammation with cardiovascular disease. *Journal of Nature and Science*, *3*(4), Article e341.

Mainous, A. G., Rooks, B. J., Wu, V., & Orlando, F. A. (2024). Poverty, chronic inflammation, and risk of cardiovascular disease and cancer mortality. *Journal of the American Board of Family Medicine*, *37*(1), 32–40.

Mizushima, Y. and Kobayashi, M. (1968). Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. Journal of Pharmacy and Pharmacology, 20(3), 169-173.

Naz, R., Ayub, H., Nawaz, S., Islam, Z. U., Yasmin, T., Bano, A., Wakeel, A., Zia, S., & Roberts, T. H. (2017). Antimicrobial activity, toxicity and anti-inflammatory potential of methanolic extracts of four ethnomedicinal plant species from Punjab, Pakistan. BMC Complementary and Alternative Medicine, 17(1), 302.

Onaolapo, A. Y., & Onaolapo, O. J. (2021). Plants with anti-inflammatory activity: A review of progress and future prospects. *Journal of Herbal Medicine*, *27*, Article 100423.

Onwuka, G. I. (2005). Food analysis and instrumentation: Theory and practice. Naphthali Prints.

Oyedapo, O. O., & Famurewa, A. J. (1995). Antiproteolytic and membrane-stabilizing activities of extracts of Fagara zanthoxyloides, Olax subscorpioides and Tetrapleura tetraptera. *International Journal of Pharmacognosy*, *33*(1), 65–69.

Sakat, S., Juvekar, A. R., & Gambhire, M. N. (2010). In vitro antioxidant and anti-inflammatory activity of methanol extract of Oxalis corniculata Linn.

International Journal of Pharmacy and Pharmaceutical Sciences, *2*(1), 146–155.

Shinde, U. A., Phadke, A. S., Nair, A. M., Mungantiwar, A. A., Dikshit, V. J., & Saraf, M. N. (1999). Membrane stabilizing activity—a possible mechanism of action for the anti-inflammatory activity of Cedrus deodara wood oil. *Fitoterapia*, *70*(3), 251–257.

Singh, S., Singh, S. K., & Yadav, A. (2013). Pharmacognostical and phytochemical investigation of Piliostigma reticulatum. *International Journal of Pharmaceutical Sciences and Research*, *4*(4), 1503–1509.

Yahfoufi, N., Alsadi, N., Jambi, M., & Matar, C. (2018). The immunomodulatory and anti- inflammatory role of polyphenols. *Nutrients*, *10*(11), Article 1618.