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Anti-inflammatory Effects of *Vigna subterranea* Methanol Seed Extract and N-Acetyl cysteine in Experimental Paw Edema Models

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ABSTRACT

Acute inflammation is associated with edema and membrane destabilization. Medicinal plants and antioxidant compounds are increasingly explored as safer anti-inflammatory agents. Bambara groundnut, *Vigna subterranea,* is commonly used in folkloric medicine. Scientific validations of the anti-inflammatory activity of *Vigna subterranea* combined with N-acetylcysteine (NAC), an antioxidant, are scarce. This study evaluated the anti-inflammatory effects of *Vigna subterranea* methanol seed extract, alone and in combination with N-acetylcysteine, using paw edema models in rats. Twenty-four male Wistar rats were randomly assigned into six groups (n = 4): normal saline control, *Vigna subterranea* extract (200 and 400 mg/kg), NAC (400 mg/kg), NAC (400 mg/kg) + extract (200 mg/kg), and diclofenac (100 mg/kg). Anti-inflammatory activity was evaluated using carrageenan- and formalin-induced paw edema models. In addition, the membrane-stabilizing potential of test agents was investigated by osmotic fragility. Data were expressed as mean ± SEM and analyzed using one-way ANOVA followed by LSD post hoc, with $p < 0.05$ considered significant. In the carrageenan-induced model, neither *Vigna subterranea* extract nor NAC produced a significant reduction in paw edema. However, both agents demonstrated significant anti-inflammatory activity in the formalin-induced paw edema model, particularly at the third hour post-induction ($p<0.05$). Diclofenac showed significant inhibition across both models. Furthermore, the extract and NAC enhanced erythrocyte membrane stability, suggesting protective effects against hypotonic-induced hemolysis. *Vigna subterranea* methanol seed extract and N-acetylcysteine exhibit significant anti-inflammatory effects in the formalin-induced paw edema model and demonstrate membrane-stabilizing activities. These findings suggest a possible role in modulating subacute inflammation and preserving membrane integrity.

Keywords: *Vigna subterranea*; N-acetylcysteine; paw edema; inflammation; membrane stability

INTRODUCTION

Inflammation is an important basic pathological process in trauma, infection, and diseases of internal organs such as furuncle, carbuncle, pneumonia, gastritis, hepatitis, and nephritis. It is not only an adaptive response of the body but also a protective process of the body, including the immune system. It prevents damaging factors from spreading while repairing the damaged tissue[1]. Inflammatory responses can help in healing but can also cause harm if it persists[2]. Drugs used to manage inflammation, including the non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, are effective, the adverse effects associated with their long-term use remain a concern in public health. Therefore, continual bioprospecting of effective, safer, and more affordable anti-inflammatory drugs is essential.

Drug discovery from natural products, especially plant materials, is historical. Important drugs such as quinine, morphine, digoxin, and artemisinin are sourced from plant materials. *Vigna Subterranea* (Bambara groundnut) is a grain legume crop indigenous to Africa and exceptionally rich in nutrition. It is regarded as an orphan crop representing a neglected and under- researched plant genetic resource[2]. The plant is widely cultivated in northern Nigeria and used traditionally for managing various disease conditions and symptoms, including pain and inflammation[3]. Its seed consists of 49%-63.5% carbohydrate, 15%-25% protein, 4.5%-7.4% fat, 5.2%-6.4% fiber, 3.2%-4.4% ash, and 2% mineral, compared to whole fresh cow milk: 88% moisture,

4.8% carbohydrate, 3.2% protein, 3.4% fat, 0.7% ash, and 0.01% cholesterol[4]. The secondary metabolites contained in Bambara groundnut may exert anti-inflammatory activity through modulation of various signaling pathways, including inhibition of cyclooxygenase (COX) and lipoxygenase enzymes[5]. In particular, the polyphenolic compounds flavonoids and saponins have shown significant anti-inflammatory activity in several experimental models[6]. This may be attributed to inhibition of pro-inflammatory mediators such as cytokines and prostaglandins[7]. *Vigna subterranea* is widely cultivated in sub-Saharan Africa, including northern Nigeria, and therefore can provide a sustainable source of pharmaceutical raw materials and phytochemicals[8]. However, limited studies exist on the anti-inflammatory activity of *Vigna subterranea.*
Oxidative stress and erythrocyte fragility are essential mechanisms in the pathophysiology of inflammation. Inflammation is accompanied by the generation of reactive oxygen species, which promote lipid peroxidation and impair membrane stability. N-Acetylcysteine is the acetylated variant of the amino acid L-cysteine and a precursor of glutathione, the body's major antioxidant. It is widely used as the specific antidote for acetaminophen overdose, prevention of chronic obstructive pulmonary disease exacerbation, prevention of contrast-induced kidney damage during imaging procedures, attenuation of illness from the influenza virus when started before infection, treatment of pulmonary fibrosis, and treatment of infertility in patients with clomiphene-resistant polycystic ovary syndrome[9]. N-Acetylcysteine enhances the body's antioxidant and nitric oxide systems during stress, infections, toxic assault, and inflammatory conditions[10]. Combined efficacy of NAC and plant-based bioactive in reducing inflammation has not been extensively studied.

Therefore, this study aims to investigate possible synergistic effects of Methanol Seed Extract of *Vigna Subterranea* (MEVS) and NAC to modulate inflammatory responses using carrageenan- and formalin-induced paw edema models in rats. Furthermore, the study evaluated the involvement of erythrocyte membrane stability as a possible mechanism of the inflammatory activity of the test agents.

MATERIALS AND METHODS

Plant collection, authentication and extraction

The seeds of *Vigna subterranea* were collected during the harvesting season from Kwami Village Plantation in Kwami Local Government of Gombe State, Nigeria. It was identified and authenticated by the plant taxonomist in the Department of Pharmacognosy and Drug Development, Gombe State University, where a specimen was submitted. About 5 kg of the deshelled seeds were dried in a ventilated room (at 27 °C) for 25 days and ground into fine pieces using a mortar and pestle.

Furthermore, 4.22 kg of the seed material was macerated in 3.7 liters of 70% methanol for a period of 72 hours with regular shaking and turning. The extraction process was repeated 2 times for exhaustive extraction. The methanol extract was filtered and concentrated by air drying at 27 °C for 10 days. The choice of methanol for the extraction is informed by its high extraction efficiency for polar and moderately polar phytochemicals. Furthermore, the methanol was completely evaporated before administration to the experimental animals. The percentage yield of the methanol extract was determined; the extract was stored in clean containers at room temperature until needed for experimental procedures.

The percentage yield of the methanol extract was calculated as follows:

$$\text{Percentage yield} = \frac{\text{Weight of extract (g)}}{\text{weight of dry powder of seed (g)}} \times 100$$

$$\frac{98.4}{4218.1} \times 100 = 2.33\%$$

Apparatus and equipment

Weighing machine, permanent markers, tape, syringe, gloves, Winchester bottle, white thread, transparent ruler, centrifuge, Jenway 6051 colorimeter, distiller.

Drugs

N –acetylcysteine, diclofenac manufactured by Martindale, USA.

Experimental animals, groupings and dosing

All experimental animals were obtained from the animal house, Human Physiology Department, Gombe State University. They were allowed to acclimatize for two weeks and fed with standard pellets and clean water *ad libitum*. The animals were housed in plastic cages with wooden shavings as bedding, at room temperature under standard environmental conditions (12 hours light/dark cycle), and received a standard rodent diet (Lives Feed Plc, Gombe, Nigeria) and drinking water *ad libitum*. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. In addition, the protocol of the study was approved by the Ethics Committee of the Faculty of Basic and Allied Medical Sciences, Gombe State University (GSU/COMS/FBMAS/R&EC/24/P016). Doses were selected based on our preliminary findings from an acute toxicity study.

A total of 24 Wistar rats (male) with an average weight of 180 g (for paw edema models) and 12 female Swiss mice of average weight 25-35 g (for acute toxicity) were used for the study. Rats were used for the edema models because their larger paws allow more precise measurement of diameter, while mice were used for the acute toxicity study because they are easy to handle, more affordable, and widely accepted as toxicity models.

For the edema models, the rats were randomly assigned into six groups (n=4).

Group 1 (negative control): 10 mg/kg normal saline
Group 2,3,4 and 5 (test groups): given 200, 400 mg/kg *Vigna Subterranea* extract (MVES) alone, 400 mg N-Acetylcysteine alone, and 400 mg/kg N-Acetylcysteine + 200 mg/kg MVES, respectively.
The group 400 mg/kg N-Acetylcysteine + 200 mg/kg MVES was included to evaluate possible enhancement of the submaximal dose of the extract by a pharmacologically active dose of NAC. Hence, this allows investigation of the possible synergistic effect of the combination.
Group 6 (positive control): Diclofenac 100 mg/kg

Acute toxicity test

The acute toxicity test, carried out intraperitoneally, was carried out using Lorke's method[12]. The study was conducted in two phases. In the first phase, nine healthy Swiss mice weighing between 25–35 g were randomized into three groups of three mice each. Group I received 10mg/kg of the extract, while groups II and III received 100 and 1000 mg/kg extract intraperitoneally, respectively. The mice were observed for symptoms of toxicity and mortality for twenty-four hours and then for 7 days following treatment. In the second phase, three mice were divided into three groups of one mouse each; the first received the extract at a dose of 1600 mg/kg while the second and third groups received 2900 and 5000mg/kg extract, respectively. The mice were also observed for 24hours and then for 6 days following treatment (no mortality recorded). The final LD_{50} was calculated as the square root of the product of the lowest lethal dose and the highest non-lethal dose, the geometric mean of consecutive doses for which 0 and 100% survival rates were recorded.

$$LD50 = \sqrt{D_o \times D_{100}}$$

Where D_0 is the highest tested dose with 0% mortality, and D_{100} is the lowest dose with 100% mortality.

Carrageenan-induced paw edema model

This test was carried out to evaluate the effect of MVES on acute inflammation induced by carrageenan[13]. Twenty-four male Wistar rats fasted for 12 h were randomly assigned to 6 groups (n=4).

One-hour post-treatment, 100 µL carrageenan 1% v/w in normal saline was subcutaneously injected into the right hind paw. Changes in paw circumference were recorded for 5 hours and at 24 h post-carrageenan injection[14]. Drugs and extracts were administered 1hr before the commencement of the experiment. Baseline diameter of the right paw was measured and recorded, then 0.1ml of carrageenan was injected in the plantar region of the animals. Then the diameter of the injected right paw was measured hourly for 5 hours and at hour 24 to evaluate the progression of the edema.

Formalin-induced paw edema model

After 48 hours of the carrageenan model and the paw edema volume was back to normal, the diameter of the left paws of the same 24 rats in 6 groups was recorded as the paw baseline diameter before injection of formalin 0.1 ml into each left paw. Thereafter, establishment and progression of edema was monitored hourly for 5 hours and at hour 24.

Then the percentage inhibition of edema was calculated by the following formula:

$$\text{Percentage Inhibition } \frac{(Ec-Et)}{Ec} \times 100$$

Ec= mean edema in control group;
Et= mean edema in treatment group

Edema E= D_t-D_0
D_t= diameter of paw after injection at a time t
D_0= diameter of paw before injection (at baseline)

Erythrocyte osmotic fragility test

The erythrocyte osmotic fragility test was carried out according to the method of Wintrobe *et al*. [15]. Graded hypotonic saline 0.1% to 1% solutions (5 mL) were placed in clean test tubes. A drop of blood sample by ocular collection from the experimental animal was then placed in each test tube and mixed with the containing solution. The samples were centrifuged using a Centurion Scientific Machine (C2 series) for five (5) minutes at a speed of 2000 revolutions per minute (RPM). It was allowed to settle for 15 min, and the hemolysis across decreasing NaCl concentrations (1%-0.1%) was measured using a Jenway 6051 Colorimeter. Results are expressed as fraction hemolysis.
The animals were euthanized by exposure to an excess dose of ether after the completion of experiments by terminal retro-orbital blood collection. This complies with the institutional animal ethics guidelines to avoid animal reusage for other experimental purposes.

Data analysis

All values were expressed as mean ± standard error of the mean. The data were analyzed using SPSS version 20. The analysis was carried out using one-way ANOVA followed by Tukey post hoc test. P value < 0.05 was considered statistically significant.

RESULTS

Percentage yield of methanol seed extract

The cold maceration of *Vigna subterranea* in methanol produced a dark brown gelatinous extract. The yield is 2.33%.

Acute toxicity (LD_{50}) study of methanol extract of *Vigna subterranea*

There was no mortality observed in any of the groups up to 5000 mg/kg of the extract; LD50 >5000 mg/kg (Table 1). In addition, no serious symptoms of toxicity were recorded.

Table 1: Acute Toxicity (LD_{50}) Study of Methanol Extract of *Vigna subterranea*

Phase/Group	Dose (mg/kg)	Mortality
	Phase I	
Group 1	10	0/3
Group 2	100	0/3
Group 3	1000	0/3
	Phase II	
Group 1	1600	0/1
Group 2	2600	0/1
Group 3	5000	0/1

Effects of methanol seed extract of *Vigna subterranea* and n-acetylcysteine on Carrageenan-induced paw edema in Wistar rats

The mean paw diameters of the experimental animals before induction of edema (baseline) with carrageenan ranged from 2.3 to 3.7 mm (Table 2). The responses to extract and NAC were generally not significant across the treatment groups at any time. There was low or no inhibition of edema across the treatment groups at all times, except for NAC (400 mg/Kg) + MEVS (200 mg/Kg), which reduced edema by 3.00±1.34 at the 4th hour (Table 2). However, diclofenac significantly reduced edema, especially at the 4th hour of the experiment (Figure 1).

Percentage inhibition of edema

Percentage inhibition was generally low in the treatment groups except for NAC (400 mg/Kg) + MEVS (200 mg/Kg), which exhibited a moderate 57.14% inhibition at the 4th hour (Table 3). However, diclofenac significantly reduced edema, especially at the 4th hour of the experiment (92.86% reduction).

Effects of methanol seed extract of *Vigna subterranea* and N-acetylcysteine on formalin-induced paw edema in Wistar rats

The mean baseline paw diameters of the rats are 2.30, 2.35, 2.55, 2.50, 2.55 and 2.50 mm, respectively, across the treatment groups (Table 4). The result of the percentage inhibition of edema by the *Vigna subterranea* extract and NAC post-induction by formalin revealed a significant reduction in paw edema especially at the 3rd hour of experiment (Figure 2). The percentage inhibitions by MEVS 200 mg/Kg, MEVS 400 mg/kg, NAC 400 mg/Kg, MEVS 200 mg/kg + NAC 400 mg/Kg and Diclofenac 100 mg/Kg are 44.44, 66.67, 55.56, 88.89 and 77.78 respectively (Table 5). In addition, diclofenac sustained a 100% inhibition at hour 5 (Table 5).

Effect of methanol seed extract of *Vigna subterranea* and N-acetylcysteine on erythrocytes osmotic fragility

The study revealed a descending order of hemolysis in the normal control group at 0.1-0.5% tonicity (hypotonic); 0.95±0.00, 0.85±0.25, 0.60±0.29, 0.50±0.00 and 0.42±0.00 (Table 6). Similarly, in the positive control group (Diclofenac), complete hemolysis was recorded at these tonicities except at 0.3% (0.66±0.33). NAC (400 mg/kg) provided almost complete membrane stability in the hypotonic media except at 0.2% (0.25±0.25). Both doses of the test extract (200 mg/kg and 400 mg/kg) provided moderate membrane stability at the hypotonic percentages of NaCl (0.25 and 0.5), except at 0.5% (Table 6), when the 400 mg/kg MEVS lost its membrane protective activity (1.0). However, the NAC (400 mg/kg) + MEVS (200 mg/kg) combination exhibited low membrane protective activity (0.77, 0.75, 0.75 and 0.50) except at 0.5%, which gave 0.25 hemolysis (Table 6).

Table 2: Effects of methanol seed extract of *Vigna subterranea* (mevs) and n-acetyl cysteine on Carrageenan-induced paw edema in Wistar rats

	Mean paw diameters at specific times						
Treatment	Baseline (mm)	1hr	2hr	3hr	4hr	5hr	24hr
NS (10 mg/Kg)	2.3	2.50±0.35	3.00±1.35	2.75±0.45	3.00±0.55	2.50±0.25	2.00±0.16
MEVS (200 mg/Kg)	2.35	2.50±0.24	2.75±0.25	2.60±0.25	2.90±0.25	2.50±0.25	2.00±0.21
MEVS (400 mg/Kg)	2.65	2.80±1.25	3.00±0.25	3.00±0.25	3.25±0.25	3.00±0.25	2.00±2.34
NAC (400 mg/Kg)	2.55	3.00±0.15	3.00±0.25	3.00±0.25	3.00±0.25	2.75±0.25	2.15±0.25
NAC (400 mg/Kg) + MEVS (200 mg/Kg)	2.7	3.00±0.23	3.00±0.53	3.00±0.19	3.00±1.34	2.85±0.38	2.25±0.20
DCL (100 mg/Kg)	3.1	3.15±0.25	3.20±0.35	3.25±0.50	3.15±0.15	3.15±0.10	2.85±0.32

† Values are expressed as mean ± S.E.M
MEVS: methanol seed extract of *Vigna subterranea*; NAC: N-acetylcysteine; DCL: Diclofenac

Table 3: Percentage inhibition of edema by *Vigna subterranea* and NAC in Carrageenan model

	% Inhibition at specific times					
Treatment (mg/Kg)	1hr	2hr	3hr	4hr	5hr	24hr
MEVS (200 mg/kg)	25	42.86	44.44	21.43	25	-16.67
MEVS (400 mg/kg)	25	50	22.22	14.29	-75	-116.67
NAC (400 mg/Kg)	-125	35.71	0	35.71	0	-33.33
NAC (400 mg/Kg) + MEVS (200 mg/Kg)	-50	57.14	33.33	57.14	25	-50
DCL (100mg/Kg)	75	85.71	66.67	92.86	75	16.67

† MEVS: methanol seed extract of *Vigna subterranea*; NAC: N-acetylcysteine; DCL: Diclofenac

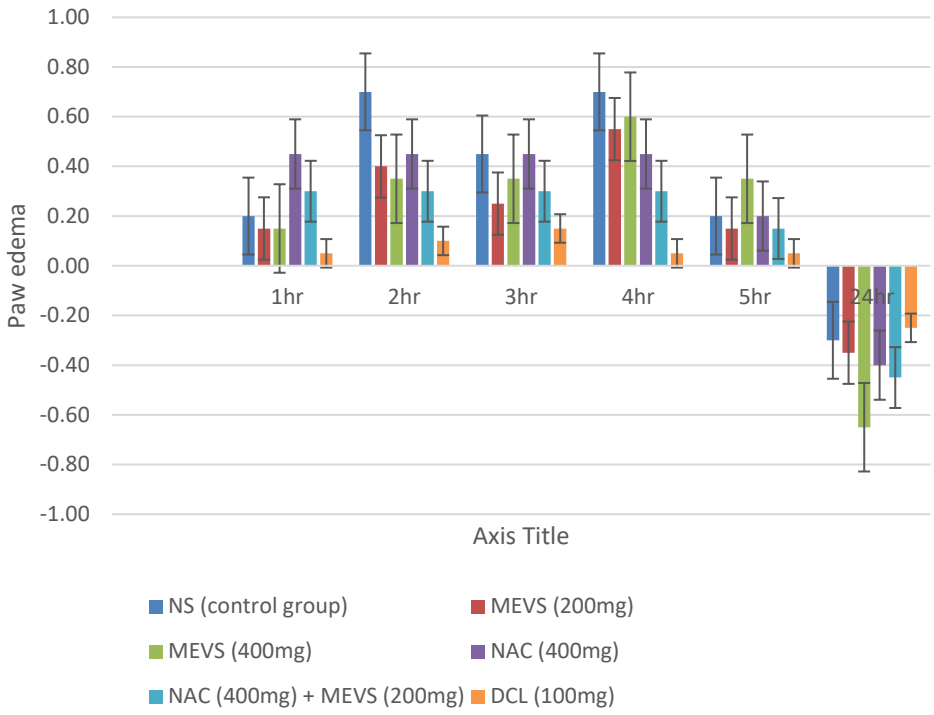

Figure 1: Effect of *Vigna subterranea* and NAC on Carrageenan-induced paw edema

Table 4: Effect of *Vigna subterranea* and NAC on paw diameter in formalin edema model

	Paw diameters at specific times in formalin-induced paw edema model						
Treatment (mg)	Baseline paw diameter (mm)	1hr	2hr	3hr	4hr	5hr	24hr
NS (control group)	2.3	2.5	3	2.75	3	2.5	2
MEVS (200mg)	2.35	2.5	2.75	2.6	2.9	2.5	2
MEVS (400mg)	2.55	3	3.2	3	3	3	2
NAC (400mg)	2.5	3.3	3	3	3	2.75	2.5
NAC (400mg) + MEVS (200mg)	2.55	3	3	2.7	3	2.7	2.25
DCL (100mg)	2.5	3	3	2.6	2.75	2.5	2

† Values are expressed as mean ± S.E.M

MEVS: methanol seed extract of *Vigna subterranea*; NAC: N-acetylcysteine; DCL: Diclofenac

Table 5: Percentage inhibition of edema by *Vigna subterranea* and NAC in formalin model

	% Inhibition at specific times in formalin-induced paw edema model					
Treatment (mg)	1hr	2hr	3hr	4hr	5hr	24hr
MEVS (200mg)	25	42.86	44.44	21.43	25	-16.67
MEVS (400mg)	25	50	66.67	50	25	-183.33
NAC (400mg)	-50	42.86	55.56	42.86	25	66.67
NAC (400mg) + MEVS (200mg)	-75	50	88.89	50	75	-33.33
DCL (100mg)	75	42.86	77.78	64.29	100	-66.67

† MEVS: methanol seed extract of *Vigna subterranea*; NAC: N-acetylcysteine; DCL: Diclofenac

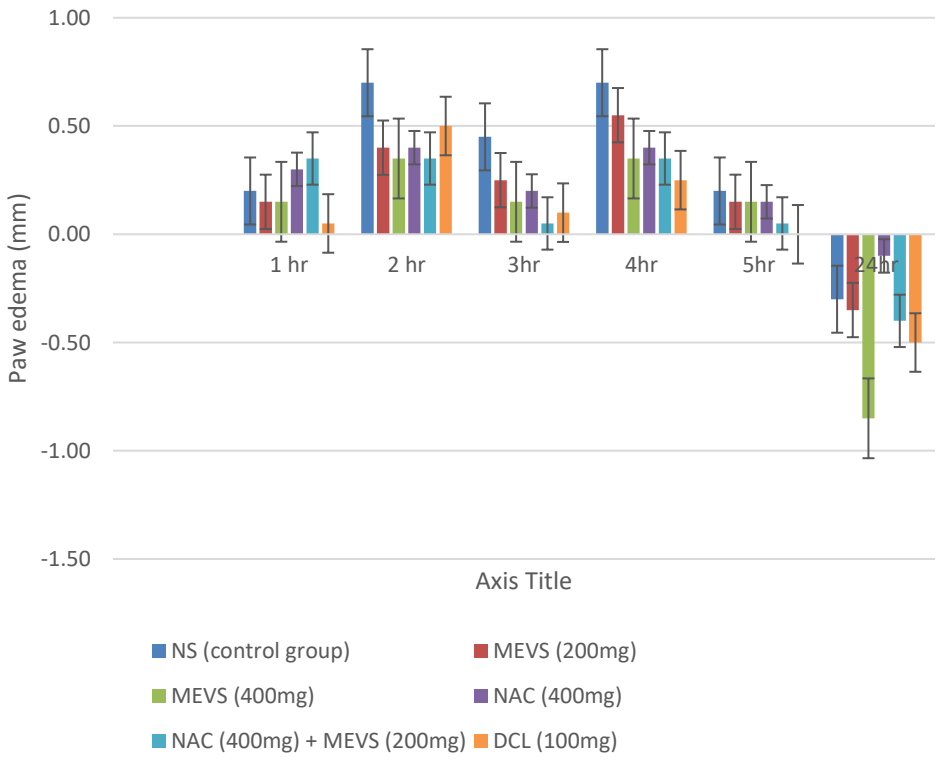

Figure 2: Effect of *Vigna subterranea* and NAC in formalin-induced paw edema model

Table 6: Effect of Methanol Seed Extract of *Vigna subterranea* and N-Acetylcysteine on Erythrocyte Osmotic Fragility

GROUPS (mg/Kg)	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**	**0.7%**	**0.8%**	**0.9%**	**1.0%**
NS (10)	0.95±0.00	0.85±0.25	0.60±0.29	0.50±0.00	0.42±0.00	0.37±0.00	0.35±0.25	0.25±0.29	0.20±0.29	0.0±0.25
MEVS (200)	0.50±0.29	0.25±0.25	0.25±0.25	0.25±0.25	0.50±0.29	0.50±0.29	0.75±0.25	0.75±0.25	0.75±0.25	0.75±0.25
MEVS (400)	0.25±0.25	0.25±0.25	0.25±0.25	0.50±0.29	1.00±0.00	0.75±0.25	1.00±0.00	0.50±0.29	0.75±0.25	1.00±0.00
NAC (400)	0.00±0.00	0.25±0.25	0.00±0.00	0.00±0.00	0.00±0.00	0.25±0.25	0.00±0.00	0.25±0.25	0.25±0.25	0.25±0.25
NAC (400) + MEVS (200)	0.77±0.29	0.75±0.29	0.75±0.25	0.50±0.29	0.25±0.25	0.50±0.29	0.4±0.25	0.37±0.29	0.35±0.29	0.25±0.25
DCL (100)	1.00±0.00	1.00±0.00	0.66±0.33	1.00±0.00	1.00±0.00	0.66±0.33	0.60±0.00	0.53±0.33	0.50±0.00	0.50±0.00

DISCUSSION

Inflammation is a complex biological response triggered by injurious stimuli, including pathogens, damaged cells, and irritants. It aims to protect the body from further injury[16]. Paw edema models in rodents are widely accepted protocols for studying inflammation[17]. This study evaluated the modulatory role of *Vigna subterranean* and N-Acetylcysteine (NAC) in carrageenan- and formalin-induced paw edema models of inflammation in Wistar rats. The results showed varying degrees of significance across the treatment groups and models. The effects of the test extract and NAC on the Osmotic Fragility Test also provide insight into the potential of the interventions to cause membrane stabilization.

The carrageenan-induced paw edema is mediated by histamine, serotonin, bradykinin, and prostaglandins. In this study, the lack of significant difference in the paw edema inhibition among treatment groups for the carrageenan-induced edema model is in contrast to the findings of Oyeyinka *et al*.[18]. The variation may be attributed to differences in dosage, method of extraction, or bioavailability. While Oyeyinka *et al*. tested medium to high doses of carrageenan, the current study used medium doses of the extract. Similarly, it may indicate that the extract possesses relatively weak activity against the acute inflammatory mediators in the carrageenan-induced edema or that the administered doses were insufficient to suppress the cascade of mediator release. Similar observations have been reported for several plant extracts that demonstrated modest or delayed anti-inflammatory effects in carrageenan-induced inflammation despite possessing antioxidant and membrane-stabilizing properties. In another study, N-Acetylcysteine decreased edema and neutrophil infiltration in the carrageenan model. N-Acetylcysteine decreases pleural fluid and lung inflammation, and it enhances the anti-inflammatory effect of diclofenac in carrageenan-induced inflammation[18]. The early phase of carrageenan-induced inflammation in rat paw (0–2 h) is primarily mediated by the release of histamine, serotonin, and bradykinin, while the late phase (2–6 h) is associated predominantly with prostaglandins, nitric oxide, lysosomal enzymes, cytokines, and neutrophil infiltration.

The results of our study suggest that the synergistic anti-inflammatory effect of the methanol extract of *Vigna subterranea* may not be revealed at the early phase of inflammation. This is clearly indicated by the low percentage inhibitions exhibited by the NAC and Methanolic extract of Vigna subterranea at the early hour of both models.

However, at later hours, Vigna subterranea treatment alone and in combination with NAC exhibited the highest anti-inflammatory potential in both carrageenan- and formalin-induced paw edema. This could be due to the synergistic potential of the combined treatment in the study. This is in agreement with studies of Elberry *et al*.[19] who reported the anti-inflammatory potential of NAC in formalin-induced paw edema by significantly decreasing paw edema thickness through modulation of serum nitric oxide synthase, C-reactive protein, cyclooxygenase-2 levels, IL-1β, TNFα, IL-8, IL-6, IL-10, and NF-κB p65[19-20]. While studies on the anti-inflammatory potential of NAC have demonstrated the modulation of oxidative stress and inflammatory pathways[20], there is no study on the anti-inflammatory potential of Vigna *subterranea* in the literature, demonstrating the novelty of the current study and the need to further investigate the anti-inflammatory potential of the extract.

The activity shown at the 24th hour may not essentially be due to the extract but rather an indication of the normal resolution of acute inflammation. It may suggest a selective activity against mediators involved in the later phase of inflammation. The plant extract might contain phytochemicals that have a long onset and short duration of anti-inflammatory action.

The formalin test assesses both neurogenic and inflammatory pain responses. Formalin-induced inflammation is characterized by tissue-mediated inflammatory responses involving histamine, serotonin, prostaglandins, bradykinin, and leukocyte infiltration, with the late phase being particularly associated with cyclooxygenase products and oxidative stress. Therefore, the observed activity at the third hour may indicate interference with prostaglandin synthesis, cytokine production, or reactive oxygen species generation. This finding suggests that the anti-inflammatory activity of the extract may be more pronounced in sustained or subacute inflammatory conditions rather than in the immediate acute phase.

The anti-inflammatory effect observed may be attributed to the phytochemical constituents present in *Vigna subterranea* seeds. The methanolic seed extract of Vigna Subterranea has been reported to contain higher flavanol and flavonol content as well as higher *in vitro* antioxidant activities[21]. Phenolic compounds and flavonoids are capable of inhibiting cyclooxygenase and lipoxygenase pathways, scavenging reactive oxygen species, suppressing pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins, and stabilizing lysosomal membranes[22]. These mechanisms may collectively contribute to the attenuation of edema formation observed in the present study.

The membrane stabilization demonstrated by the extract and N-Acetylcysteine in the osmotic fragility test further supports their anti-inflammatory potential. Stabilization of erythrocyte membranes is considered an indicator of stabilization of lysosomal membranes because both membranes share structural similarities. During inflammation, destabilization of lysosomal membranes leads to the release of proteolytic enzymes and inflammatory mediators that aggravate tissue

injury. Therefore, agents capable of preventing membrane lysis may reduce inflammatory tissue damage by limiting the release of lysosomal constituents. The protective effect observed in the osmotic fragility assay suggests that the extract may preserve membrane integrity under stress conditions, thereby contributing to its anti-inflammatory activity.

In addition, the activity of N-Acetylcysteine observed in this study may be related to its well-established antioxidant properties. N-acetylcysteine acts as a precursor for glutathione synthesis and directly scavenges reactive oxygen species, thereby reducing oxidative stress and inflammatory signaling[23]. Previous studies have shown that N-acetylcysteine suppresses activation of nuclear factor kappa B (NF-κB), decreases production of inflammatory cytokines, and attenuates edema formation in experimental inflammation[23]. The combined treatment group containing N-acetylcysteine and the extract may therefore have exerted synergistic or additive antioxidant and membrane-stabilizing effects, although this effect was not sufficiently pronounced in the carrageenan model.

The discrepancy between the outcomes of the carrageenan and formalin models may also reflect differences in the mechanisms underlying the two models. While carrageenan-induced inflammation is predominantly acute and exudative, formalin-induced inflammation involves more complex neurogenic and tissue-mediated inflammatory processes. Previous studies reported that NAC administration decreased paw thickness and neutrophil infiltration through inhibition of cyclooxygenase products such as prostaglandin, further buttressing the anti-inflammatory effects of NAC[24]. The pharmacological activity reported for *Vigna subterranea* in the current study is consistent with our earlier research on the plant, where we investigated both the central and peripheral analgesic activities of the methanol seed extract[4]. The methanol seed extract of *Vigna subterranea* (MEVS) reduced central and peripheral nociception[4] and inhibited writhing in a dose-dependent manner[4].

Erythrocyte osmotic fragility assesses red blood cell (RBC) membrane stability, which can be affected by oxidative stress and inflammatory mediators. The study found that *Vigna subterranean* and NAC may enhance RBC membrane stability, with the strongest membrane-stabilizing effect preventing hemolysis exhibited by NAC. This is in agreement with a recent study by Eligini *et al*.[25]. who found that NAC preserved albumin redox status, limited free haemoglobin accumulation, attenuated storage-induced proteomic changes and preserved RBC osmotic and rheological properties related to osmotic fragility and hydration[25]. This further supported the antioxidant and anti-inflammatory potentials of NAC as reported in numerous studies[19-20]. Moreover, the effect of the methanolic seed extract of Vigna subterranea was also in collaboration with the finding of Anorue *et al*.[26]. who found that *Vigna subterranea* extract reversed the sickled cells, reduced the rate of polymerization, maintained RBC membrane stability, increased beta globin synthesis, and increased the oxy-haemoglobin concentration in sickle cell beta thalassemia[26].

The significant inhibition observed in formalin-induced edema and the protection against erythrocyte membrane lysis support the potential therapeutic value of the extract in inflammatory conditions associated with oxidative stress and membrane destabilization. Further studies involving isolation of active constituents, cytokine analysis, antioxidant enzyme assays, and molecular pathway investigations are recommended to elucidate the precise mechanisms responsible for the observed pharmacological effects.

Conclusion

Based on the findings of the current study, we surmise that *Vigna subterranea* and NAC combinations may possess a synergistic anti-inflammatory activity. Furthermore, *Vigna subterranea* may demonstrate higher activity in sub-acute inflammatory conditions rather than acute inflammation.

Conflict of interest

'The Authors have no conflicts of interests to declare'

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Authors' contributions: MMS developed the concept, supervised the models and writing, analyzed the data. OO prepared the extract, helped in experimental procedures and interpreted the data. FA and ZY conducted the laboratory work. ASI supervised writing of the manuscript and interpretation of data.

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