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Original research article

Preclinical investigation on the antinociceptive, antipyretic, anti-inflammatory and antioxidant activities of *Ananas comosus* leaf extract

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ABSTRACT

Ananas comosus is a well-known medicinal plant used in traditional medicine for various therapeutic purposes, including the treatment of pain, fever and inflammation. This study aimed to evaluate the antinociceptive, anti-inflammatory, antipyretic and antioxidant activities of the ethanolic leaf extract of *A. comosus* and the mechanism by which it produces its antinociceptive effects in mice. The antinociceptive activity of ethanolic leaf extract of *A. comosus* was evaluated using an acetic acid-induced writhing test and a formalin-induced pain test. The mechanism of anti-nociception was determined by pretreating mice with various antagonists (naloxone, glibenclamide, atropine, nifedipine and theophyline) before administration of the extract (800 mg/kg) during the acetic acid-induced writhing test. The antipyretic effect was evaluated using yeast-induced pyrexia in mice, while formalin-induced inflammation was used to evaluate the anti-inflammatory effects. The extract demonstrated potent antinociceptive activity compared to the control group in the acetic acid-induced writhing test and in the formalin-induced pain models. Naloxone, glibenclamide, atropine and nifedipine did not block the antinociceptive activity. However, theophylline significantly reversed the antinociceptive effect in the acetic acid-induced writhing test. The extract also showed antipyretic and anti-inflammatory effects, while the results of the antioxidant study showed that it has high antioxidant properties. The study results showed that the extract has potent antinociceptive, antipyretic, anti-inflammatory and antioxidant effects.

Keywords: *Ananas comosus*, antinociceptive, antipyretic, anti-inflammatory, antioxidant

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1. Introduction

Pain management is one of the world's greatest public health challenges. The unpleasant emotional and sensory experience associated with or similar to potential or actual tissue damage is commonly referred to as pain.[1] Inflammation is a normal physiological response of mammalian tissue to the presence of microorganisms or other factors that result in tissue damage. During this process, blood cells and plasma accumulate in the affected area, causing local edema. Inflammatory mediators are normally released in the body due to a cascade of processes that result in stimulation of mast cells, which activate nociceptors and cause pain in the body.[2] Activation of G protein-coupled receptors also plays a role in the development of inflammation, pain and fever.[3] Fever is a physiological sign primarily associated with a sustained increase in body temperature during periods of fatigue, lethargy, and anorexia.[4] Nonsteroidal anti-inflammatory drugs (NSAIDs) are currently the most commonly used agents to treat fever, pain, and inflammation. However, the undesirable effects of these medications, such as ulcers and other associated side effects have been a cause of great concern regarding their use in recent years.[5] Therefore, there is a need to develop new active ingredients with negligible harmful effects. Medicinal plants and their bioactive derivatives have long been used in traditional medicine worldwide for prophylaxis and treatment of various diseases.[6] In addition, medical chronicles have shown that medicinal plants are among the main sources of orthodox medicines currently used in modern medicine. *Ananas comosus* is a well-known monocotyledonous perennial medicinal plant from the Bromeliaceae family.[7] It is used in traditional Nigerian medicine for various therapeutic purposes, including the treatment of diarrhea and skin rashes as an antibacterial as well as a hemostatic, analgesic and antipyretic agent. It is also used to treat arthritis, burns, and abortion.[8] Recent studies have revealed the possible antioxidant potential of *A. comosus* which may play a significant role in some of its therapeutic effects. Antioxidants are compounds known for their ability to prevent oxidative stress by neutralizing free radicals which are implicated in various diseases and inflammatory processes.[9] Antioxidants not only help in mitigating oxidative damage but also enhance the overall efficacy of anti-inflammatory treatments by potentially reducing inflammation related pain. However, it is important to scientifically evaluate the therapeutic potential recognized in traditional medicine. Therefore, this study aimed to evaluate the antinociceptive, anti-inflammatory, antipyretic and antioxidant activities of the ethanolic leaf extract of *A. comosus* and the mechanism by which it produces its antinociceptive effects.

2. Materials and Methods

2.1 Plant material

Fresh leaves of fully grown *A. comosus* plants (about 13 months old, cultivated from May 2022 to June 2023) were harvested from a pineapple farm plantation in Oton community in Sapele Local Government Area, Delta State, Nigeria. The harvested plant leaves were then separated from possible impurities. They were rinsed under running water to remove dirt. The plant species was verified by Mr. Jeffery Azilla, a botanist at the Herbarium Unit, Federal College of Forestry, Jos.

2.2 Extraction

The plant leaves were air-dried (at room temperature) for four weeks, and pulverized into fine powder using an electric motorized grinder. The pulverized plant material was macerated in 70% ethanol using standard procedures previously described by Handa et al.[10] The pulverized leaves were completely macerated in 70% ethanol at room temperature for 72 h with periodical shaking to ensure complete interaction of menstruum and pulverized plant. Thereafter, the micelle solution was then firstly filtered through a mesh sieve, then through a cotton plugged funnel and lastly through Whatman filter paper No. 1. The collected filtrate was concentrated using a water bath set at 40°C.

2.3 Phytochemical analysis

Qualitative phytochemical screening was conducted to identify the bioactive chemicals present in *A. comosus*. Tests for various phytochemicals including alkaloids, saponins, tannins, flavonoids and phenols were carried out using standard methods outlined by Sofowora[11] and Harborne.[12]

2.4 Drugs and chemicals

The following drugs and chemicals were used: acetic acid (BDH, Poole, UK), aspirin (Prestige Brands, USA); formalin (Balaji Formalin Pvt. Ltd., India); dihydrocodeine (Accord Pharmaceuticals, Nepal); diclofenac (Embassy Pharmaceutical and Chemicals Ltd., Nigeria); glibenclamide (Sygen Pharmaceuticals Ltd., Nigeria); nifedipine (Unicure Pharmaceutical Co. Ltd., Nigeria); atropine (Ancalima Life-sciences Ltd., India); naloxone (Jackson Laboratories Pvt. Ltd., India); theophylline (Eminent Drugs and Pharmaceutical Ltd., Nigeria). All the drugs used were of high standard grade and solutions from these were freshly prepared daily before use. All treatments used for the study were prepared using normal saline as solvent.

2.5 Animals

Both sexes of Swiss albino mice (weight range 20-28 g) housed at the animal facility of the Department of Pharmacology, Novena University were used for the study. The animals were housed in mouse cages, under standard laboratory conditions of room temperature and humidity, with free access to water and mice feed. Animal studies were performed in conformity with the "NIH revised guidelines for laboratory animal care and use"[13] and Novena University ethical codes and regulations for laboratory animal use.

2.6 Evaluation of antinociceptive effects

2.6.1 Acetic acid-induced writhing test

Acetic acid-induced writhing tests were carried out using the method described previously by Woode et al.[14] Twenty-five swiss albino mice of either sex were used for the study. They were randomly assigned to five groups of five animals each. The animals were pre-treated orally with the vehicle, extract or standard drug. Group 1 (control) was administered 10 ml/kg normal saline (vehicle), groups 2-4 received 200, 400 and 800 mg/kg *A. comosus* extract, respectively, while group 5 received 150 mg/kg aspirin. One hour after the respective administrations, the animals were injected with acetic acid (0.6%, 0.1 ml/10 g body weight *i.p*). The mice were placed in individual cages and the number of writhes was counted for each mouse for 15 min after a 5-min latency period. The percentage inhibition of writhes was calculated using the following formula:

% inhibition = [(Mean number of writhes control - Mean number of writhes treated)/ Mean number of writhes control] × 100

2.6.2 Formalin-induced pain

Formalin testing was carried out according to the method of Hunskaar and Hole.[15] Thirty Swiss albino mice of both sexes randomly assigned to six groups of five animals each were used for the study. The animals were pre-treated orally with the vehicle (normal saline), extract or standard drugs. Group 1 (control) was administered 10 ml/kg normal saline, groups 2-4 received 200, 400 and 800 mg/kg *A. comosus* extract, respectively, group 5 received 30 mg/kg dihydrocodeine, while group 6 received 10 mg/kg diclofenac. One hour after the respective administrations, each mouse was injected with 0.02 ml of 2.7% formalin into the right hind paw. The animals were placed individually in transparent cages and observed for 30 min after injection of formalin, and the amount of time (in seconds) spent licking and biting the injected hind paw was recorded as an indicator of nociceptive behaviour. The nociceptive scores were taken at 0-5 min (representing the early phase/ neurogenic pain) and 15-30 min (representing the late phase/ peripheral pain) after formalin injection, respectively.

2.6.3 Mechanism of antinociception

The acetic acid-induced writhing test was employed for determining the possible involvement of certain pathways in the process

by which *A. comosus* ethanolic extract elicits its anti-nociceptive effect. Thirty-five mice of either sex were randomly assigned to seven groups of five mice each. The animals were pre-treated with five different inhibitors/ antagonists: naloxone (a nonselective opioid receptor antagonist), glibenclamide (an ATP sensitive potassium channel inhibitor), atropine (a nonselective muscarinic receptor antagonist), nifedipine (L-type voltage-gated calcium channel blocker) and theophylline (a non-selective adenosine receptor antagonist). The mice in the test groups (groups 2–6) were pre-treated with these antagonists 15 min (*i.p.*) and 30 min (*p.o.*) before the administration of *A. comosus* extract (800 mg/kg *p.o.*). The animals in groups 1 and 7 served as negative and positive controls and were not pretreated with any of the antagonists but were treated with normal saline (10 ml/kg *p.o.*) and aspirin (150 mg/kg), respectively. One hour after the administration of normal saline, aspirin or extract, the animals were administered acetic acid (0.6% 0.1 ml/10 g body weight *i.p.*). The mice were placed in individual cages five minutes after acetic acid administration and the number of writhes was counted for each mouse for 15 min after a 5-min latency period. The percentage inhibition of writhes was calculated using the following formula:

% inhibition = [(Mean number of writhes control – Mean number of writhes treated)/ Mean number of writhes control] × 100

2.6.3.1 Involvement of the opioid system

Experimental animals in group 2 were pre-treated with naloxone (2 mg/kg, *i.p.*) 15 min before the administration of *A. comosus* extract (800 mg/kg, *p.o.*) and their nociceptive responses were observed.

2.6.3.2 Involvement of ATP-sensitive K^+ channels

Experimental animals in group 3 were pre-treated with glibenclamide (8 mg/kg, *p.o.*) 30 min before the administration of *A. comosus extract* (800 mg/kg, *p.o.*) and their nociceptive responses were observed.

2.6.3.3 Involvement of the muscarinic cholinergic system

Experimental animals in group 4 were pre-treated with atropine (5 mg/kg, *i.p.*) 15 min before the administration of *A. comosus extract* (800 mg/kg, *p.o.*) and their nociceptive responses were observed.

2.6.3.4 Involvement of voltage-gated calcium channels

Animals in group 5 were pre-treated with nifedipine (10 mg/kg, *p.o.*) 30 min before the administration of *A. comosus extract* (800 mg/kg, *p.o.*) and their nociceptive responses were observed.

2.6.3.5 Involvement of the adenosinergic system

Group 5 animals were pre-treated with theophylline (10 mg/kg, *p.o.*) 30 min before the administration of *A. comosus extract* (800 mg/kg, *p.o.*) and their nociceptive responses were recorded.

2.7 Evaluation of antipyretic effects

The antipyretic activity of the plant extract was evaluated using Brewer's yeast induced pyrexia as described by Sobeh *et al*.[16] Twenty-five Swiss albino mice were used for the study. The baseline rectal temperature was taken for each animal using a digital thermometer. Then, fever was induced by administering aqueous suspension of Brewer's yeast in normal saline (30% w/v) subcutaneously (10 ml/kg) to each mouse. After 18 h of yeast suspension administration, the rectal temperature of the animals was taken again to verify development of fever. The criterion for inclusion was an elevation of rectal temperature by 0.5°C and above. The animals were randomly assigned to five groups of five animals each and were treated orally with the vehicle (normal saline), extract or standard drug. Group 1 was administered 10 ml/kg normal saline, groups 2-4 received 200, 400 and 800 mg/kg *A. comosus* extract doses, respectively, while group 5 received 150 mg/kg paracetamol. The post-treatment rectal temperatures of each mouse were taken at 0.5, 1, 1.5, 2, 2.5 and 3 h, respectively, after treatment.

2.8 Evaluation of anti-inflammatory effects

The formalin-induced inflammation test was carried out using the method described previously by Malmberg and Yalsh.[17] Twenty-five Swiss albino mice of both sexes were used for the study. Initial paw volume was measured using a plethysmometer (Ugo Basile® 37140), this served as the baseline. The animals were randomly assigned to five groups of five animals each. The animals were pre-treated orally with the vehicle, extract or standard drug. Group 1 (negative control) was administered normal 10 ml/kg saline, groups 2-4 received 200, 400 and 800 mg/kg *A. comosus* extract, respectively, while group 5 received 10 mg/kg diclofenac. One hour after the respective administrations, each mouse was injected with 0.02 ml of 2.7% formalin into the right hind paw. The paw edema was then measured up to the ankle and recorded 1, 2, 3, 4, 5 and 24 h post-treatment using a plethysmometer to determine the edema volume.

2.9 Evaluation of antioxidant activity

2.9.1 Determination of DPPH free radical scavenging activities

The free radical scavenging ability of the extract against DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical was estimated using the method described by Ursini et al.[18] The extract at different concentrations (100, 200, 300 and 400 µg/mL) was diluted with 3 ml ethanol and mixed with 3 ml DPPH solution. The resultant mixture was shaken and then incubated at room temperature in the dark for 30 min. The absorbance of the solution was measured against a blank at 517 nm. The concentrations were prepared in triplicates and the percentage inhibition of DPPH was calculated using following equation:

$$\% \text{ Inhibition} = [(A0 - A1)/A0] \times 100$$

Where A0 is the absorbance of the blank sample and A1 is the absorbance of the tested sample.

2.9.2 Total flavonoid assay

Total flavonoid content was determined using the method described by Ebrahimzadeh et al.[19] Five millilitres (5 ml) of 2% aluminium (III) chloride ($AlCl_3$) in methanol was mixed with 5 ml of different concentrations of the extract (100, 200, 300 and 400 µg/mL). Absorbance was read at 415 nm after 10 min against a blank sample consisting of 5 ml extract solution with 5 ml methanol without $AlCl_3$. The total flavonoid content was calculated using a standard curve with rutin (0-100 mg/L) as the standard.

2.9.3 Total phenolic content

Total phenolic content was determined using the method described by Dewanto et al.[20] Zero point five millilitres (0.5 mL) of various concentrations (100, 200, 300 and 400 µg/mL) of *A. comosus* ethanolic extract was dissolved in 100 µl of Folin-Ciocalteau reagent and 6 ml of distilled water. The resultant solution was vortexed for 1 min and 2 ml of 15% Na_2CO_3 was added and the mixture vortexed again for 30 sec. The solution was made up to 10 ml with distilled water. After 1.5 h, the absorbance of the samples was read at 750 nm with a UV spectrophotometer. Gallic acid solution was used for the preparation of calibration curve. Total phenolic contents of samples were expressed as milligrams of gallic acid equivalent (mg GAE)/100 g of dry weight.

2.9.4 Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using the method described by Benzie and Strain.[21] The FRAP reagent was prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM TPTZ (tripyridyltriazine) solution and 20.0 mM $FeCl_3 \cdot 6H_2O$ solution in a ratio of 10:1:1 in volume. Then 0.5 ml of different concentrations (100, 200, 300 and 400 µg/mI) of the extract was added to 3 ml of FRAP reagent. The resultant mixture was incubated at 37°C for 30 min. The absorbance at 593 nm was then measured using a colorimeter.

$$\text{Ferric reducing antioxidant power} = (A1 - A2)/A1] \times 100$$

Where A1 is the absorbance of the control, and A2 is the absorbance of the sample.

An absorbance curve was used to determine the Ferric reducing antioxidant power **(**FRAP) in µM Fe (II)/g.

2.9.5 Total antioxidant capacity (TAC)

The total antioxidant capacity of the extract was estimated by the method described by Prieto et al.[22] Different concentrations (100, 200, 300 and 400 µg/mL) of the extract (0.1 mL) were added to 1 mL of reagent solution (28 mmol/L Na_3PO_4, 4 mmol/L ammonium molybdate and 0.6 mol/L H_2SO_4) in test tubes. The tubes were incubated in a thermal block at 95°C for 90 min. The mixture was allowed to cool at room temperature. The absorbance was measured at 695 nm against blank using a colorimeter. Antioxidant capacity was stated as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0-500 mg/ml.

2.10 Statistical analysis

Data obtained from the study was expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Dunnet's post hoc test were used to test for significance, $P<0.05$ was considered significant. GraphPad Prism 8.0 (San Diego, California, USA) was used for the analysis.

3. Results

3.1 Phytochemical screening

Preliminary phytochemical screening results revealed the presence of alkaloids, cardiac glycosides, steroids, tannins, phenols, saponins and flavonoids in the extract (Table 1).

3.2 Acetic acid-induced writhing test

The intraperitoneal injection of acetic acid to mice in this experiment elicited a characteristic nociceptive behaviour known as writhing (abdominal constrictions, pelvic rotation and hind limb stretching). The oral administration of the extract (200-800 mg/kg) one hour before the administration of 0.6 % acetic acid significantly ($P<0.001$) inhibited abdominal writhes compared to the control group (Fig. 1). The extract attenuated acetic acid induced writhes by 54.0, 62.9 and 69.1% at 200, 400 and 800 mg/kg, respectively (Fig. 1). In a similar way, the oral administration of the peripherally acting non-steroidal anti-inflammatory drug (NSAID), aspirin, 60 min before injecting the 0.6% acetic acid significantly ($P<0.001$) reduced writhes in comparison to the negative control by 66.5% at a dose of 150 mg/kg (Table 2).

3.3 Formalin-induced pain

The intraplantar injection of 0.02 mL of 2.7% formalin into the right hind paw of mice induced a nociceptive response characterized by the biting and licking of the injected paw. All drug-treated groups displayed reduction in nociceptive scores in both phases of pain (phases 1 and 2) when compared to the control (Fig. 2a and b). The standard drug dihydrocodeine had a better effect in both phases, eliciting 82.2% and 100%, respectively. Diclofenac showed 9.6% and 62.3% pain reduction in the respective phases. the test doses of the *A. comosus* ethanolic extract (200, 400 and 800 mg/kg) showed 28.2%, 28.4% and 31.8% pain reduction, respectively, for phase 1 and 39.4%, 69.5% and 56.2%, respectively, for phase 2 (Table 3). The anti-nociceptive effect of 400 and 800 mg/kg extract doses was significant ($P<0.05$ - $P<0.01$) in phase 2 pain (inflammatory pain). However, the effect was non-dose dependent as 400 mg/kg had a more pronounced pain inhibitory effect compared to the other test doses (Fig. 3a - b).

3.4 Mechanism of antinociception

Fig. 4. shows the effects of pre-treatment of mice with five different antagonists on the anti-nociceptive activity of *A. comosus* ethanolic extract (800 mg/kg) in acetic acid induced writhing test. Pre-treatment of the mice with naloxone (2 mg/kg), glibenclamide (8 mg/kg), atropine (5 mg/kg) and nifedipine (10mg/kg) could not block the anti-nociceptive activity of *A. comosus* ethanolic extract. However, theophylline (10 mg/kg) markedly reversed the anti-nociceptive effects of *A. comosus* ethanolic leaf extract in acetic acid induced writhing test (Table 4, Fig. 4).

Table1. Phytochemical constituents of *A. comosus* ethanolic leaf extract.

Phytochemical	Inference
Tannins	+
Saponins	+
Alkaloids	+
Cardiac glycosides	+
Steroids	+
Phenols	++
Flavonoids	++

+ = Present, ++ = Abundantly present

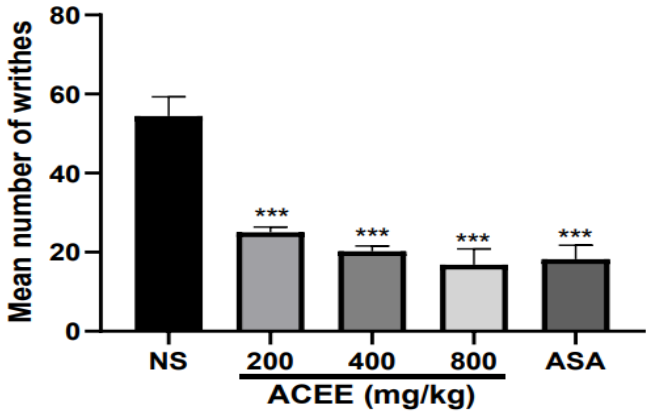

Fig. 1. Antinociceptive activity of *A. comosus* in acetic acid-induced writhing test. Values are expressed as Mean ± SEM, where n = 5, ***significant at $P<0.001$ using one-way ANOVA. NS = normal saline, ASA = aspirin, ACEE = *A. comosus* ethanolic leaf extract.

Table 2 Antinociceptive effect of *A. comosus* leaf extract in acetic acid-induced writhing test.

Treatment	Dose	Antinociceptive effect (%)
Normal saline	10 ml/kg	-
ACEE	200 mg/kg	54.0
	400 mg/kg	62.9
	800 mg/kg	69.1
ASA	150 mg/kg	66.5

ASA = Aspirin, ACEE = *A. comosus* ethanolic leaf extract

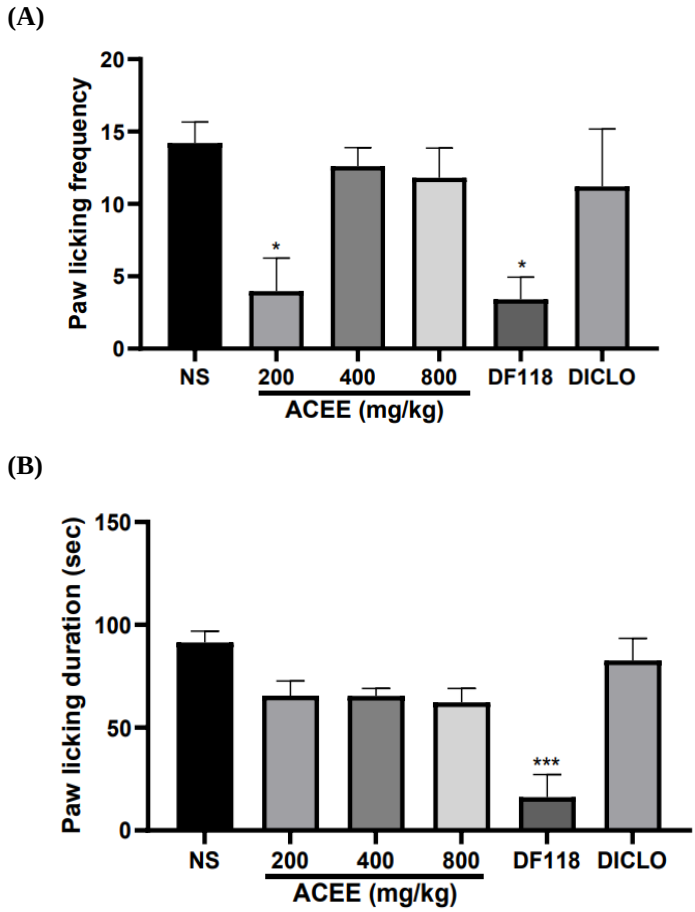

Fig.2. Effect of *A. comosus* extract on (A) paw licking frequency and (B) paw licking duration in phase 1 (neurogenic) pain in formalin-induced pain in mice. Values are expressed as Mean ± SEM, where n = 5, *significant at $P<0.05$, ***significant at $P<0.001$ using one-way ANOVA. NS = normal saline, ACEE = *A. comosus* ethanolic leaf extract, DF118 = dihydrocodeine, DICLO = diclofenac.

Table 3. Antinociceptive effect of *A. comosus* ethanolic leaf extract in formalin-induced pain.

Treatment	Dose	Antinociceptive effect (%)	
		Phase 1	Phase 2
Normal saline	10 ml/kg	-	-
ACEE	200 mg/kg	28.2	39.4
	400 mg/kg	28.4	69.5
	800 mg/kg	31.8	56.2
DF118	30 mg/kg	82.2	100
DICLO	10 mg/kg	9.6	62.3

ACEE = *A. comosus* ethanolic leaf extract, DF118 = dihydrocodeine, DICLO = diclofenac

3.5 Antipyretic test

The results obtained from the antipyretic study revealed that the subcutaneous injection of Brewer's yeast elicited an increase in rectal temperature in the test animals. This increase in temperature was sustained throughout the study in the negative control group. All doses of *A. comosus* ethanolic leaf extract caused reduction in rectal temperature in the animals. The standard drug paracetamol also demonstrated a similar decrease in rectal temperature throughout the study (Fig. 5).

3.6 Formalin-induced inflammation

Injection of 0.02 mL of 2.7% formalin into the plantar paw caused inflammation of the hind paw of the experimental animals which caused an increase in paw volume. The standard non-steroidal anti-inflammatory drug diclofenac inhibited this inflammatory effect. All doses of *A. comosus* extract used for the study (200-800 mg/kg) had anti-inflammatory effect in the experimental animals (Fig. 6). This was observed as a decrease in the hind paw volume compared to animals in the control group.

3.7 DPPH scavenging activity

Results obtained from the test for DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity is represented in Table 6. They revealed that at 100-400 µg/mL the radical scavenging activity of the extract ranges between 68.2% - 96.2%.

3.8 Total flavonoid content

Results obtained from the test for total flavonoid content are presented in Table 5. They revealed that 400 µg/mL extract contains 201.7±13.3 mg rutin/g dw.

3.9 Total phenolic content

Results obtained from the test for total phenolic content are presented in Table 5. They revealed that 400 µg/mL extract contains 135.5±7.37 mg GAE/g dw.

3.10 Ferric reducing antioxidant power

Results obtained from the test for ferric reducing antioxidant power are presented in Table 5. They revealed that 400µg/mL extract contains 66.7±5.4 µM Fe (II)/g.

3.11 Total antioxidant capacity (TAC)

Results obtained from the test for total antioxidant capacity are presented in Table 5. They revealed that 400 µg/mL extract contains 541.6±6.9 mg GAE/g.

4. Discussion

This study was designed to evaluate the antinociceptive, anti-inflammatory and antipyretic activities of *A. comosus* ethanolic leaf extract using established *in vivo* mice models. The scope of study also covers the *in vitro* assessment of the antioxidant activity of the extract. Preliminary phytochemical screening to identify the active principles in the extract revealed the presence of alkaloids, cardiac glycosides, steroids, tannins, phenols, saponins and flavonoids, which is consistent with a previous report by Paixao et al.[23] Several phytochemicals, such as alkaloids and their derivatives, have been isolated from plant sources and used for their analgesic and anti-inflammatory effects. [24] Saponins and flavonoids have also been reported to have antinociceptive, anti-inflammatory and antipyretic properties.[25] Hence, the pharmacological activity of the extract may be attributed to the presence of these bioactive phytochemicals.

The acetic acid-induced writhing test is among the models employed in evaluating the anti-nociceptive activity of extracts. This model is very sensitive and able to detect anti-nociceptive effects of test agents at dose levels that may appear to be inactive with other methods like the tail flick test.[26] In the acetic acid-induced writhing test, intraperitoneal injection of mice with acetic acid (0.6%, 10 mL/kg) elicits a characteristic nociceptive behaviour known as writhing (abdominal constrictions, pelvic rotation, and hind limb stretching). These behaviours result from the action of endogenous inflammatory mediators such as serotonin, histamine, bradykinin and prostaglandins which sensitize C fibres and contribute to pain perception.[27] Various antinociceptive agents exert their effects through different mechanisms that directly or

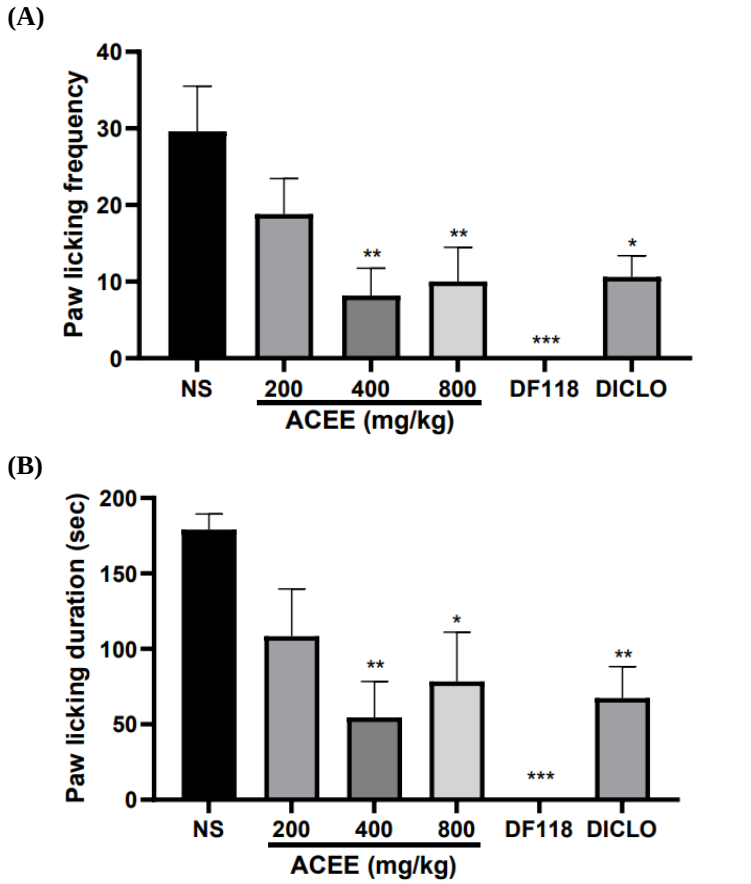

Fig. 3. Effect of *A. comosus* extract on (A) paw licking frequency and (B) paw licking duration in phase 2 (inflammatory) pain in formalin-induced pain in mice. Values are expressed as Mean ± SEM, where n = 5, *significant at $P<0.05$, ***significant at $P<0.001$ using one-way ANOVA. NS = normal saline, ACEE = *A. comosus* ethanolic leaf extract, DF118 = dihydrocodeine, DICLO = diclofenac.

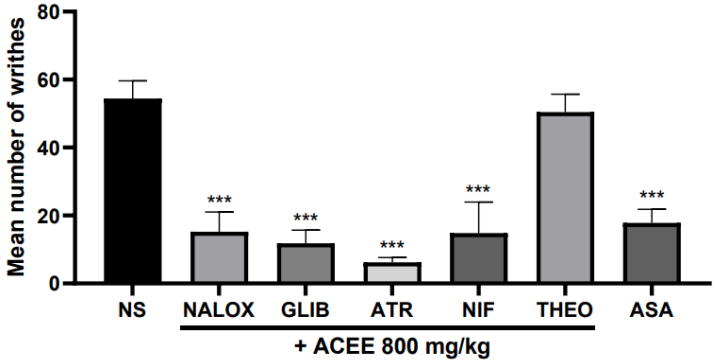

Fig. 4. Effect of *A. comosus* in presence of antagonists in acetic acid-induced writhing test for possible mechanism of action. Values are expressed as Mean ± SEM, where n = 5. Using one-way ANOVA, ***significant at $P<0.001$. NS = normal saline, ACEE = *A. comosus* ethanolic leaf extract, NALOX = naloxone, GLIB = glibenclamide, ATR = atropine, NIF = nifedipine, THEO = theophylline, ASA = aspirin.

Table 4. Antinociceptive effect of *A. comosus* in presence of antagonists in acetic acid-induced writhing test for possible mechanism of antinociception.

Treatment	Antinociceptive effect (%)
Normal saline	-
NALOX 2mg/kg + ACEE 800 mg/kg	72.1
GLIB 8mg/kg + ACEE 800mg/kg	78.3
ATR 5mg/kg + ACEE 800mg/kg	88.6
NIF 10mg/kg + ACEE 800mg/kg	72.8
THEO 10mg/kg + ACEE 800mg/kg	7.4
ASA 150 mg/kg	67.3

ACEE = *A. comosus* ethanolic leaf extract, NALOX = naloxone, GLIB = glibenclamide, ATR = atropine, NIF = nifedipine, THEO = theophylline, ASA = aspirin

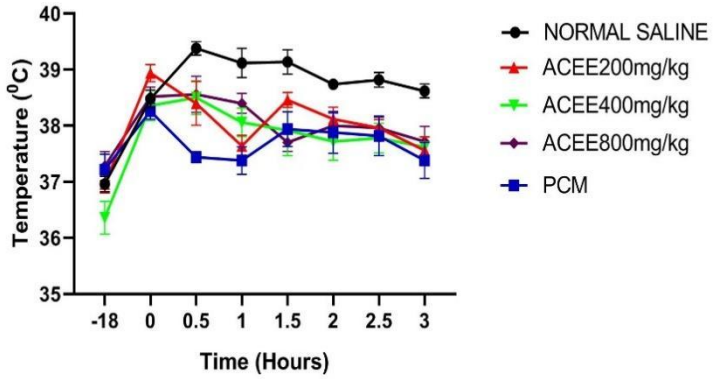

Fig. 5. Effect of *A. comosus* extract on body temperature in Brewer's yeast-induced pyrexia in mice. ACEE = *A. comosus* ethanolic leaf extract, PCM = paracetamol.

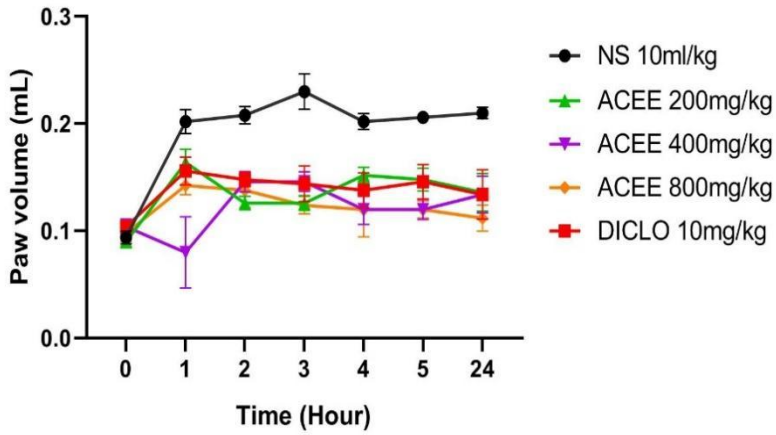

Fig. 6. Effect of *A. comosus* ethanolic leaf extract on paw volume in formalin-induced inflammation in mice. ACEE = *A. comosus* ethanolic leaf extract, DICLO = diclofenac.

Table 5. Antioxidant property of *A. comosus* ethanolic leaf extract.

Concentration (µg/mL)	Total flavonoids content (mg rutin/g dw)	FRAP content (µM Fe (II)/g)	TAC (mg GAE/g dw)	DPPH (% inhibition)	Total phenol content (mg GAE/g dw)
100	165.9±44.83	45.84±5.32	553.8±17.66	68.27±3.02	113.2±6.60
200	172.2±25.98	52.83±3.36	532.0±11.97	77.41±5.28	115.0±8.67
300	187.5±5.77	61.21±4.34	539.3±25.92	68.19±15.08	128.0±5.56
400	201.7±13.28	66.68±5.38	541.6±6.90	96.19±2.01	135.5±7.37

n = 3, Values expressed as Mean ± SEM

indirectly influence these mediators. Observation from the study indicates that the extract attenuated acetic acid-induced writhes by 54.0%, 62.9% and 69.1% at doses of 200, 400 and 800 mg/kg, respectively. Aspirin demonstrated a similar effect, attenuating writhing by 66.5% at a dose of 150 mg/kg. The anti-nociceptive activity of the extract was dose-dependent, with the highest dose eliciting a slightly stronger effect (69.1%) compared to aspirin (66.5%). Aspirin primarily exerts its antinociceptive effect through the irreversible inhibition of cyclooxygenase (COX) enzymes. This inhibition is a result of the acetylation of these enzymes and prevents the conversion of arachidonic acid to prostaglandins and thromboxanes, both of which are key mediators of pain.[28] However, results from the study suggests that the extract elicits its antinociceptive activity through mechanisms different from that of aspirin.

The formalin-induced pain model was also used to evaluate its anti-nociceptive effect. This model elicits biphasic pain forms (neurogenic and inflammatory). It is widely known as a highly predictive pain model and mimics acute clinical pain due to tissue injury.[29] The test result showed that the extract was effective against both pain forms, although it had a stronger effect against phase 2 pain, as all test doses (200-800 mg/kg) of the extract showed significant activity when compared with the control. The first, neurogenic, phase occurs almost immediately after formalin injection and is elicited by the direct chemical activation of central nociceptive afferent terminals of the Aδ nerve fibres. The second, inflammatory, phase is due to central sensitization in the dorsal horn and the direct stimulation of chemical nociceptors which results in an increase in the input from C fibres. It is known that centrally acting analgesics like dihydrocodeine, inhibit both the neurogenic and inflammatory phases of the formalin test. Peripherally acting analgesics, which include NSAIDS like diclofenac, only effectively inhibit the late or inflammatory phase.[30] The result suggests the extract possesses both central and peripheral antinociceptive activity but the peripheral effect may be higher compared to the central effect.

The mechanism of antinociception was evaluated with five different antagonists including naloxone, glibenclamide, atropine, nifedipine and theophylline on the anti-nociceptive activity of *A. comosus* ethanolic extract (800 mg/kg) in acetic acid induced writhing test. Pre-treatment of the mice with naloxone (2 mg/kg), glibenclamide (8 mg/kg), atropine (5 mg/kg) and nifedipine (10 mg/kg) did not block the anti-nociceptive activity of *A. comosus* ethanolic extract as the anti-nociceptive activity of the extract remained significant (P<0.001) even in the presence of these antagonists. However, theophylline (10 mg/kg) reversed the anti-nociceptive effects of *A. comosus* ethanolic extract in the acetic acid induced writhing test. The anti-nociceptive activity of the extract was reduced to only

7.4% in the presence of theophylline. The number of writhes recorded for the group pre-treated with theophylline before administration of the extract was similar to the number of writhes recorded in the negative control group. This gives a clear indication that the mechanism by which the extract elicits its anti-nociceptive activity may involve adenosinergic pathways. It is well known that theophylline, an adenosine antagonist blocks A_1 and A_2 receptor subtypes. The activation of A_1 receptor peripherally and spinally has been found to produce significant anti-nociceptive effect.[31-32] The anti-nociceptive effect may thus be due to activation of A_1 receptors and/or an elevation in endogenous adenosine centrally or peripherally.

Antipyretic agents are substances recognized for their capability to lower elevated body temperature, particularly in pathological conditions. The administration of Brewer's yeast suspension to mice has been shown to increase rectal temperature, which is attributed to an enhancement in prostaglandin synthesis.[33] Hence the use of yeast-induced pyrexia model for evaluating the antipyretic potential of the extract in this study. Results obtained from the antipyretic study showed time-dependent changes in rectal temperature in mice injected with Brewer's yeast. Mice in the control group maintained an increase in rectal temperature above 38.4°C throughout the study. However, the extract and standard drug paracetamol inhibited this sustained elevated rectal temperature seen in the control group. The effect of 400 mg/kg extract was similar to that of the 800 mg/kg extract. Paracetamol had a similar effect but with earlier attainment of peak antipyretic action. Antipyretic agents elicit their activity through different biochemical pathways primarily inhibiting enzymes, modulating inflammatory processes and by direct activity on the hypothalamus. The standard drug, paracetamol, is known to elicit its antipyretic activity via the central nervous system and inhibition of prostaglandin synthesis. However, alkaloids and flavonoids from plants are known to elicit potent antipyretic activity primarily by prostaglandin synthesis inhibition, inflammatory pathway modulation and direct central nervous system activity. Hence, they may be the components of the extract responsible for the antipyretic activity observed in this study.[34]

The anti-inflammatory activity was assessed using the formalin induced inflammatory model in mice. The results revealed that all extract doses used for the study demonstrated a potent anti-inflammatory effect. The extract also demonstrated potent in vitro antioxidant properties. It showed high ferric reducing antioxidant power, high total antioxidant capacity and high DPPH scavenging activity. Also, the total phenolic and flavonoid contents where high. The antioxidant activity may be responsible for its anti-inflammatory effects since agents with high antioxidant activity are known to decrease inflammation.[35] It may have the capacity of inhibiting reactive oxygen species (ROS) generating oxidases *in vivo* and may also increase endogenous antioxidants or even directly inhibit enzymes catalyzing oxidation of cell/tissue components.

5. Conclusion

This study revealed that *A. comosus* ethanolic leaf extract has potent anti-nociceptive, antipyretic, anti-inflammatory and antioxidant activities. The mechanism for antinociception may involve interaction with adenosinergic pathways. *A. comosus* ethanolic leaf extract may thus be recommended as candidate for further studies purposed towards employing these therapeutic benefits for clinical use.

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Conflicts of Interest

The authors declare no conflict of interest.

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