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Original Research Article

ANTI-INFLAMMATORY ACTIVITY OF PARTIALLY PURIFIED COLUMN CHROMATOGRAPHY FRACTIONS OF *Acanthus montanus* ROOTS

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ABSTRACT

Acanthus montanus Nees T. Anderson (Acanthaceae) has traditionally been used in traditional African medicine to treat pain and inflammation. The study aimed to evaluate the anti-inflammatory effects of purified active fractions (designated AMC-1 and AMC-2) in murine models. Using 100% methanol, the dried whole plant was macerated to obtain a methanol extract (ME), which was further fractionated into pet ether (PEF), chloroform (CF), and methanol (MF) fractions. AMC-1 and AMC-2 were isolated from PEF using a bioassay-guided assay and tested for anti-inflammatory activity in mice using xylene-induced ear oedema, agar-induced rat paw oedema, and leukocyte migration models. Topical application of AMC-1 and AMC-2 significantly ($p < 0.05$) inhibited acute ear oedema induced by xylene, with inhibitions of 56% and 41%, respectively. AMC-1 and AMC-2 at 400 mg/kg significantly ($p < 0.05$) inhibited acute paw oedema. AMC-1 had a higher inhibition of 87%, whereas AMC-2 had an inhibition of 60%. The leukocyte migration count of AMC-2 (92.8, -76%) was significantly higher ($p < 0.05$) than that of AMC-1 (86.4, -30%) and the standard treatment, indomethacin (61.6, -17%). There is good biological activity associated with AMC-1 and AMC-2, which can be promising as anti-inflammatory agents. Further research is ongoing to validate the results.

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INTRODUCTION

As a natural response to foreign invasion, inflammation serves as a protective mechanism that enhances the body's ability to detect and counter deleterious stimuli, such as chemical toxicants, bacterial pathogens, and cancerous cells, partly through increased blood flow to the site of injury. The host immune response facilitates the removal of harmful stimuli and promotes the healing of damaged tissue. Clinically, inflammation is characterised by pain, swelling, redness, and heat, which arise from complex vascular and cellular events [1,2]. Several processes regulate the initiation, progression, and resolution of inflammation. During this process, secretory cells play a key role by releasing a wide range of mediators, including pro-inflammatory and cytotoxic cytokines, prostaglandins, reactive oxygen species, and nitric oxide (NO), all of which contribute to the inflammatory cascade [3]. However, excessive production of these mediators can lead to tissue damage and disease progression. Although inflammatory conditions are

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commonly treated with non-steroidal anti-inflammatory drugs (NSAIDs), their use is limited due to associated adverse effects [4]. This limitation underscores the need for safer and more effective alternative therapies.

Medicinal plants remain an important source of bioactive compounds with therapeutic potential. In many parts of Africa, traditional medicine continues to play a vital role in healthcare delivery, with numerous plants used to manage inflammatory conditions [5,6]. One such plant is *Acanthus montanus* Nees T. Anderson (Acanthaceae), widely used in ethnomedicine to treat pain and inflammation. The plant is broadly distributed across West and Central Africa, ranging from Ghana to Nigeria and extending to Angola and the Democratic Republic of Congo [7,8]. It is commonly used as a cough remedy, particularly for women and children [9], and has also been employed to treat urethral discharge [10]. The young shoots, often prepared with groundnuts or the kernel butter of *Irvingia gabonensis*, are used during pregnancy to alleviate stomach upset and morning sickness [11]. Inflammatory conditions are also treated with *A. montanus* through scarification with its thorns, a practice reported among the Gabonese (Geviya) [12] and Cameroonian (Aguambu-Bamumbu) communities [13].

Previous pharmacological studies on *A. montanus* have demonstrated a broad spectrum of biological activities, including antimicrobial [14–16], analgesic and antipyretic [17,18], antihelminthic [19], antifertility and fetotoxic [20], antidiabetic [21], anticonvulsant and sedative [22], and toxicological effects [23]. Despite extensive pharmacological studies, a gap remains in identifying and evaluating specific bioactive fractions responsible for its anti-inflammatory activity. In particular, few studies have focused on bioassay-guided isolation of active constituents. Accordingly, this study aims to evaluate the anti-inflammatory effects of purified fractions isolated from *A. montanus* roots using established *in vivo* models.

MATERIALS AND METHODS

Animals

Forty-eight adult Wistar albino rats (150 to 200 g) and twenty-four mice (20-30 g) of either sex were obtained from the animal house of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. The animals were kept under standardised conditions (temperature 22 ± 2 ◦C; humidity 60 ± 4%; natural lighting), fed a standard diet, and provided with water *ad libitum.* The handling of animals used for experimental and other scientific purposes complied with institutional regulations, as well as with the EEC regulations (O.J. of EEC L 358/1 12/18/1986). All experimental tests were authorized by the Institutional Ethics Committee on Animal Research, UNN (FPSRE/UNN/21/00200)

Plant material

Fresh roots of Acanthus montanus Nees T. Anderson were collected from Nsukka, Enugu State, Nigeria. The plant sample was identified and authenticated by Mr. Alfred Ozioko of the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nigeria, where the voucher specimen (InterCEDD/035).

Extraction and purification

The dried roots (4.5 kg) were extracted for 48 h by cold maceration with 100% methanol at room temperature. The extract was concentrated under reduced pressure to remove the methanol, yielding the crude methanol extract (ME, 196.92 g, 4.37% w/w) at a temperature below 45 °C. The ME (196.92 g) was subjected to solvent-guided fractionation on a silica gel column (60–120 mesh size; 135 cm × 7.5 cm) using successive elution with petroleum ether, chloroform, and methanol. This yielded the petroleum ether fraction (PEF, 8.4 g, 4.26% w/w), chloroform fraction (CF, 6.29 g, 3.19% w/w), and methanol fraction (MF, 133.93 g, 68% w/w). The yield (%) of the extraction and fractionation processes was calculated using the following relation:

$$Yield\ of\ extract\ (\%) = \frac{\text{Weight of extract (g)}}{\text{Weight of plant material macerated (g)}} \times 100$$

$$\text{Yield of fraction (\%)} = \frac{\text{Weight of fraction (g)}}{\text{Weight of extract fractionated (g)}} \times 100$$

The original ME, PEF, CF, and MF were assessed. Through a bioactivity-guided activity study using the rat paw oedema model of acute inflammation as an activity guide, PEF was identified as the most active fraction. The PEF (8.03 g) was chromatographed on a silica gel (70–230 mesh) glass column (97 × 4 cm; L x W) and eluted with 100% n-hexane and gradient mixtures of n-hexane and ethyl acetate (19:1-1:19), respectively. Eight fractions (I–VIII) (50 mL each) were collected and pooled based on thin-layer chromatography (TLC) profiles. Thin-layer chromatography (TLC) was performed using pre-coated silica gel 60 F254 TLC plates (Merck,

Darmstadt, Germany). Eluates showing similar TLC profiles based on the number of spots, similarity of Rf values and colour of spots were pooled into three sub-fractions, Fr A_1 (I), Fr A_2 (II) and Fr A_3 (III-VIII). The active fraction A3 was chromatographed on a silica gel flash column and eluted with a gradient solvent system, starting with n-hexane (100 %), n-hexane + ethyl acetate (90:10), n-hexane + ethyl acetate (100%), and 100% ethyl acetate to afford 25 sub-fractions (B_1-B_{25}) of 25 mL volume each. The sub-fractions (B_1 – B_{25}) were analysed by TLC and pooled together based on the density of the spots with Rf values and subsequently subjected to preparative TLC using silica gel (1 cm thick) coated chromatoplates developed with a 2:8 mixture of ethyl acetate and n-hexane to yield two isolates, AMC-1 (530 mg) and AMC-2 (400 mg).

Anti-inflammatory activity test

Xylene-induced mouse ear oedema

In this experiment, topical acute inflammation was studied using the standard method [24, 25], modified by Okoli and Akah [26]. Briefly, albino mice (24) of either sex were randomly assigned into four groups (n=6). Both AMC-1 and AMC-2 were dissolved in 2% Tween 80 and given to groups I and II as treatment. As controls, groups III and IV received 0.05 ml of 2 % Tween 80 and indomethacin (0.05 ml/ear), respectively, both dissolved in distilled water. After applying topical inflammation to the anterior surface of the right ear for 1 h, xylene (0.05 ml) was applied to the posterior surface of the same ear. Mice were sacrificed 2 h after the xylene application, following an overdose of ether anaesthesia. Both ears were removed, and the mice were sacrificed. The oedema was quantified by measuring the weight difference between two earplugs. A circular section of 7 mm diameter was removed and weighed from both ears (the right treated and the left untreated). The anti-inflammatory activity was evaluated as the magnitude (%) of reduction in oedema in treated animals compared to control animals using the following formula:

[1] $$\% \text{ oedema reduction} = \frac{100 \times (Rt - Lt)}{(Rc - Lc)}$$

Rt = mean weight of the right ear plug of treated animals

Lt = mean weight of the left ear plug of treated animals

Rc = mean weight of the right ear plug of control animals

Lc = mean weight of the left ear plug of control animals

Agar-induced rat paw oedema

The experiment on rat paw oedema was performed using the method of Winter et al. [27], with slight modifications. Using sub-plantar agar injection, acute inflammation was measured in terms of the volume change of the rat's hind paw. Briefly, adult albino rats (24) of either sex were randomly assigned into four groups (n = 6). As a treatment, groups 1 and 2 received 400 mg/kg of AMC-1 and AMC-2, respectively. The control groups (3 and 4) received 2% tween 80 at 5 ml/kg, and indomethacin (10 mg/kg) dissolved in distilled water. An acute paw oedema was induced in rats 1 h after treatment by injecting 0.1 mL of freshly prepared agar into the sub-plantar region of the right hind paw. The animal's hind paw was immersed in a narrow-calibrated vessel of distilled water not filled to the brim, and the volume of liquid level was measured before (0.5 h) and at 1, 2, 3, and 4 h after induction of oedema. The difference between the volume of liquid levels at zero time (Vo) and the volume of liquid levels at various times (Vt) following the induction of oedema was used to estimate inflammation. The level of inhibition of oedema was calculated using the equation below [28, 29].

[2] $$\text{Inhibition of oedema (\%)} = \frac{100\ (1 - [a - x]}{b - y}$$

a = mean paw volume of treated rats at various times after agar injection

x = mean paw volume of control rats at various times before agar injection

b = mean paw volume of control rats at various times after agar injection

y = mean paw volume of control rats at various times before agar injection

Leukocyte migration test

The experiment on leukocyte migration *in vivo* was conducted using the method described by Ribeiro et al. [30]. Briefly, adult albino rats (24) of either sex were randomly assigned into four groups (n = 6). Groups 1 and 2 were given 400 mg/kg of AMC-1 and AMC-2, respectively, while group 3 received indomethacin (10 mg/kg) orally. Animals received an intraperitoneal injection of Agar (2.8% w/v, 1 mL) 1 h post-treatment. Animals were sacrificed after 4 h using ether anaesthesia. An incision was made on the peritoneal cavity of each rat, which was

washed with 5 mL of 10% ethylenediaminetetraacetic acid (EDTA). After staining with Wright's stain, total and differential leukocyte counts (%) in the peritoneal wash were determined using a manual cell counter [3]

Inhibition (%) = $\frac{1-T\times100}{C}$

Where T is the mean total leukocyte count of treated animals, and C is the mean total leukocyte count of control animals.

Statistical analysis

The results are presented as mean ± SEM. The percentage of inhibition of oedema was calculated for each animal group compared with a vehicle-treated control group. The significance of differences between the groups was calculated with GraphPad Prism (5.03), using One-Way ANOVA and Dunnett's post hoc tests. P < 0.05 was considered statistically significant.

RESULTS

Yield of extraction and fractionation

The extraction process of fresh roots of *Acanthus montanus* yielded 196.92 g (4.37%.w /w) of methanol extract (ME). Fractionation of ME yielded Pet ether (8.4 g, 4.26% w/w), chloroform (6.29 g, 3.19% w/w), and methanol (133.93 g, 68% w/w) fractions.

Physicochemical parameters of AMC-1 and AMC-2

Bioassay-guided fractionation of the methanol extract of *Acanthus montanus* roots yielded two purified fractions, AMC-1 and AMC-2. AMC-1 was obtained as a pale yellow, needle-like semi-solid with a weight of 530 mg, while AMC-2 appeared as a light orange, thick oily liquid with a weight of 430 mg. Chromatographic analysis revealed Rf values of 0.33 for AMC-1 and 0.50 for AMC-2 in an 8:2 solvent system (Table 1).

Table 1: Physicochemical parameters of AMC-1 and AMC-2

Compound	Rf Values (8:2)	Appearance	Texture	Weight (mg)	Colour
AMC-1	0.33	Needle-like semi-solid	Oily	530	Pale yellow
AMC-2	0.50	Thick Liquid	Oily	430	Light orange

Effects of AMC-1 and AMC-2 on xylene-induced mouse ear oedema

Topical application of AMC-1 and AMC-2 significantly (p < 0.05) inhibited acute oedema induced by xylene in the mouse ear, resulting in an inhibitory effect of 56 and 41%, respectively. The reference drug, indomethacin (10 mg/kg), caused 37% inhibition (Table 2).

Table 2: Effects of AMC-1 and AMC-2 on xylene-induced mouse ear oedema

Treatment	Dose (mg/ear)	Oedema (mg)	Inhibition (%)
AMC-1	5	1.37 ± 0.32*	56
AMC-2	5	1.86 ± 0.46*	41
Indomethacin	5	2.00 ± 0.38*	37
Control	-	3.14 ± 0.34	-

* p < 0.05 compared to the control (ANOVA; Dunnet's post hoc test). Values are expressed as the mean ± SEM (n=6).

Effects of AMC-1 and AMC-2 on agar-induced rat paw oedema

Oral administration of AMC-1 and AMC-2 significantly (p < 0.05) inhibited the development of acute oedema of the rat paw induced by agar at 400 mg/kg. AMC-1 exhibited higher inhibition of 87%, while that of AMC-2 was 60% at the 4th h. Indomethacin, which was the reference drug, (10 mg/kg) caused an inhibition of 93% (Table 3).

Effects of AMC-1 and AMC-2 on agar-induced leukocyte mobilization

AMC-1 and AMC-2 increased the total leukocyte. The leukocyte count of AMC-1 was (68.4, -30%) and AMC-2 was (92.8, -76%) compared to indomethacin, which was (61.6, -17%). Only AMC-2 exhibited a significant (p < 0.05) increase compared with the control (Table 4).

Table 3: Effects of AMC-1 and AMC-2 on agar-induced rat paw oedema

Treatment	Dose (mg/kg)	Oedema (mL)				
		0.5 h	1 h	2 h	3 h	4 h
AMC-1	400	0.24 ± 0.01 (4)	0.16±0.03 (41)	0.07±0.02* (67)	0.06±0.02* (62)	0.02±0.01 (87)
AMC-2	400	0.28±0.01 (12)	0.21±0.03 (22)	0.18±0.05 (15)	0.15±0.03* (62)	0.06±0.01* (60)
Indomethacin	10	0.28±0.03 (12)	0.24±0.04 (11)	0.09±0.01* (57)	0.07±0.02* (56)	0.01±0.01* (93)
Control	-	0.25±0.03	0.27±0.03	0.21±0.02	0.16±0.01	0.15±0.02

* $p < 0.05$ compared to the control (ANOVA; Dunnet's post hoc test). Values are expressed as the mean ± SEM (n=6), while those in parentheses represent percent inhibition.

Table 4: Effects of AMC-1 and AMC-2 on agar-induced leukocyte mobilization

Treatment	Dose (mg/kg)	TLC ($x10^4$)	Inhibition (%)
AMC-1	400	68.4±2.5	-30
AMC-2	400	92.8±10.4*	-76
Indomethacin	10	61.6±2.58	-17
Control	-	52.6±5.88	-

* $p < 0.05$ compared to the control (ANOVA; Dunnet's post hoc test). TLC: Total leukocyte count. Values expressed as mean ± SEM (n=6).

DISCUSSION

Studies in ethnomedicine are crucial for identifying new drugs from indigenous plants, given the extraordinary chemical diversity of plant extracts and natural products [31]. These natural compounds remain important sources of leads for anti-inflammatory drug development, particularly given the adverse effects associated with conventional therapies. Previous studies on *Acanthus montanus* have demonstrated that its crude extracts possess anti-inflammatory properties [14]. The present study builds on these findings by evaluating purified fractions, AMC-1 and AMC-2, thereby providing insight into the specific constituents responsible for anti-inflammatory activity.

As a well-established model, the xylene-induced ear oedema model provides a reliable means of assessing the effects of compounds on acute topical inflammation, particularly early vascular events such as vasodilation and increased permeability [32]. Acute inflammation is characterised by enhanced vascular permeability, leading to the leakage of plasma proteins and fluid into surrounding tissues, resulting in oedema [33]. The ability of AMC-1 and AMC-2 to suppress oedema formation in this model suggests that these fractions interfere with early inflammatory processes, possibly by inhibiting the release or action of mediators such as histamine, serotonin, and phospholipase A_2-derived products. This inhibition of vascular permeability is indicative of an antiphlogistic effect [34]. The comparatively stronger activity of AMC-1 suggests that it may contain more potent bioactive constituents capable of stabilising vascular responses and limiting exudative processes associated with acute inflammation.

The agar-induced paw oedema model further demonstrated the systemic anti-inflammatory activity of AMC-1 and AMC-2 and is widely used for evaluating agents active against acute inflammation [35]. This model involves both early and late phases of inflammation, initially mediated by histamine and serotonin, followed by prostaglandins, nitric oxide, and cytokines. Cell migration in this model is often associated with increased nitrite levels, reflecting nitric oxide production during inflammation [36]. Excessive nitric oxide, particularly from inducible nitric oxide synthase (iNOS), contributes to oxidative stress and

tissue damage. The ability of AMC-1 and AMC-2 to attenuate oedema progression suggests interference with these mediators, possibly through inhibition of cyclooxygenase pathways or modulation of nitric oxide production. The stronger and more sustained effect of AMC-1 indicates that it may possess more potent constituents capable of suppressing key inflammatory mediators, thereby supporting its systemic anti-inflammatory potential.

In contrast to the inhibitory effects observed in the oedema models, investigation of both fractions on leukocyte migration induced by agar revealed that AMC-1 and AMC-2 modulated cellular components of inflammation. Leukocyte recruitment is a fundamental aspect of the inflammatory response and is triggered by changes in vascular permeability, blood flow, and microvascular dynamics [36,37]. These processes are regulated by signalling pathways such as nuclear factor-kappa B (NF-κB), which promotes the production of pro-inflammatory cytokines. Neutrophils, as the first line of defense, play a crucial role in combating pathogens, while leukocytes in general contribute to the neutralisation of harmful agents and tissue repair [38]. The enhanced leukocyte migration observed, particularly with AMC-2, suggests stimulation of immune cell mobilisation or increased responsiveness to chemotactic signals. Although excessive leukocyte infiltration may exacerbate tissue damage, controlled recruitment is essential for host defense. This indicates that AMC-2, and to a lesser extent AMC-1, may exert immunomodulatory effects rather than purely anti-inflammatory actions.

The differing effects of AMC-1 and AMC-2 across the experimental models highlight the complexity of their pharmacological activities. It is likely that these fractions contain multiple bioactive constituents acting through distinct but complementary mechanisms. While AMC-1 appears to exert a stronger inhibitory effect on inflammatory mediators and vascular responses, AMC-2 may have a more pronounced influence on immune cell mobilisation. Such dual activity is common among plant-derived compounds, which can simultaneously suppress excessive inflammation while supporting immune function. Phytochemicals such as flavonoids, alkaloids, and terpenoids are known to inhibit inflammatory signalling pathways, reduce oxidative stress, and stabilise biological membranes, thereby limiting tissue damage [39,40].

CONCLUSION

The results of this study showed that AMC-1 and AMC-2 act at multiple levels of the inflammatory response, including vascular permeability, mediator release, and cellular recruitment. These results provide a pharmacological basis for the traditional use of *Acanthus montanus* in inflammatory conditions. AMC-1 demonstrated a stronger anti-inflammatory profile than AMC-2 and may represent a major contributor to the observed activity. However, the precise mechanisms underlying these effects remain to be fully elucidated. Further studies are required to characterise the active constituents and investigate their molecular targets in order to fully establish their therapeutic potential.

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AUTHORS' CONTRIBUTION

COO, BCO: Conceptualised the problem and designed the study. ELU, BCO, CAE: Drafted the manuscript. ELU, CAE: Performed statistical analysis on the data generated. ELU, BCO, CAE, COO: Proofread the manuscript before final submission

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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