Anti-nociceptive and anti-inflammatory potentials of Vernonia amygdalina leaf extract via reductions of leucocyte migration and lipid peroxidation

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ABSTRACT

Background: Vernonia amygdalina is well known as a medicinal plant in folk medicine as antidiabetic, anthelmintic, antimalarial, laxative/purgative, and expectorant among others. Aim: This study was conducted to investigate the antinociceptive and anti-inflammatory effects of V. amygdalina. Materials and Methods: Methanol extract of V. amygdalina leaf (MEVA) was evaluated for antinociceptive effect and possible mechanisms of action in the presence of naloxone (1 mg/kg), atropine (2 mg/kg), and prazosin (1 mg/kg) using acetic acid writhing test in mice. The anti-inflammatory effect was evaluated in carrageenan hind paw edema and carrageenan air pouch models. Protein concentration, malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) assays were carried out for its antioxidative activities in inflammation. Hematoxylin and eosin staining was used to assess the level of inflammation. Results: From the acetic acid writhing test results, MEVA (50, 100 mg/kg) showed significant antinociceptive effect. Naloxone, atropine and prazosin did not significantly reverse the antinociceptive effect of MEVA (50 mg/kg). MEVA (50, 100, and 200 mg/kg) showed dose-dependent inhibition of edema (41.4, 63.0, and 68.6%) at 4 h post-carrageenan injection. In the carrageenan air pouch model, MEVA (200 mg/kg) significantly (P < 0.05) reduced infiltrating leukocytes, protein concentration and MDA levels, while GSH and SOD were unaffected. The histological study showed a reduction in the infiltration of inflammatory cells in MEVA-treated groups. Conclusion: V. amygdalina showed antinociceptive activity and anti-inflammatory effect via reductions of leukocyte migration and lipid peroxidation.

KEY WORDS: Anti-inflammatory, antinociceptive, malondialdehyde, Vernonia amygdalina
 appetizer and the water extract as a digestive tonic. It is well known as a medicinal plant in folk medicine as antidiabetic, antihelmintic, antimalarial, laxative/purgative, expectorant, worm expeller and fertility inducer in subfertile women, antipyretic, and recently for a non-pharmacological solution to persistent fever, headache, and joints pain associated with AIDS [9,10]. Studies have also been conducted to establish its antinociceptive, anti-inflammatory, and antioxidant properties of \textit{V. amygdalina} among others [9].

However, the exact mechanisms underlying the therapeutic actions of \textit{V. amygdalina} on pain and inflammation are yet to be elucidated. Hence, this experiment was performed to elucidate the mechanisms of action of the antinociceptive and anti-inflammatory potentials of \textit{V. amygdalina}.

**MATERIALS AND METHODS**

**Reagents and Drugs**

The chemicals and drugs include: Formalin, acetic acid, naloxy hydrochloride dehydrate (Sigma-Aldrich, USA), atropine, prazosin, carrageenan, indomethacin (Sigma-Aldrich, USA), morphine, and distilled water.

**Experimental Animals**

Swiss mice weighing (25-30 g) and Wistar rats weighing (180-200 g) of both sexes were used for this study. They were purchased, housed and bred at the Pre-clinical Animal House, College of Medicine, University of Ibadan, Nigeria where this study was conducted. Animals were housed in 5 cages and in at a temperature of 22±2°C and 45-65% relative humidity environment under a 12 h light/12 h dark cycle (8:00 a.m. - 8:00 p.m.). The animals were acclimatized for 2 weeks with unrestricted access to food and water before the experiment. All experimental procedures on rodents were conducted in accordance with established protocols under the guidelines of the Principle of Laboratory Animal Care (NIH publication No. 85-23) [11] and ethical guidelines for investigation of experimental pain in conscious animals by Zimmerman [12].

**Collection and Extraction of \textit{V. amygdalina} Leaves**

Fresh leaves of \textit{V. amygdalina} were collected from the Arobo-Makinde area, Arulogun, Ojoo, Ibadan, Nigeria, which was authenticated at the Forestry Research Institute of Nigeria, Ibadan Oyo state. The voucher number: FHI - 110415 was assigned. Fresh leaves of \textit{V. amygdalina} were collected and air dried after which they were blended into a powdery form, and then 2.26 kg of \textit{V. amygdalina} was macerated in 10 L of methanol at room temperature for 48 h. It was then decanted with a filter paper. The process was repeated 3 times for exhaustive extraction. The extract was concentrated with a rotary vacuum evaporator at 40°C to produce a methanol extract of \textit{V. amygdalina} (MEVA). The extract was further concentrated in a vacuum oven at a temperature of 40°C.

**In vitro Antinociceptive Studies in Acetic Acid-induced Abdominal Writhing Test in Mice**

This test was carried out using the modified method [13]. The mice were pre-treated, orally, with the vehicle, MEVA of 50 mg/kg, 100 mg/kg, and 200 mg/kg for 3 days and indomethacin (10 mg/kg), once. Mice were injected with 0.2 ml (i.p.) of 3% acetic acid solution, 1 h after treatment with the extract, which induced the characteristic writhing.

**Mechanisms of Action: Evaluation of the Mode of Action of \textit{V. amygdalina} Extract for Antinociceptive Activity**

This was designed to assess the possible participation of different systems in the antinociceptive effect of MEVA, (50 mg/kg), mice were pre-treated with naloxone (1 mg/kg, i.p.), a non-selective opioid receptor antagonist; atropine (2 mg/kg, i.p.), a non-selective muscarinic receptors antagonist; and prazosin (1 mg/kg i.p.), an alpha-1- adrenoreceptor antagonist.

**Carrageenan-induced Hind Paw Edema Model in Rat**

A total of 30 rats were divided into five groups, and they were pre-treated with MEVA for 3 days before the experiment. The doses given include 50 mg/kg, 100 mg/kg, and 200 mg/kg. Control animals received 1% tween 80 (10 ml/kg) and indomethacin (5 mg/kg) was used as a reference drug. Carrageenan was injected 1 h after the past treatment. Paw edema was induced by right subplantar injection of 0.1 ml/paw of 1% freshly prepared carrageenan suspension in distilled water into the right hind paw of each rat. The paw edema volume was measured using the Ugo basile plethysmometer before and as well as at 1, 2, 3, and 4 h after the injection of carrageenan [14].

**Carrageenan-induced Air Pouch Model in Rats**

Air pouch was induced in rats as described [15]. Briefly, rats were anesthetized with ketamine (100 mg/kg, i.p.) and air cavities were produced by subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back (1st day). An additional 10 ml of air was injected into the cavity on the 4th day [16]. Rats were divided into three groups (n = 6); carrageenan (1% tween 80; 10 ml/kg), MEVA 200 mg/kg, and indomethacin (5 mg/kg) and orally pre-treated for 3 days before induction of inflammation. On the 6th day, 2 ml of 2% carrageenan solution dissolved in sterile saline was injected into the pouch cavity to induce inflammatory responses. 24 h after the carrageenan injections, rats were anesthetized with deep ether anesthesia and the pouch was carefully opened by a small incision. 2 ml of 0.9% normal saline was given to wash out the cavity.

**Determination of Exudates Volume and Leukocyte Infiltration**

The pouch cavity was opened with a small incision; the exudates were harvested and their volumes were measured [17]. The leukocytes in the fluid were counted using a hemocytometer. The exudates were transferred to ice-cold tubes. Cell-free
exudate was achieved by centrifugation at 3000 revolutions per minute (rpm) at 4°C for 15 min, and was stored at 4°C to prevent degeneration of cells. The supernatant was collected and stored at 4°C.

**Determination of Protein Concentration**

The protein concentrations of the exudates from the air pouch experiment were determined by the means of Biuret method [18]. 50 µl of the supernatant was added to 1.950 ml of distilled water. 3 ml of Biuret reagent was also added to the content in a test tube. Then, the whole sample was incubated at room temperature for 50 min. The absorbance was read at 540 nm and the concentration was determined from the standard curve.

**Determination of Reduced Glutathione (GSH) Level**

The method of Beutler and co-workers [19] was used in estimating the level of reduced glutathione (GSH) in air pouch exudate. 0.1 ml of test sample (supernatant) was diluted in 0.9 ml of phosphate (PO₄) buffer. 1 ml of 20% trichloroacetic acid (TCA) was added and allowed to stand for 20 min before centrifugation at 10,000 rpm for 10 min. 0.25 ml of the supernatant was removed and added to 0.75 ml of phosphate buffer. 2 ml of 0.0006 M of 5, 5'-Dithiobis (2-nitrobenzoic acid) was added and incubated for 10 min. Absorbance was read at 412 nm.

**Determination of Malondialdehyde (MDA)**

MDA is a presumptive biomarker for lipid peroxidation in plasma, live organisms, and cultured cells. 0.1 ml of test sample was dissolved in 1.9 ml of Tris-potassium chloride buffer. 0.5 ml of 30% TCA was added and 0.5 ml of 75% of thiobarbituric acid was also added. The whole mixture was incubated at 80°C for 45 min. It was allowed to cool on ice and then centrifuged at 4000 rpm for 10 min. Absorbance was read at 532 nm [20].

**Determination of Superoxide Dismutase (SOD)**

The level of SOD activity was determined by the method of Misra and Fridovich [21]. Superoxide (O₂⁻) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O₂⁻ introduced increased with increasing pH and also increased with increasing concentration of epinephrine. 10.8 mg of adrenaline was dissolved in 20 ml of 0.1 M of hydrochloric acid to yield 0.3 mm. 0.1 ml of test sample (supernatant) was dissolved in 2.6 ml of carbonate buffer. Then, it was allowed to equilibrate for about 5-10 min. The content was poured into cuvettes and the 0.3 mm of prepared adrenaline was added. Increase in absorbance for 60 s, 120 s, and 180 s (0, 1, 2, and 3 min) was read.

**Histological Analysis**

The pouch area was excised and fixed in 10% formalin in 0.01 M phosphate buffer (pH 7.4) and embedded into paraffin wax blocks. Sections were stained with hematoxylin and eosin.

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**RESULTS**

**Effects of MEVA on Acetic Acid-induced Writhing Test in Mice**

Figure 1 shows acetic acid-induced writhing response in mice which serve as an indication of antinociceptive activities of MEVA. Intraperitoneal injection of acetic acid produced 40.4 ± 4.7 mean number of writhes in the group administered with the vehicle. MEVA at 50, 100, and 200 mg/kg significantly inhibits writhes response by 63.9, 50.9, and 32.5%, respectively. Positive control drug (Indomethacin, 10 mg/kg) significantly inhibits writhing response by 84.7%.

**Mechanism of Action of V. amygdalina Involving Opioidergic, Adrenergic, Cholinergic Receptors in Acetic Acid-induced Writhing Test in Mice**

Figure 2 shows the results of the effect of pretreatment of mice with various antagonists of the antinociceptive activity MEVA (50 mg/kg). The results showed that pretreatment with naloxone (1 mg/kg) insignificantly (P > 0.05) reversed the antinociceptive activity of MEVA (50 mg/kg) by increasing the number of writhes in mice. Pretreatment of mice with atropine (2 mg/kg) or prazosin (1 mg/kg) did not prevent the antinociception caused by MEVA in mice.

**Effects of MEVA on Carrageenan-induced Hind Paw Edema in Rats**

Hind paw edema was induced by injecting carrageenan (1%) in the left hind paw after administration. The result of the inhibitory effect of MEVA on increase in hind paw volume (edema) is shown in Figure 3. The increase in paw volumes in animal pretreated with MEVA (50, 100, and 200 mg/kg) for 3 days before injection with carrageenan was significantly (P < 0.001) less than animals pretreated with vehicle. MEVA (50, 100, and 200 mg/kg) showed dose-dependent inhibition of edema (41.4%, 63.0%, and 68.6%) at 4 h post carrageenan injection. At the same time, rats pretreated with indomethacin...
(5 mg/kg) showed a more significant inhibition (78.7%; \( P < 0.001 \)) on the increase in paw volume at the 4 h.

**Anti-inflammatory Effects of *V. amygdalina* in Carrageenan-induced Air Pouch Model of Inflammation**

The volume of exudates recovered from the carrageenan-induced air pouch revealed that pretreatment with MEVA caused a reduced but insignificant (\( P > 0.05 \)) fluid exudation compared to carrageenan alone [Figure 4a]. Indomethacin (5 mg/kg) however caused a significant reduction in fluid exudates. Leukocytes migration into the air pouch was increased 24 h post carrageenan injection. The results revealed that pretreatment with MEVA (200 mg/kg) and indomethacin (5 mg/kg) caused a significant (\( P < 0.001 \)) reduction in total leukocytes accumulation in the air pouch compared to carrageenan alone [Figure 4b]. The protein concentration in fluid exudates was significantly (\( P < 0.05 \)) reduced by MEVA (200 mg/kg) and indomethacin (5 mg/kg) [Figure 4c].

**Antioxidant Effects of MEVA in Carrageenan-induced Air Pouch in Rats**

Carrageenan injection into the pouch induces rapid recruitment of leukocytes with concomittant elevation in the lipid peroxidation reactions as measured by the MDA level as well as the reduction of GSH and SOD. Pretreatment with MEVA (200 mg/kg) and indomethacin (5 mg/kg) for 3 days before induction significantly prevented the carrageenan-induced lipid peroxidation by 48.01% and 73.0%, respectively, when compared to vehicle-treated controls [Table 1]. While MEVA inhibited MDA significantly (\( P < 0.05 \)), it prevents depletion of GSH and \( z \) (\( P > 0.05 \)). Meanwhile indomethacin (5 mg/kg) significantly increase GSH levels (23.3%; \( P < 0.05 \)) and SOD activities (47.9%; \( P < 0.001 \)) when compared to vehicle treated controls.

**Histological Findings**

**Effect of MEVA on Carrageenan-induced Air Pouch in Rat**

The anti-inflammatory effects of MEVA were further assessed by examining the pouch tissue histologically [Figure 5]. The pouch tissue appeared thickened (characteristics of edema) due to infiltration of leukocytes after carrageenan injection in the air pouch [Figure 5a]. The treated groups which received MEVA (200 mg/kg) and indomethacin (5 mg/kg) in Figure 5b and Figure 5c, respectively, showed a reduced in infiltration of inflammatory cells when compared with the vehicle-treated group.

**DISCUSSION**

This study investigated the antinociceptive and anti-inflammatory effects of the MEVA leaf and its possible mechanisms of action through the investigation of the involvement of the adrenergic, cholinergic, and opioidergic systems. To investigate its antinociceptive effect, the acetic acid-induced writhing in mice considered as a model for visceral pain was selected.

The acetic acid-induced writhing model has been known to produce tissue necrosis by a chemical irritant in the peritoneal region in laboratory rodents. It has been suggested that acetic acid acts by releasing endogenous inflammatory mediators of the nociceptive neurons [22,23], such as bradykinin, prostaglandins, and pro-inflammatory cytokines, when injected intraperitoneally [24,25]. The main cytokines involved in nociception, induced by acetic acid, are TNF-\( \alpha \), interleukin-1\( \beta \), and interleukin 8, and they are released from resident peritoneal macrophages and mast cells [23,25]. From the acetic acid writhing test results, the doses of 50 mg/kg and 100 mg/kg of MEVA were observed to be more statistically significant in bringing about antinociception. These results suggest that 50 mg/kg may be the effective dose of MEVA in carrying out its antinociceptive property. The inhibitory effect carried out by MEVA against the nociceptive activity of acetic acid suggests the presence of active antinociceptive phytochemical present in *V. amygdalina* leaf.

Furthermore, from the acetic acid writhing tests, naloxone (a non-selective opioid antagonist) co-administered with MEVA was observed to have a more reversible effect on its antinociceptive property in comparison with atropine (a...
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Table 1: Effect of MEVA on MDA, GSH, and SOD in carrageenan-induced air pouch model of inflammation

| Treatment      | MDA (nM of MDA/mg protein) | GSH (µM GSH/ml exudate) | SOD (mIU/mg protein) |
|----------------|----------------------------|-------------------------|----------------------|
| Carrageenan    | 58.56±9.68                 | 0.89±0.04               | 22.2±1.41            |
| MEVA (200 mg/kg) | 30.44±5.81***              | 0.98±0.06               | 28.52±2.71           |
| Indo (5 mg/kg)  | 15.82±2.71***              | 1.16±0.08*              | 42.62±3.80***        |

MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase, MEVA: Methanol extract of V. amygdalina leaf, Data are expressed as mean±SEM (n=5). Comparisons were made using one-way ANOVA followed by post-hoc Newman-Keuls test. *P<0.05, **P<0.01, ***P<0.001 compared with the carrageenan group.

Figure 4: Anti-inflammatory effects of methanol extract of Vernonia amygdalina (200 mg/kg) in carrageenan-induced air pouch in rats. (a) Exudate volume, (b) leukocytes count, and (c) protein concentration. Data are expressed as mean ± SEM (n=5). Comparisons were made using one-way ANOVA followed by post-hoc Newman-Keuls test. *P<0.05; **P<0.01; ***P<0.001 compared with the carrageenan group.

Figure 5: (a-c) Representative photomicrographs of H and E stained section of pouch tissue in the carrageenan-induced air pouch (magnification ×100).

our findings on the antiinflammatory property might involve the opioidergic system with little or no contribution of the adrenergic and cholinergic systems.

Carrageenan-induced rat paw edema is also a suitable experimental animal model for evaluating the anti-edematous effect of natural products and is believed to be biphasic [9]. The first phase (1 h) involves the release of serotonin and histamine while the second phase (over 1 h) is mediated by prostaglandins, the cyclooxygenase products, and the continuity between the two phases is provided by kinins [9,26]. Carrageenan-induced paw edema has been increasingly used to test new anti-inflammatory drugs as well as to study the mechanisms involved in inflammation [27]. From the results of this study, it was observed that MEVA (50-200 mg/kg) significantly inhibited the carrageenan-induced rat paw edema at the 4th h. Indomethacin, used as the reference drug, is shown to have a statistically significant inhibition at the 4th h post carrageenan-induction. Based on the results of this study, it could be suggested that the anti-inflammatory effect of this extract may be attributed to the inhibition of prostaglandin release or synthesis. This is
also in tandem with the mechanism of action of nonsteroidal anti-inflammatory drugs in the inhibition of inflammatory processes. Agents that suppressed carrageenan-induced paw edema particularly in the second phase are good candidates for further anti-inflammatory screening.

We further evaluated the anti-inflammatory mechanism of MEVA in carrageenan-induced air pouch model in rats. Carrageenan air pouch model was used because of the flexibility in its usage for other assessments and biochemical assays. The carrageenan air pouch model has been extensively used for the study of various types of inflammation and inflammatory processes. This model has distinct advantages over other models of inflammation because of the ability to perform biochemical analysis of both exudates and inflammatory cells together with the histological and angiogenesis analysis of the air pouch lining [17]. The results of this study indicated that after injection of the carrageenan solution into the air pouch, the pouch fluid volume, the total number of infiltrating leukocytes in the pouch fluid and distinct granulation of the pouch wall lining increased significantly.

From the results obtained, it was observed that there was a significant increase in the amount of exudate collected from the pouches of animals of the carrageenan only when compared with indomethacin-treated animals. MEVA (200 mg/kg) slightly reduced fluid exudation in the air pouch. We also observed a significant reduction in the infiltrating leukocytes, this was evidenced in the total leukocytes number in the exudates as well as the histological analysis of the pouch tissues. The protein concentration estimation of the exudates collected from the air pouches of the animals was carried out to ascertain the extent of increased vascular permeability that causes increased exudation of plasma protein into the pouch. MEVA reduced protein concentration in the air pouch. Carrageenan-induced inflammation is associated with protein leakage in the air pouch, mediated by inflammatory factors such as bradykinin, serotonin, histamine, prostaglandins [28]. Furthermore, leukocytes recruitment is characterized by infiltrating neutrophils which account for further release of potent pro-inflammatory molecules [29]. Since indomethacin (standard nonsteroidal anti-inflammatory drugs) demonstrated a similar profile, it is possible that both MEVA and indomethacin are acting through common pathways.

The main cells involved in the inflammatory response are monocytes/macrophages, polymorphonuclear leukocytes, and endothelial cells. When these cells become activated, they aggregate and infiltrate tissues where they undergo a respiratory burst, increasing their oxygen use and production of cytokines, reactive oxygen species (ROS), and other mediators of inflammation. These events can initiate and also perpetuate inflammatory cascades and cause subsequent tissue damage [30]. In the MDA content estimation of the exudates from the pouches of the animals, it was observed that the MDA level was significantly increased in the animals’ group that received carrageenan only. MDA is a biomarker for lipid peroxidation. Lipid peroxidation is the oxidative degeneration of membrane lipids of endoplasmic reticulum. These lipids are rich in polyunsaturated fatty acids. These leads to the formation of lipid peroxides which in turn gives product such as MDA that can cause damage to the membranes.

Carrageenan injection into air pouch was hypothesized to cause increase in lipid peroxidation mediated via the increase in NO radical production [31]. The increased NO might be accompanied with the superoxide radical which induce a strong oxidant and peroxynitrite that results in acute endothelial dysfunction and thus activation of inflammation [32]. In our study, the increased level of MDA in the group of animals that received no treatment suggests that the antioxidant defense mechanism was compromised. MEVA and indomethacin, however, protected the animals against carrageenan-induced lipid peroxidation, hence reducing the oxidative stress levels in the animals.

GSH is a water-soluble tripeptide composed of the amino acids glutamine, cysteine, and glycine. GSH plays such an important role in the detoxification of a variety of electrophilic compounds and peroxides through catalysis by GSH S-transferases and GSH peroxidases [33]. Being a major endogenous non-enzymic antioxidant produced by the cells, it participates in neutralization of free radicals and ROS. From the results obtained, it was observed that the groups that received no treatment after carrageenan injection had a reduction in GSH level when compared with the group of animals that received normal saline only. Furthermore, the group of animals that received treatment of 200 mg/kg dose of MEVA had a slight increase in GSH level compared with the positive control group. The indomethacin group of animals also had a slight increase in GSH level. SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. From the results, it was observed that the MEVA slightly increased SOD activities but significantly increased in indomethacin - treated animals. This suggests the fact that the generation of oxidative stress was slightly reduced by the antioxidant potentials of the MEVA.

Taken together, these findings showed that MEVA prevented the alteration on oxidative stress markers, it significantly decreased MDA level in exudate and prevented the depletion in reduced GSH levels and SOD activities induced by carrageenan. These results demonstrated the antioxidant action of MEVA in this model and suggest the effect as a possible mechanism of MEVA anti-inflammatory action.

Histological study showed that carrageenan-induced air pouch inflammation is characterized with infiltration of inflammatory cells, production of prominent artifactual granules, granuloma formation of the epidermal and dermal layer, and keratinization of the epidermal layer. From the results, it was observed that there was a reduction in the infiltration of inflammatory cells into the dermis of skin tissues of animals pre-treated with MEVA. This suggests that MEVA was able to reduce the migration of pro-inflammatory cells such as neutrophils into the pouches of the animal’s skin. The Stratum basale appears normal, but the Stratum corneum and Granulosum appear mildly distorted unlike those which received no treatment.
CONCLUSION

V. amygdalina leaf possesses antinociceptive and anti-inflammatory properties. This justifies its use as a medicinal plant in some parts of the world. However, the mechanism through which it carries out its antinociceptive property is unlikely to involve the opioidergic, cholinergic, and adrenergic system. Its anti-inflammatory mechanism is mediated in part through reducing inflammatory leukocytes migration to inflammatory focal point and lipid peroxidation.

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