**Anti-inflammatory of n-hexane, ethyl-acetate and aqueous-methanol fractions of *Harungana Madagascriensis* leaves on Wistar albino rats: A Comparative study**

**ABSTRACT**

Background

Inflammation is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. When inflammation is inappropriately directed against self-tissues or is not adequately controlled, it becomes the cause of injury and disease. Thisscientific investigation could help in the search for new drugs especially those which can be used in treatment and management of several ailments, especially inflammatory diseases.

**Methods**

Plant extract and albino Wister rats were used for the study. Fractioning of the extract was prepared using n-hexane, ethyl acetate and aqueous-methanol. Rats were treated with different concentrations of the fractions.

**Results**

The n-hexane, ethyl acetate and aqueous-methanol fractions of *H. madagascariensis* demonstrated substantial anti-inflammatory activity which suppressed paw oedema in both the early and later phases of inflammation. In acetic acid-induced writhing test, the fractions exhibited significant (P<0.05) decreases in the number of writhes in comparison to the control. The n-hexane fraction exhibited a dose dependent significant reduction (27.1, 42.9 and 65.7%) in the *in vivo* leukocyte mobilization. Contrary to the n-hexane fraction, there was a non-dose dependent significant reduction (P<0.05) in the percentage inhibition of mobilization by the ethyl acetate (32.9, 63.3, and 58.6%) and aqueous-methanol (54.5, 63.3, and 62.1%) fraction. In all the fractions, the proportion of neutrophils in the perfusate was higher than lymphocytes and macrophages at different doses. The fractions significantly (P<0.05) decreased granuloma formation induced by cotton pellet. The fractions at all doses significantly (P<0.05) inhibited the activity of phospholipase A2 (PLA2) and significantly protected the human erythrocyte membrane against lysis induced by hypotonic solution. Comparatively, the aqueous-methanol fraction had a better activity than n-hexane, and ethyl acetate fractions.

**Conclusion**

*H.madagascariensis* leaf extract possesses potent anti-inflammatory properties and could be used for the effective treatment of inflammation and inflammatory related diseases

Key words: Inflammation, *Harungana Madagascariensis*, Fractions, n-Hexane, Ethyl-acetate, Aqueous-methanol

1. **INTRODUCTION**

Inflammation is an important biological response of the body’s immune system to harmful stimuli, such as damaged cells, pathogens and irritants (Gusev & Zhuravleva 2022). It serves as a protective mechanism intended to eliminate the initial cause of cell injury, clear dead cells and tissues and initiate tissue repair. It is protective attempts by the organism to remove the injurious stimuli as well as initiate healing process for the tissue (Burgess, et al., 2022). The factors that can stimulate inflammation include microorganisms, physical agents, chemicals, inappropriate immunological responses, and tissue death (Oronsky, et al., 2022).Inflammation can also result when tissues die from a lack of oxygen or nutrients, a situation that often is caused by loss of blood flow to the area (Ji, et al., 2023). The four cardinal signs of inflammation include: redness (rubor), [heat](https://www.britannica.com/science/heat) (calor), swelling (tumor), and pain (dolor). Heat results from increased blood flow through the area and are experienced only in [peripheral](https://www.merriam-webster.com/dictionary/peripheral) parts of the body such as the skin. Fever is brought about by chemical mediators of inflammation and contributes to the rise in temperature at the injury. Swelling, called [edema](https://www.britannica.com/science/edema), is caused primarily by the accumulation of fluid outside the blood vessels. The pain associated with inflammation results in part from the distortion of tissues caused by edema, and it also is induced by certain chemical mediators of inflammation, such as bradykinin, [serotonin](https://www.britannica.com/science/serotonin), and the [prostaglandins](https://www.britannica.com/science/prostaglandin). A fifth consequence of inflammation is the loss of function of the inflamed area. Loss of function may result from pain that [inhibits](https://www.merriam-webster.com/dictionary/inhibits) mobility or from severe swelling that prevents movement in the area (Ahmad, & Ahsan 2022).

Mechanisms designed to destroy foreign invaders and necrotic tissues have an intrinsic ability to injure normal tissues. When inflammation is inappropriately directed against self-tissues or is not adequately controlled, it becomes the cause of injury and disease (Yasmeen, et al., 2024; Nair, et al., 2021) Inflammation is typically categorized into two types: acute, is the initial response of the body to the harmful stimuli and is achieved by the increase movement of plasma and leukocytes from the blood to the injured site, tissues. It is a short-term process, usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimuli, and chronic, which persists over time and may lead to tissue damage(Raziyeva, et al., 2021).It is characterized by simultaneous destruction and healing of the tissue from the inflammatory process (Soliman, & Barreda 2022; Kumar, et al., 2024).

*Harungana madagascariensis*is a tropical much-branched shrub to small tree growing up to 12 m tall commonly known as ‘Dragon’s blood tree. The Plant has many common names such as; English (orange-milk tree, blood tree); Hausa (alillibar); Igbo (oturu); Yoruba (elepo). Ethno-medicinal applications include its utilization in the cure for leprosy, jaundice, ulcers, asthma etc (Khatun, et al., 2022). The bark and root decoctions are remedy for dysentery and piles. It relieves stomach-ache, painful menstruation menstrual problems, dysmenorrhea, menstrual irregularity, miscarriage, sterility and haematuria (Asadu, et al., 2005). *H. madagascariensis* has been shown to have hypoglycaemic effects, lowering blood glucose levels in diabetes mellitus, and others like antioxidant, anti-hepatotoxicity and antimicrobial activities (Khatun, et al., 2022;Roy, et al., 2025, ].Thus, many researchers have dedicated their efforts to search for safer drugs as well as natural products with less adverse effects for inflammatory diseases.

**AIM OF THE STUDY**

This study was aimed at evaluating the anti-inflammatory activities of different fractions *Harungana madagascariensis* leaves and*,* the underlying mechanisms of action of the plant.

1. **MATERIALS AND METHODS**

**2.1 Plant Material**

The leaves of *Harungana madagascariensis* were used for this study. They were collected from Adada river in Nsukka, Enugu State and identified by Mr. Alfred Ozioko of Bioresource Development and Conservation Programme (BDCP) Research Centre, Nsukka, Enugu State Nigeria.

**Experimental Animals**

Adult Wistar rats and adult swiss albino mice of both sexes were used for the experiments. The animals were obtained from the Animal house of the Faculty of Biological Sciences, University of Nigeria, Nsukka and acclimatized for 7 days under standard environmental conditions prior to the experiment. They were grouped as follows:

* Group 1: Rats were treated with 3%tween80 (2 ml/kg)
* Group 2: Rats were treated with 100 mg/kg body weight of fraction
* Group 3: Rats were treated with 200 mg/kg body weight of fraction
* Group 4: Rats were treated with 400mg/kg body weight of fraction
* Group 5: Rats were treated with a known amount of standard drug

**Blood samples**

Human blood used for phospholipase A2 and membrane stabilisation was collected from healthy individual free from anti-inflammatory drugs.

**Chemicals**

All chemicals used in this study were of analytical grade and products of Sigma Aldrich, (USA), British Drug House (BDH) England, Burgoyne, (India), Harkin and Williams, (England), Qualikems (India), Fluka (Germany), May and Baker, (England).

**2.2 Methods**

 **2.2.1 Preparation of extract**

The fresh leaves of *Harungana madagascariensis* were air-dried, milled into coarse powder and macerated in a mixture of methanol and chloroform (2:1) for 48 hours. The solution was filtered with Whatman No.1 filter paper and fractioned with 20% distilled water of the total volume of the filtrate to obtain two layers that were separated using a separation funnel. The upper layer was designated methanol extract and the lower layer designated chloroform extract. They were concentrated differently using a rotary evaporator at an optimum temperature range of 45–50OC.

 **Fractionation of the methanol extract**

Fractionation of methanol extract was done using fractioning method. Fractioning of the methanol extract was done using n-hexane, ethyl acetate and aqueous-methanol (90:10 v/v) in a separation funnel. Methanol extract (20g) was dissolved in 250 ml of aqueous-methanol (90:10) and poured into a separation funnel. 250 ml of n-hexane was poured and shaken vigorously and allowed to stand. Two layers were formed; upper layer and lower layers. The upper layer was collected and designated n-hexane fraction. The lower layer was poured back to the funnel and re-washed with n-hexane. Ethyl acetate was introduced into the aqueous portion, and treated same as n-hexane. After re-washing with ethyl acetate, the last portion was aqueous methanol fraction. The fractions were concentrated using rotary evaporator and used for inflammatory tests.

**2.3 ANTI-INFLAMMATORY ASSAYS**

**2.3.1 Egg albumin-induced oedema in rats**

The rat paw oedema method of Winter *et al*. 1962 was used. Increase in the right hind paw volume induced by the sub-plantar injection of fresh egg albumin was used as a model for acute inflammation. For each of the (n-hexane, ethyl acetate and aqueous-methanol), thirty-two (32) adult wistar rats of either sex (120-200g) were divided into eight groups of four rats each. They were fasted and deprived of water for 18 hours before the experiment. Deprivation of water was to ensure hydration and to minimize variability in oedematous response. After the fasting and deprivation, the right hind paw volume of the rats was measured at time zero (t =0) using Vernier calliper. Then group 1 received 2 ml/kg b.w of 3% tween 80, group 2 and 3 received 100 and 400 mg/kg of n-hexane respectively, group 4 and 5 received 100 and 400 ethylacetate fraction and group 6 and 7 received aqueous-methanol fraction respectively while group 8 received 10 mg/kg indomethacin intraperitoneally. Acute inflammation was induced one hour after administration of fractions, by injecting 0.1 ml of undiluted fresh egg albumin into the sub-plantar of the right hind paw of the rats. Paw volumes were measured at 0.5, 1, 2, 3, 4 and 5 hours after egg albumin injection. Oedema formation was assessed by finding the difference in the zero-time paw circumference and its circumference at the different times after egg albumin injection. This was used to calculate the percentage inhibition of inflammation:

$$ \% inhibition of inflammation =\frac{\left(Vt-Vo\right)control-\left(Vt-Vo\right)treated groups}{\left(Vt-Vo\right)control}x 100$$

Where Vt = volume of oedema at time (0.5,1,2,3,4 and 5 hours)

 Vo= volume of oedema at time zero (initial time)

**2.3.2 Acetic acid induced writhing in mice**

The method described by Koster *et al*. 1959 [16] and Taber *et al*. 1969 were used. Twenty mice were fasted for six hours and divided by into five groups of four animals per group for each fraction. Groups 3-5 received the fractions intraperitoneally (100, 200 and 400 mg/kg respectively) while the groups 1 (vehicle control) and 2 (treatment control) received 2 mg /kg b.w of 3% Tween 80 and Indomethacin (10 mg/kg b.w) respectively, 30 minutes before administration of 1% acetic acid solution (0.1 ml/kg i.p). The writhes (each of which is characterized by a wave of contraction of abdominal musculature followed by extension of the hind limbs) were counted 5 minutes after acetic acid injection for the period of 20 minutes. The percentage protection against writhing was taken as an index of analgesia and calculated using the following formula:

$$\% Analgesic activity =\frac{Mean writhing count(control group-treated group)}{Mean wrthing count of control group}x 100$$

**2.3.3 Leukocyte mobilization in rats**

The effects of the fractions (n-hexane, ethyl acetate and aqueous-methanol) of the *H. madagascariensis* leaf on *in vivo* leukocyte mobilization induced by inflammatory stimulus (agar) were investigated using the method of Rebeiro *et al*. 1991. Wistar rats of either sex divided into five groups of four rats each were used for each fraction. Group 1 (control) was administered 2 ml of 3% tween 80, group 2, 3 and 4 was administered 100, 200 and 400 mg/kg of the different fractions respectively while group 5 received 10 mg/kg b.w of indomethacin. Each rat in the groups received intraperitoneal injection of 05 ml of 3% (w/v) agar in normal saline one hour after oral administration of the test substance. The rats were sacrificed four hours after agar injection and their peritonea washed with 5 ml phosphate buffered saline (PBS) containing 0.5 ml of 10 % EDTA. The peritoneal fluid was recovered and used for evaluation of total and differential leukocyte count (TLC and DLC) on the perfusates after staining with Leishmannʼs stain. The percent inhibition of leukocyte migration was calculated the following formula:

Percentage $\% leukocyte inhibition=1(\frac{T}{C})x 100$

Where T represents the leukocyte count of the treated group and C represent the leukocyte count of the control group.

**2.3.4 Cotton pellet induced granuloma test in rats**

The effect of *H. madagascariensis* leaf on chronic inflammation was evaluated using cotton-pellet granuloma in rats according to the method of Mosquera *et al*. 2011. For each of the fraction, twenty (20) adult Wistar rats of either sex (120-200g) were divided into five groups of four rats each. On day 1(one), groups 2, 3 and 4 of the rats received orally graded doses of (100, 200 and 400 mg/kg) of the fractions respectively. The control and reference groups (1 and 5 respectively) received equivalent volume of 3% tween 80 and indomethacin (10 mg/kg) respectively. Thirty minutes later, two autoclaved cotton pellets were aseptically implanted under the skin of previously shaved back of anaesthetised rats. The fraction doses, vehicle and reference were administered once daily for the following seven (7) days. On the 8 day, the animals were killed by overdose of ether and the pellets dissected out from the extraneous tissues. The wet pellets were weighed and dried in a hot air oven overnight at 40 oC. The dried pellets were weighed and increases in the dry weight of the pellets were taken as a measure of the granuloma tissue formed around each pellet.

The level of granuloma tissue formation was calculated thus:

$(\frac{Tc-Tt}{TC})x 100$ = level of granuloma tissue.

Where Tc represent weight of granuloma tissue of control group

 Tt represent weight of tissue of treated group.

**2.3.4 Determination of phospholipase A2 activity**

The preparation of phospholipase A2 from *Aspergillus niger* and assay of the effects of the on its activity was performed using the method of Vane 1971.

Fresh whole blood (5 ml) each was collected from healthy volunteers who were free from drugs for at least two weeks, the blood was centrifuged at 3000 rpm for 10 minutes and the supernatant (plasma) was discarded. The red cells obtained were re-suspended in a volume of normal saline equal to that of plasma, re-centrifuged at 3000 rpm for 10 minutes and the supernatant discarded. The red cells were reconstituted as a 40 % (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4) and this served as the substrate for phospholipase.

Fungal enzyme preparation was obtained from *Aspergillus niger* which was cultivated in a sabouraud dextrose broth for three days with intermittent shaking. The culture was transferred into a test tube containing 3 ml phosphate buffer saline and centrifuged at 3000 rpm for 10 minutes. The fungal cells settled at the bottom of the test tube while the supernatant contained the enzyme preparation that was used for enzyme assay. A volume of phosphate buffered saline (1 ml) was added into each sets of test tubes for both “test” and “blank”. Aliquots (0.2 ml) of red blood cells suspension and CaCl2 solution (0.2 ml) were added into each set of test tubes. While each test tube labelled “test” was treated with 1 ml of free enzyme, the test tubes labelled “blank” were treated with 1 ml of boiled enzyme separately. Aliquots (1 ml) of varying concentrations of each fraction (0.2-1.0 mg/ml) and 0.4 mg/ml of indomethacin in phosphate buffered saline were dispensed into the respective test tubes. The control red blood cell suspension, CaCl2 and free enzyme. The set up was incubated for 1 hour at room temperature. The incubates were then centrifuged at a speed of 3000 rpm for 10 minutes and absorbance taken at a wavelength of 418 nm using spectrophotometer.

$$\% inhibition of enzyme activity =\frac{Ac-At}{Ac}x 100$$

**2.3.5 Determination of hypotonicity-induced haemolysis of human red blood cell**

The effects of the on haemolysis of human red blood cells (HRBC) induced by hypotonic solution (distilled water) was evaluated using the method of Shinde *et al*. 1999.

Samples of each used in this assay were dissolved in distilled water (hypotonic solution) and normal (isotonic solution). The hypotonic solution (5 ml) containing graded doses of each fraction (0.2-1.0 mg/ml) were put into two sets of centrifuge tubes. The isotonic solution (5 ml) containing graded concentrations of each (0.2-1.0 mg/ml). was also put into duplicate (per dose) of the centrifuge tubes. Controls tubes contained 5 ml of distilled water and 5 ml of 0.4 of indomethacin respectively. Erythrocyte suspension (0.1) was added to each of the tubes and mixed gently. The mixtures were incubated for 1 hour at room temperature, and afterwards, centrifuged at 1300 g for 3 minutes. The absorbance (OD) of the haemoglobin content of the supernatant was determined at a wavelength of 418 nm using a spectrophotometer. The percentage haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100 %. The percentage inhibition of haemolysis by each fraction was calculated thus:

$$\% inhibition of haemolysis =1-\frac{OD2-OD1}{OD3-OD1}x 100$$

Where OD1= absorbance of test sample in isotonic solution

OD2= absorbance of test sample in hypotonic solution

OD3= absorbance of test sample in hypotonic solution

**2.4 STATISTICAL ANALYSIS**

The biochemical data obtained from the study were analyzed using a statistical program SPSS, version 22. The results were expressed as Mean ± SD. A one-way / two-way ANOVA was employed for comparison among the groups followed by Dunnet’s Post-Hoc Multiple Comparisons tests. *P=0.05* was considered as statistically significant.

**3.0 RESULTS**

**3. 1 EFFECT OF DIFFERENT FRACTIONS ON EGG ALBUMIN INDUCED RAT PAW OEDEMA**

Results in Table 1 show that the paw volumes of the rats increased significantly (P<0.05) 30 minutes after egg albumin induction. Subsequently, there was significant decrease in paw oedema in rats treated with n-hexane, ethyl acetate and aqueous-methanol fractions of methanol extract in a time dependent manner compared with rats in the negative control those administered with (3% tween 80). The reduction in paw volume (oedema) was greatest at the 5th hour in all the fractions. Maximum oedema inhibition of 69.49 % and 72.88 % was observed in rats administered with 100 and 400 mg/kg bw of aqueous-methanol fraction respectively at 5th hour. Data in Table 1 also show that the percentage inhibitory effect and decrease in paw volume of all ethyl acetate and aqueous methanol fractions were comparable with that of the standard anti-inflammatory drug, indomethacin.

**TABLE 1: EFFECT OF DIFFERENT FRACTIONS ON EGG ALBUMIN INDUCED RAT PAW OEDEMA**

|  |  |
| --- | --- |
| Groups | ∆ Paw volume(oedema) mm and % inhibition of oedema |
|  | 30 mins | 1 hr | 2hrs | 3hrs | 4hrs | 5hrs |
| Control | 0.48 ± 0.01e | 0.50 ± 0.01e | 0.53 ± 0.01e | 0.55 ± .01f | 0.57 ± 0.01f | 0.59 ± 0.01f |
| 100mg/kg n-hexane  | 0.39 ± 0.02d 18.75% | 0.38 ± 0.02d24% | 0.36 ± 0.01d32.07 | 0.34± 0.02d38.18% | 0.32 ± 0.01c43.86% | 0.30 ± 0.01c49.15% |
| 400mg/kg n-hexane | 0.32 ±0.03c33.33% | 0.30 ± 0.02c40% | 0.28 ± 0.02c50% | 0.27 ± 0 .02bc50.90% | 0.25 ± 0.02b56.14% | 0.23 ± 0.1b61.01% |
| 100mg/kg ethyl acetate | 0.44 ± 0.03e8.33% | 0.43 ± 0.03e14% | 0.41 ± 0.03e22.64% | 0.35 ± 0.1d36.36% | 0.37 ± 0.04d35.08% | 0.35 ± 0.04d40.67% |
| 400mg/kg ethyl acetate | 0.39 ± 0.02d18.75% | 0.38 ± 0.03d24% | 0.36 ± 0.03d32.07% | 0.35 ± 0.03d36.36% | 0.32 ± 0.04c43.85% | 0.29 ± 0.05c50.84% |
| 100mg/kgAqueous-methanol | 0.27 ± 0.01b43.75% | 0.25 ± 0.01b50% | 0.24 ± 0.01b54.71% | 0.22 ± 0 .02ab60% | 0.20 ± 0.01a64.91% | 0.18 ± 0.02a69.49% |
| 400mg/kgAqueous-methanol | 0.23 ± 0.02a52.08% | 0.22 ± 0.02a56% | 0.20 ± 0.02a62.26% | 0.19 ± 0 .02a65.45% | 0.18 ± 0.02a68.42 % | 0.16 ± 0.02a72.88% |
| 10mg/kgIndomethacin | 0.39 ± 0.02d18.75 | 0.37 ± 0.01d26% | 0.34 ± 0.02d35.84% | 0.32 ± 0.01cd41.81% | 0.30 ± 0.01c47.38% | 0.27 ± 0.02bc54.24% |

∆ = change in paw circumference

Values are presented as mean ± SD, n=4, p < 0.05

Percentage inhibition of oedema was calculated relative to control

Mean values with different letters of alphabet down to the column are significantly different (P < 0.05) while mean values with the same letters of alphabet down the column are not- significantly different (P > 0.05).

**3.2 EFFECT OF FRACTIONS ON ACETIC ACID INDUCED NOCICEPTIVE (ANALGESIC)**

As shown in Table 2, all the fractions (n-hexane, ethyl acetate and aqueous-methanol) as well as the standard drug, indomethacin exhibited a significant (P<0.05) inhibition of writhing induced by acetic acid though not dose dependently in n-hexane fraction. The inhibitory effect observed in groups treated with aqueous-methanol fraction was found to be higher than that observed in n-hexane and ethyl acetate fractions.

**TABLE 2: EFFECT OF FRACTIONS ON ACETIC ACID INDUCED NOCICEPTIVE (ANALGESIC)**

|  |  |  |  |
| --- | --- | --- | --- |
| Groups | Dose (mg/kg) | No of writhing (counts/20 mins) | Percentage (%) inhibition |
|  |  | N-hexane | Ethyl acetate | Aqueous-methanol | N-hexane | Ethyl acetate | Aqueous-methanol |
| 1 | 2 ml | 56.80 ± 7.90d | 56.8 ± 7.90d | 56.8 ± 7.90d | - | - | - |
| 2 | 100 | 34.00 ± 2.16c | 30.25 ± 2.6b | 33.5 ± 3.4c | 40.1 | 46.7 | 41 |
| 3 | 200 | 29.00 ± 8.79bc | 27.25 ± 6.4b | 25.3 ± 2.2b | 48.9 | 51.9 | 55.4 |
| 4 | 400 | 24.25 ± 4.35b | 24.00 ± 3.4b | 20.0 ± 3.7ab | 39.2 | 57.0 | 64.8 |
| 5 | 10 | 14.50 ± 2.30a | 14.5 ± 2.3a | 14.5 ± 2.3a | 74.4 | 74.4 | 74.4 |

Values are presented as mean ± SD, n=4, p < 0.05

Percentage inhibition was calculated relative to control

Mean values with different letters of alphabet down to the column are significantly different (P < 0.05) while mean values with the same letters of alphabet are not- significantly different (P > 0.05).

**3.3 EFFECT OF FRACTIONS ON AGAR INDUCED *IN VIVO* LEUCOCYTE MOBILISATION**

As shown in table 3, the n-hexane fraction caused a significant reduction in the in vivo leukocyte migration to the inflammation area induced by agar. The percentage inhibitions of migration were lower than that obtained for the groups treated with indomethacin. The proportion of neutrophils in the perfusate was higher than lymphocytes and macrophages in all the groups administered with n-hexane fraction compared to the control. All the doses of ethyl acetate fraction significantly reduced (p < 0.05) the agar induced in vivo leukocyte migration to the inflamed area, though not dose dependently. The percentage inhibitions of migration obtained with were 32.9, 63.3 and 58.6 % respectively and were lower than the 69.9 % inhibition obtained for the group treated with indomethacin. While the aqueous-methanol fraction significantly reduced (p < 0.05) the agar induced in vivo leukocyte migration to the inflamed area. The percentage inhibition of migration obtained were 54.5, 63.3 and 62.1 % respectively and was lower than the 69.9 % inhibition obtained for the group treated with indomethacin. The proportion of neutrophils in the perfusate was higher than lymphocytes and macrophages compared to the control. Comparatively, the total leukocyte migrations produced by the n-hexane and ethyl acetate treated groups were higher than that obtained for the aqueous-methanol fraction. Conversely, the aqueous-methanol fraction showed a greater inhibition of leukocyte migration to the flamed area than was obtained with n-hexane and ethyl acetate doses except for groups treated with n-hexane 400 mg/kg.

**TABLE 3: EFFECT OF FRACTIONS ON AGAR INDUCED *IN VIVO* LEUCOCYTE MOBILISATION**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Treatment | Dose (mg/kg) | TLC (mm3) | % inhibition | Differential Leucocyte mobilization (%) |
|  |  |  |  | N | L | M | B | E |
| Control |  | 5716.7±189d |  - | 70.33c | 19.67a | 5.67b | 2.0b | 2.33b |
| N-hexane | 100 | 4166.7 ± 136c | 27.1 | 64a | 21.66b | 5.0b | 1.0ab | 0.6a |
|  | 200 | 3266.7 ± 152b | 42.9 | 68.33a | 32.09ab | 3.33ab | 0.33a | 0.33a |
| 400 | 3450 ± 304b | 65.7 | 57.33a | 37.33ab | 3.33ab | 0.67ab | 1.33ab |
| Ethyl acetate | 100 | 2633.3 ± 196b | 32.9 | 61.67b | 36.67b | 1.0a | 0.33a | 0.33a |
|  | 200 | 2100 ± 264b | 63.3 | 55.67ab | 41.67bc | 1.3a | 0.67a | 0.67a |
| 400 | 2366.7 ± 251b | 58.6 | 50.57a | 47c | 1.3a | 0.67a | 0.33a  |
| Aqueous-methanol | 100 | 2600 ± 173b | 54.5 | 68c | 24.33ab | 5.67b | 0.67a | 1.33b |
|  | 200 | 2100 ± 264a | 63.3 | 66abc | 28.66b | 4.33b | 0.33a | 0.67ab |
| 400 | 2166.7 ± 251a | 62.1 | 60.33ab | 38c | 1.33a | 0.33a | 0.0a  |
| Indomethacin | 10 | 1750 ± 150a | 69.9 | 56.33a | 42a | 1.0a | 0.33a | 0.33a |

Values are presented as mean ± SD, n=4, p < 0.05

Percentage inhibition was calculated relative to control

Mean values with different letters of alphabet down to the column are significantly different (P < 0.05) while mean values with the same letters of alphabet are not- significantly different (P > 0.05).

**3.4 EFFECT OF N-HEXANE, ETHYL ACETATE AND AQUEOUS METHANOL FRACTIONS ON COTTON PELLET INDUCED GRANULOMA**

All the doses of n-hexane fraction significantly inhibited the cotton pellet induced granuloma tissue in rats in a dose dependent manner. Granuloma tissue weights of group treated with n-hexane fraction were significantly p < 0.05 lower than that of their control (Table 4). n-hexane fraction exhibited 33.3, 40 and 43.3 % inhibition of granuloma formation at different doses, whereas indomethacin showed 71.7% inhibition when compared to the control. Different doses of Ethyl acetate fraction, significantly inhibited the granuloma formation though, not dose dependent by 50, 46.7 and 51.7 % respectively. While aqueous-methanol fraction significantly inhibited the granuloma formation by 58.3, 63.3 and 66.7% respectively, while the standard anti-inflammatory drug, indomethacin (10 mg/kg) inhibited granuloma tissue formation more than the varied doses of the different fractions.Comparatively, aqueous-methanol fraction (400 mg/kg) had the highest inhibition compared with n-hexane and ethyl acetate fractions

**TABLE 4: EFFECT OF N-HEXANE, ETHYL ACETATE AND AQUEOUS METHANOL FRACTIONS ON COTTON PELLET INDUCED GRANULOMA**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Treatment |  Dose(mg/kg/day) |  Wet weight(mg) |  Dry weight (mg) |  Granuloma weight (mg)  |  % inhibition |
| Control (3% tween 80) |  2ml | 247± 8.7e | 187±4.5e | 60±4.5e |  - |
|  n-hexane  |  100 | 117 ± 3.1d | 77±9.0c | 40±3.2 c | 33.3 |
|  200 | 141± 3.5b | 105±7.6b | 36±9.0b | 40 |
|  400 | 127±2.5a | 93±2.0b | 34±2.0b | 43.3 |
| Ethyl acetate |  100 | 207 ± 9.1d | 117±3.8b | 30±3.8b | 50 |
|  200 | 193±5.1c | 161±4.5b | 32±4.5b | 46.7 |
|  400 | 164±3.6b | 135±2.3b | 29±2.3b | 51.7 |
| Aqueous-methanol |  100 | 193 ± 2.9d | 168±5.2d | 25±5.8d | 58.3 |
|  200 | 172±4.6c | 150±2.9c | 22±2.9c | 63.3 |
|  400 | 157±8.2b | 137±2.9b | 20±2.9b | 66.7 |
| Indomethacin |  10 | 133±2.0a | 116±4.0a | 17±4.0a | 71.7 |

Mean values with different alphabet down the column are significantly different (p < 0.05). n=4,

**3.5 EFFECT OF N-HEXANE, ETHYL ACETATE, AND AQUEOUS METHANOL FRACTIONS ON PHOSPHOLIPASE A2 ACTIVITY**

Results in Table 5, reveal that n-hexane, ethyl acetate and aqueous-methanol fractions as well as the standard drug diclofenac inhibited the release of haemoglobin into the medium by PLA2. This was shown by the significant (P < 0.05) decreases in optical densities in the varying concentrations of the fractions and diclofenac, indicating reduced haemoglobin. The percentage inhibition calculated relative to control shows that the highest concentration (1.0 mg/ml) had 59.9, 51.9 and 66.5 % exhibited a higher % inhibitory activity against the lowest concentration (0.2 mg/ml) had 29.9, 32.7 and 40.5 % in the entire fraction. Comparatively the aqueous-methanol fraction exhibited the highest activity.

**TABLE 5: EFFECT OF N-HEXANE, ETHYL ACETATE, AND AQUEOUS METHANOL FRACTIONS ON PHOSPHOLIPASE A2 ACTIVITY**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Treatment | Conc (mg/ml) | Absorbance(418 nm)n-hexane | % inhibitionPLA2 activity | Absorbance (418 nm)Ethyl acetate | % inhibitionPLA2 activity | Absorbance (418 nm)Aq-MeOH | % inhibitionPLA2 activity |
| Control | - |  1.359±0.01 |   |    |   |   |   |
| Fractions | 0.2 |  0.953±0.01\* |  29.9 |  0.914±0.02\* |  32.7 |  0.808±0.03\* |  40.5 |
|   | 0.4 |  0.791±0.07\* |  41.8 |  0.894±0.01\* |  34.2 |  0.662±0.04\* |  51.3 |
|   | 0.6 |  0.715±0.01\* |  47.4 |  0.764±0.10\* |  43.8 |  0.512±0.01\* |  62.3 |
|   | 0.8 |  0.626±0.01\* |  53.9 |  0.675±0.02\* |  50.3 |  0.498±0.01\* |  63.4 |
|   | 1.0 |  0.554±0.04\* |  59.9 |  0.654±0.00\* |  51.9 |  0.455±0.02\* |  66.5 |
| Diclofenac | 0.4 |  0.365±0.03\* |  73.1 |  0.365±0.03\* |  73.1 |  0.365±0.03\* |  73.1 |

Absorbance values are presented as mean±SD n=3.P<0.05 is significantly different compared with control

**3.6 EFFECT OF N-HEXANE, ETHYL ACETATE, AND AQUEOUS-METHANOL FRACTIONS ON MEMBRANE STABILIZATION OF HUMAN RED BLOOD CELL**

Results in Table 6, show that the n-hexane, ethyl acetate and aqueous-methanol fractions inhibited lyses of HRBC subjected to hypotonic solution. This was shown by the decrease in optical densities (OD) of hypotonic solution when compared with the control. The decrease in OD implies less haemoglobin in the medium which indicates inhibition of lyses of HRBC. The n-hexane, ethyl acetate and aqueous-methanol fractions had a concentration dependent inhibition of haemolysis, with increased concentration. The percentage inhibition (77.6 %) obtained for aqueous-methanol fraction (1.0 mg/ml) was comparable to that of the standard drug, diclofenac (81.9 %) at 0.4 mg/ml (Table 6).

**TABLE 6: EFFECT OF N-HEXANE, ETHYL ACETATE, AND AQUEOUS-METHANOL FRACTIONS ON MEMBRANE STABILIZATION OF HUMAN RED BLOOD CELL**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Treatment | Conc (mg/ml) | Absorbance(418 nm)n-hexane | % inhibition ofHaemolysis HRBC | Absorbance (418 nm)Ethyl acetate | % inhibitionHaemolysis HRBC | Absorbance (418 nm)Aq-MeOH | % inhibitionHaemolysis HRBC |
| Control | - | 2.178±0.01 |   |    |   |   |   |
| Extract | 0.2 |  1.697±0.02\* |  22.1 |  1.628±0.12\* |  25.3 |  0.775±0.04\* |  64.4 |
|   | 0.4 |  1.536±0.00\* |  29.5 |  1.303±0.02\* |  40.2 |  0.663±0.01\* |  69.6 |
|   | 0.6 |  1.209±0.01\* |  44.5 |  1.079±0.07\* |  50.5 |  0.613±0.01\* |  71.9 |
|   | 0.8 |  1.114±0.00\* |  48.9 |  0.893±0.02\* |  59 |  0.564±0.01\* |  74.4 |
|   | 1.0 |  0.953±0.00\* |  56.2 |  0.764±0.01\* |  64.9 |  0.468±0.02\* |  77.6 |
| Diclofenac | 0.4 |  0.394±0.05\* |  81.9 |  0.394±0.05\* |  81.9 |  0.394±0.05\* |  81.9 |

Absorbance values are presented as mean ±SD n=3.

\*P<0.05 is significantly different compared with control.

Percentage inhibition of haemolysis was calculated relative to control

**4.0 Discussion**

A medicinal plant is any plant in which, one or more of its organs, contains substances that can be used for therapeutic purposes or are precursor for the synthesis of useful drugs. The medicinal values of these plants lie in bioactive phytochemical constituents that produce definite physiological action on the human body. Bioactive principles are responsible the therapeutic activities of medicinal plants such as antioxidant, anti-inflammatory, anti-diabetic, antimalarial activities and antimicrobial properties (Aldughaylibi, et al., 2022).The search for alternative anti-inflammatory therapies devoid of deleterious effects on the integrity of gastric mucosa makes the research of natural products with simultaneous anti-inflammatory and anti-ulcerogenic activity important.

Inflammation is a pathophysiological process of plasma-derived and cellular events in response to infection and tissue injury. The primary mediators of inflammation are vaso-active amines and eicosanoids (prostaglandins), released from mast cells. These can cause vasodilation, fever and pain in many disorders. Oedema induced by phylogistic agent (egg albumin) is a widely accepted model for the evaluation of anti-oedema and anti-inflammatory effect of drugs. Egg albumin-induced oedema has been commonly used as an acute inflammatory models and it is believed to be biphasic. The early phase (1-2) of the oedema is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The later phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells, and prostaglandins produced by tissue macrophages (Alharbi, et al., 2023). In the present study, the fractions (n-hexane, ethyl acetate and aqueous-methanol) at various concentration and different time intervals afforded protection against egg albumin-induced paw oedema. The fractions could have prevented the release of histamine and serotonin (5-HT), the two mediators that are released by egg albumin. Its anti-inflammatory activity may be attributed to its high concentration of flavonoid, which has been reported to possess potent inhibitory effect on enzymes involved in the production of the chemical mediators of inflammation (Al-Khayri, et al.,2022; Soliman, & Barreda 2022) .

Acetic acid-induced writhing is a well recommended protocol in evaluating medicinal agents for their analgesic properties. The writhing induced by chemical substances, as acetic acid, is due to sensitization of nociceptors by prostaglandins. The characteristic of pain activity generated by intraperitoneal injection of acetic acid is presented with contraction of abdominal muscle followed by extension of hind limbs and elongation of body part and such constriction is thought to be mediated by local peritoneal receptor. Studies have revealed the increase in prostaglandins especially PGE2, PGF2, PGI2, lipoxygenase products and peritoneal mast cells in peritoneal fluids treated with algogenic acetic acid (Kushwaha, et al., 2024). Acetic acid further potentiates the pain through capillary permeability (Sultana, et al., 2022). The major contribution of prostaglandins to eliciting pain response is mainly due to interaction with endogenous mediators like histamine, serotonin, bradykinin and substance which further stimulate the sensitization of pain receptors to these mediators (Wang & Thyagarajan 2022). It is well established that NSAIDs relieve the pain response peripherally by inhibiting production of prostaglandins, thromboxane and other inflammatory mediators acting on cyclooxygenase enzymes. Any substance lessening the number of constrictions induced by acetic acid can be considered to have analgesic potential*. H. madagascariensis* fractions significantly (P<0.05) reduced the number of writhings in dose dependent manner, with highest inhibition (64.8%) recorded for aqueous-methanol fraction at 400 mg/kg as shown in Table 2. The analgesic effect of the fractions as seen in this experiment may therefore be due to either to their action on visceral receptors sensitive to acetic acid, to inhibition of the production of algogenic substances or the inhibition at the central level of the transmission of painful messages. This strongly suggests that the plant under study possess peripheral analgesic property, possibly mediated through the same mechanism of inhibition of prostaglandins generating pathway and local peritoneal inflammation.

The various doses of *H. madagascariensis* fractions evoked significant (P<0.05) inhibition of agar induced leukocyte migration into the peritoneum. At the onset of an inflammation, a number of different cells become activated and are recruited into an inflammatory area, where they release inflammatory mediators that cause vasodilation and increased permeability of plasma proteins and fluids into the tissues. The vessel become engorged and dilated allowing large numbers of neutrophils to extravagate and appear within the junctional epithelium and underlying connective tissue. These cells are responsible for the inactivation and removal of invading infectious agents and damaged tissues. Chemotactic movement of leukocytes towards the foreign body is the first and most important step in phagocytosis (Zhou & Sun 2022). Leukocytes are rapidly mobilized from the bone marrow into the blood during infections, acute inflammatory reactions and in the superficial surface of a lesion during sub-acute or chronic inflammation. They function as phagocytes of bacteria, fungi and viruses and detoxifiers of toxic proteins that may result from allergic reactions and cellular injury. Recruitment of leukocytes from circulation to sites of inflammation involves numerous soluble factors that mediate communication and interaction between circulating leukocytes and vascular endothelium. Of these soluble mediators, chemokines play a pivotal role in the process of adhesion and directional migration of leukocytes. Chemokines are produced by a variety of cell types, such as those of hematopoietic and non hematopoietic origins, in response to antigens, polyclonal stimulants, cell irritants and cytokines. The effect of the fractions on in-vivo leukocyte mobilization leads to decrease in total leukocyte count. The anti-inflammatory effects of *H. madagascariensis* might have been possible through the alteration of the activation of inflammatory cells or by reducing the process of recruiting soluble inflammatory mediators, adhesion and migrating of leukocyte. In differential leukocyte mobilization, all the tested doses neutrophil, lymphocyte, basophil, macrophages and eosinophil of the fractions showed decrease in number when compared with the control. Eosinophils and basophils are predominant when inflammation is initiated by immediate allergic reactions or parasites. The neutrophils being higher in percentage than the lymphocytes probably might have led to the engulfing, and elimination of the foreign body and to the alteration in the migration of the inflammatory cells. The inhibition of leukocyte migration by fractions therefore showed that the plant could alter the action of the endogenous factors that are involved in the migration of these cells to the site of inflammation, thereby reducing the inflammatory process.

The cotton pellet granuloma model has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation. There are three phases in the inflammatory response in the cotton pellet induced granuloma. In the first phase, inhibition of fluid containing low protein takes place at the site of cotton implantation. In the second phase, after 2-3 days of pellet implantation, exudation of fluid containing protein takes place. In the third phase, that is the proliferative phase, appearance of collagen mucopolysaccharide synthesis, and increase in the number of fibroblasts around the cotton pellet occurs. The amount of newly formed connective tissue can be measured after removing and weighing the dried pellets. *H. madagascariensis* leaves significantly (P<0.05) decreased the final dry weight of the cotton pellets that is, it decreased the amount of granulomatous tissue, suggesting that it has the capability of reducing the synthesis of mucoopolysaccharides and collagen and the number of fibroblasts, which are natural proliferative events of granulation in tissue formation (Anosike et al., 2012a). *H. madagascariensis* leaves decreased the weight of granuloma tissue in a dose dependently, confirming its activity in the chronic phase of inflammation.

This study revealed the mechanisms of anti-inflammatory action of the anti-inflammatory action of the fractions have been established using inhibition of phospholipase A2 and membrane stabilization. It revealed that the fractions inhibited PLA2 activity in a concentration dependent manner. Phospholipase A2 is an enzyme that cleaves free fatty acids from membrane phospholipids. Arachidonic acid released from these phospholipids is acted upon by cyclooxygenase (COX) and lipoxygenase (LOX) both of which lead to the de novo synthesis of lipid mediators. The action of COX and on arachidonic acid produces mediators such as TXA2, prostaglandin E2, D2, and I2, while the action of 5-LOX on arachidonic acid releases leukotrienes such as leukotrienes B4 (LTB4). The fractions of *H. madagascariensis* leaves (n-hexane, ethyl acetate and aqueous-methanol) from 0.2-1.0 mg/ml exhibited significant (p<0.05) and concentration dependent inhibition of PLA2 activity. This inhibition by the fractions PLA2 activity by the fractions implies that it was able to suppress the release of free fatty acids from the red blood cell (RBC) membrane phospholipids and consequent deprivation of COX and LOX precursors for the synthesis of inflammatory mediators, hence limiting their effects such as vasodilation, vascular permeability, chemotaxis and pain, thereby preventing inflammation. The mechanism of PLA2 inhibition by the fractions could be direct inhibitory action of Glucocorticoids, which might be attributed to the presence of flavonoids in the fractions. Glucocorticoids exert their multiple metabolic functions by binding to cytoplasmic receptors and subsequent induction of catalytic or regulatory proteins (Yamamoto, et al., 2015). Their anti-inflammatory properties have been attributed in part to the liberation and enhanced synthesis of proteins, collectively lipocortins, which inhibits phospholipase A2 (Dar, et al., 2012).

To further investigate the mechanisms underlying the anti-inflammatory activities of the plant, the inhibition of membrane lyses in rats was examined. From the data shown in Table 6, the fractions of *H. madagascariensis* leaves significantly (p<0.05) protected the human erythrocyte membrane against lyses induced by hypotonic solution compared to the control. This leads to decrease in haemoglobin released, indicated by the reduction in absorbance values of the extract relative to control (hypotonic solution). The highest percentage inhibitions (56.2, 64.9 and 77.6%) of haemolysis were obtained at 1.0 mg/ml of the fractions respectively. This study demonstrated the capability of the plant fractions to inhibit lyses of human red blood cells (HRBCs) membrane induced by hypotonic solution. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the fractions may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated leucocytes such as, bactericidal enzymes and proteases, which upon release cause further tissue inflammation and damage (Wang & Thyagarajan, 2022). It has also reported that the cell volume of erythrocytes is closely related to the intracellular content of calcium (Dar, et al., 2012). Both membrane stabilization and phospholipase A2 activity are related to low calcium availability. Hence, it could be speculated that the cytoprotective effect of the fractions on the erythrocyte membrane might be due the ability of the fractions to alter the influx of calcium into the erythrocyte.

**Conclusion**

This study revealed that *H. madagascariensis* leavespossess anti-inflammatory properties. The possible underlining mechanism by which the extract inhibited inflammation include: inhibition of PLA2 activity, and stabilization of lysosomal membrane. This result could be attributed to the abundant phyto-constituents present in the plant suggesting the use of *H. madagascariensis* in treatment of pain and inflammatory- related diseases. However, Aqueous-methanol fraction had a better activity than the other fractions.

**ETHICAL APPROVAL**

Animal Ethic committee approval has been collected and preserved by the author(s)

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

**COMPETING INTERESTS**

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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