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RESEARCH ARTICLE

Effects of *Hoslundia opposita* Leaf, a Traditional Remedy for Furuncles, on Cell-mediated Responses to Inflammation and Immunostimulation

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Abstract

Background: In Nnobi, southeast Nigeria, crushed leaf of *Hoslundia opposita* Vahl (*Lamiaceae*) is applied on hard furuncles/boils as a ripening agent to evoke formation of pus. **Aim:** This research aimed to assess the effects of *H. opposita* leaf on cell-mediated responses to inflammation and immunostimulation. **Methods:** The effects of the methanol: dichloromethane (1:1) extract (HOE) of *H. opposita* leaf, and its hexane (HOHF), ethyl acetate (HOEF) and methanol (HOMF) fractions on cell-mediated responses to inflammation and immunostimulation were studied using *in vivo* and *in vitro* methods. The extract and fractions were subjected to *in vivo* acetic acid-induced increase in vascular permeability and carrageenan-induced leucocyte migration tests. The effects of the extract and fractions on heat- and hypotonic solution-induced haemolysis of erythrocytes, and phagocytic activity of neutrophils were evaluated *in vitro*. **Results:** The results showed that the HOE significantly suppressed acetic acid-induced increase in vascular permeability ($P<0.05$), and heat- and hypotonic solution-induced haemolysis of erythrocytes ($P<0.001$). The HOE and fractions also decreased total leucocyte migration ($P<0.05$), inhibited both phagocytic activity of neutrophils ($P<0.001$) and intracellular reduction of nitroblue tetrazolium dye to formazan in phagocytosing neutrophils ($P<0.001$). **Conclusion:** The results obtained indicate that the efficacy of *H. opposita* leaf in managing furuncles may primarily stem from its ability to curb cell-mediated responses to inflammation and immunostimulation.

Major Findings: The efficacy of crushed leaf of *H. opposita* in the treatment of furuncles is primarily mediated through suppression of increased vascular permeability, leucocyte migration and phagocytosis, along with membrane stabilisation effect.

Keywords: Endotoxin-activated Plasma, Leucocyte Migration, Membrane Stabilisation, Neutrophils, Nitroblue Tetrazolium, Phagocytosis, Vascular Permeability

1. Introduction

A furuncle, or boil, is a painful, pus-filled lump under the skin resulting from infected and inflamed hair follicles or a cut in the skin. A furuncle (boil) develops as a deep-seated lump in the skin, sometimes presenting a central 'head' full of pus. Pus comprises living, dead and disintegrated neutrophils, living and dead microorganisms, and cell debris, all suspended in the inflammatory exudate. A furuncle is initially very small, but in about 2 weeks grows into a highly painful, pus-filled large-sized nodule, as white blood cells accumulate at the site to fight the infection, which, on maturation, oozes the accumulated pus. However, some boils last longer than 2 weeks without a head/accumulation of pus. Symptoms can include signs of inflammation such as redness, swelling, and pain; a yellow or white tip/centre and oozing; skin weeping

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and crusting. Acute inflammation of a hair follicle may present as a furuncle in which there is a focal collection of neutrophils and tissue debris, producing pus – suppuration[1,2]. A localised accumulation of pus, which is a creamy fluid, yellow or blood-stained and often viscous, constitutes an abscess[1,2]. Abscess formation/suppuration is a common sequela of acute inflammation[3-5].

The inflammatory reaction, the physiological response of living tissues to cell injury due to diverse agents, is an important aspect of the body's defence mechanism. The inflammatory reaction serves to localise and eliminate the causative agent, limit tissue injury, and restore the tissue to normality (or as close to normality as possible). The immune system shields the body from a variety of environmental threats, principally microbes[6,7]. In the inflammatory response, which is essentially a protective and salutary process, there are complicated interactions involving the vascular system, the immune system and repair mechanisms. Inflammation is an effector mechanism of immunity and one of the first responses of the immune system to infection or irritation[6,8,9]. Inflammation has immune recognition components, and inflammatory responses are mediated by the immune system. Neutrophils, lymphocytes, macrophages, eosinophils, and some other cells are involved in both inflammatory and immune responses[3-7]. There is an interplay of the body's responses to inflammation and stimulation of the immune system: both have some common humoral and cellular mediators; inflammation occurs in the immunological response due to activation of mast cells, basophils, etc., and action of mediators and complement products; and they share some processes such as phagocytosis, chemotaxis, and inflammation.

Besides its salutary effects, the inflammatory response may have injurious effects on the organ or host; frequently, there may be a need for an intervention to limit the injurious effects. Such intervention includes drainage of abscess, use of antibiotics and anti-inflammatory agents. One way to facilitate drainage of pus is the use of boil ripening agents.

Several herbs, such as *Curcuma longa* (turmeric), *Allium sativum* (garlic), are used as home or traditional remedies to treat boils or facilitate the accumulation of pus in boils. One of such herbal remedies is *Hoslundia opposita* Vahl (*Lamiaceae*) leaf. *Hoslundia opposita*, commonly known as orange berry, has widespread natural distribution, occurring in both tropical and sub-tropical open woodland[10,11]. It is commonly found throughout Africa, from Senegal, Nigeria, to Sudan, Ethiopia, Tanzania and southern Africa. The morphology has been described[12,13]. The tasty fruits are edible and serve as food for rabbits in Ogun state (southwest Nigeria). In Africa, various parts of the plant are popular traditional remedies. The aerial parts are used to treat gonorrhea, cystitis, hookworm, cough, wounds and bilharzia[14-16]. The leaf infusion is widely used as a purgative, diuretic, febrifuge, antibiotic and antiseptic[17]. To relieve indigestion, the leaves are combined with the leaves of *Ocimum basilicum*, boiled and the decoction consumed in northwestern Tanzania[18]. It is used to treat anaemia in northern and south-eastern Cote d'Ivoire[19]. The $GABA_A$ receptor stimulatory, insecticidal, and antioxidant activities of *H. opposita* leaves have been demonstrated[20-23]. The leaves' essential oil has been demonstrated to have antidyslipidemic, antidiabetic, antimicrobial, insect repellent, and acaricidal activities[22, 24-27].

In Nnobi, southeast Nigeria, *H. opposita* leaf is applied on hard furuncles/boils as a boil ripening agent to evoke the formation of pus/pus head and ripening of the boil; hence, they call it *ulu ntu* (Igbo term that literally means boil ripening/softening agent). The fresh leaves are crushed and applied to the furuncle, which bursts and releases pus after about 30 minutes following the appearance of pus close to the surface of the furuncle.

This study evaluated the effects of *H. opposita* leaf extract and fractions on cellular inflammatory and immunological responses, which may underlie its traditional use as a boil ripening agent.

2. Materials and Methods

2.1 Animals

Adult conventional grade UN-FERH: NS outbred strain of albino mice (*Mus musculus*) (19–29 g) and Sprague Dawley rats (*Rattus novergicus*) (120-170 g) of either sex bred in the laboratory animal facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, were deployed for the study. The animals were fed on standard pellets and

water. The National Research Council Guide for the Care and Use of Laboratory Animals was followed in every investigation involving animals[28]. The Ethics Committee of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, approved the experimental protocol (FPSRA/UNN/24/0082).

2.2 *Candida albicans* Culture

Laboratory strain of *Candida albicans* was cultured in a Sabouraud agar plate and transferred before use to Sabouraud broth, under aseptic conditions at the Department of Microbiology, University of Nigeria, Nsukka.

2.3 Preparation of Extract

Fresh *H. opposita* leaves were gathered in December, from Mbu in Isiuzo Local Government Area of Enugu State, Nigeria. The plant was identified by a Taxonomist at the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, where a voucher specimen was deposited (specimen No. InterCEDD/831).

After being cleaned and allowed to dry in the shade for five days, the leaves were ground into a coarse powder using a grinding machine. The pulverised plant material (8.3 kg) was macerated in a combination of methanol and dichloromethane (1:1) at room temperature (26±1°C) for 48 h and filtered. Fresh solvent was used to continually wash the plant material until the filtrate turned clear. The filtrate was evaporated in a rotary vacuum evaporator, under reduced pressure at 40°C to afford the concentrate, which was subsequently dried to a constant weight to obtain 395.25 g of the methanol: dichloromethane extract (HOE; 4.76% w/w).

2.4 Fractionation of HOE

The HOE (300 g) was fractionated in a silica gel (70–230 mesh size) column successively eluted with n-hexane, ethyl acetate and methanol in order of ascending polarity. The eluents were evaporated in a rotary vacuum evaporator under reduced pressure at 40°C to afford the different concentrates which were subsequently dried to a constant weight to obtain the hexane (HOHF; 24.95 g; 8.32% w/w), ethyl acetate (HOEF; 51.53 g; 17.12% w/w) and methanol (HOMF; 85.46 g; 28.49% w/w) fractions.

2.5 Phytochemical Analysis

Using established methods, phytochemical analyses were performed on HOE and fractions[29-31].

2.6 Acute Toxicity and Lethality Test

Acute toxicity and lethality (LD_{50}) of HOE were evaluated using an established protocol[32], with slight modifications. In stage one, mice were assigned to three groups (n=3) at random and given 10, 100, and 1000 mg/kg of HOE, respectively, through the oral route. The mice were carefully watched for 24 h for manifestations of toxicity and death. Since no death occurred after 24 h in stage one, 1600, 2900 and 5000 mg/kg were orally administered to another set of mice at one dose per animal (n=1) in stage two. The mice were watched for 24 h for the manifestation of toxicity and mortality.

2.7 Pharmacological Tests

2.7.1 *In Vivo Studies*

2.7.1.1 *Acetic acid-induced Vascular Permeability*

The technique described by Whittle was deployed to assess the effect of HOE on vascular permeability[33]. Mice were randomly placed in groups (n = 6) to receive HOE (200, 400, or 800 mg/kg) orally. Animals in control groups received 10% Tween 80 (5 ml/kg) or piroxicam (50 mg/kg). After an hour, each mouse received an intravenous injection of 0.2 ml of Evans blue dye (0.25% in normal saline) via the tail vein. The mice were then administered an intraperitoneal acetic acid solution (0.6% v/v) (1 ml/100 g) after 30 min. After 30 min of stimulus injection, the animals were sacrificed and the peritoneal cavity washed with normal saline (5 ml) into heparinized tubes. The peritoneal washout was centrifuged at 3000 rpm for 30 min, and the dye content measured at 610 nm using a spectrophotometer, SPECTRO 21D™ (PEC MEDICALS, USA).

2.7.1.2 *Leucocyte Migration Induced by Inflammatory Stimulus*

A modification of the methods described by Ribeiro *et al.*,[34] and Ezike *et al.*,[35] was utilized to evaluate the effects of HOE and fractions on leucocyte migration *in vivo*. The rats were randomly assigned to groups (n=6) to receive 200, 400 or 800 mg/kg of HOE, HOHF, HOEF and HOMF, respectively, through oral route. Control groups received 10% Tween 80 (2.5 ml/kg), or piroxicam (50 mg/kg). One hour later,

each rat was given 0.5 ml of 2% (w/v) carrageenan suspension in normal saline intraperitoneally. The rats were sacrificed four hours later, and the peritoneal cavity washed with 5 ml of phosphate buffer saline (PBS) containing 0.5 ml of 10% EDTA. Total and differential leucocyte counts of the peritoneal fluid were determined.

2.7.2 In Vitro Studies

2.7.2.1 Membrane Stabilisation Activity

2.7.2.1.1 Preparation of Erythrocyte Suspension

Fresh whole ox blood (20 ml) was collected from the Abattoir in Ikpa Market, Nsukka, into EDTA tube (containing 0.5 ml of 10% EDTA). About 10 ml was withdrawn and centrifuged at 4000 rpm for 5 min. The supernatant was decanted, and the packed cells washed four times with equal volume of normal saline and reconstituted to a 40% v/v suspension of erythrocytes with isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4)[36]. The isotonic buffer solution contained the following (g/L) - NaCl (9.00), Na_2HPO_4 (1.15) and $NaH_2PO_4.2H_20$ (0.26).

2.7.2.1.2 Heat-induced Haemolysis

This was done as previously described by Ezike *et al*[35]. Test concentrations of HOE (50, 100, 200 and 400 µg/ml) dissolved in 0.01% v/v Tween 80 in isotonic buffer were prepared, and 5 ml of each was put into two pairs of test tubes. In addition, four test tubes containing 5 ml of hydrocortisone (50 µg/ml), four test tubes containing 5 ml of piroxicam (50 µg/ml), and two control test tubes containing 0.01% Tween 80 in isotonic buffer were prepared. The erythrocyte suspension (0.05 ml) was added to each test tube and gently mixed. A pair of each set of test tubes (containing a particular test substance or control) was maintained at 0–4°C in a freezer for 20 min, while the other pair was incubated at 54°C in a regulated water bath for 20 min. Following incubation, the contents of each test tube was centrifuged at 4000 rpm for 5 min and the absorbance (OD) of the supernatant measured at 540 nm using a spectrophotometer, SPECTRO 21D™ (PEC MEDICALS, USA). This procedure was done in triplicate.

Inhibition of haemolysis was calculated using the relation[35]:

Inhibition of haemolysis (%) = $100[1-(OD_2 - OD_1)/(OD_3 – OD_1)]$

where OD_1 = absorbance of test sample unheated; OD_2 = absorbance of test sample heated; OD_3 = absorbance of control sample heated.

2.7.2.1.3 Hypotonic Solution-induced Haemolysis

This was done as previously described by Ezike *et al*[35]. The HOE was dissolved in 0.01% v/v Tween 80 in isotonic buffer or distilled water to give test concentrations of 50, 100, 200 and 400 µg/ml in isotonic and hypotonic solutions, respectively. Each test concentration (5 ml) was put into two pairs of test tubes, each pair containing isotonic and hypotonic solutions, respectively. In addition, two pairs of test tubes containing 5 ml of hydrocortisone (50 µg/ml) in isotonic or hypotonic solution per pair, and a pair of control test tubes containing 5 ml of 0.01% v/v Tween 80 in distilled water (hypotonic solution) were prepared. The erythrocyte suspension (0.05 ml) was added to each tube and gently mixed. Following 1 h of incubation at room temperature (29±1°C), the mixture was centrifuged at 4000 rpm for 5 min and the absorbance (OD) of the supernatant measured at 540 nm using a spectrophotometer, SPECTRO 21D™ (PEC MEDICALS, USA). This procedure was done in triplicate.

Inhibition of haemolysis was calculated using the relation[35]:

Inhibition of haemolysis (%) = $100[1-(OD_2 - OD_1)/(OD_3 – OD_1)]$

where OD_1 = absorbance of test sample in isotonic solution; OD_2 = absorbance of test sample in hypotonic solution; OD_3 = absorbance of control sample in hypotonic solution.

2.7.2.2 Phagocytic Activity of Neutrophils

2.7.2.2.1 Preparation of Candida albicans Suspension

Laboratory strains of *Candida albicans* culture incubated overnight in Sabouraud broth was centrifuged at 3000 rpm for 15 min to form the cell button. Subsequently, the supernatant was discarded and the cell button rinsed with sterile Hanks Balanced Salt Solution (HBSS) and centrifuged at 3000 rpm for 15 min. This was repeated three times. Subsequently, the cell count was adjusted to 1 x 10^8 cells/ml using the 0.5 McFarland Standard. The resulting cell suspension

was centrifuged and the cell button obtained was suspended in a mixture of sterile HBSS (0.8 ml) and rat serum (0.2 ml) in 4:1 proportion and used for the experiment as the *C. albicans* suspension.

2.7.2.2.2 Assay of Phagocytic Activity

Appropriate dilutions of HOE, HOHF, HOEF and HOMF (dissolved with 3% Tween 80 in PBS) were done to give test concentrations of 5, 10, 25, 50 and 100 µg/ml. Levamisole (10 and 25 µg/ml) and 3% Tween 80 in PBS were used as controls. Each test concentration (0.04 ml) was introduced into a test tube containing 0.4 ml of rat blood. The test tubes were incubated at 37°C for 15 min. Subsequently, 0.04 ml of the *C. albicans* suspension was added to each test tube and incubated at 37°C for 1 h. Afterwards, 5 µL of the contents of each test tube was smeared on a microscope slide, air-dried for 48 h to fix, stained with Leishman stain, and allowed to dry. This procedure was done in triplicate, using blood from different rats.

2.7.2.2.3 Evaluation of Phagocytosis

The mean number of *Candida* cells phagocytosed by neutrophils per 100 granulocytes on each slide was established microscopically using morphological criteria, this was used as the phagocytic index (PI). The PI of each test group was compared with PI of control. Inhibition (%) of phagocytic activity was calculated using the relation:

Inhibition (%) = 100(1-x/y); where: x = PI of test, y = PI of control.

2.7.2.3. Nitroblue Tetrazolium (NBT) Test

The nitroblue tetrazolium (NBT) test was done using a modification of the method described by Ganachari *et al* [37]. Sheep blood collected in a heparin tube was centrifuged, and a suspension of the leucocytes was made from the buffy coat. The concentration of the leucocyte suspension was adjusted to 5 x 10^6 /ml with PBS, and 0.5 ml was put into each assay test tube. Subsequently, 0.1 ml of 5, 10, 25, 50 and 100 µg/ml of each of HOE, HOHF, HOEF and HOMF dissolved using 3% Tween 80 in PBS were introduced singly into the test tubes. The 3% Tween 80 in PBS (0.1 ml) and endotoxin-activated plasma (0.1 ml) served as controls. Exactly 0.2 ml of freshly prepared 0.2% NBT solution was put into each test tube and incubated at 37°C for 20 min. The contents of each test tube were centrifuged at 3000 rpm for 5 min and the supernatant was discarded. Subsequently, a thin film was made with 5 µL of the contents of each assay test tube on a slide, dried for 48 h, fixed by heating and counterstained with dilute carbol fuchsin for 30 s. The slide was washed under tap water, dried and viewed under oil immersion (100x). The NBT positive cells containing dark blue granules in two hundred neutrophils were counted in four different fields, and the average was taken. The experiment was done in triplicate.

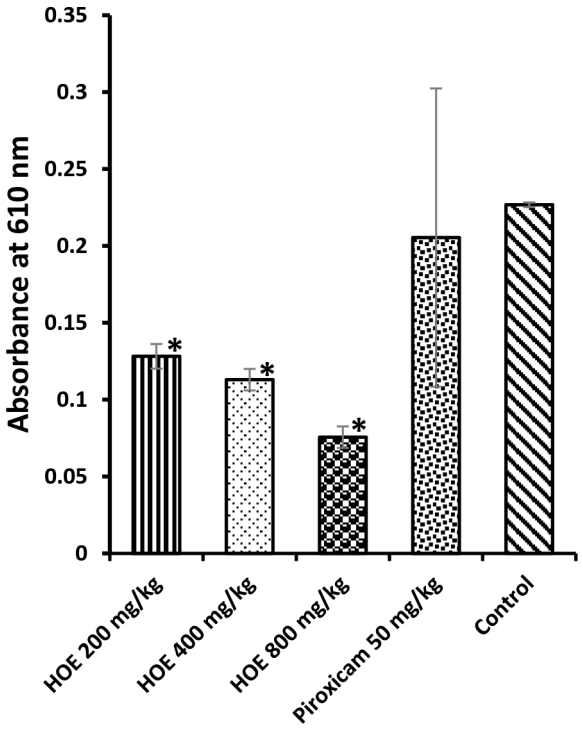
2.8 Statistical Analysis

The results obtained were analysed using One-way ANOVA in GraphPad Prism 8.3.0 and subjected to Dunnett's Post hoc test. Differences between the means of treated and control groups were considered significant at $P<0.05$ and $P<0.001$, as appropriate. The results were presented as mean ± SEM.

3. Results

3.1 Phytochemical Constituents of *H. opposita* Leaf Extract and Fractions

The HOE tested positive for alkaloids, flavonoids, resins, saponins, tannins, steroids, and terpenoids; the



n = 6; * P<0.05, compared to control (Dunnett's); HOE = *H. opposita* leaf methanol:dichloromethane (1:1) extract

Figure 1. Effect of *H. opposita* leaf extract on acetic acid-induced increase in vascular permeability.

Table 1. Effects of *H. opposita* leaf extract and fractions on leucocyte migration

Treatment	Dose (mg/kg)	TLC	Neutrophil count	Lymphocyte count	Monocyte count
HOE	200	9.38±0.36 (21.77)	2.69±0.096 (NI)	4.37±0.20 (15.15)	2.22±0.21* (57.06)
	400	5.46±0.59* (54.46)	1.31±0.15 (14.38)	2.52±0.27* (51.07)	1.25±0.15* (75.82)
	800	4.13±0.39* (65.55)	0.76±0.083* (50.33)	1.87±0.18* (63.69)	1.36±0.15* (73.69)
HOHF	200	5.62±0.88* (53.13)	0.83±0.13* (45.75)	2.53±0.41* (50.87)	2.17±0.32* (58.03)
	400	3.96±0.30* (66.97)	0.68±0.062* (55.56)	1.91±0.14* (62.91)	1.31±0.11* (74.66)
	800	3.98±0.38* (66.81)	0.75±0.056* (50.98)	1.83±0.20* (64.47)	1.37±0.14* (73.50)
HOEF	200	7.48±0.83* (37.61)	1.53±0.19 (0.00)	3.90±0.48* (24.27)	2.03±0.18* (60.74)
	400	7.52±0.54* (37.28)	1.60±0.13 (NI)	3.76±0.21* (26.99)	2.06±0.25* (60.15)
	800	8.03±0.82* (33.03)	1.03±0.13 (32.68)	4.32±0.44* (16.12)	2.62±0.26* (49.32)
HOMF	200	8.03±1.17* (33.03)	1.76±0.27 (NI)	2.84±0.40* (44.85)	3.32±0.50* (35.78)
	400	7.76±0.52* (35.28)	1.74±0.16 (NI)	2.64±0.19* (48.74)	3.28±0.18* (36.56)
	800	8.09±0.71* (32.53)	1.60±0.14 (NI)	3.39±0.32* (34.17)	3.02±0.28* (41.59)
Piroxicam	50	6.19±0.42* (48.37)	1.08±0.073 (29.41)	2.42±0.16* (53.01)	2.65±0.24* (48.74)
Control	2.5 ml/kg	11.99±1.18	1.53±0.15	5.15±0.58	5.17±0.54

n=6; *P<0.05 compared to control (Dunnett's multiple comparison test); values in parentheses indicate inhibition of leucocyte migration (%) relative to control; HOE methanol: dichloromethane extract; HOHF hexane fraction; HOEF = ethyl acetate fraction; HOMF = methanol fraction; NI = no inhibition

HOHF was positive for resins, steroids, and terpenoids; HOEF gave positive reactions for flavonoids, resins, and tannins; while HOMF tested positive for alkaloids, flavonoids, resins, saponins, tannins.

3.2 Acute Toxicity and Lethality

Oral administration of HOE caused no death at 10 – 5,000 mg/kg; also, no obvious indicators of acute toxicity were seen. The LD_{50} of HOE was more than 5,000 mg/kg.

3.3 Effect of *H. opposita* Leaf Extract on Acetic acid-induced Increase in Vascular Permeability

The HOE elicited dose–related and significant (P<0.05) suppression of acetic acid-induced increase in vascular permeability (Figure 1). The HOE 200, 400 and 800

Table 2. Effects of *H. opposita* leaf extract on heat- and hypotonic solution-induced haemolysis of red blood cells

Treatment	Concentration (µg/ml)	Inhibition of heat-induced haemolysis (%)	Inhibition of hypotonic solution-induced haemolysis (%)
HOE	50	62.27*	31.23*
	100	65.37*	31.71*
	200	65.26*	33.30*
	400	63.46*	38.70*
Hydrocortisone	50	70.93*	42.45*
Piroxicam	50	83.98*	NT

* P < 0.001; Inhibition (%) of haemolysis shown were derived from absorbance values; HOE = dichloromethane:methanol extract of *H. opposita* leaf; NT= not tested

Table 3. Effects of *H. opposita* leaf extract and fractions on *in vitro* phagocytic activity of neutrophils

Treatment	Concentration (µg/ml)	Phagocytic Index	Inhibition (%)
HOE	5	15.67±0.33*	59.12
	10	18.33±1.86*	52.18
	25	12.67±0.67*	66.94
	50	4.33±0.67*	88.70
	100	0.33±0.33*	99.14
HOHF	5	14.33±1.33*	62.61
	10	16.67±2.17*	56.51
	25	22.00±0.58*	42.60
	50	6.67±0.33*	82.60
	100	6.33±1.33*	83.49
HOEF	5	13.00±1.53*	66.08
	10	9.00±1.16*	76.52
	25	4.67±0.33*	87.82
	50	2.33±0.33*	93.92
	100	6.67±0.33*	82.60
HOMF	5	11.33±0.33*	70.44
	10	16.00±0.58*	58.26
	25	9.0±0.58*	76.52
	50	4.67±0.33*	87.82
	100	0.67±0.33*	98.25
Levamisole	10	14.00±1.16*	63.48
	25	20.67±2.028*	46.07
Control	0.04 ml	38.33±0.67	—

n=3; *P<0.001 compared with control (3% Tween 80 in PBS) (Dunnett's multiple comparison test); HOE methanol: dichloromethane extract; HOHF = hexane fraction; HOEF = ethyl acetate fraction; HOMF: methanol fraction

mg/kg evoked 43.46, 50.17 and 66.66% inhibition of acetic acid-induced increase in vascular permeability, respectively. The inhibition produced by HOE was greater than that of piroxicam (9.42%).

3.4 Effects of *H. opposita* Leaf Extract and Fractions on Carrageenan-induced Leucocyte Migration

The HOE and fractions elicited significant (P<0.05) inhibition of total leucocyte (21.77 to 66.97%, with HOHF eliciting the greatest inhibition); lymphocyte (15.1 to 64.47%, with HOHF eliciting the greatest inhibition); and monocyte counts (35.78 to 74.66%, with HOHF eliciting the greatest inhibition), respectively, compared to control. The HOE and HOHF produced dose-related inhibition (Table 1).

The HOE and HOHF also significantly inhibited neutrophil count, while HOMF elicited an increase in neutrophil count. Administration of lower doses of HOEF (200 and 400 mg/kg) caused an increase in neutrophil count, while 800 mg/kg reduced neutrophil count (Table 1).

3.5 Effects of *H. opposita* Leaf Extract on Membrane Stabilisation

The HOE elicited significant (P<0.05) inhibition of heat- and hypotonic solution-induced haemolysis of erythrocytes (Table 2), with greater protection against heat-induced haemolysis (Table 2).

3.6 Effects of HOE and Fractions on *in vitro* Phagocytic Activity of Neutrophils

The HOE, fractions, and levamisole elicited significant (P<0.001) inhibition of phagocytic activity of neutrophils (Table 3).

3.7 Effects of HOE and Fractions on Intracellular Reduction of Nitroblue Tetrazolium

The extract and fractions significantly decreased the intracellular reduction of NBT to formazan (deep blue compound) by the neutrophils, as shown by a significant reduction (P<0.001) in the proportion (%) of NBT-positive cells compared to control (Table 4).

4. Discussion

Results of the study demonstrated that *H. opposita* leaf extract and fractions suppressed the increase in vascular permeability, modulated total leucocyte migration, elicited membrane stabilisation effects, and inhibited both phagocytic activity and intracellular killing capacity of neutrophils. These observed effects of *H. opposita* leaf constituents suggest anti-inflammatory and immunosuppressive actions. Notwithstanding the salutary effects of inflammation, anti-inflammatory agents are needed and deployed to limit the injurious effects of the inflammatory response.

The inflammatory response consists of complicated interactions involving the vascular system, the immune system, and repair mechanisms. Also, inflammation can be immune-mediated mediated[6,7]. The cellular phase of inflammation consists of exudation, leucocyte migration, and phagocytosis. In the acute inflammatory response, there are three major vascular events: vasodilatation and increased blood flow, increased

Table 4. Effects of *H. opposita* leaf extract and fractions on intracellular reduction of NBT

Treatment	Concentration (µg/ml)	NBT Positive cells per 200 cells	NBT Positive cells (%)	Reduction in NBT positive cells (%)
HOE	5	29.00±1.16	14.50±0.58	9.38
	10	26.50±0.29	13.25±0.14	17.19
	25	20.00±0.58	10.00±0.29*	37.50
	50	16.33±0.88	8.17±0.44*	48.94
	100	8.67±0.33	4.34±0.17*	72.88
HOHF	5	27.33±1.67	13.67±0.83	14.56
	10	27.00±0.58	13.50±0.29	15.63
	25	16.33±2.03	8.17±1.01*	48.94
	50	7.67±1.20	3.83±0.60*	76.06
	100	4.00±0.00	2.00±0.00*	87.50
HOEF	5	27.33±1.86	13.67±0.92*	14.56
	10	20.00±0.00	10.00±0.00*	37.50
	25	15.50±0.29	7.75±0.14*	51.56
	50	8.33±0.33	4.17±0.17*	73.94
HOMF	5	30.33±0.33	15.17±0.17	5.19
	10	19.00±0.00	9.50±0.00*	40.63
	25	11.67±0.88	5.84±0.44*	63.50
	50	7.00±0.58	3.50±0.29*	78.13
	100	4.50±0.29	2.25±0.14*	85.94
Endotoxin-activated plasma	0.1 ml	66.50±0.87	33.25±0.43*	NR
Control	0.1 ml	32.00±0.00	16.00±0.00	—

n=3; *P<0.001 compared with control (3% Tween 80 in PBS) (Dunnett's multiple comparison test); Reduction in NBT positive cells (%) was calculated relative to control; NR = no reduction; HOE = methanol: dichloromethane extract; HOHF hexane fraction; HOEF = ethyl acetate fraction; HOMF: methanol fraction

vascular permeability, and leucocytosis. Acetic acid causes an immediate, sustained increase in vascular permeability that is prolonged over 24 h[1]. The *H. opposita* leaf extract suppressed the upsurge in vascular permeability elicited by acetic acid, demonstrating reduction in exudation and its sequelae, and inhibition of inflammation.

Vasodilatation and increased vascular permeability usher in the cellular phase of acute inflammation, wherein migration of leucocytes, principally neutrophils, from the circulation into the site of tissue injury occurs. Leucocytes are part of the immune system, and include neutrophils, lymphocytes, monocytes, eosinophils and basophils. Monocytes eventually leave the bloodstream and become tissue macrophages, which engulf and digest pathogens, such as cellular debris, cancer cells, microbes, and other foreign substances. Neutrophils, the most abundant circulating leucocytes, are an essential component of the inflammatory response and the innate immune system. Generally, neutrophils comprise the first line of body defence and are the dominant cells in acute inflammatory exudate in the first 24 h, while monocytes/macrophages appear in the next 24-48 h; though there are exceptions. Neutrophils, monocytes and macrophages are phagocytic. Intraperitoneal administration of carrageenan in rodents elicits acute inflammation characterised by accumulation of fluids and migration of many leucocytes into the peritoneal cavity. Extract and fractions modified carrageenan-induced migration of leucocytes into the peritoneum, as shown by the reduced total leucocyte count.

Though HOHF and higher doses of HOE reduced neutrophil count, HOMF and lower doses of HOEF and HOE elicited no effect or an increase in neutrophil count. The observation that the administration of *H. opposita* leaves constituents reduced or increased neutrophil count suggests the ability to both suppress and slightly stimulate neutrophil migration. These apparently paradoxical effects, which have been observed elsewhere, are known to depend on the procedure, timing, dose administered and host genetic background[38]. For instance, levamisole has been demonstrated to elicit both immunosuppressant and immunostimulant actions[38]. The ability of extracts and fractions to affect the migration of cellular components of the inflammatory response and the immune system, such as neutrophils, lymphocytes, and monocytes, to the site of injury suggests immunoregulatory activity. However, results suggest the predominant activity is immunosuppression and anti-inflammatory activity as shown by the ability of HOE and all fractions to reduce total leucocyte, monocyte and lymphocyte counts.

Activated neutrophils release their lysosomal constituents (e.g. bactericidal enzymes and proteases) at sites of inflammation, triggering more tissue impairment and inflammation[3-5]. The membranes of intracellular lysosomes and erythrocytes (red blood cells; RBC) are analogous[39]; therefore, the extract's capacity to stabilise erythrocyte membranes suggests potential to stabilise lysosomal membranes. Stabilisation of the membrane inhibits lysis and release of the lysosomal contents, consequently reducing inflammation and tissue damage. Damage to the RBC membrane makes the cell become fragile and rupture easily. Heating and increase in temperature, hypotonic solution, mechanical damage, drugs and other chemicals, oxidising agents, membrane defects, among other factors, have been demonstrated to cause haemolysis[4,5,40]. Erythrocytes in mammalian blood spontaneously haemolyze when heated above body temperature[41-43]. Mechanisms of heat-induced haemolysis include fragmentation, increase in both fluidity and permeability of the RBC membrane, and a change in sphericity coefficient[44,45]. Also, studies have demonstrated that heating and increasing temperatures affect RBC membrane mechanical stability[41], with resultant loss of RBC deformability[41,46,47]. When put in a hypotonic solution, RBC swell and increase in cell volume with a decrease in surface–to-volume ratio. Therefore, the RBC gets spheroidal, less deformable and more fragile, leading to lysis[40,48]. The HOE inhibited heat- and hypotonic solution-induced lysis of ox red blood cells *in vitro*, substantiating the membrane stabilisation effect. Inhibition of the increase in vascular permeability and migration of inflammatory cells, stabilising cellular membranes to avert lysis and the resultant discharge of the cytoplasmic contents, will result in significant suppression of inflammation and immune response. Acetylsalicylic acid has been demonstrated to stabilise lysosomes[49,50], and this contributes to its anti-inflammatory effect. It is worth noting that the reference anti-inflammatory agents used in the experiment, piroxicam and hydrocortisone, also elicited membrane-stabilising effects. The extract elicited greater inhibition of heat-induced haemolysis. Inhibition of heat-induced haemolysis suggests preservation of the fluidity, permeability, and mechanical stability of the RBC membrane, while inhibition of hypotonic solution-induced haemolysis suggests preservation of the cell surface to volume ratio as mechanisms of membrane stabilisation. The extract may act through these mechanisms to elicit membrane stabilisation, ultimately suppressing body responses to inflammation and immunostimulation.

Once neutrophils accumulate at the site of inflammation, they are involved in clearing the injurious agent by the process of phagocytosis. Phagocytosis is mainly a defensive reaction against invasion of the body by antigens, microbes and other foreign substances. Phagocytosis is the process of engulfment and internalisation of solid particulate material, including invading microorganisms, damaged cells and tissue debris by specialised cells called phagocytes or phagocytic cells. The phagocytic cells include neutrophils, monocytes and macrophages. Phagocytosis involves three sequential cellular events: recognition and attachment of the particle to be ingested; its engulfment with ensuing formation of a phagocytic vacuole; and intracellular killing, degradation, or digestion of the ingested material[3-5]. Extracts and fractions significantly inhibited the phagocytic function of neutrophils, suggesting possible suppression of inflammation and immunological responses.

Once attached to the cell membrane of the phagocyte, the particle is engulfed and subsequently exposed to degradative lysosomal enzymes. Hence, the final step in the elimination of infectious agents, necrotic cells and other foreign substances is their killing, digestion or degradation within phagocytic cells. The killing of microorganisms by neutrophils depends on oxygen-dependent and oxygen-independent mechanisms[1,3-5]. Though both mechanisms likely co-occur during the phagocytosis of most organisms, the oxygen-dependent mechanism is of greater importance. The oxygen-dependent annihilation of microorganisms is due to reactive oxygen species/metabolites (H_2O_2, superoxide anion, hydroxyl radical, singlet oxygen) formed during the respiratory/oxidative burst, which follows activation of cell membrane NADPH oxidase by phagocytosis [1,3-5]. The NBT test, based on the formation of a deep blue/blue black/purple formazan precipitate following neutrophil-mediated reduction of the NBT dye, is a commonly used method to test for neutrophil respiratory burst activity[51-53]. Nitroblue tetrazolium is reduced by superoxide/free oxygen radicals to produce formazan[54-56], hence the NBT test is used to measure reactive oxygen species-generating activity in the cytoplasm of neutrophils. Neutrophils that phagocytose NBT and have an oxidative burst produce large formazan deposits in the cytoplasm, which can be visualised using a microscope and quantified. The HOE and fractions elicited a significant reduction in the number of NBT-positive cells, indicating inhibition of intracellular reduction of the phagocytosed NBT dye to formazan. This inhibition of the reduction of NBT indicates the ability to interfere with the metabolic functions of the neutrophils after phagocytosis of the foreign NBT dye. The ability of the extract and fractions to curb the metabolic activity of activated neutrophils indicates a suppression of the intracellular killing capacity of neutrophils. The extract and fractions elicited varying degrees of inhibition of the migration, phagocytic activity and intracellular killing capacity of neutrophils, suggesting suppression of defensive functions of neutrophils during inflammation and immunostimulation.

5. Conclusion

This study demonstrated that constituents of *H. opposita* leaf inhibit the activities of cells that mediate responses to inflammation and immunostimulation. In conclusion, the efficacy of *H. opposita* leaf in the treatment of furuncles may be primarily due to its anti-inflammatory and immunosuppressive activities mediated through suppression of increased vascular permeability, leucocyte migration and phagocytosis, along with membrane stabilisation effect. The results validate the use of the plant in the treatment of furuncles.

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7. Author Contribution

Adaobi Chioma Ezike: Conceptualisation. Adaobi Chioma Ezike and Ebere Nwamaka Okonkwo: Methodology, data analysis, writing, review and editing. Adaobi Chioma Ezike: Supervision. All authors have read and agreed to the published version of the manuscript.

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