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Evaluation of the analgesic and anti-inflammatory activities of methanol leaf extract of *Heterotis rotundifolia*

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Abstract

The aerial part of *Heterotis rotundifolia* is used in ethnomedicine to relieve pain and inflammatory conditions. This study investigated the analgesic and anti-inflammatory effects of the methanol leaf extract of *Heterotis rotundifolia* (MLEHR) in albino rats. Air-dried pulverized leaves of *H. rotundifolia* (400 g) were extracted by cold maceration in two litres of 90% hydromethanol. An oral acute toxicity test of the extract was done following the up-and-down method. The analgesic potential of MLEHR (200, 400 and 800 mg/kg) was tested using the hot plate, tail flick and acetic acid-induced writhing tests. Its anti-inflammatory activity was investigated by means of the carrageenan-induced paw oedema and xylene-induced ear oedema models. The preliminary phytochemical analysis of MLEHR showed presence of flavonoids, alkaloids, saponins, tannins, carbohydrates, fats and oils. There was neither death nor signs of toxicity after 14 days of observation in the acute toxicity test, and the LD_{50} was above 4,000 mg/kg body weight. The results of the hot plate model and acetic acid induced writhing showed a significantly ($p < 0.05$) higher pain reaction time and a significantly ($p < 0.05$) lower number of abdominal constrictions, respectively in the treated groups when compared to the untreated control. The tail flick test only showed significantly ($p < 0.05$) higher pain reaction time in the group treated with 800 mg/kg of the extract when compared to the untreated control. The extract at all the doses used in the treatment led to significantly ($p < 0.05$) lower oedema formation induced by carrageenan and xylene. The effects of treatment with MLEHR as recorded in this study validate the folkloric use of *Heterotis rotundifolia* to alleviate pain and inflammation.

Keywords: *Heterotis rotundifolia;* analgesic; anti-inflammatory; methanol extract.

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Introduction

Pain and inflammation are non-specific symptoms of many diseases and disorders of humans and animals (Shojaii *et al*., 2015; Madubuike *et al*., 2021). Effective and efficient management of pain and inflammation is therefore a very important aspect of general health care delivery. Narcotic analgesics (opiates) and the non-steroidal anti-inflammatory drugs (NSAIDs) have been the major therapeutic agents used against nociceptive and inflammatory conditions (Ezeja *et al*., 2011; Madubuike and Asuzu, 2015). Treatment with these drugs is usually associated with numerous adverse effects such as: gastrointestinal ulceration, nephrotoxicity, central nervous system depression, tolerance and physical dependence (Domaj *et al*., 1999; Farshchi *et al*., 2009; Harvey and Champe, 2009). Recently, there is a growing interest in the discovery of novel pain killers and anti-inflammatory drugs that may be safer and possibly more cost effective than the currently available ones (Madubuike *et al*., 2021).

Over the years, natural products of plant origin have proven to be rich sources of novel therapeutic agents (Woode *et al*., 2008; Ainooson *et al*., 2009). In Nigerian ethnomedicine, the aerial parts of *Heterotis rotundifolia* is used to treat a number of diseases associated with pain and inflammation (Abere *et al*., 2009; Nwodo *et al*., 2015). The plant is a perennial decumbent herb that belongs to the *Melastomataceae* family, and widely grows in tropical Africa (Wagner *et al*., 1990). Its common name is "pink lady". In Nigeria, different tribes identify it with different names: it is called 'nkpisi-nku' in Igbo; 'awede' in Yoruba; 'balli' in Hausa; 'ebafo' in Benin and 'akpaliihie' in Ikwere (Ogunka-Nnoka *et al*., 2020).

Decoction of the leaves of *H. rotundifolia* is used by rural and sub-urban dwellers in Nigeria to treat rheumatism, stomach ache, diarrhea, cough, conjunctivitis (Chukwuma *et al*., 2015) and trypanosomosis (Nwodo *et al*., 2015). It is also used in East Africa to treat bilharzias and manage dysentery (Abere *et al*., 2009). Its antimicrobial activity has also been reported (Abere *et al.*, 2010; Dougnon *et al*., 2017). The present study investigated the analgesic and anti-inflammatory activities of *H. rotundifolia* methanol extract in the albino rat model.

Materials and Methods

Plant collection and extract preparation: Fresh leaves of *Heterotis rotundifolia* were collected from Eket in Akwa Ibom State, Nigeria. The plant was identified at the Botany Department of Michael Okpara University of Agriculture, Umudike (MOUAU). A voucher specimen with identification number: MOUAU/VPP/2018/14 was deposited in the Institution's herbarium. The leaves were air-dried and reduced to coarse powder using an electric blender. Four hundred grams of the coarse powder was extracted with two litres of 90% methanol, by cold maceration. The extract was oven-dried (40°C) after concentration with a rotary evaporator, and stored as methanol leaf extract of *Heterotis rotundifolia* (MLEHR) at 4 °C until time of use (Madubuike *et al*., 2012).

Preliminary phytochemical analysis of MLEHR: A qualitative phytochemical analysis of the extract was done, following the methods of Harbourne (1991) and Trease and Evans (1996).

Animals: One hundred and seventy albino rats were used for the study. They were procured at eight-weeks of age, from the Experimental Animal Unit of the College of Veterinary Medicine, MOUAU. The mean weight of the rats was 98.5 ± 4.07 g. They were housed in stainless steel rat cages and fed *ad libitum* with standard pelleted rat chow. The experimental protocol was approved by the institution's Research Ethics Committee

(Approval No.: MOUAU/CVM/REC/201917). The rats were handled in accordance with the guidelines of the National Institute of Health (NIH) for the Care and Use of Laboratory Animals (NIH, 2011).

Acute toxicity test: A modified up-and-down acute toxicity test procedure was employed for the determination of the oral acute toxicity of MLEHR. Three (3) albino rats were dosed orally with 4000 mg/kg of MLEHR. Another three rats received equivalent volume of water orally and served as untreated control. The rats had free access to feed and drinking water for 14 days during which they were monitored for evidence of toxicity and death (OECD, 2008).

Hot plate test: Thirty albino rats, randomly assigned into five groups (n = 6), were used for the hot plate test. Group A rats served as the untreated control and received distilled water (10 ml/kg). Group B rats served as the standard control and were given 200 mg/kg of acetylsalicylic acid (ASA) orally. Groups C, D and E were treated orally with MLEHR at 200, 400 and 800 mg/kg body weight, respectively. One hour later, the rats were placed on a hot plate maintained at temperature of 55^{o}C ± 1^{o}C and their pain reaction time recorded. The response time was noted as the time at which the animals reacted to the pain stimulus by jumping, raising or licking the hind limb. A cut-off time was set at 20 seconds to protect the paws from thermal injury (Shalheen *et al*., 2000).

Tail flick/immersion test: The rats used for the tail flick/tail immersion test were assigned into five groups of six rats each. Group A rats served as untreated control and received distilled water (10 ml/kg). Group B rats served as standard control and were given ASA (200 mg/kg) *per os*. Groups C, D and E rats were treated orally with 200, 400 and 800 mg/kg body weight of MLEHR, respectively. After one hour, about 3 cm length of the tail of each rat was dipped into a water bath maintained at 50^{o}C ± 1^{o}C. The response time was noted as the time it took for the rats to withdraw their tail from the hot water (Pandurangan *et al*., 2013).

Acetic acid-induced writhing response: The method described by Ganeshpurkar and Rai (2013) was adopted for this experiment. Five groups of six randomly selected rats per group, were used for this test. Group A rats received distilled water (10 ml/kg); Group B rats received ASA (200 mg/kg); Groups C, D and E were given 200, 400 and 800 mg/kg body weight of the MLEHR, respectively. The drug (ASA) and the extract (MLEHR) were administered orally. One hour later, 0.7% acetic acid (10 ml/kg) solution was injected intraperitoneally to all the rats. The number of writhes (abdominal constrictions) occurring within 30 minutes after acetic acid injection were counted. A significant reduction of writhes in treated animals compared to those in the control group was considered as an antinociceptic response. Analgesic activity was calculated using the formula: Percentage Analgesia = (Mean number of writhes in the untreated control group – Mean number of writhes in a treated group) / Mean number of writhes in the untreated control group (Madubuike and Asuzu, 2015).

Carrageenan-induced paw oedema: The carrageenan-induced paw oedema test for assessing the anti-inflammatory activity of MLEHR was done based on the method of Ganeshpurkar and Rai (2013). Thirty rats were randomly assigned to five groups of six rats each. The untreated control group (Group A) received distilled water (10 ml/kg) orally. The reference group (Group B) was treated orally with ASA (200 mg/kg). Following the oral route also, MLEHR was administered to the other groups (C, D and E) in doses of 200, 400 and 800 mg/kg, respectively. All the treatments were administered one hour before the induction of oedema, which was achieved by administering 0.1 ml of 1% w/v carrageenan in saline into the plantar surface of the right hind

paw of the rats. Oedema formation was quantified as foot volume increase and measured by water displacement using a calibrated glass tube into which the right hind paws of the rats were dipped. The anti-inflammatory activity was calculated as: Percentage inhibition = 100 (1- $\frac{a-x}{b-y}$), where: a = the mean paw volume of the test rats after carrageenan injection; b = the mean paw volume of the control rats after carrageenan injection; x = the mean paw volume of the test rats before carrageenan injection; and y = the mean paw volume of the control rats before carrageenan injection (Madubuike and Asuzu, 2015)

Xylene-induced ear oedema: The xylene-induced ear oedema experiment was carried out according to the method of Sadeghi *et al*. (2014). Thirty rats were randomly assigned to five groups of six, per group. Cutaneous inflammation was achieved by topical application of xylene (30 µL) on the dorsal surface of the right ear of the rats. The untreated control group (Group A) receive only xylene (30 µL/ear). The reference group (Group B) was treated topically with ASA (100 µL/ear) and xylene (30 µL/ear), both applied together on the dorsal surface of the right ears of the rats. The extract (MLEHR) was locally administered to the other groups (C, D and E) at 100, 200 and 400 µL/ear, respectively, together with the irritant (xylene 30 µL/ear) as described earlier. Two hours later, the animals were euthanized and two ear punches of both ears (6 mm diameter) were taken from each rat and weighed on a Metler analytic balance. The increase in the weight of the right (treated) ear punch compared to the left (untreated) ear indicated the oedema. The anti-inflammatory activity was evaluated as the percent oedema reduction in the rats treated with the extract/drug, with respect to the untreated control rats given the irritant only, using the formula: Percentage Inhibition = 100(1 - X/Y); where X = the mean oedema of the test group; and Y = the mean oedema of the control group (Madubuike and Asuzu, 2015).

Data Analysis: Data obtained from the study were subjected to one-way analysis of variance (ANOVA), using the SPSS version 24. Variant means were separated by the least significant difference (LSD) method and differences were considered significant at $p < 0.05$. Summaries of the results were presented as means ± standard error of mean (SEM) in tables, bar charts and line graphs.

Results

Preliminary phytochemical analysis: The preliminary phytochemical analysis of the MLEHR revealed that it contains the following constituents: flavonoids, tannins, alkaloids, saponins, fats and oils and carbohydrates. Alkaloids were present in high concentrations. Flavonoids, fats and oils and saponins were moderately present, while tannins and carbohydrates were present in low concentrations.

Oral acute toxicity: The extract administered at 4000 mg/kg resulted in no death, and no sign of toxicity was observed within the 14 days observation period. The LD_{50} was considered to be above 4000 mg/kg.

Effect of MLEHR on the pain reaction time of albino rats (hot plate test): The results of the hot plate test showed that treatment with MLEHR at all the doses used for the treatment, led to significantly ($p < 0.05$) higher pain reaction time in the treated groups when compared to the untreated control group (Figure 1).

Effects of treatment with MLEHR on the pain reaction time of albino rats (tail flick test): The results of the tail flick test showed that treatment with MLEHR evoked a significantly ($p < 0.05$) higher pain reaction time in all the treated groups, when compared to the untreated control group, and this was dose-dependent (Table 1).

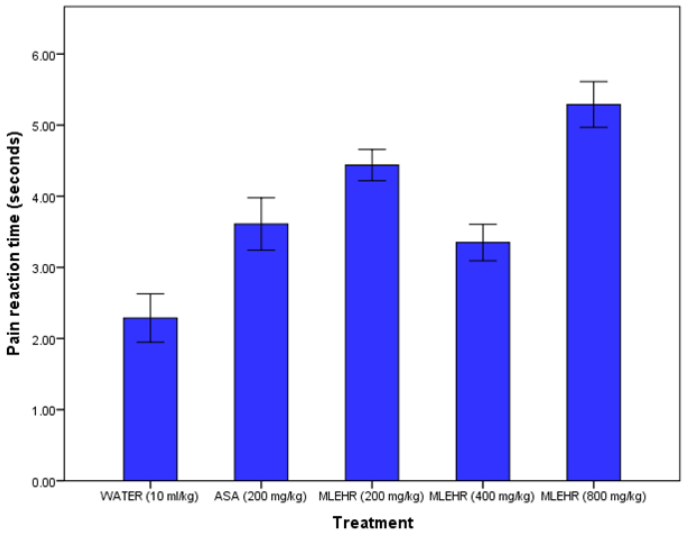

Figure 1. Effect of treatment with methanol leaf extract of *Heterotis rotundifolia* (MLEHR) on the pain reaction time of albino rats (hot plate test). [MLEHR = Methanol leaf extract of *Heterotis rotundifolia*; ASA = Acetylsalicylic acid]

Table 1. Effect of treatment with methanol leaf extract of *Heterotis rotundifolia* (MLEHR) on the pain reaction time of albino rats (tail flick test). [MLEHR = Methanol leaf extract of *Heterotis rotundifolia*; ASA = Acetylsalicylic acid]

Groups	Treatment	Pain reaction time (seconds)
A	Distilled water 10 ml/kg	2.39 ± 0.13
B	ASA 200 mg/kg	3.66 ± 0.19*
C	MLEHR 200 mg/kg	3.17 ± 0.18*
D	MLEHR 400 mg/kg	3.50 ± 0.11*
E	MLEHR 800 mg/kg	4.42 ± 0.15*

* P < 0.05 when compared with untreated control group

Effect of treatment with MLEHR on the acetic acid-induced writhing response of albino rats: Treatment with the extract, at all doses used and the reference drug (ASA) led to significantly ($p < 0.05$) lower number of writhes (abdominal constrictions) in the treated groups when compared to the untreated control group. This effect of the extract was dose-related, with treatment with the highest dose (800 mg/kg) recording the lowest number of writhes (Table 2).

Effect of treatment with MLEHR on carrageenan-induced paw oedema in albino rats: Treatment with MLEHR ameliorated paw oedema in the treated groups. The reduction in paw oedema was significant ($p < 0.05$) 4 h post-induction of inflammation (Figure 2). The maximum anti-inflammatory activity (74%) was recorded for the rat group treated with 800 mg/kg MLEHR, followed by the reference drug (ASA), which caused a 73% anti-inflammatory activity, 4 h after carrageenan

administration. Treatment with the extract at 400 and 200 mg/kg caused 43% and 32% anti-inflammatory activity respectively, four hours post oedema induction.

Effects of treatment with MLEHR on xylene-induced ear oedema: The different treatment doses (100, 200 and 400 µg/ear) of MLEHR led to significantly ($p < 0.05$) lower xylene-induced ear oedema in the rats, exhibiting 51, 73 and 81% anti-inflammatory activity, respectively (Table 3). The effect of MLEHR was dose-dependent, and all doses compared favourably above that of the reference drug (ASA), which achieved 22% inhibition of xylene-induced ear oedema in the rats.

Table 2. Effect of treatment with methanol leaf extract of *Heterotis rotundifolia* (MLEHR) on the acetic acid-induced writhing response of albino rats. [MLEHR = Methanol leaf extract of *Heterotis rotundifolia*; ASA = Acetylsalicylic acid]

Groups	Treatment	Number of writhes	% Analgesia
A	Distilled water 10 ml/kg	25.80 ± 1.92	0
B	ASA 200 mg/kg	3.80 ± 0.45*	85.3
C	MLEHR 200 mg/kg	10.60 ± 2.61*	58.9
D	MLEHR 400 mg/kg	8.20 ± 2.28*	68.2
E	MLEHR 800 mg/kg	2.60 ± 0.55*	89.9

* $P < 0.05$ when compared with untreated control group

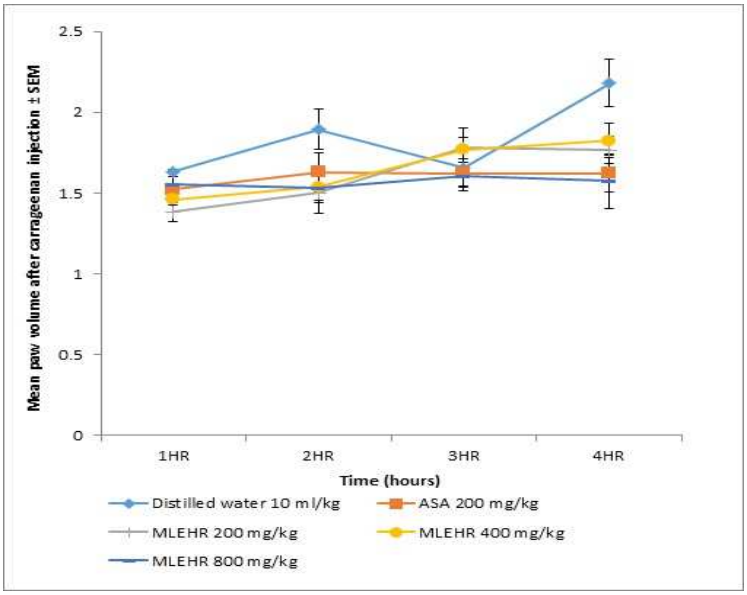

Figure 2. Effect of treatment with methanol leaf extract of *Heterotis rotundifolia* (MLEHR) on carrageenan-induced paw oedema of albino rats. [MLEHR = Methanol leaf extract of *Heterotis rotundifolia*; ASA = Acetylsalicylic acid]

Table 3. Effects of treatment with methanol leaf extract of *Heterotis rotundifolia* (MLEHR) on xylene-induced ear oedema in albino rats. [MLEHR = Methanol leaf extract of *Heterotis rotundifolia*; ASA = Acetylsalicylic acid]

Group	Treatment	Weight difference (mg) between right and left ear punches ± S.E.M.	Percentage anti-inflammatory activity
A	Xylene 30 µL/ear	1.34 ± 0.04	0
B	ASA (100 µg/ear) + Xylene (30 µL/ear)	1.04 ± 0.03*	22
C	MLEHR (100 µg/ear) + Xylene (30 µL/ear)	0.66 ± 0.03*	51
D	MLEHR (200 µg/ear) + Xylene (30 µL/ear)	0.36 ± 0.01*	73
E	MLEHR (400 µg/ear) + Xylene (30 µL/ear)	0.26 ± 0.02*	81

* $P < 0.05$ when compared with untreated control group

DISCUSSION

The result of the preliminary phytochemical analysis of MLEHR agrees with the reports of Roko *et al*., (2020) and Ogunka-Nnoka *et al*., (2020) who showed the presence of alkaloids, flavonoids, tannins, carbohydrates and essential oils in *H. rotundifolia*. The essential oils identified by Ogunka-Nnoka *et al*., (2020) include: N-Hexadecanoic acid, Stigmasterol and 12-Octadecadienoic acid. These essential oils have been reported to possess anti-inflammatory and antioxidant properties (Adeoye-Isijola *et al*., 2018; Ogunka-Nnoka *et al*., 2020), hence their presence in MLEHR may have contributed to the analgesic and anti-inflammatory activities exhibited by MLEHR in this study. In addition, many plants that are rich in alkaloids and flavonoids have been profiled as analgesics (Farouk *et al*., 2008; Shoaib *et al*., 2016; Madubuike *et al.,* 2021) and anti-inflammatory (Madubuike and Asuzu, 2015) agents. It is also possible that one or some of these phytochemicals (alkaloids or flavonoids) that are present in MLEHR, are responsible for the analgesic and anti-inflammatory activities of the extract as observed in this study.

The absence of mortality and morbidity in oral acute toxicity study is an indication that the LD_{50} of MLEHR is greater than 4000 mg/kg. The extract was therefore considered acutely safe and well tolerated even at the highest dose of 4,000 mg/kg that was administered.

The hot plate and the tail immersion tests are selective test models for centrally-acting analgesics (Woolfe and Macdonald, 1994; Ezeja *et al.*, 2011; Shoaib *et al*., 2016). The results of treatment with MLEHR in these two models suggest that MLEHR may also contain analgesic principles that act via the central nervous system. Roko *et al*., (2019) also reported significant analgesic activity of ethanol extract of *Heterotis rotundifolia* in rats subjected to the tail immersion test.

Acetic acid-induced writhing reflex is a popular model for testing substances with analgesic potential (Madubuike *et al.*, 2021). Pain in this model is triggered by localized inflammatory response, leading to cyclooxygenase-mediated prostaglandins synthesis (Onasanwo and Elegbe, 2006; Ahmed *et al.*, 2006; Madubuike and Asuzu 2015). These prostaglandins stimulate the nociceptive neurons (Dhara *et al*., 2000) thereby inducing pain sensation. The abdominal constriction response may also result from the activation of local peritoneal receptors (Bentley *et al*., 1983) which involves

prostanoids mediators. The analgesic activity of NSAIDs is due to inhibition of cyclooxygenase pathway and synthesis of prostaglandins (Vane and Botting 2003; Biswal *et al*., 2003; Rang *et al*., 2006). It is thought that the antinociceptive effect of MLEHR, which was expressed by significantly lower number of abdominal constrictions in the treated groups, could probably be due to inhibition of the cyclooxygenase pathway of arachidonic acid metabolism (which yields prostaglandins) and subsequent down-regulation of inflammatory mediators such as cytokines and interleukins (Reibero *et al*., 2000).

Carragenan-induced paw oedema test is the most widely used model for testing of non-steroidal anti-inflammatory agents (Tasleem *et al.*, 2014). Inhibition of inflammation in this model is believed to be biphasic (Kaushik *et al*., 2012; Eidi *et al*., 2016). The early phase had been reported to last between one to two hours, and it is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings, while the late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Antonio and Souza, 1998; Gupta *et al*., 2006). Since MLEHR significantly inhibited paw oedema induced by carrageenan in the second phase, it is most likely that the anti-inflammatory activity of MLEHR is mediated via inhibition of cyclooxygenase enzyme, which is the major mechanism of action of the NSAIDs.

The xylene-induced oedema is one of the classic models for acute inflammation test (Sun *et al*., 2018; Singsai *et al*., 2020). The inhibition by MLEHR of ear oedema induced by xylene is a demonstration of the ability of the extract to also down-regulate local inflammation (Madubuike and Asuzu, 2015; Anyasor and Ijitayi, 2018).

Conclusion: The methanol leaf extract of *Heterotis rotundifolia* in this study exhibited significant analgesic and anti-inflammatory activities. This result justifies the ethnopharmacological use of the plant for treating pain and inflammatory conditions.

Conflict of interest

The authors declare that no conflict of interest was associated with this work.

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