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Quantitative Analysis, Anti-Inflammatory and Analgesic Effects of Ethanol Leaf Extract and Fractions of *Microsorium scolopendria* (Burm. f.) Copel. in Mice

Uwemedimo Francis Umoh[1,*], Ekikere Ezekiel Ubengama[2], Gina Ramos[3], Victor Chubueze Onyido[4]
[1]Department of Pharmacognosy and Natural Medicine, Faculty of Pharmacy, University of Uyo, Uyo, Ibom State, Nigeria.
[2]Department of Pharmacognosy, Faculty of Pharmacy, University of Calabar, Calabar, Cross River State, Nigeria.
[3]Department of Pharmacognosy, Faculty of Pharmacy, Madonna University, Nigeria, Elele, Rivers State, Nigeria.
[4]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Madonna University, Nigeria, Elele, Rivers State, Nigeria.

Corresponding author*
uwemedimoumoh@uniuyo.edu.ng

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Abstract

Microsorium scolopendria, a fern was studied for phytochemicals, anti-inflammatory, and analgesic effects to add credence to its various folkloric applications. Phytochemicals were analyzed quantitatively, anti-inflammatory experiment was carried out with egg albumin-induced paw oedema and xylene-induced ear oedema models while analgesic effects were studied using formalin-induced paw licking, acetic acid-induced writhing and hot plate-induced pain models. The results revealed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, and phenols with alkaloids (14.6 %w/w) as the most predominant phytoconstituent. In anti-inflammatory experiments, *M. scolopendria* extract reduced oedema caused by egg albumin and xylene in a dose related manner and comparable to standard agents. Also, in analgesic experiments, the extract reduced pain induced by formalin, acetic acid, and hot plate. These reductions were statistically ($p \leq 0.05$) significant. Considering the fractions in all experiments, butanol and ethyl acetate fractions were prominent in anti-inflammatory effect while ethyl acetate fraction was at top in reduction of pain. This study supports the use of *M. scolopendria* in ethnomedicinal practice.

Keywords: *Microsorium scolopendria*; Quantitative analysis; Anti-inflammatory; Analgesic; Mice.

INTRODUCTION

Microsorium scolopendria, commonly known as "wart fern" of the Polypodiaceae family, studied with synonyms such as *Phymatosorus scolopendria, Polypodium scolopendria, Microsorum parksii, Polypodium scolopendria,* and *Microsorum parksii* (Snogan et al., 2007; Wunderlin et al., 2024) is reported to be indigenous to Polynesian Islands of Fuji, Tahiti, Hawaii, Rapa Nui, and Madagascar (Ramanitrahasimbola et al., 2005) and natively distributed in tropical Africa, Ceylon, Indochina, Malaysia to Polynesia and Australia (Holttum, 1954).

Ethnomedicinally, it is reported to be helpful in management of asthma, inflammatory diseases, cancer, abscesses (where a paste of ground leaves is usually mix with earth from a wasp's nest), wounds, insanity, coughing fits and as enema (Fernández et al., 2011). Other reported folkloric uses are; as a purgative, treatment of antibacterial, gastric and renal infections, stomach aches, gastrointestinal aches and as a diuretic. Its sweet smelling fronds are used in perfumery and clothing industries (Jofré et al., 2016; Ho et al., 2015). Its attributable properties are associated with high contents of polyphenols in the plant tissues (Xia et al., 2014; Shuvalov et al., 2020; Wang et al., 2023). Apart from its ethnomedicinal attributes, it is a valuable air-purifying ornamental plant (Snogan et al., 2007).

Pharmacological reports have it that, it has adaptogenic and anabolic effects (Hunyadi *et al.*, 2016: Ambrosio *et al.*, 2020), neuroprotective effect (Ho et al., 2007), improved cognitive impairment and protection against brain injury and antioxidant effects (Xia et al., 2014; Baroni *et al.*, 2021; Balada et al., 2022; Wang et al., 2023). Scientific data on this specie is scanty compared to reported traditional uses. More so, oral reports from the indigenous people of Ikot Ekpene Local Government Area of Akwa Ibom State, Nigeria have it that *M. scolopendria* is used for the management of cough, headache, stomach aches and gastrointestinal aches. This study is justified by the fact that there is no report on the anti-inflammatory and analgesic properties of *M. scolopendria*, thus lending scientific credence to its numerous traditional uses.

Figure 1. *M. scolopendria* in a natural habitat.

MATERIALS AND METHODS

Plant collection and Identification

The plant, *M. scolopendria* was collected from Ikot Ekpene local Government, Akwa Ibom State, Nigeria. It was identified by Dr. Imoh Imeh Johnny of the Department of Pharmacognosy and Natural Medicine, Faculty of Pharmacy, University of Uyo, Uyo and authenticated by Prof (Mrs.) M. E Bassey of the Department of Botany and Ecological Studies, Faculty of Science, University of Uyo, Uyo, Nigeria.

Preparation of Extract

The leaves were separated from the stems, air-dried and reduced to powder with hammer mill. 1 kg of the powdered leaf was macerated with 70% ethanol with intermittent agitation and filtered after 72 hours. The filtrate was concentrated using a rotary evaporator and further dried in a water bath at a temperature of 40℃ to obtain an extract free from extraction solvent and stored in a refrigerator for use in the study.

Quantitative Phytochemical Screening of Extract

Quantification of Alkaloid Content: The ethanol leaf extract (1mg) of *M. scolopendria* was dissolved in dimethyl sulphoxide (DMSO) and 1mL of 2 N HCl was added, filtered, and transferred to a separating funnel. Bromocresol green solution (5 mL) and phosphate buffer (5 mL) were added. The mixture was shaken with 1 mL, 2 mL, 3 mL, and 4 mL chloroform and collected in a 10 mL volumetric flask, and adjusted to the required volumes with chloroform. Atropine (20, 40, 60, 80, and 100 µg/mL) as reference standard was prepared in the same manner as the test material above. The absorbance for both the test and standard solutions were determined against the reagent blank at 470 nm with UV/Visible spectrophotometer and the total alkaloid content expressed as mg of atropine equivalent per gram (AE/g) of extract (Fazel *et al*., 2008; Enema et al., 2024).

Quantification of Total Flavonoid Content: Aluminium chloride colorimetric assay method was adopted for this study**.** A reaction mixture in a volumetric flask (10 mL) consisting of 1 mL of *M. scolopendria* extract, 4 mL of distilled water, 0.30 mL of 5 % sodium nitrite and 0.3 mL of 10 % aluminium chloride (after 5 minutes) was added and mixed thoroughly. Also, after another 5 minutes, 2 mL of 1M sodium hydroxide was treated and adjusted to 10 mL with distilled water. Quercetin (20, 40, 60, 80 and 100 µg/mL), prepared in the similar way as the extract was used as a reference standard. The absorbance of both the test and standard solutions was determined against the reagent blank at 510 nm using UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of quercetin equivalent per gram (QE/g) of extract **(**Lee et al., 2012; Enema et al., 2024**)**.

Quantification of Saponin Content: The method described by Ameen et al., (2021) was adopted for this study. 1 g of *M. scolopendria* leaf extract was measured into a 250 mL beaker and 100 mL of isobutyl alcohol was added. This mixture was swirled for 5 minutes, filtered, and transferred into a 100 mL beaker made up of 20 mL of 40% saturated solution of $MgCO_3$. The colourless filtrate (1 mL) was pipetted into a volumetric flask (50 mL), 2 mL of 5% $FeCl_3$ solution was added and adjusted to marked level with distilled water. This mixture was allowed to stand for 30 minutes for a blood red colour to develop. Percentage saponin was calculated using the formula:

$$\%\, Saponin = AS\ x\ AG\ x\ DF\ /\ Wt\ of\ sample\ x\ 100$$

Where:

AS	: Absorbance of sample
AG	: Average gradient
DF	: Dilution factor
Wt of sample	: Weight of sample

Quantification of Tannin Content: The tannin constituents of *M. scolopendria* leaf extract were determined by a method described by Rajeev (2012) using insoluble polyvinyl- polypirrolidone (PVPP). To 0.1 mL of the extract (dissolved in 1% methanol), 100 mg PVPP was added and vortexed, left for 15 min at 4°C, and centrifuged for 10 min at 3,000 rpm. Using the clear supernatant, the non-tannin phenolic content was determined as total phenolic content. The difference between total phenolic and non-tannin phenolic content in the ethanol leaf extract of *M. scolopendria* was taken as the tannin quantification.

Quantification of Total Terpenoid content: The total terpenoid was determined by the method described by Elsayed *et al*., (2019). Stock standard solution of each terpene: α-pinene, (−)-β-pinene, myrcene, (R)- (+)-limonene, terpinolene, linalool, α-terpineol, β-caryophyllene, α-humulene, and caryophyllene oxide was prepared in ethyl acetate. The standard terpenes were mixed and the concentration of each terpene was

adjusted to 1.0 mg/mL from where serial dilutions were made to prepare the individual points of the calibration curves. N-tridecane (100 µg/ mL), a C13 hydrocarbon, was selected as the internal standard (IS) and added to all calibrations and sample solutions. Nine calibration points ranging from 0.75 – 100 µg/mL were prepared from the previously mentioned stock standard solutions (0.75, 1.0, 2.0, 5.0, 10, 25, 50, 70, and 100 µg/mL) and n-tridecane. The concentration of IS at each calibration point was 100 µg/mL and these solutions were used to construct individual terpene calibration curves.

Quantification of Total Phenol Content: The total phenolic content of the fractions was determined spectrophotometrically with Folin – Ciocalteu reagent and the procedure was repeated thrice. To the 0.5 mL (1 mg/mL) ethanol leaf extract (0. 5 mL) of *M. scolopendria*, was added 2.5 mL of 10% Folin Ciocalteu reagent and 2 mL of Na_2CO_3 (7%). The resulting mixture was vortexed for 15 seconds and incubated at 40ºc for 30 minutes for colour development. The absorbance of the samples was measured at 765 nm wavelength. For the garlic acid calibration curve, 2.5 mL of distilled water was added to different concentrations and the total phenolic content was calculated from the calibration curve and results were expressed as milligrams per garlic acid equivalent (mgGA/g) dry weight of extract (Kaur and Kpoor, 2002).

Animal handling

Albino mice (20-30 g) were procured from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Uyo, Akwa Ibom State, Nigeria. They were kept under standard conditions, starved of food, twenty four (24) hours before the experiment and given access to only water.

Acute Toxicity Study

The OECD/OCDE guideline [2002] was adopted to determine the LD_{50}. The LD_{50} was achieved by administering of 2000 mg/kg orally to three mice. After no mortality occurred within 24 hours, this dose was again administered to another set of three mice and observed for the manifestation of physical signs of toxicity such as writing, reduced motor activity, decreased respiration and death within twenty four hours intervals.

Anti-inflammatory study

Egg Albumin-induced Oedema: In this model, albino mice of either sex were randomized and divided into five groups of five animals each. Group one animals were pretreated with distilled water (10 mL/kg), groups 2 to 4 were pretreated with 500 mg/kg, 1000 mg/kg and 1500 mg/kg of the ethanol leaf extract of *M. scolopendria* (Ms), respectively, thirty (30) minutes before the induction of oedema with fresh egg albumin while group five mice were administered with 100 mg/kg of acetyl salicylic acid (ASA). The linear circumference of the injected paws was measured with venier caliper at t=o and at thirty (30) minutes intervals for 5 hours following the administration of egg albumin (Okokon et al.,2008; Umoh *et al*., 2020). This experiment was repeated for the fractions: dichloromethane (DCM), ethyl acetate (EtoAC), butanol (But) and aqueous fractions (Aq) at 1000 mg/kg.

Xylene-induced Ear Oedema: Twenty five (25) albino mice were grouped into five groups of five animals per group. Mice in group one were administered with distilled water (10 mL/kg), those in groups 2 to 4 (500 mg/kg, 1000 mg/kg, and 1500 mg/kg) group five mice received dexamethasone, 4 mg/kg, thirty (30) minutes before the topical application of 50 microliter of xylene to the anterior and posterior surfaces of the right ears while the left ears served as control. Fifteen minutes following xylene application, the mice were sacrificed by chloroform anesthesia, both ears removed and weighed. The average weight differences between the two ears were taken to measure the inflammatory response (Atta and Alkofahi, 1998).

Analgesic Study

Acetic Acid-induced Writhing in Mice: Albino mice of either sex were selected, divided and pretreated like the egg albumin model thirty (30) minutes before the intraperitoneal injection of 2% acetic acid. Analgesic activity was expressed as reduced abdominal constrictions between control animals administered with distilled water (10 mL/kg) and mice pretreated with the extracts (Nwafor and Okwuasaba, 2003). This procedure was repeated for the fractions (DCM, EtoAc, But and Aq) at 1000 mg/kg.

Formalin-induced Paw Licking in Mice: This method was similar to the one described by Nwafor and Okwuasaba (2003). Albino mice of either sex were randomized and divided into five groups of five animals each and pretreated with distilled water (10mL/kg for group 1), ethanol leaf extract of *M. scolopendria* (500 mg/kg, 1000 mg/kg and 1500 mg/kg for groups 2 to 4) and ASA (100 mg/kg for group 5). Twenty microliters of 2.5% formalin solution (formaldehyde) made up to phosphate buffer was administered subcutaneously under the surface of the right hind paw. The time the animals spent in licking the injected paw was noted and taken as an indication of pain with the first phase of response at 5 minutes and second phase (15-30 minutes) following formalin injection. This procedure was repeated for the fractions (DCM, EtoAc, But and Aq) at a dose of 1000 mg/kg.

Hot Plate –induced Pain: The effect of the ethanol leaf extract of *M. scolopendria* on hot plate-induced pain was investigated using adult mice. The mice were grouped and pretreated as earlier mentioned in formalin–induced paw licking model. Hot plate connected to electricity was maintained at a temperature of 45℃ ±

1℃. The mice were placed into a glass beaker of 50 cm diameter on the heated surface of the hot plate and the time(s) between placement and licking of the paw were recorded (Nwafor & Okwuasaba, 2003).

Statistical Analysis

Data collected were expressed as Mean ± standard error of the mean (SEM) and significance of data taken at $p \leq 0.05$.

RESULTS

Quantitative phytochemical screening of *M. scolopendria*: The result of quantitative phytochemical screening of the ethanol leaf *M. scolopendria* is presented in Figure 1. This result revealed the various percentages (w/w) of secondary metabolites present in the ethanol extract of the *M. scolopendria* (Figure 1).

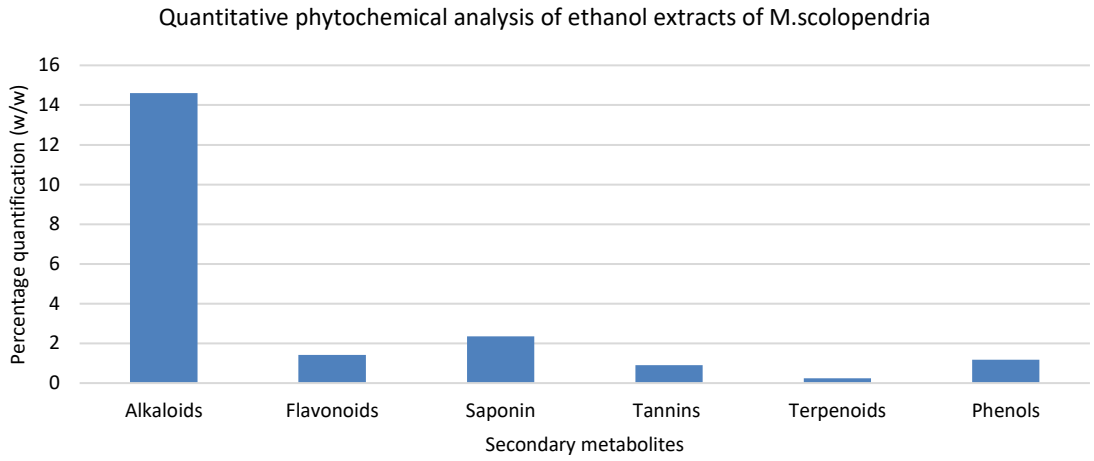

Figure 1. Quantitative phytochemical analysis of ethanol extract of *M. scolopendria.*

Acute Toxicity Study

Following the administration of 2000 mg/kg twice to two groups of mice with no mortality, the LD_{50} was determined to be 5000 mg/kg and the three doses employed for the study were 500 m/kg, 1000 mg/kg and 1500 mg/kg.

Anti-inflammatory study

The effect of ethanol leaf extract of *M. scolopendria* on egg albumin-induced oedema in mice is presented in Table 1 while the effect of the partitioned fractions is in Table 2. The result showed the various responses of the extract and fractions to oedema caused by egg albumin between 1 hour to 5 hour intervals while the consequence of xylene-induced topical oedema in mice is presented in Table 3.

Table 1. The effect of the ethanol extracts of *M. scolopendria* on egg albumin-induced oedema in mice.

Treatment (mg/kg)	Time (hours)				
	1	2	3	4	5
Dist. Water 10 mg/kg	1.66 ± 0.02	1.10 ± 0.01	0.97± 0.01	0.52± 0.02	0.52± 0.02
Ms 500	0.99±0.01*	0.50±0.01*	0.33±0.01*	0.34±0.01*	0.25±0.01*
Ms 1000	1.12±0.01	0.57±0.01*	0.53±0.01*	0.43±0.01	0.25±0.01*
Ms 1500	1.02±0.01*	0.56±0.01*	0.41±0.01*	0.35±0.01*	0.30±0.01*
ASA 100	0.55±0.01*	0.45±0.01*	0.34±0.01*	0.24±0.01*	0.16±0.01*

Values are expressed as mean ± SEM, where n=5 and *$p \leq 0.05$ is considered significant

Table 2. The effect of the partitioned fractions of *M. scolopendria* on egg albumin-induced oedema in mice.

Treatment (mg/kg)	Time (hours)				
	1	2	3	4	5
Dist. Water 10 mL/kg	1.66 ± 0.02	1.70 ± 0.01	1.97± 0.01	1.52± 0.02	0.90± 0.01
DCM 1000	1.21±0.01	1.11±0.02	0.93±0.01*	0.74±0.01*	0.58±0.01*
EtoAC 1000	1.24±0.01	1.03 ±0.01*	0.96±0.01*	0.82±0.01*	0.69±0.01*
But. 1000	0.91±0.01*	1.09±0.01*	0.99±0.01*	0.80±0.01*	0.59±0.01*
Aq. 1000	1.28±0.02	1.19±0.02	1.33±0.01	1. 01±0.01	0.85±0.01
ASA 100	0.55±0.01*	0.45±0.01*	0.34±0.01*	0.24±0.01*	0.02±0.01*

Values are expressed as mean ± SEM, where n=5 and *p≤0.05 is considered significant

Table 3. Effect of ethanol extract of *M. scolopendria* on xylene induced oedema in mice.

Treatments (mg/kg)	Weight difference (mm)
Distilled water 10 mL/kg	0.059 ± 0.06
Ms 500	0.036 ± 0.03*
Ms 1000	0.034 ± 0.03*
Ms 1500	0.042 ± 0.01
Dexamethazone 4	0.016 ± 0.03*

Values are expressed as mean ± SEM, where n=5 and *p≤0.05 is considered significant

Analgesic study

The result of the effects of the ethanol leaf extract and fractions of *M. scolopendria* on formalin-induced paw licking is presented in Tables 4 and 5 while the effect of the ethanol extract of ethanol of *M. scolopendria* on hot plate-induced pain is presented in Table 6.

Table 4. The effect of the ethanol extracts of *M. scolopendria* on formalin-induced paw licking in mice.

Treatments (mg/kg)	Mm	Time	(Minutes)			
	5	10	15	20	25	30
Dist. Water 10 mL/kg	26.4 ± 5.60	13.00±1.05	9.80 ±2.46	6.80± 3.38	4.80± 3.95	6.60± 4.89
Ms 500	14.67±0.90*	1.33±0.02*	4. 00±0.02*	2.33±0.01*	0.33±0.01*	1.33±0.02*
Ms 1000	10.75±0.05*	0.33±0.02*	0.00±0.00*	4.33±0.07*	1.33±0.02*	1.67±0.02*
Ms 1500	11.67±0.06*	3. 00±0.04*	3.67±0.05*	3.20±0.74*	2.80±1.11*	3.40±1.50*
ASA 100	14.60 ±1.60*	2.80 ± 1.39*	2.60 ± 0.68*	0.67±0.02*	1.00±0.03*	0.33±0.01*

Values are expressed as mean ± SEM, where n=5 and *p≤0.05 is considered significant

Table 5. The effect of the partitioned fractions of *M. scolopendria* on formalin-induced paw licking in mice.

Treatments (mg/kg)	Time		(Minutes)			
	5	10	15	20	25	30
Dist. Water 10 mL/kg	26.4 ± 5.60	13. 00 ±1.05	9.80 ±2.46	6.80± 3.38	4.80± 3.95	6.60± 4.89
DCM 1000	23.33±0.90	18.60±0.10	6.33±0.10*	16.00±0.11	12.33±0.10	9.33±0.13
EtoAC 1000	18. 00±0.10	7.67±0.10*	4.00±0.10*	1.33±0.08*	1.66±0.10*	6.33±0.12
But. 1000	18.33±0.10	14.33±0.11	8. 00±0.90	7.33±0.02	5.33±0.12	1.33±0.06*
Aq. 1000	18.33±0.12	13.00±0.10	11. 00±0.40	1.00±0.02*	6.00±0.11	2.33±0.10*
ASA 100	14.60±1.60*	2.80 ± 1.39*	2.60 ± 0.68*	3.20±0.74*	2.80±1.11*	3.40±1.50*

Values are expressed as mean ± SEM, where n=5 and *p≤0.05 is considered significant.

Table 6. The effect of ethanol extract of *M. scolopendria* on thermal-induced pain in mice.

Treatments (mg/kg)	Time (seconds)
Distilled water 10 mL/kg	11.35 ± 1.90
Ms 500	18.84 ± 1.20
Ms 1000	33.57 ± 1.83*
Ms 1500 mg/kg	29. 21± 1.50*
ASA 100 mg/kg	29. 21± 1.50*

Values are expressed as mean ± SEM, where n=5 and *p≤0.05 is considered significant

Discussion

The result of the quantitative phytochemical analysis (Figure 1) of the ethanol leaf extract of *M. scolopendria* revealed the presence of alkaloids (14.6), flavonoids (1.42), saponins (2.35), tannins (0.91), terpenoids (0.25) and phenols (1.18). These quantifications were done spectroscopically with garlic acid used as standard for tannin and total phenolic determinations (Appendices 3 and 6) and quercetin, for total flavonoid content. From the result, alkaloids were the highest, while terpenoid

constituents were the least. Plants are known to owe their medicinal properties to the presence of secondary metabolites in them (Babu *et al*., 2021). Alkaloids, saponins and flavonoids are reputed for their involvement in the reduction of oedema and pains (Gonfa *et al*., 2023; Sun and Shahrajabian, 2023; Hassan *et al.,* 2011). The intrinsic anti-inflammatory and analgesic abilities of the ethanol extract and fractions of *M. scolopendria* may be linked to these secondary metabolites.

Acute toxicity is the measure of the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short period. Using the OECD/OCDE guideline (2002), 2000 mg/kg of the ethanol extract was administered to two sets of three mice without any death, and a lethal dose (LD_{50}) of 5000 mg/kg was established. The three doses employed for the study were 500 m/kg, 1000 mg/kg and 1500 mg/kg which represented the lower, median and high doses. A lethal dose of 5000 mg/kg and above are considered practically non- toxic and therefore, the ethanol extract of *M. scolopendria* may be regarded as safe on a short period of administration and when given orally (Erhirhie *et al*., 2018).

In the anti-inflammatory study, the ethanol extract of *M. scolopendria* was able to reduce oedema caused by egg albumin in a dose dependent manner. This observed effect was statistically ($p \leq 0.05$) significant when compared to distilled water (10 mL/kg), and similar to that produced by acetyl salicylic acid (ASA) 100 mg/kg. The partitioned fractions of the ethanol extract were also able to reduce oedema caused by egg albumin. Comparing the fractions (Table 4.2), the butanol fraction (But) was the most potent followed by ethyl acetate (EtoAc) with the least being dichloromethane fraction. The induction of oedema using egg albumin is linked to the release of histamine and serotonin. The observed anti-inflammatory effect observed with the administration of ethanol extract and fractions of *M. scolopendria* could be due to their ability to inhibit these two mediators of inflammation. Table 4.3, which is the effect of the ethanol extracts of *M. scolopendria* in mice revealed that the extract at doses of 500 mg/kg and 1000 mg/kg was able to reduce topical oedema caused by xylene significantly ($p \leq 0.05$) when compared to distilled water and the effect was similar to that of dexamethasone (4 mg/kg). Xylene as a phlogistic agent is known to cause inflammation by the action of phospholipase A_2, hence the ability of the two doses of the extracts *M. scolopendria* in reducing oedema by xylene may be linked to their effect in blocking the release of phospholipase A_2 (Okokon *et al*., 2008; Umoh *et al*., 2020)

The effect of the ethanol extract and fractions of *M. scolopendria* on formalin induced paw licking in mice (Tables 4 and 5) revealed that the extract was able to reduce the number of times the mice licked their paws at the first five minutes and even throughout the entire duration of the experiment. This reduction was significant ($p \leq 0.05$) compared to distilled water and ASA, a standard drug. Although at time (5 minutes), the extract's action looked better than that of ASA, this was not sustained from time (10 -30 minutes). For the fractions, the ethyl acetate fraction was more potent than the aqueous fraction. Considering the thermal pain induction using a hot plate, the extract also prolonged the time the mice stayed on the hot plate in a dose-dependent manner significantly ($p \leq 0.05$) when compared to distilled water and similar to ASA. The mechanism of formalin pain is both neurogenic and inflammatory while that of hot plate gives information on their ability to behave as narcotics. Thus, the capacity of the ethanol extract and fractions on *M. scolopendria* to reduce pains induced by formalin and hotplate may be attributed to these mechanisms (Nwafor and Okwusaba, 2003; Umoh *et al*., 2020)

CONCLUSION

This study lends credence to the numerous applications of the leaf of *M. scolopendria* in folkloric medicine.

***Competing Interests*:** The authors declare that there are no competing interests.

Ethics: The handling of animals was done with humane care in accordance with best practice and supervised by the Faculty of Pharmacy Ethics Committee.

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