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CHEMICAL CHARACTERIZATION, PHYTOCHEMICAL EVALUATION, ANALGESIC AND ANTIPYRETIC ACTIVITIES OF *HARUNGANA MADGASCARIENSIS*

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ABSTRACT

This study aims at investigating the phytochemical constituents, GC-MS profile, the analgesic and antipyretic activity of organic and aqueous extracts of the stem bark of *Harungana madagascariensis* Lam. This plant is widespread in different parts of Africa and has vast ethnopharmacological applications. The results of the phytochemical analysis identified alkaloids, flavonoids, phenolic compounds, tannins, saponins, triterpenoids/steroids, anthraquinone and saponins as the classes of compounds contained in the stem bark. The GC-MS evaluation indicated the following important five compounds: β-Pinene, Hexadecanoic acid, methyl ester, 6-Octadecenoic acid, methyl ester, (Z)-, 9,15-Octadecadienoic acid, methyl ester, (Z,Z)- and Octadecanoic acid, 11-methyl-, methyl ester as the bioactive compounds responsible for the analgesic and antipyretic activities. The present study is a randomized control study. Acetic acid induced writhing was employed for analgesic testing. Acetic acid was used to induce writhing in Wistar rats which were divided into six (6) groups. The groups were administered organic & aqueous extracts of the plant (200 mg/kg and 400 mg/kg). The animals were observed for number of writing movements and the percentage writhing was calculated. Baker's yeast induced pyrexia was employed for the antipyretic testing. The animal groups were administered extracts of the plant (200 mg/kg and 400 mg/kg), with Paracetamol as the standard drug (100 mg/kg) and Normal saline (control) for both experiments. The body temperature of the rats was measured rectally over a period of four (4) hours. The results showed that the 400mg/kg doses were significantly more active than the standard drug Paracetamol. The results of this study validates the indigenous use of *Harungana madagascariensis* in the treatment of fever and thus; the plant warrants further therapeutic investigations and has potentials as an antimalarial.

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INTRODUCTION

The demand for new drugs is on the rise given the accelerated rate of increase in the emergence of new diseases. Medicinal plants have assumed a pivotal role in the search for new therapies. Africa is home to a wide array of medicinal plants which overtime has proved potent in many disease conditions. *Harungana madagascariensis* Lam., ex. poir) (family Hypericaceae) is a tropical plant native to Africa, Mauritius and Madagascar [1]. The plant is an edible medium sized tree growing to a height of 5-10 m and is often found adorning gardens. The documented ethnopharmacological uses are numerous in various African communities. The leaves are used to treat urogenital infections and chest pains [2]. The stem bark is employed in the treatment of malaria,[3] fever, anemia, asthma, tuberculosis, angina, diarrhea, dysentery, syphilis, gonorrhea, and wound [4]. Fruits are used as an abortive [5], while the gummy exudent is used as enema to treat enteritis, leprosy and parasitic skin diseases. Other documented uses include river blindness, ulcer, asthma, hepatitis, dysmenorrhea, and toothache [6]. All parts of the plant (leaves, stem bark, roots) have been reported to possess several bioactivities which includes:, antifungal, antiprotozoan, antiviral, antibacterial, pains [7,8,9,10] antioxidant;[11] antidiarrhoeal, antiamoebic, and spasmolytic; [12] . Phytochemical studies carried out on this plant have identified, anthraquinones, flavonoids, alkaloids, saponins, glycosides, and tannins as secondary metabolites in *Harungana madagascariensis* [13,14,15,16].

The overall objective of the study is to investigate the analgesic and antipyretic activity of *H. madagascariensis* and determine the bioactive compounds.

MATERIALS AND METHOD

Materials Collection, Identification and Authentication *H. madagascariensis* (stem bark) was sourced from a bio reserve in Nigeria. The plant was authenticated by a botanist, Dr. Suleiman Mikailu of the Department of Pharmacognosy & Phytotherapy, University of Port Harcourt.

EQUIPMENT AND INSTRUMENTS

Electronic weighing balance (model WT6002A), Maceration jars, Thermostat bath (HH-6; Techmel and Techmel, USA), Lypholiser (Harvest right scientific freeze dryer), Beakers, Glass funnels, Measuring cylinders, Conical flask, Rotary evaporator (R-205), Desiccator, Spatula, Crucibles, Filter papers, Syringes and Digital thermometers.

REAGENTS

Methanol, Dichloromethane of Analytical grade (SigmaAldrich). Chloroform, Diethyl Ether, Acetic Anhydride, Glacial acetic acid, Sodium picrate, 2% 3,5-Dinitrobenzoic acid, Picric acid, Iodine solution, Dimethylsulfoxide, (JHD company, Guangdong. GuanghuaSci-Tech. Co. Ltd. China), Hydrochloric acid, Million's reagent, Benedict's solution, Wagner's reagent, Sodium Hydroxide, Ferric chloride solution, Saturated lead acetate solution, Dragendorff's reagent, Kedde reagent, Ammonia solution, 7.5% Potassium Hydroxide, Fehling's solution A and B (Sigma Aldrich Chemicals, St Louis, USA), Distilled water, Deionized water (Pharmaceutical Chemistry Lab, University of Port Harcourt).

METHODS EXTRACTION OF THE PLANT MATERIALS

The plant materials were extracted according to the American National Cancer Institute (NCI) method of extraction [17].

200g of the pulverized plant material was macerated in a 1: 1 mixture of 500ml of dichloromethane and 500ml of methanol for 24 h. The ratio of plant material to solvent used was 1:5. This ratio was maintained for all weighed amount of plant materials used. The obtained solution containing the extracts was decanted off and 500ml of methanol was added to the

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residue and allowed to stand for another 24 h. The solution of the extract was collected by filtration and 1 liter of deionized water was added to the residue. The aqueous extract was collected after 24 h of maceration. The methanol extraction was combined with the 1:1 dichloromethane and methanol extraction to yield the organic extract. This extraction solution was evaporated to dryness on a rotary evaporator at a temperature of 40˚C. The obtained dry extracts were further dried in a desiccator to remove any trace of solvent. The aqueous extraction was dried using a lyophilizer to obtain a solid sample. These two extracts were investigated for anti-pyretic and analgesic activity.

PHYTOCHEMICAL SCREENING

The plant extract (crude extract of the stem bark) was subjected to preliminary analysis using the method described by Trease and Evans [18].

GC-MS ANALYSIS OF THE ORGANIC EXTRACTS

The gas chromatography mass spectrometry (GC-MS) analysis of the DSE was quantitatively determined using an Agilent 7890B GC system coupled with an Agilent 5977A MSD with a Zebron-5MS column (ZB-5MS 30 m × 0.25 mm × 0.025μm) (5%-phenylmethylpolysiloxane). The GC-grade helium served as the carrier gas at a constant flow rate of 2 mL/min. The DSE was dissolved with ethanol and filtered before use. The column temperature was maintained at 60°C and gradually increased at 10°C per minute until a final temperature of 300°C was reached. The time taken for the GC-MS analysis was 30 min. The compounds were identified based on computer matching of the mass spectra with the NIST 11 MS library (National Institute of Standards and Technology library).

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EXPERIMENTAL DESIGN

This study was designed in line with the ethically approved experimental protocols adopted by the department of Experimental Pharmacology and Toxicology, of the Faculty of Pharmaceutical Sciences, University of Port Harcourt. Healthy adult Wistar albino rats irrespective of sex, weighing between 170-200 grams were selected for the study. These animals were obtained from the animal house of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu state, Nigeria. The animals were exposed to 12 hours of dark light cycle and kept under room temperature and humidity. After the animals were selected for study, they were separated in cages and were given food and water. The animals were allowed to acclimatize to laboratory conditions for 14 days prior to the experiments.

ANTIPYRETIC ACTIVITY BAKER'S YEAST INDUCED HYPERTHERMIA IN RATS

The method established by Tomazetti et al, 2005 [19], with some modifications was employed in the antipyretic activity evaluation, with fever induced by Brewer's yeast in rats. The basal rectal temperature of each rat was recorded at zero hour using clinical digital thermometer.

Pyrexia was induced by subcutaneous injection of 15% w/v suspension of Brewer's yeast in distilled water at a dose of 10 ml/kg body weight. In order to ensure uniform spreading of the suspension beneath the skin, the injection site was massaged. Immediately after yeast administration, food was withdrawn but access to water was still maintained. After 18 hours of Brewer's yeast injection the rise in rectal temperature was recorded and only animals showing an increase in temperature of at least 0.6°C (or 1°F) were selected for the study. The mean increment recorded was 0.96°C after 18 h of administration. The animals were randomly divided into 6 groups, each group containing five rats. Group I received normal saline orally. Group II was given standard drug Paracetamol at the dose of 100 mg/kg per-oral. The remaining Groups were Treated Orally as Follows; • Groups III and IV received organic extract of the plant (H.

madagascariensis) at oral dose of 200 mg/kg and 400 mg/kg respectively. While Groups V and VI received aqueous extracts at doses of 200 and 400 mg/kg respectively.
After the treatment, the temperature of all the rats in each group was recorded at 0, 1, 2, 3 and 4 hours.

ANALGESIC ACTIVITY ACETIC ACID INDUCED WRITHING TEST
The method described by Koster et al, 1959 [20], was used for the evaluation of analgesic activity in rats. The experimental animals were weighed and randomly divided into 6 groups consisting of 5 rats in each. Group I (control) received normal saline (10 ml/kg) orally. Group II (positive control) received standard drug Paracetamol at oral dose of 100 mg/kg. Remaining groups were treated orally as follows:
• Groups III and IV received organic extract at doses of 200 and 400 mg/kg respectively.
• Groups V and VI received aqueous extracts at doses of 200 and 400 mg/kg respectively. All treatments were administered orally. 45 minutes after administration of standard drug and test samples, each mouse was injected with 0.7% acetic acid at the dose of 10 ml/kg body weight intraperitoneally. The number of writhing responses manifested by each mouse was recorded for 30 minutes commencing just 5 minutes after acetic acid injection.

The percentage analgesic activity was calculated as follows:

% inhibition of writhing = [wc-wt/wcX100]

Where W is number of writhing, Wc is control, and Wt is test.

STATISTICAL ANALYSIS
All values were expressed as the mean ± standard error of the mean (SEM) and the results were analyzed statistically by one-way analysis of variance (ANOVA) for analgesic activity and for antipyretic effect through time by Graphpad prism version 8. $P < 0.05$ was considered to be statistically significant.

RESULTS
The results of the phytochemical screening are presented in Table 1

Table 1. The phytochemical screening results

TEST	HARUNGANA
1. Carbohydrate	
a. Molisch test	-
b. Fehlings test	-
2. Anthraquinone	
a. Free (Bomtrager's test)	+
3. Triterpenoids/Steriods	
a. Liebermann-Buchard Test	+
b. Salkwoski's Test	+
4. Phenolics test	

a.	**$FeCl_3$ test**	**+**
5.	**Tannin test**	
a.	**Phlobatannins test**	**+**
6.	**Flavonoids**	
a.	**Shinoda Reduction Test**	**+**
b.	**$AlCl_3$ test**	**+**
7.	**Alkaloids**	
a.	**Dragendorff's (orange colour)**	**+**
b.	**Mayer's test (cream)**	**+**
c.	**Hager's test (yellow ppt)**	**+**
8.	**Saponin**	
a.	**Frothing Test**	**+**
b.	**Emulsion Test**	**-**

Key: + = present, - = absent

The results presented in Table 1 showed that the organic extract of *H. madagascariensis* contained alkaloids, flavonoids, phenolic compounds, tannins, saponins, triterpenoids/steriods, anthraquinone. This study is in consonance with the work of other researchers who have investigated the phytochemical constituents of *H. madagascariensis*. These earlier researchers identified, anthraquinones, flavonoids, alkaloids, saponins, glycosides, and tannins as secondary metabolites in Harungana madagascariensis [13,14,15,16].

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The GC-MS chromatogram of the organic extract of *H. madagascariensis* is presented in Figure 1. A total of 46 peaks were recorded.

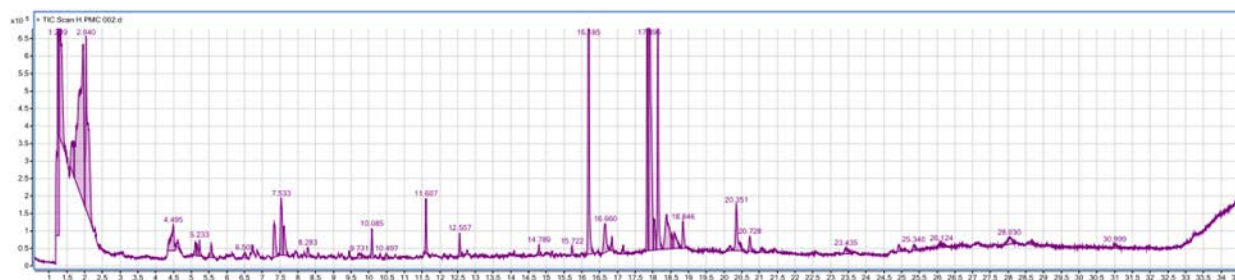

Figure 1. GC-MS Chromatogram of the organic extract of *H. madagascariensis.*

The GC-MS chemical characterization of the organic extract of *H. madagascariensis* was carried out and the results presented in Table 2. The interpretation of GC-MS mass-spectra was based on the NIST library of the equipment. The individual spectrum were matched with that of the library and the following parameter; molecular weight, structure, retention time and fragmentation patterns compared. Five bioactive compounds were ascertained from the spectral match.

Table 2: GC-MS analysis of *H. madagascariensis* stem-bark

Peak	RT	Area %	MW	MF	Name of Compound	Documented Bioactivity
1	4.341	14.03	136	$C_{10}H_{16}$	β-Pinene	Antimicrobial and Antibacterial [21,22]
2	16.580	8.39	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester	Antifungal and Antioxidant, Antimicrobial, hypocholesterolemic, nematicidal, pesticidal, antiandrogenic flavour, haemolytic, 5-Alpha reductase inhibitor [23]
3	17.873	11.41	296	$C_{19}H_{36}O_2$	6-Octadecenoic acid, methyl ester, (Z)-	Antipyretic [24]
4	18.811	0.85	294	$C_{19}H_{34}O_2$	9,15-Octadecadienoic acid, methyl ester, (Z,Z)-	
5	20.688	1.72	312	$C_{20}H_{40}O_2$	Octadecanoic acid, 11-methyl-, methyl ester	Antimicrobial [25]

The results of the anti-pyretic evaluation of the extracts *H. madagascariensis* are presented in Table 3 and Figure 2 below. The results showed a rapid onset of activity by the 200 mg/kg and 400 mg/kg doses of the extracts that were comparable to the standard drug PCM at 1 h. The 400 mg/kg organic extract yielded very significant activity ($P<0.05$) than PCM; an indication of superior anti-pyretic activity against PCM at 2 h.

Table 3. Mean Reduction in Pyrexia in Rats

Treatment	Time Interval					
	37	0h (After 18h)	1h	2h	3h	4h
Control	36.6±1.4	39.8±1.7	39.7±1.5	39.4±1.9	39.1±1.2	38.8±1.1
PCM	36.5±1.1	39.5±1.8	37.6±1.3	37.1±1.5	36.4±1.4	35.4±1.6
HM org 200mg/kg	37±0.9	37.3±1.6	37.2±1.0	37.0±1.4	36.5±1.0	36.0±1.4
HM org 400mg/kg	36.4±1.3	39.1±1.2	37.5±1.0	36.5±1.5*	37.0±0.7	36.5±1.3
HM aq 200mg/kg	37.4±1.0	38.5±1.0	37.9±1.3	37.9±1.9	37.6±0.9	36.8±1.6
HM aq 400mg/kg	36.8±0.6	39.1±1.3	37.7±1.1	37.5±2.0	36.9±0.5	36.3±1.0

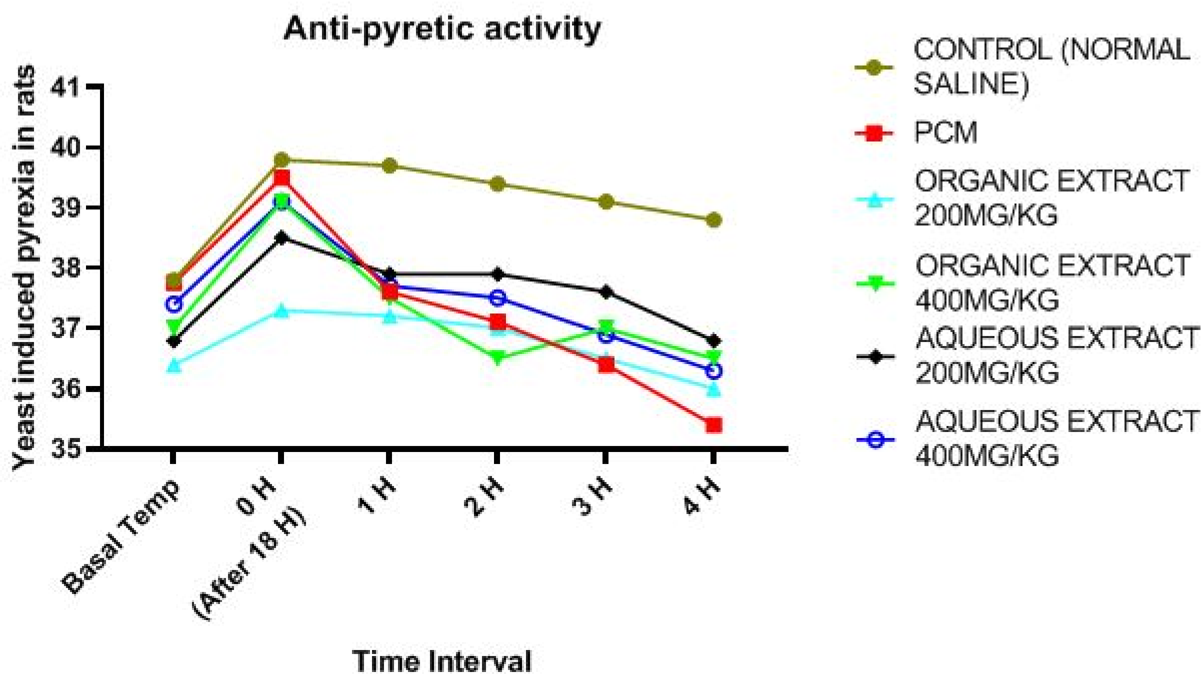

Figure 2. A Line Graph Showing the Antipyretic Effect of the Extracts *H. madagascariensis* on Yeast Induced Pyrexia

Further evaluation of the anti-pyretic activities of the extracts were carried out by calculating the percentage reduction of pyrexia as seen in Figure 3 below:

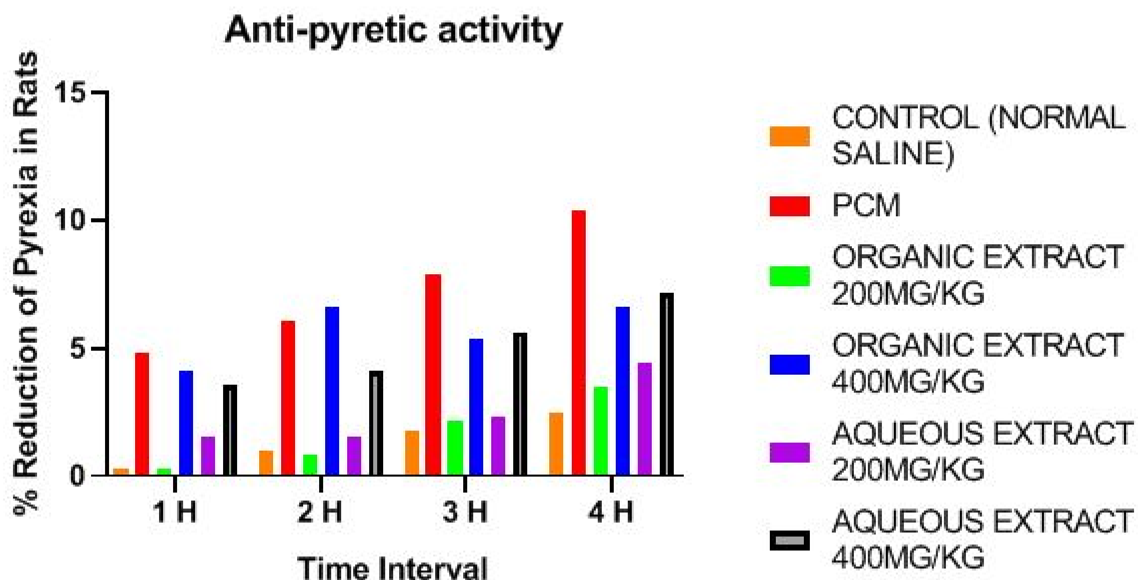

Figure 3. A Bar Chart Showing the % Reduction of Pyrexia in Rats after administration of the Extracts of *H. madagascariensis* on Yeast Induced Pyrexia.

A break-down of the percentage reduction of pyrexia in the rats displayed in Figure 3 was in consonance with the observations made in Table 3 and Figure 2 previously; that the extracts and fractions had their maximum % reduction between 3-4 h; an indication that the herbal extracts

had potent activity. The results at 1-2 h of the 400 mg/ kg dose of the organic extracts and PCM showed that the activities were comparable. Also, the 400 mg/kg dose of the organic extract had a very significant activity (*P*<0.05) at 2 h, which surpassed that of PCM and the other extracts. But at the 3rd and 4th hour respectively PCM significantly inhibited pyrexia with the maximum % reduction being observed at the 4th hour of the experiment.

The analgesic effects of the extracts *H. madagascariensis* were investigated and the results obtained are presented in Table 4.

Table 4. Analgesic Effect of Extracts of *H. madagascariensis* on Acetic Acid Induced Writhing Test.

Treatment	% Writhings
PCM	84.4%
HM organic extract 200 mg/kg	82.8%
HM organic extract 400 mg/kg	85.4%
HM aqueous extract 200 mg/kg	85.6%
HM aqueous extract 400 mg/kg	91.0%

The results of the analgesic experiments carried out are presented in Table 4. There are strong indications that *H. madagascariensis* possess significant analgesic activities. This is evident from the dose-dependent and time-dependent significant activities recorded for the extracts. The analgesic results again validate the anti-pyretic activities of the extracts. It could be observed that all the doses were comparable or superior to PCM. These results clearly support the reported ethnopharmacological use of this plant in the treatment of malaria and fevers. Previous studies have linked the presence of alkaloids, flavonoids, tannins, terpenoids, saponins and steroids with good anti-pyretic activities. Saponins are known to inhibit the enzymes involved in the formation of pyrexia, while flavonoids hinders the synthesis of PG2 by inhibiting tumour necrosis factor –α responsible for the induction of fever [26,27].

DISCUSSION

Mankind's dependence on medicinal plants cannot be over-emphasized as most drugs in clinical use today originated from plants. Over 80% of the third world's populations rely on medicinal plants for their healthcare [28]. *H. madagascariensis* is a plant well-distributed in different parts of African and some regions in Asia. The plant has a plethora of traditional uses; some of which are yet to be validated. In this present work, the analgesic and antipyretic activities have been investigated. A correlation of these activities with the phytochemistry and chemical characterization of the bioactive compounds was carried out. The results of the phytochemical screening showed the presence of flavonoids and saponins which may explain this plant's antipyretic and analgesic activities. The bioactive classes of phytoconstituents such as alkaloids, tannins and glycosides were also identified in the plant. Documented studies conducted on medicinal plants have associated the presence of metabolites such as flavonoids and alkaloids to antiinflammatory, analgesic and antipyretic properties [26]. Flavonoids have been implicated in the attenuation of arachidonic acid release through inhibition of neutrophils degranulation [29] leading to the suppression of prostaglandins and leukotrienes responsible for inflammation, pain, and fever. The extracts of *H. madagascariensis* significantly reduced the number of writhing in a dose dependent manner. The extracts at the dose of 200 mg/kg and 400 mg/kg produced significant reductions in the number of writhing ($P < 0.05$) produced by acetic acid in rats when compared to the control group. The aqueous extract at a dose of 400 mg/kg showed the greatest reduction in writhing, 91.0% ($P< 0.05$) compared to the standard drug (paracetamol) which showed a reduction in writhing of 84.4%. The extracts of *H. madagascariensis* were also

assessed for antipyretic activity against yeast induced fever which is an indicator of pathogenic fever. The yeast induced fever remains an economic and versatile method for testing antipyretic drugs [19], as the proteins contained in yeast induce fever by stimulation of inflammation [30]. The production of endogenous pyrogens such as pro-inflammatory cytokines (interleukin [IL-1β and IL-6], interferons [IFN-α] and tumor necrosis factors [TNF-α]) [31] and prostaglandins (PGE2 and PGI2) [32] are responsible for increasing the temperature of the body by acting on the hypothalamus in the brain [33]. The extracts of the plant and paracetamol reduced the rectal temperature in a time dependent manner. The presence of four highly potent compounds in *H. madagascariensis* could be attributed to the observed analgesic and antipyretic activities. One of the compounds; 6-Octadecenoic acid, methyl ester, (Z)- is a documented antipyretic agent. Similarly, an analogue of 9,15-Octadecadienoic acid, methyl ester, (Z,Z)- , which is 9-Octadecenoic acid (Z)-methyl ester is reported to possess a wide range of activities which are; anticarcinogenic, antioxidant activity and acts as endogenous peroxisome proliferator activated receptor ligand, [23,34]. This suggest that 9,15-Octadecadienoic acid, methyl ester, (Z,Z)- may possess antioxidant and anticarcinogenic properties.

CONCLUSION

An investigation of the chemical characterization, phytochemical evaluation, analgesic and antipyretic activities of *H madgascariensis* has been carried out. The results show that the stem barks possess potent analgesic and antipyretic activities. The presence of seven secondary metabolites was detected from the phytochemical analysis of the plant. The GC-MS analysis gave five bioactive compounds which from previous studies have demonstrated good activities. Thus, it may be said that these compounds are responsible for the observed analgesic and antipyretic activities of *H. madgascariensis*. This research work has validated the indigenous use of the plant in the treatment of fever.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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