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Phytochemical Screening and Evaluation of Anti-Inflammatory Activity Studies of Ethyl Acetate Leaves Extract from *Ocimum gratissimum (L.)*

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Abstract: *The present communication attempts to investigate the possible phyto-constituents and evaluate the anti-inflammatory activities of Ocimum gratissimum leaves extracts. Phytochemical screening was carried out on n-hexane, ethyl acetate and methanol fractions of the leaves extracts from Ocimum gratissimum. Acute toxicity test and anti-inflammatory studies were carried out on ethyl acetate fractions of the extract. Ethyl acetate extract (at doses of 50,100 and 200 mg/kg body weight) were tested for their anti-inflammatory activity against formalin-induced paw edema in rats. The extracts were subjected to preliminary phytochemical screening tests for various constituents. This revealed the presence of: steroids, triterpenoids, cardiac glycosides, and anthraquinones in the n-hexane extract. Saponins, steroids and triterpenoids were found to be present in the ethyl acetate extract. The methanol fraction was found to contain saponins, phenolic compounds, tannin, flavonoids, steroids, triterpenoids, and cardiac glycosides. The phytochemical screening tested negative for alkaloids in all the 3 fractions. Ethyl acetate extract was found to have a significant ($P < 0.01$) inhibitory effect on the formalin-induced oedema in rats at the doses of 50, 100, and 200mg/kg body weight (orally) tested in rats when compared to the normal saline (negative control). The extract also competed significantly and favorably with standard drug, prednisolone- positive control ($p<0.05$). The activity of the extract resides more at the highest dose 200 mg/kg body weight (orally), which was found to have the highest percentage inhibition (78.7 %) at 1-hour. Inhibitory effect of prednisolone was rapid within the first 3 hours with % inhibition of 78.7, 78.4 and 77.8 at 1-hour, 2-hour and 3-hour respectively and slight decline in the inhibitory effect was noticed at 4 and 5-hour, yielding % inhibition of 76.8 and 76.4 respectively. This shows that ethyl acetate extract possess longer inhibitory activity than prednisolone. The intra-peritoneal median lethal dose (LD_{50}) in rats was calculated to be above 5000 mg/kg body weight (orally), hence the plant's leaves may be safe when consumed. The results confirmed that ethyl acetate extract contains pharmacologically active principles and are in agreement with the local applications of the plant in the treatment of inflammatory-related disease conditions.*

Keywords: *Ocimum gratissimum, anti-inflammatory, edema, phlogistic agent, prednisolone.*

I. Introduction

Plant plays an important role in human life as the main source of food medicine, wood, oxygen producer and lots more. Plants contribution to the medicinal field is largely owing to the activity of the plant derived drugs. Plant derived drugs are those of the biologically active substances which are isolated or purified from plants (Arnason *et al.*, 1995). Today about 25% of commercial drugs such as aspirin, atropine, quinine, morphine, vincristine, and vinblastine are plant-derived. Aspirin which is consumed virtually on routine basis originated from *Salix alba* (white willow). Its anti-inflammatory activities make it extremely useful in relieving fever, pain and related anti-inflammatory ailments. Anti-coagulant activity of aspirin (vasoprin) was also discovered and it is widely administered to prevent strokes and heart attack in calibrated doses (Gilani and Atta-urRahman, 2005). Aspirin prevent aggregation of blood platelets and enhances the blood supply to the heart and brain (Anarson *et al.*, 1995). *Atropa belladonna*'s atropine is used in alleviating pains and to treat cerebral palsy associated with Parkinson's disease (Anarson *et al.*, 1995). Quinine isolated from *Cinchona officinalis* and *Cinchona pubescens* bark is used to treat malaria infection (Anarson *et al.*, 1995). Morphine from *Papaver somniferum* capsules act as pain-killer (Anarson *et al.*, 1995). However, wrong administration of these drugs could lead to addiction and casually fatal. Vincristine and vinblastine from *Catharanthus roseus* are used to treat Hodgkin's disease, chorio carcinma, childhood leukemia and breast cancer (Sumner *et al.*, 2000).

Ocimum gratissimum (L.) is an aromatic perennial herb. It is widely grown in Nigeria, and it is used in the treatment of epilepsy, and high fever, (Okwu and Josiah, 2006). *O. gratissimum* (L.) is commonly known as scent leaf; "Nchuanwa" (Igbo), "Effirin" (Yoruba) "Dodoya'' (Hausa), (Orwa *et al.*, 2009; Okoli *et al.*, 2009). The plant is used as food spices (Okwu and Josiah, 2006), and for the treatment of ailments such as malaria,

diabetes, respiratory and urinary tract infections, cough, fever, and diarrhea disease (Janssen *et al.,* 1989; Nakamura *et al.,* 1999; Ngassoum *et al*., 2003; Offiah and Chikwendu, 1999). Its ability to prevent inflammation of the gum (gingivitis) and mouth (stomatitis) makes *Ocimum gratissimum* leaf extract a good mouth wash to improve oral hygiene (Aguiyi et *al.,* 2000). Bioactivities such as hypoglycemic, anti-inflammatory, antidiarrhoea, antihelminthic and anti-microbial of the plant extracts confirmed its broad spectrum of applications in traditional medicine, (Dubey *et al.,* 2000; Okoli *et al.,* 2009; Pessoa *et al.,* 2002). *O. gratissimum* essential oil is a principal anti-diabetic and anti-inflammatory medicinal plant (Dubey *et al.,* 2000; Okoli *et al.,* 2009; Pessoa *et al.,* 2002). The objectives of the research are to carry out preliminary phytochemical screening on fractions of the plant's extract and to evaluate the anti-inflammatory activity of the crude extract of the plant.

II. Materials And Methods

Collection of Plant Materials

Fresh leaves and stems of *O. gratissimum* were collected from the Usmanu Danfodiyo University Teaching Hospital Staff Quarters in the month of August 2014, in a garden, where they were grown as economic plants.

Identification of Plant Materials

The plant was identified by a Taxonomist at the herbarium of the Department of Pharmocognosy and Ethno-pharmacy, Usmanu Danfodiyo University, Sokoto. A voucher specimen with no. Pcg/udus/0001 was assigned and deposited at the herbarium for reference purpose.

Sample Treatment

The leaves were washed with water to remove dust and other contaminants and then shade-dried for 3 weeks. The dried leaves were ground using mortar and pestle and then sieved using 2 mm mesh to obtain fine powder. The powdered sample was stored in a sealed sample container for required usage.

Extraction of Plant Materials

The plant materials were extracted gradiently using *n*-hexane, ethyl acetate and methanol using the method described by Arnason *et al., (*1995). The resulting fractions of the extract were concentrated to dryness their percentage yields calculated and noted.

Preliminary Phytochemical Screening

Phytochemical screening of the crude extract of n- hexane, ethyl acetate and methanol fractions of the leaves of *O. gratissimum* was carried out using standard methods (Arnason *et al., 1995;* Khandelwal, 2006) to identify the presence of major secondary metabolites.

Animal Management

Male adult Wistar rats weighing between 150-250 g were used for the experiments. The animals were obtained from animal house of Faculty of Pharmaceutical Sciences Usmanu Danfodiyo University Sokoto). The animals were maintained under standards environmental conditions of temperature, illumination of light and dark cycle. The animals were fed with Standard Laboratory feed and water). Food was withdrawn 12 hours before and during the experimental Hours. Ethical clearance for handling the animals and the procedures used in the study was obtained from the institution of animal and ethical committee.

Experimental Procedures

Phytochemical Screening of Plant Extracts

Phytochemical screening was performed to assess the qualitative chemical compositions of the three different extracts.

Determination of Acute Toxicity (LD_{50})

Acute toxicity study was carried out using New Approach to Practical Acute Toxicity Testing (Lorke, 1983). The median lethal dose (LD_{50}) was calculated from the second phase and recorded.

Determination of Anti-inflammatory Activity

Formalin-induced Paw Licking in Rats

The rats were randomly divided into 5 groups of 5 animals per group. Formalin (0.1 mL/kg of 2.5 % v/v) was injected into the plantar surface of the left hind-paw of the rats 60 minutes after treatment with ethyl acetate extract and standard drug (10 mg/kg prednisolone). The test was carried out in a transparent plastic

chamber (30 x 30 x 30) cm with a mirror placed at the bottom (base) of the chamber to allow for unobstructed visibility of the rats behavioural responses. The time that the animals spent licking the injected paws was measured as an index of inflammation. Flinching and licking of the paws were noted for the phase I response (0-5 minutes after injection) and phase II responses (20-60 minutes) respectively.

Formalin-induced Paw Oedema in Rats

An increase in the rat hind paw diameter induced by sub-planter injection of a phlogistic agent was used as the measure of acute inflammation. The phlogistic agent employed in this study was formalin 0.1 mL/kg of 2.5 %. The rats were randomly divided into 5 groups of 5 animals per group. Thirty minutes before the injection of formalin, the groups were treated orally as follows. group 1: 0.9 % (w/v) normal saline 10 cm^3/kg, (negative control), group 2: 10 mg/kg prednisolone (as positive control), groups 3, 4 and 5 received ethyl acetate leaves extract of *O. gratissimum* at the doses of 50, 100 and 200 mg extract mg/kg respectively. Paw thickness was measured hourly after formalin injection. Paw diameter (mm) was measured at 1, 2, 3, 4 and 5 hours later using digital caliper, and percentage inhibitions determined.

$$\text{Inhibition (\%)} = \frac{\text{Mean paw diameter (control)} - \text{Mean paw diameter (treated)}}{\text{Mean paw diameter (control)}} \times 100$$

Data Analysis

All data in the experiment was expressed as mean ± standard error of the mean (S.E.M), while statistical significance between groups was done using student's T-test. Differences in mean were considered to be significant when $p < 0.05$ and 0.01 (Duncan *et al.*, 1977).

III. Results

Phytochemical Screening of *O. gratissimum* Leaves Extracts

The results of the phytochemical screening showed that the crude methanol extract contains saponins, phenolic compounds, tannin, flavonoids, steroids, triterpenoids, and cardiac glycosides; the ethyl acetate extract contains saponins, steroids and triterpenoids while the *n*-hexane fraction contains steroids, triterpenoids, cardiac glycosides, and anthraquinones (Table1).

Table1: Qualitative Phytochemical Analysis of *O. gratissimum* Leaves Extracts

S/No.	Constituents /Test	N-hexane Extract	Ethyl acetate Extract	Methanol Extract
1	**Saponnin**			
	frothing test	-	+	+
2	**Steroids /Triterpene**			
	Salkowski's test	+	+	+
	Lieberman Burchard's test	+	+	+
3	**Flavonoids**			
	Shinoda's test	-	-	+
	NaOH	-	-	+
4	**Alkaloids**			
	Meyer's test	-	-	-
	Wagner's test	-	-	-
	Dragendorff's test	-	-	-
5	**Tannin**			
	Lead acetate	-	-	+
6	**Phenolic compounds**			
	($FeCl_3$)	-	-	+
7	**Cardiac glycosides**			
	kellers- killernin's test	+	-	+
8	**Anthroquinones**			
	Borntrager's test	+	-	-

Key: + = Present **⁻ = Absent**

Percentage Yield of *O. gratissimum* Leaves Extracts

Table 2 shows the resulting fractions *n*-hexane; ethyl acetate and methanol leaves extract from *O. gratissimum*, concentrated to dryness and gave the percentage yields of 4.93, 4.48 and 9.60 % w/w respectively.

Table 2: Mass and %Yield of Fractions of the Extract

Fractions	**Yield % (w/w)**
n-hexane	4.93
Ethyl acetate	4.48
Methanol	9.60

Acute Toxicity Test of Ethyl acetate Leaves Extract.

Table 3 represents the outcome of experiment conducted on 12, mature and healthy rats which were provided for acute toxicity experiment in two successive phases (I & II). The outcome of the first phase which required utilization of 9 rats, and the final stage which involved 3 rats, were summarized on the table. Median lethal dose (LD_{50}) in rats was calculated to be above 5000 mg/kg body weight (orally).

Table 3: Acute Toxicity Test of Ethyl acetate Extract at Phase I and II

Extract dose mg/kg		Log dose		No. of death	
Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
10	1600	1	3.2	0/3	0/1
100	2900	2	3.4	0/3	0/1
1000	*5000	3	3.7	0/3	0/1
No. of animals used				9	3
Total no of rats used				12	

***LD $_{50}$ > 5000 mg / kg body weights**

Formalin-induced Paw Licking Test

Ethyl acetate extract (at 50, 100 and 200 mg/kg body weight) reduced the paw licking time on formalin-induced paw licking in Wistar rats as shown in figure 1 A and B. Ethyl acetate extract, 200 mg/kg body weight, showed the most potent anti-inflammatory effect which competed favourably with standard drug.

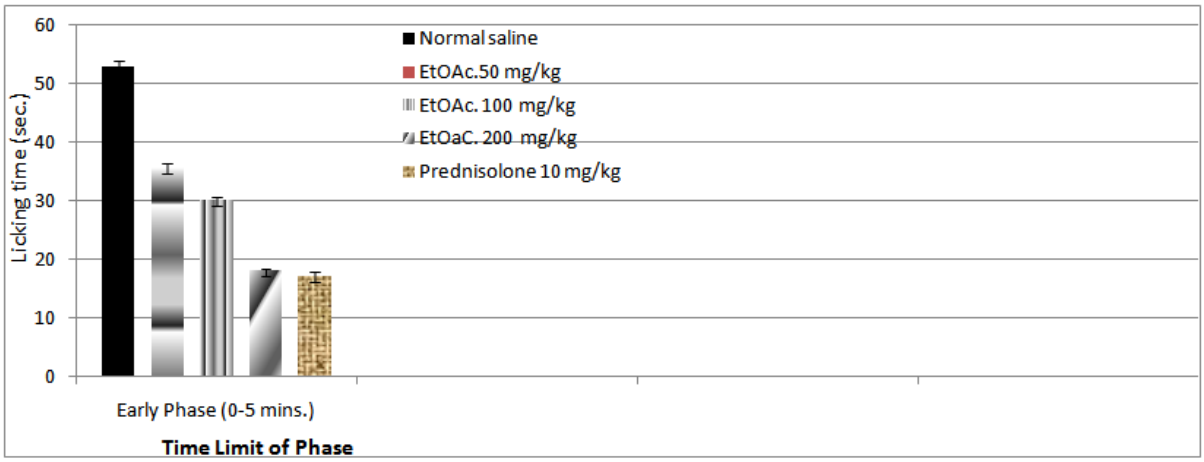


Figure 1 A

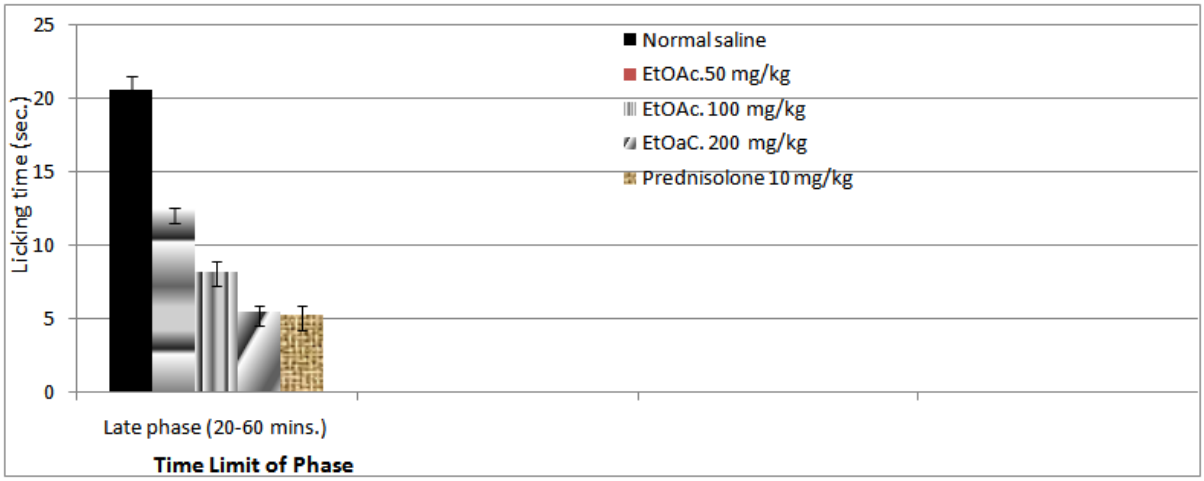


Figure 1B

Effect of graded-doses of ethyl acetate extract on formalin-induced paw licking on Wistar rats (Figure 1 A and 1B): $P < 0.05$ compared to standard drug; $P < 0.01$ when compared to normal saline. Values are expressed as mean ± S.E.M (Standard Error of the Mean) of five rats using Student's T-test

Anti-Inflammatory Study of Ethyl acetate Extract

Results of anti-inflammatory study was conducted and summarized below.

Table 4A: Effects of Normal saline (0.9 w/v), Ethyl acetate, and Prednisolone on Formalin-induced Paw Oedema in Rats

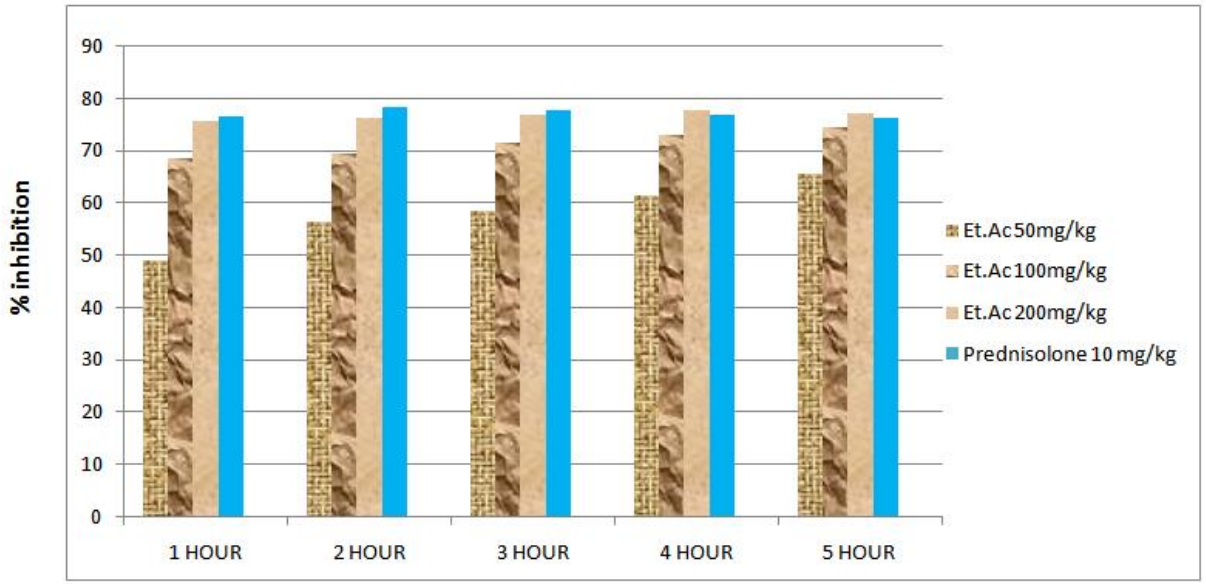
Group	Dose mg/kg	Change in hind paw diameter (mm) 1hr	2hr	3hr	4hr	5hr
Normal saline n=5	10 mL/kg	12.46 ±0.04	12.34 ±0.06	11.66 ±0.04	9.78 ±0.05	8.69 ±0.04
Ethyl acetate n=5	50	6.17 ±0.03	5.42 ±0.05	5.27 ±0.05	4.92 ±0.03	4.38 ±0.05
SEthyl acetate n=5	100	3.86 ±0.04	3.78 ±0.04	3.62 ±0.04	3.45±0.05	3.25 ±0.04
Ethyl acetate n=5	200	3.00 ±0.04	2.97 ±0.04	2.94 ±0.03	2.84 ±0.05	2.88 ±0.04
Tab Prednisolone n=5	10	2.63 ±0.03	2.54 ±0.04	2.81 ±0.05	2.97 ±0.05	3.00 ±0.04

An increase in the rat hind paw diameter induced by sub-planter injection of formalin was used as the measure of acute inflammation. Values are expressed as mean ± S.E.M (Standard Error of the Mean) of 5 rats using Student's T-test

Table 4B: % inhibition of Normal saline (0.9 w/v), Ethyl acetate, and Prednisolone on Formalin-induced Paw Oedema in Rats

Group	Dose mg/kg	Percentage(%) inhibition 1hr	2hr	3hr	4hr	5hr
Normal saline n=5	10 mL/kg					
Ethyl acetate n=5	50	49.1	*56.5	*58.5	*61.5	*65.5
Ethyl acetate n=5	100	*68.7	*69.5	*71.4	*73.0	*74.4
Ethyl acetate n=5	200	**75.7	**76.2	**76.8	**77.8	**77.3
Prednisolone n=5	1.0	**76.7	**78.4	**77.8	**76.8	**76.4

An appreciable significant hourly inhibition of formalin when compared to standard drug was observed: *$P < 0.05$; **$P < 0.01$. Values are expressed as ± S.E.M (Standard Error of the Mean) of 5 rats using Student's T-test



Time after extract/drug administration (min.)

Figure 2: An appreciable significant hourly inhibition of formalin when compared to standard drug was observed: P < 0.05. Values are expressed as mean ± S.E.M of five rats using Student's T-test.

IV. Discussions

The presence of saponins, steroids and triterpenoids in ethyl acetate fraction of the plant extract of *O. gratissimum* (Table 1) supports the claim that these compounds have anti-inflammatory properties since saponins, steroids and triterpenoids have been found in other natural products with anti-inflammatory properties (Yeonju *et al*, 2012). The resulting fractions of *n*-hexane, ethyl acetate and methanol leaves extract from *O. gratissimum*, concentrated to dryness and gave the percentage yields of 4.93, 4.48 and 9.60 % w/w respectively; methanol extract showed the highest yield (9.60 %). The high polarity of methanolic extract may be responsible for the relatively high yield (Arnason *et al.*, 1995; Harborne, 1973).The formalin-induced licking response was used as a model for evaluating anti-inflammatory activity (Hunskar *et al.*, 1985). Rats were subcutaneously administered with 0.1 mL/ of 2.5 % v/v formalin on the dorsal part of the rats' hind paw; this would definitely tell whether the licking response was genuinely due to formalin injected into the paw because at times, the animals lick the forepaw under normal physiological condition. In the formalin-induced paw licking test, the highest dose (200 mg/kg) significantly reduced licking time when compared with other doses (50, 100 mg/kg); this showed that activity of the extract is dose-dependent. The inhibition at the early and late phase of 200 mg/kg of the extract competed favourably with the standard drug ($P < 0.01$); this showed that the extract is effective and may be considered as an alternative choice if formulated into drugs. The test possesses two distinct phases, possibly reflecting different stages of pain. Formalin produced a distinct biphasic response. The early phase reflects a direct effect of formalin on nociceptors (non-inflammatory pain), whereas the late phase reflects inflammatory pain (Elisabetsky *et al*, 1995; Hunskar and Hole, 1997). All the extracts produced very significant anti-inflammatory effects in both phases ($P < 0.05$ and 0.01 respectively). This suggests that the ant-inflammatory effect of the extract was mediated by both neurogenic and inflammatory mechanisms. The LD_{50} of the plant above 5000 mg/kg observed in this study seems to suggest that the plant's leaves may be safe for consumption because it did not affect any of the parameters measured (Lorke 1983). All the extract doses above 50 mg/kg were found to significantly ($P < 0.05$) inhibit the formalin-induced rat paw oedema, a test which has significant predictive value for anti-inflammatory agents which act by inhibiting the mediators of acute inflammation (Mossa et al., 1995). The extract inhibited the formalin-induced inflammation test with the high dose showing the highest percentage inhibition. The activity of the extract resides more at the highest dose 200 mg/kg body weight orally which was found to have the highest percentage inhibition (77.8%) of formalin-induced paw edema in rats at 5-hour. The effective peak of standard drug (prednisolone) resides at 1-hour with percentage inhibition of 78.7; which the inhibitory effect declined sharply after an hour of oral administration against formain-induced oedema.

V. Conclusion

In the course of the study, preliminary phytochemical screening was successfully carried out on various fractions *Ocimum gratissimum* leaves extract. Phyto-constituents which may be responsible for the anti-inflammatory activity of the plant were thoroughly investigated and further analysed using ethyl acetate extract.

Ethyl acetate extract of *Ocimum gratissimum* plant's leaves experimentally showed that the plant leaves possess anti-inflammatory activity. In the light of this research, further studies to systematically extract the active metabolites from the leaves of *O. gratissimum* would be needed. This will help to ascertain the actual compound(s) responsible for the anti-inflammatory properties. Also, the mechanism of action of this plant at the molecular level will throw more light on its mode of action.

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