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Anti-inflammatory, Anti-nociceptive and Total polyphenolic Content of Hydroethanolic Extract of *Ocimum gratissimum* L. Leaves

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Abstract

Ocimum gratissimum has been reported in several ethnopharmacological surveys as a plant readily accessible to the communities and widely used with a lot of therapeutic potentials. In this study, we aimed to experimentally evaluate the anti-inflammatory effects of hydro-ethanolic extract in animal models of inflammation and nociception and membrane stabilization assay.

O gratissimum leaves hydroethanolic extract was subjected to phytochemical screening and spectrophotometric quantification of polyphenolics. The extract was investigated for anti-inflammatory effects in carrageenan –induced paw oedema and cotton pellet - induced granuloma in rats. The antinociceptive effects were investigated in acetic acid –induced writhing in mice and formalin test in rats. Animals were randomly divided into groups; negative control, extract treated (200 -800 mg/kg) and indomethacin (10 mg/kg) standard reference groups. In- vitro anti-inflammatory activity was performed by testing for membrane stability in heat/hypotonic solution –induced rat erythrocytes destabilization assay.

Phytochemical screening revealed presence of saponins, tannins, alkaloids, flavonoids, terpenoids, and cardenolides. Quantification of the polyphenolic content revealed the presence of appreciable quantities of phenolics and flavonoids. Carrageenan-induced paw oedema, cotton-pellet granuloma, acetic acid –induced writhing and formalin induced paw licking tests showed that hydroethanolic extract of *O gratissimum* possess anti-inflammatory and anti-nociceptive effects. The extract did not induce gastric lesion formation in stomach of cotton-pellet granuloma rats. The extract was more efficient at reducing membrane destabilization than indomethacin in the membrane stability assay.

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These results suggest that hydroethanolic extract of *O gratissimum* leaves exhibits anti-inflammatory and anti-nociceptive effects in the animals.

Keywords

hydroethanolic; polyphenolic; carrageenan; erythrocytes; *Ocimum gratissimum*

Introduction

Ocimum gratissimum is a sub-shrub and perennial plant. It belongs to the family Lamiaceae and originated from Eastern India, and spread to virtually all-tropical areas of the globe [1]. *Ocimum gratissimum* has been reported in several ethnopharmacological surveys as a plant widely used in traditional medicine and readily accessible to the communities. It is a shrub commonly found around village huts and gardens [2, 3]. It is known as Efinrin nla in Yoruba, Ebavbokho in Bini, Dadoya in Hausa, and Nchanwu in Igbo speaking peoples of Nigeria [4]. In Uganda, it is known as Omujaja [3]. Extensive phytochemical and pharmacological studies into the activity of the leaves of *O gratissimum* have demonstrated its riches of both the oil and solvent extracts in bioactive phytochemicals. Reports have shown its detoxification property in the liver and also its anti-oxidant activities [5, 6]. The anti-inflammatory, antifungal and antibacterial properties of its essential oil have been well demonstrated [7, 8, 9]. Ocimum oil has been shown to calm overactive gastro-intestinal tracts in diarrhea [10]. Nangia et al., [11] reported the inhibition of breast tumor growth and angiogenesis by the crude extract of the plant.

Inflammation is a silent marauder responsible for most aches and pains. Persuasive experimental, pathological and epidemiological evidence implicates inflammation as the culprit in most debilitating diseases in several age-associated diseases such as obesity, metabolic syndrome, neurodegenerative diseases, rheumatoid arthritis, and cancer [12]. Inflammatory diseases affect many people in the world and represent the greatest collective burden of suffering and economic cost. Anti-inflammatory herbal medicines and their constituents are being proven to be potent protectors against various pro-inflammatory mediators in both acute and chronic inflammations. Although there is a growing interest in anti-inflammatory activity of plant extracts by the pharmaceutical as well as the herbal industry, a high proportion of the plant species have not been examined in detail [13].

In this study, we aimed to experimentally evaluate the anti-inflammatory effects of hydroethanolic extract of *O gratissimum* leaves (OGEE) in in- vitro and in vivo models of inflammation and nociception.

Materials and Methods

Plant collection and extract preparation

Seeds of O *gratissimum* were collected and grown in a well maintained domestic garden in Ishaka, Western Uganda. Taxonomic identification was conducted by a botanist from the Makerere University herbarium. A voucher specimen of the plant was deposited in the herbarium of the Pharmacognosy unit of School of Pharmacy, KIUWC, Ishaka, Bushenyi,

Uganda. Fresh leaves were harvested from the fully matured plants and air dried at room temperature. Dried leaves were ground into powder using a mechanical grinder. 100g of the dried powder leaves was soaked in 500 ml 70% ethanol and shaken for 48 hours. The aqueous ethanol was evaporated over a water bath set at 50°C and the extract concentrated to dryness in an oven set at 40°C. The extract was designated as OGEE and stored at 4°C in a refrigerator until used.

Experimental Animals

Male wistar rats weighing 150 to 200g and albino mice weighing 20 – 25g were used for the pharmacological assays. The animals were maintained in ordinary animal cages under constant 12 h/12 h light/dark cycle. They were acclimatized in the laboratory environment for one week, and were fed with standard pellet diet and water *ad libitum*. All experiments were carried out with strict compliance to The “Principle of Laboratory Animal Care” (NIH Publication No. 85-23) [14] and ethical guidelines for investigation of experimental pain in conscious animals by Zimmerman [15].

Phytochemical screening

Conventional standard protocols described by Evans [16], Harborne [17], Odebiyi and Sofowora [18] for detecting the presence of different chemical constituents in the plant extract were employed. Secondary metabolites tested for include; alkaloids, anthraquinones, tannins, saponins, flavonoids, cardenolides, and terpenoids. The presence of the compounds tested was rated as positive (+) or negative (-).

Determination of caffeic acid derivatives content in hydroethanolic extract of *O gratissimum* leaves

The caffeic acid derivatives in the hydroethanolic extract were determined using the spectrophotometric method with Arnow's reagent. 0.2 ml of OGEE (1 mg/ml) was dispensed into three separate test tubes, hydrochloric acid (1 ml, 0.5 N), Arnow's reagent (1 ml) and sodium hydroxide solution (1 ml, 1 N) were added to the test tubes and allowed to stand for 5 min. The absorbance was read at 500nm using (Spectronic 21D Milton Roy) Spectrophotometer. Caffeic acid was used as standard for the calibration curve. The total caffeic acid derivatives content of OGEE was estimated quantitatively from a linear regression curve ($y = 1.543x + 0.008$, $r2 = 0.999$) of caffeic acid, a standard phenolic, and presented in milligram caffeic acid equivalents per gram of sample (mg CAE/g sample).

Determination of total flavonoids content in hydroethanolic extract of *O gratissimum* leaves

The total flavonoid content (TFC) in the hydroethanolic extract was determined by the aluminium chloride spectrophotometric method. 1mL of OGEE (1 mg/ml) was dispensed into three separate test tubes. After that, 0.3 ml of 10% (w/v) $NaNO_2$ was added to the test tubes, and left to react for 5 minutes. Then, 0.3 ml of 10% (w/v) $AlCl_3$ was added and left for 1 minute to react. Then, 2 ml of 1M NaOH was added and the mixtures shaken. Aliquot of the mixtures were transferred to a cuvette, and the absorbance values measured using spectrophotometer (Spectronic 21D Milton Roy) at 510 nm. A mixture of 1ml of 80% (v/v)

methanol, 4ml of deionized water, and 0.3 ml of 10% (w/v) $NaNO_2$, 0.3 ml of 10% (w/v) $AlCl_3$ and 2 mL of 1M NaOH was prepared and used as the blank. Rutin was used as a standard for the calibration curve. The total flavonoid content of OGEE was estimated quantitatively from a linear regression curve (y = 1.494x – 0.005, r2 = 0.985) of rutin, a standard flavonoid, and presented in milligram rutin equivalents per gram of sample (mg RE/g sample).

Anti inflammatory effect on carrageenan – induced rat paw oedema

The carrageenan – induced rat paw oedema test is used in predicting the efficacies of anti-inflammatory agents that act by inhibiting the mediators of acute inflammation [19]. Pedal inflammation in Wistar rats of either sex (weighing 150 – 200g) was produced according to the method described by Winter et al., [20]. The crude hydroethanolic extract was screened for anti-inflammatory activities. In this experiment, the rats were divided into five groups of six rats. The test groups of rats received 200, 400 and 800 mg/kg of crude extracts orally, 30 mins before injection of 0.1ml of 1% carrageenan into the right hind foot of each rat under the subplantar aponeurosis. At the same time, the control group received 10ml/kg distilled water, while the reference group received 10mg/kg indomethacin. Paw volumes were measured hourly for 5 hours after carrageenan injection with a LETICA (Spain) digital plethysmometer (LE 7500). The change in paw oedema was plotted against time.

Inhibitory activity was calculated with the formula below:

$$\text{Percentage inhibition} = \frac{(C_t - C_o)_{\text{control}} - (C_t - C_o)_{\text{treated}}}{(C_t - C_o)_{\text{control}}} \times 100$$

where C_t – paw (oedema) volume at time (h) after carrageenan injection and C_o – paw (oedema) volume before carrageenan injection.

Anti inflammatory effects in Cotton pellet granuloma in rats

The rats (150 -180g) were anesthestized by intraperitoneal injection of 25 mg/kg of thiopental sodium. The groin region was carefully incised and 20mg of sterile cotton pellets inserted into the groin region. The rats were thereafter treated orally for eight consecutive days with the hydroethanolic extract of *O gratissimum* at dosages of (100, 200 or 400 mg/kg), indomethacin (5 mg/kg) and distilled water (10ml/kg). On the ninth day, the animals were sacrificed under deep anaesthesia. The cotton pellets were removed, freed from extraneous tissue, weighed and dried to a constant weight at 60°C. The mean value of wet and dry weight of cotton pellet was calculated and analyzed for statistical significance while the percentage of inhibition was derived from the mean weight of dry cotton pellet.

Ulcerogenic effect

To determine the sub acute gastric damage, the stomachs of the rats were dissected out, incised along the greater curvature; then the mucosa of the stomach was examined macroscopically with a hand lens. Erosions formed on the glandular portion of the stomachs

were counted and each one assigned a severity rating on the scale of 1-5 as described by Olajide et al., 2009[21].

Antinociceptive effect on acetic acid – induced writhing test

Acetic acid – induced writhing in mice as described by Koster et al [22] was used with slight modifications. Dilute acetic acid solution injected intraperitoneally into mice causes the animals to exhibit a characteristic writhing movement. The acetic acid-induced writhing test is a highly sensitive and useful test for analgesic drug development, and it is a model of visceral pain.

The mice were divided into four groups (n = 6) and differently pretreated with the hydroethanolic extract (200, 400 and 800 mg/kg p.o), indomethacin (10mg/kg p.o), or distilled water (10 ml/kg p.o). Thirty minutes after treatment, 1% acetic acid (10 ml/kg) was administered to the mice to induce the characteristic writhings. A writhe is described as a stretching of the whole animal so that it looks elongated with the abdomen touching the surface of the table, torsion to one side drawing up to hind limb and usually sucking in of the abdomen. The number of writhings occurring between 5 and 15 minutes after acetic acid injection was recorded. The results of the treatment groups were compared with those of vehicle treated controls.

Antinociceptive effect by formalin-induced nociception

The formalin test involves a biphasic response with the first phase, due to direct effect of formalin on nociceptors, and the second phase due to inflammation [23]. This test was carried out in five groups of six rats each. Each group was pretreated with either vehicle (distilled water 10 ml/kg) or the crude extracts (200, 400 and 800 mg/kg p.o.) and indomethacin (10 mg/kg). 50µl of 2.5% formalin solution was injected into the sub-plantar surface of the rat left hind paw 30 min after pretreatment. Severity of pain was rated using the methods of Dubuisson and Dennis [24] pain scoring measurements in the following manner: (0), normal weight bearing on the injected paw; (1), light resting of the paw on the floor; (2), elevation of the injected paw; and (3) for licking, biting and grooming of paw. These responses were observed and recorded for a total of 60 min. The first 10 min was considered as the early phase and represents aphasic pain while the period between 15 and 60 min was recorded as the late phase (representing tonic pain).

Anti-inflammatory effect by membrane stabilizing property assay

Blood was collected by cardiac puncture from healthy male wistar rats. The collected blood was mixed with equal volume of sterilized Alsever solution and centrifuged at 3000 rpm for 10 mins, packed cells were then washed with sodium phosphate buffer (0.1M, pH 7.2) and suspended in 10% (v/v) of the buffer.

Hydroethanolic extract and indomethacin (1.25, 2.5 and 5 mg/ml) was prepared in isotonic saline solution (154 mM NaCl). The assay mixture consisted of 2ml hyposaline (50mM NaCl), 1 ml sodium phosphate buffer (0.1M, pH 7.2), 0.5 ml RBC suspension (10% v/v) and 1 ml of varying concentrations of extract (1.25, 2.5 and 5 mg/ml) made up to 5ml with isosaline solution. The control was prepared as above except that the drug was not included.

The drug control was prepared similarly except that it lacked erythrocyte suspension. Each sample was prepared in triplicate. The reaction mixture was incubated at 56°C for 30 minutes in a water bath. The tubes were cooled under running water followed by centrifugation at 4000rpm for 10 minutes. Erythrocyte lysis in heated hypotonic solution was determined by the release of hemoglobin. The supernatant was collected and the absorbance of the released hemoglobin read at 560nm using a UV Spectrophotometer (Spectronic 21D Milton Roy). The percentage membrane stability was estimated using the expression:

$$\%\mathbf{Membrane\ Stability} = \mathbf{C} - \mathbf{T}/\mathbf{C} * \mathbf{100}$$

Where, C - Absorbance of Control, T - Absorbance of Test Sample

Results

Phytochemical screening results

The results revealed the presence of alkaloids, saponins, tannins, flavonoids, cardenolides and terpenoids, but the absence of anthraquinones as presented in Table 1.

Caffeic acid derivatives content & Total Flavonoid Content (TFC) in OGEE

The total caffeic acid derivatives results showed that OGEE contains 105.0 ± 0.01 mgCAE/g of sample. The amount of total flavonoid content in OGEE estimated from the rutin calibration curve was 123.7 ± 0.01 mgRE/g of the sample.

Effect of OGEE on carrageenan - induced rat paw oedema

Following carrageenan injection, the volume of rat paw increased as edema developed, indicating inflammatory activities (Figure 1). However, Pre-treatment with extract (200 – 800 mg/kg) did not produce statistically significant ($p<0.05$) inhibition of the oedematous response post carrageenan injection. Indomethacin (10 mg/kg) significantly decreased paw oedema at the 3rd, 4th, and 5th h after the injection.

Effect of OGEE on cotton granuloma in rats

OGEE and indomethacin caused a modest inhibition in granuloma weight increment as compared with control animals. The inhibition by the extract was not dose dependent as it was 15.2, 26.7 and 22.4% for 100, 200 and 400 mg/kg respectively while indomethacin (5 mg/kg) was 30%. Results as presented in table 2.

Ulcerogenic effect of sub-acute dosing of OGEE and indomethacin

In order to determine the gastrointestinal safety of OGEE after a sub-acute dosing, the stomachs of treated rats were dissected and viewed macroscopically. The result of ulcerogenic effect of OGEE and indomethacin is presented in table 3 below. The mean ulcer score in indomethacin treated animals (2.67 ± 0.21) was significantly ($p<0.05$) higher than in the extract treated groups, 0.17 ± 0.21, 0.33 ± 0.21 and 0.33 ± 0.21 for 100, 200 and 400 mg/kg respectively.

Effect of OGEE on acetic acid – induced writhing response

Figure 2 shows acetic acid –induced writhing response in mice which serve as an indication of analgesic activities of OGEE. Intraperitoneal injection of acetic acid produced 27.7 ± 3.7 mean number of writhes in the group administered with distilled water. The writhing response was reduced in mice pretreated with extract (200, 400 and 800 mg/kg) in a dose - dependent manner as 23.2 ± 0.9, 22.7 ± 0.8, and 12.0 ± 4.4 respectively. Inhibition of writhing response was statistically significant at extract (800 mg/kg) and indomethacin (10 mg/kg).

Formalin test

In the early phase, extract (200 and 800 mg/kg) treated groups showed significant changes as compared to the distilled water group (Fig 3). In the late phase (15 – 60 mins), score of pain was significantly decreased in rats pretreated with extract (200, 400 and 800 mg/kg) and indomethacin (10 mg/kg) (Fig 3).

Membrane stability—Fig 4 shows the ability of extract and indomethacin to inhibit RBC hemolysis. OGEE at concentrations of 1.25, 2.5 and 5 mg/ml protected the rat erythrocyte membrane against lysis induced by heat as is indicated by the high percentage of membrane stability. OGEE (5 mg/ml) and Indomethacin (5 mg/ml) significantly stabilized the erythrocytes membrane against hypotonic/heat – induced hemolysis.

Discussion

In the present study, we have shown that hydroethanolic extract of *O. gratissimum* leaves produces anti-inflammatory effects by decreasing the swelling in carrageenan –induced rat paw oedema; inhibition of granulocyte infiltration in cotton pellet –induced granuloma and membrane stabilization against heat/hypotonic solution-induced erythrocyte membrane destabilization. Its antinociceptive effects was also shown by decreasing number of writhing and paw licking in acetic acid –induced writhing and formalin –induced paw licking models respectively.

The phytochemical screening reveals the presence of flavonoid and tannins in the ethanolic extract. Estimations of total phenolic and flavonoid contents showed a moderate amount of total polyphenolics in the hydro-ethanolic extract. Earlier studies have shown that *O gratissimum* leaves contains mainly essential oil (Ocimum oil) and non-phlobatannins. Eugenol, thymol, and geraniol were the major volatile oil constituents found in *O. gratissimum*, while xantomicrol and cirsimaritin are the major external flavones [25]. Recently Salawu *et al.*, [26] and Chun-Ching et al., [27] reported the presence of Rutin and caffeic acid among other polyphenolics in both methanol and aqueous extracts.

In the carrageenan –induced inflammation experiment, we observed swelling of the right hind paw after injection of 1% carrageenan and found the extract (200, 400 and 800 mg/kg) and indomethacin (10 mg/kg) decreasing the level of swelling from the second to the fifth hour. The development of oedema in the rat hind paw after carrageenan injection has been previously described as a biphasic event. The early phase observed about 1 hr after carrageenan injection is reportedly due to the release of histamine, serotonin, bradykinin and

cyclooxygenase products. The late phase is due to neutrophil infiltration, as well as to the continuing production of arachidonic acid metabolites infiltration [19, 28]. Inducible cyclooxygenase (COX-2) is responsible for the late phase release of prostaglandins in carrageenan injected paw. The extract might be acting to suppress the synthesis and release of prostaglandins from the carrageenan injected paw. Some studies have demonstrated anti-inflammatory activities of aqueous extract of *O gratissimum* in agar and formalin - induced paw oedema rat models [29, 30].

Hydroethanolic extract of *O gratissimum* produced inhibitory effect in cotton –pellet granuloma test by decreasing the weight and dry weight of cotton pellet. This might be due to the inhibition of granulocyte infiltration to foreign body (cotton pellet), preventing the generation of collagen fibres and suppressing mucopolysaccharides. Repeated administrations of some non-steroidal anti-inflammatory drugs have shown evidence of gastrointestinal ulcerations due to inhibition of prostaglandin production [32]. In the cotton pellet –induced granuloma, the mean ulcer score in indomethacin treated animals was significantly higher than in the extract treated groups. Selective COX-2 inhibitors are known to be devoid of the gastrointestinal toxicities associated with other NSAIDS like indomethacin. The extract appears to have blocked inducible cyclooxygenase (COX-2) preferentially without affecting constitutive cyclooxygenase (COX-1) enzymes which produces cytoprotective prostaglandins. This implies that the extract is non-ulerogenic and therefore safe for the gastrointestinal tract. Some other studies have shown anti-ulcerative effects of *O gratissimum* extracts in ulcer models in rats [33, 34].

OGEE demonstrated significant reduction in the number of writhings in mice and the paw lickings in rats in the antinociceptive tests. The rat formalin test, which causes a local tissue injury of the paw, has been used as a model for tonic pain and localized inflammatory pain. Formalin-induced pain is caused primarily by peripheral tissue inflammation. It involves a phase of inflammation in which a variety of chemical mediators alter the functions of peripheral afferent fibers [35]. Formalin induces swelling and inflammation; it may also produce sensations other than pain, which may evoke behaviors scored in the formalin test [36]. It is well known fact that inhibition of formalin– induced paw oedema in rats is one of the most suitable test procedures to screen for anti-inflammatory and analgesic effects. Formalin injected subcutaneously into hind paw of rats produces localized inflammation and pain. The formalin test of *OGEE* demonstrated a significantly but moderate inhibition in the late phase (inflammatory phase). The essential oil has been reported to show antinociceptive activity [9], while a recent work reported that the antinociceptive activity of the essential oil may be due to the presence of its isolated active principle, eugenol or myrcene [37].

O gratissimum hydroethanolic extract protected the rat erythrocyte membrane against lysis induced by hypotonic solution and heat. During inflammation, there are lyses of lysosomes which release their component enzymes that produce a variety of disorders [38]. The lysosomal enzymes are implicated in the pathogenesis of articular tissue degradation in several rheumatic diseases [39]. Drugs by stabilizing the membrane can prevent the rupture of the lysosomes and inhibit the release of lysosomal enzymes [40, 41]. The erythrocytes membrane serves as a model for lysosomal membrane since it was observed that several agents capable of releasing hydrolytic enzymes from lysosomes also injure erythrocytes.

The membrane stabilization assay serves as a technique for the rapid screening of potential anti-inflammatory compounds based on their ability to inhibit heat –induced hemolysis of red blood cells [42]. Inhibition of membrane destabilization might lead to the prevention of leakage of serum proteins and fluids into the tissues during a period of increased permeability caused by inflammatory mediators. Our findings that pretreatment with OGEE protected rat erythrocytes from membrane destabilization, suggested a protective mechanism against lysosomal membrane destabilization.

Conclusion

The extract showed a moderate level inhibition of oedema in the carrageenan –induced oedema in rats and inhibited increase in pellet weight in the cotton pellet granuloma experiment but did not cause any significant gastric damage as compared with Indomethacin. The extract as well showed a significant inhibition of inflammatory pain in both the acetic acid-induced writhing and formalin test. Collectively, this study has shown that *O gratissimum* hydroethanolic extract possess anti-inflammatory and antinociceptive activities in the models used.

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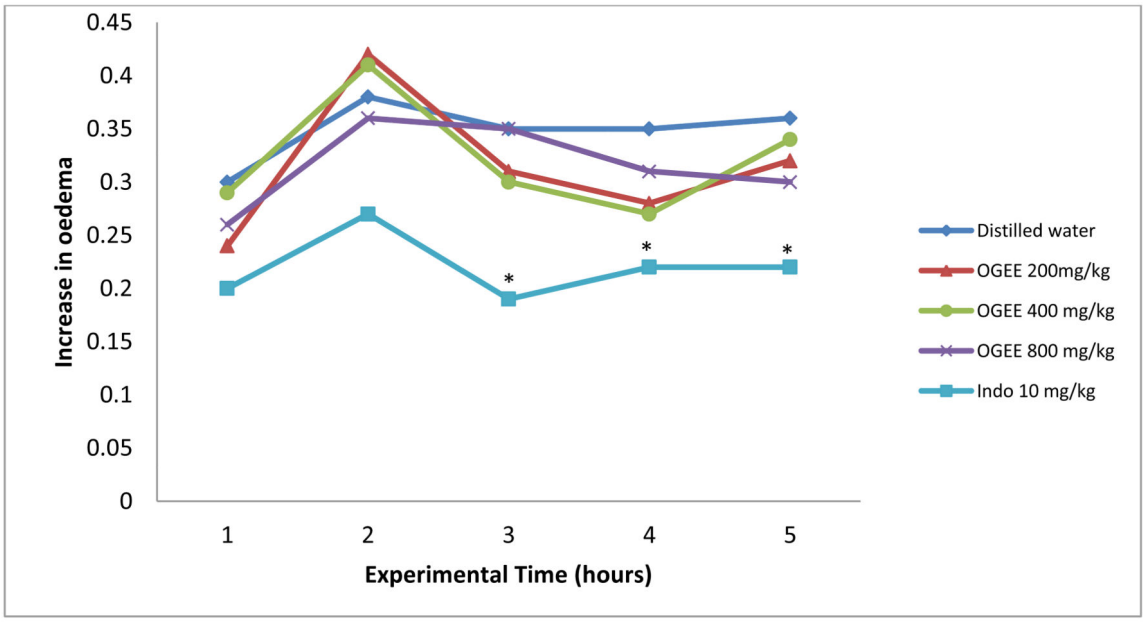

Fig 1.

Effect of hydroethanolic extract of *O gratissimum* leaves on carrageenan-induced rat paw oedema.

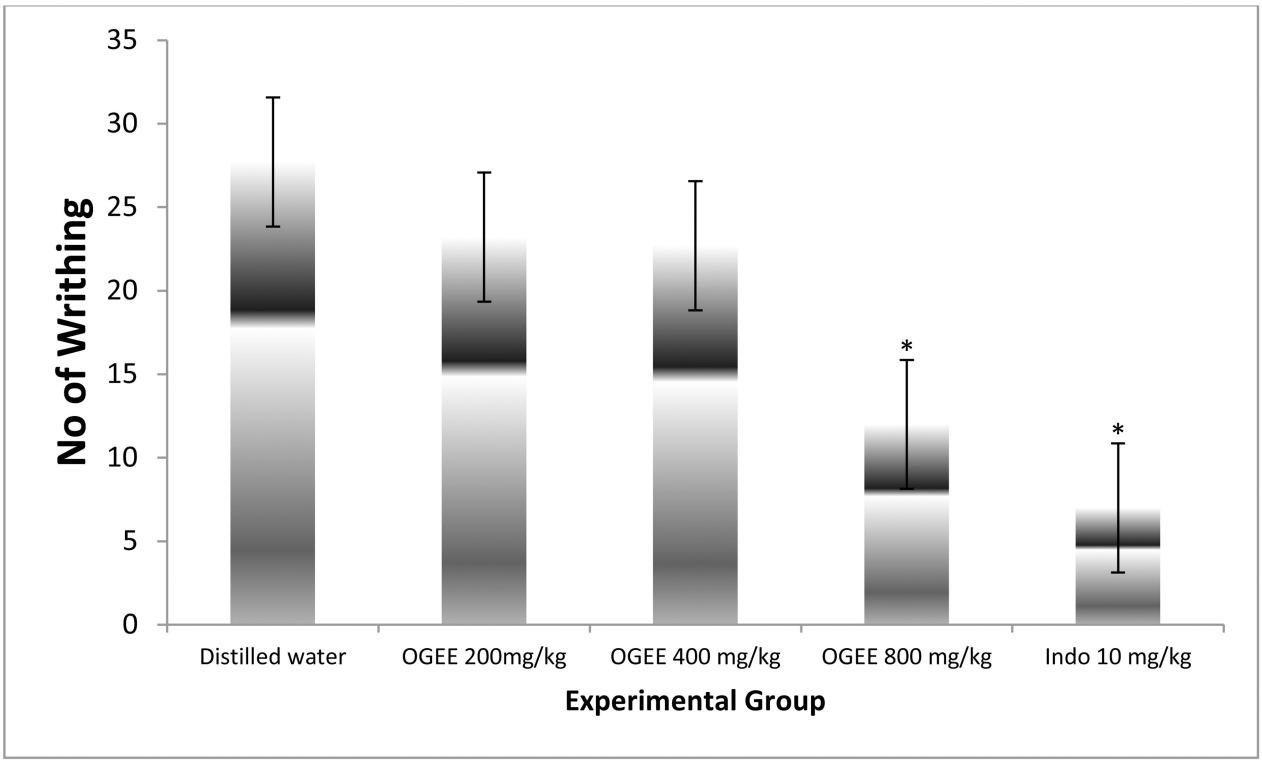

Fig 2. Effect of hydroethanolic extract of *O gratissimum* leaves in acetic acid - induced writhing in mice

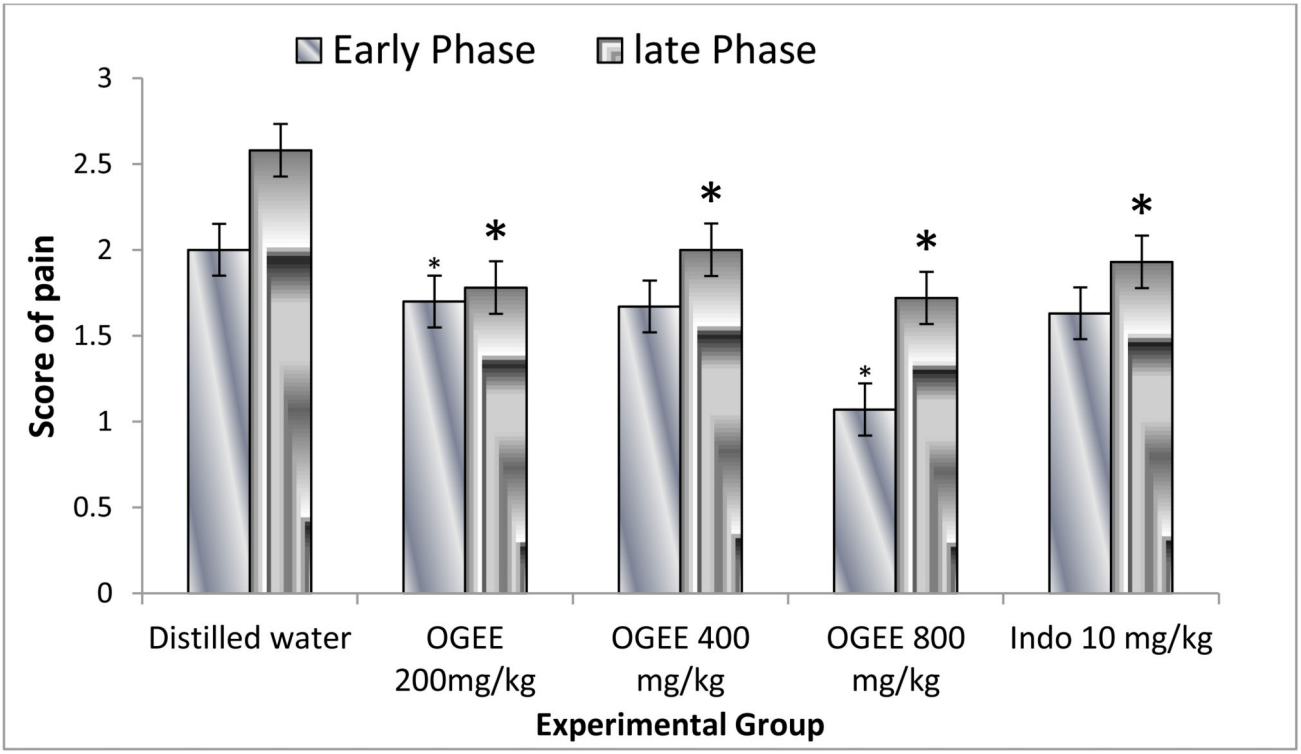

Fig 3. Effect of hydroethanolic extract of *O gratissimum* leaves on formalin -induced nociception in rats

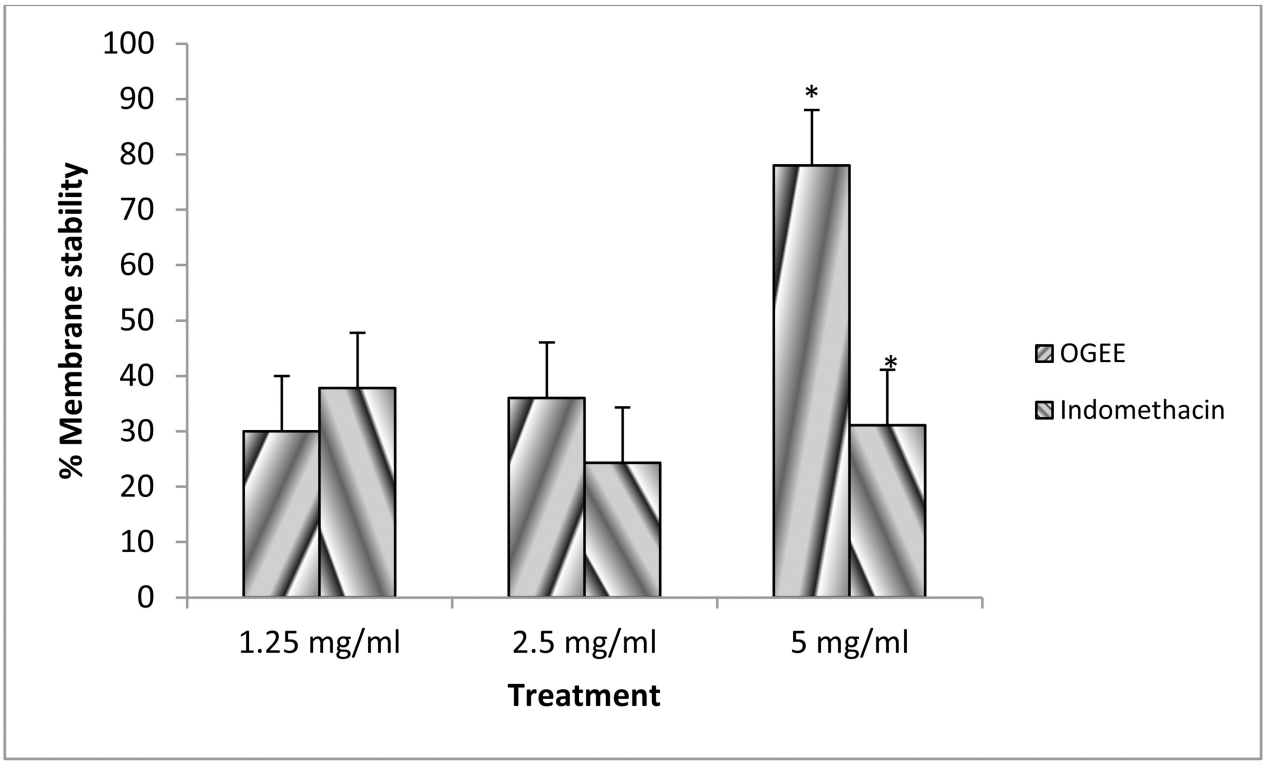

Fig 4.
Effect of hydroethanolic extract of *O gratissimum* leaves and Indomethacin on Membrane stability.

Table 1

Preliminary screening results of hydroethanolic extract of *Ocimum gratissimum* leaves.

Phytochemical constituents	Results
Alkaloids	+
Anthraquinones	-
Saponins	+
Tannins	+
Flavonoids	+
Cardenolides	+
Terpenoids	+

Key - + = present - = absent

Table 2

Effect of hydroethanolic extract of *O gratissimum* leaves on cotton pellet granuloma in rats

Treatment	Dose (mg/kg)	Granuloma weight (mg)			
		Wet (mg)	% inhibition	Dry (mg)	% inhibition
Control	10ml/kg	107.5 ± 5.7	-	32.7 ± 1.9	
OGEE	100	74.5 ± 8.2*	30.7	27.8 ± 2.3	15
OGEE	200	86.0 ± 13.8	20	22.2 ± 3.9*	32.1
OGEE	400	67.5 ± 8.2*	37.2	24.7 ± 2.0*	24.5
Indomethacin	5	65.3 ± 11.5*	39.3	23.0 ± 2.5*	29.7

Each value is the mean ± SEM of six rats (n = 6)

*P< 0.05, vs control

Table 3

Effect of hydroethanolic extract and Indomethacin on the gastric mucosa of rats in the cotton pellet granuloma experiment.

Treatment	Dose (mg/kg)	Mean ulcer score
Control	10ml/kg	0.50 ±0.22*
OGEE	100	0.17 ± 0.21*
OGEE	200	0.33 ± 0.21*
OGEE	400	0.33 ± 0.21*
Indomethacin	5	2.67 ± 0.21

Each value is the mean ± SEM of six rats (n = 6)

* P< 0.05 as compared with indomethacin