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Anti-nociceptive activity of the ethanol extract of *Eucalyptus globulus* leaf in experimental animals

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Abstract
Eucalyptus globulus has been used in African folklore for the treatment of several disease conditions like pain, fever and inflammation. The aim of the present study was to investigate the anti-nociceptive properties of *Eucalyptus globulus* and the probable mechanism using thermal and chemical models of nociception.

Acute toxicity revealed no mortality in the mice up to a dose of 5,000 mg/kg. The phytochemistry revealed the presence of alkaloids, flavonoids, saponins, tannins, cardiac glycosides and terpenoids. The extract significantly reduced pain as compared to control in the thermal and chemical models of pain employed in this study through mechanism that may involve the L-Type Voltage gated Calcium channel but ruled out the involvement of the adrenergic system and ATP sensitive K^+ channel. Moreover the anti-nociceptive activities demonstrated by the extract in this study might be due to the phytochemicals revealed in the extract.

Keywords: Anti-nociception, *Eucalyptus globulus*, calcium channel, formalin, analgesic, phytochemistry

Introduction
The folkloric use of herbal medicine therapeutically has gained increasing popularity in recent decades [1]. Majority of the inhabitants of the developing countries use one form of herbal medicine or the other [2]. In Africa traditional medicine, the use of medicinal plants has long been considered effective, affordable and reliable means of health care delivery, [3]. Plants represent the origin of modern pharmacotherapy and a good number of modern drugs have been isolated from plants [4].

Pain has been implicated in the pathophysiology of many disease conditions, which often affects the quality of life of humans. Opioids, anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs (SAIDs) are currently used in the management of pain. Though chronic administration of these drugs has shown toxic side effects to the kidney and liver.

Due to its anti-oxidant capacity [6], antimicrobial properties [5], and anti- inflammatory potentials [7], *Eukalyptus globulus* has gained recorgnition in industry and research. Eucalyptus a very large genus belongs to the family *Myrtaceae*. It has over 900 species and subspecies; and it is native to Tasmania and Australia [8]. The *Eucalyptus globulus* (among all the species of Australian Eucalyptus), was widely introduced overseas; thus, becoming largely cultivated in the Mediterranean and subtropical regions; as well as in Nigeria [9]. *E. globulus* commonly known *nkwu-ishi* by the Igbo speaking people of Eastern Nigeria; *mti-ulaya* in Swahili; *blue-gum eucalyptus* in English; *eucalyptus* in Bengali and in Hindi; and *Karpuramaram* in Tamil [10]. *E. globulus* is considerably used for the production of eucalyptus oil. It is also used in the pulp industry, as well as, extracted on commercial scale in many countries and adopted in cosmetics, perfumery, beverages, food, phytotherapy and aromatherapy [11]. The leaves of E. globulus is used in the north central Nigeria for relieve of cold and treatment of respiratory ailments [12].

Eucalyptus plants has drawn the attention of researchers worldwide due to its fast-growing ability and as a source of wood and oil used for various purposes. The oil (which is one of the principal constituents) extracted from the its leaves, bark, buds and fruits; has been reported to be possess antiseptic, anticancer, antioxidant, anti-inflammatory, and antibacterial activities [13]. All these and more; are the reasons why it has being used in the management of common cold, sinus congestion, influenza and respiratory diseases [14, 15].

However, despite the ethnomedicinal benefits of *E. globulus* leaf, there are few reports on its antinociceptive activities. Therefore, in order to establish a scientific basis for this folkloric

claim, this study was designed to investigate the anti-nociceptive properties and the possible mechanism of action of the ethanol leaf extract of *Eucalyptus globulus* in mice.

Materials and Methods

Animals

Male Swiss albino mice (22-30g), obtained from the animal house facility of College of Health Sciences, Kogi State University, Anyigba, were used in this study. They were acclimatized for two weeks, kept under standard laboratory conditions and fed on rodent cubes. This study was approved and supervised by the College of Health Sciences Research Ethics Committee of the Kogi State University, Anyigba to ensure ethical compliance.

Collection and Extraction of *Eucalyptus globulus* leaf

Eucalyptus globulus leaves were obtained from the premises of the Faculty of Agriculture, Kogi State University, Anyigba, Kogi State. The leaves were air-dried at room temperature, finely powdered with blender. The pulverized plant was macerated in ethanol for 72 hours. After the extraction, the extract was sieved and filtered. The filtrate was concentrated in the oven at 40 ºC [16]. The dried extracts were stored at 4 ºC until needed. Appropriate dose dilutions were made with distilled water.

Acute Oral Toxicity Test

The oral acute toxicity study (LD_{50}) of the ethanol leaf extract of *E. globulus* in mice was estimated according to the biphasic method of Lorke (1983). The extract 10, 100 and 1000 mg/kg were administered to three (3) groups each consisting of 3 mice in the first phase. Signs of toxicity and mortality were observed for the first 4 hours and later extended to 24 hours. Meanwhile, in the 2nd phase, the extract 1600, 2900 and 5000 mg/kg were administered to three groups each consisting of a mouse based on the result from the 1st phase. Signs of toxicity and mortality were also observed for the first 4 hours and later extended to 24 hours.

Phytochemical screening

The extract was screened to detect the presence of some phytochemicals according to the methods described by [17]

Drugs and chemicals

Diclofenac, propranolol, nifedipine, formaldehyde, ethanol, glibenclamide of high analytical grade, sstored with standard conditions were used in this study.

Antinociceptive Screening

The models of nociception used in this study were chemically and thermally dependent. Thermally dependent model used in this study is the taif flick test while the chemically dependent model is the formalin induced paw licking test in mice. These tests were carried out in strict compliance with the guidelines of the international association of pain (IASP) [18].

Formalin-induced Paw Licking Test in Mice

The procedures used in the formalin-induced paw licking test was similar to [19] with minor modification. Five groups of mice (n=5) were orally administered ethanol leaf extract of *Eukalyptus globulus* (250, 500 and 1000 mg/kg), distilled water (10 mL/kg) and Diclofenac (10 mg/kg). Sixty minutes post administration, 20 µl of 2.5% of formalin was injected into the intra plantar area of the right hind paw of each mouse. The mice were immediately placed individually in a transparent observation chamber and the time spent licking the injected paw was recorded for 30 minutes following formalin injection [first 5minutes after formalin injection (early phase) and 15–30 minutes after formalin injection (late phase)].

Tail flick Latency Assay in Mice

A water bathe maintained at 55 ºC was used and the time taken for the animal to withdraw its tail was recorded as withdrawal latency. The animals were grouped as above, the mice were orally administer distilled water (10mL/kg), diclofenac (10 mg/kg) and the extract (250, 500 and 1000 mg/kg).

Mechanism of anti-nociception of *Eukalyptus globulus*

The possible mechanism of action of *Eucalyptus globulus* was investigated by using the tail flick test. The animals were randomly divided into 5 groups of 5 animals each. The animals were orally pre-treated with a beta adrenergic blocker, propranolol (40mg/kg); L-type voltage-gated calcium channel blocker, nifedipine (10mg/kg); and ATP sensitive K^+ channel blocker, glibenclamide (8mg/kg) 60 minutes before oral administration of *Eucalyptus globulus* extract (500 mg/kg). The last 2 groups were orally administered distilled water (10mL/kg) and extract (500 mg/kg).
Sixty minutes post administration. The animals were subjected to the Tail flick test.

Statistical analysis

Data were presented as mean±SEM. Comparisons between groups were made using the one way analysis of variance (ANOVA) followed by Tukeys' *post hoc* test, 95% confidence level and at P value less than 0.05 was considered statistically significant.

Results

Acute Toxicity

The oral median lethal dose of *E. globulus* was estimated to be greater than 5,000 mg/kg in mice. No death was observed throughout the period of the experiment.

Table 1: Preliminary qualitative phytochemical analysis of the ethanol leaf extract of *Eukalyptus globulus*

Phytochemicals	Ethanol Extract
Alkaloids	+
Flavonoids	++
Saponins	+
Tannins	+
Anthraquinones	+
Terpenoids	+
Cardiac glycosides	+

(+ means present; ++ present with high percentage)

Preliminary Qualitative Phytochemical Analysis of *Eukalyptus globulus*

The phytochemical screening as shown in Table 2 revealed the presence of alkaloids, tannins flavonoids, anthraquinones, terpenoids, saponins and cardiac glycosides in the ethanol leaf extract of *Eukalyptus globule*.

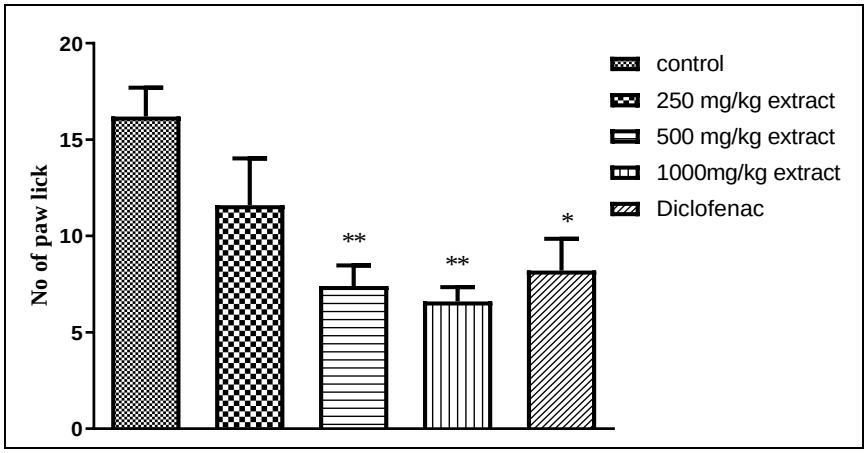

Fig 1a: Effects of graded doses of methanol extract of *Eukalyptus globulus* on early phase of formalin induced paw licking test.*p<0.05, **p<0.01 compared to vehicle treated control (One way ANOVA followed by Tukey's *post-hoc* test).

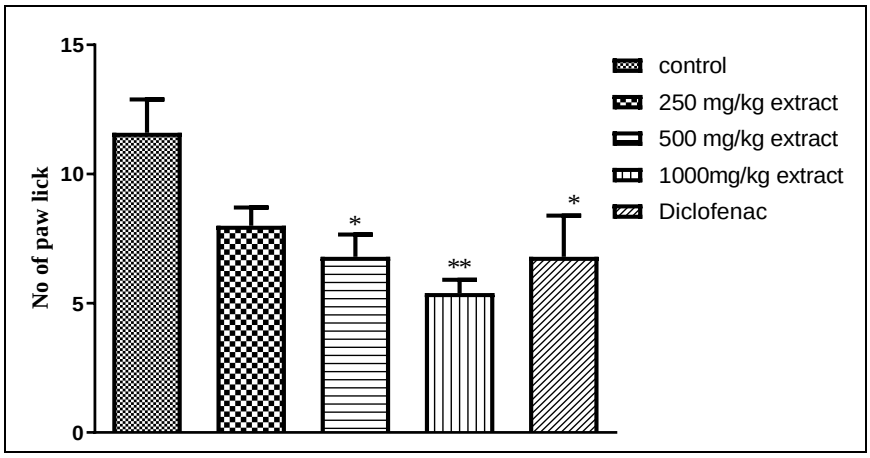

Fig 1b: Effects of graded doses of methanol extract of *Eukalyptus globulus* on late phase of formalin-induced paw licking test. *p<0.05, **p<0.01 compared to vehicle treated control (One way ANOVA followed by Tukey's *post-hoc* test).

Effects of *Eukalyptus globulus* on Formalin-induced Paw Licking Test in Mice

Eucalyptus globulus extract showed significant (p<0.05, p<0.01) reduction in the paw licking effect in both phases. In the early phase, the 500 and 1,000 mg/kg extract treated groups had better pain inhibition of 54.3 and 59.3% respectively when compared with the standard drug which has 49.4% inhibition of pain (Fig. 1a). In the late phase, the 1,000 mg/kg extract treated group had the highest pain inhibition of 53.4% while both the 500 mg/kg and the standard drug treated groups had 41.4% inhibition of pain (Fig. 1b).

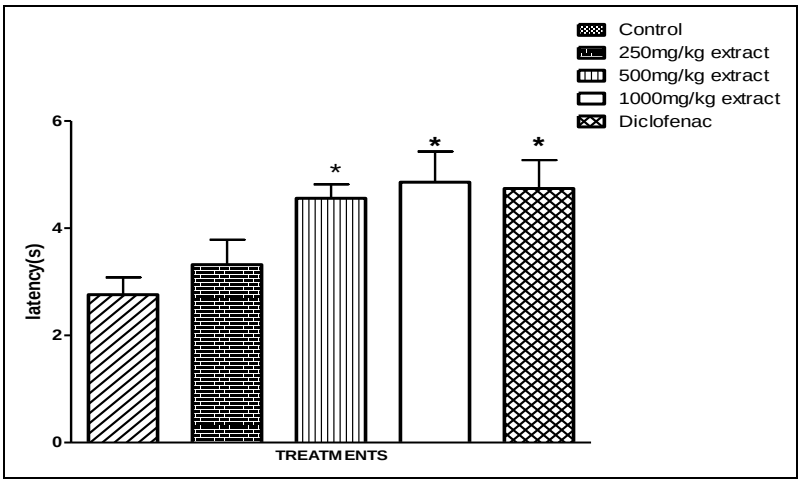

Fig 2: Effects of graded doses of methanol extract of *Eukalyptus globulus* on tail flick latencies in mice.*p<0.05 compared to vehicle treated control (One way ANOVA followed by Tukeys' post hoc test).

Effects of *Eukalyptus globulus* on Tail-flick Latency in Mice

Figure 2 shows the effects of graded doses of the ethanol extract of *Eukalyptus globulus* on tail flick latency(s). The extract (1000 and 500 mg/kg) tested showed significant (p<0.05) increase in the tail flick latency(s), the extract (250 mg/kg) was not significant compared with the control (distilled water 10 mL/kg). The efficacy of the 500 and 1000 mg/kg of the extract were comparable to the reference group (Diclofenac).

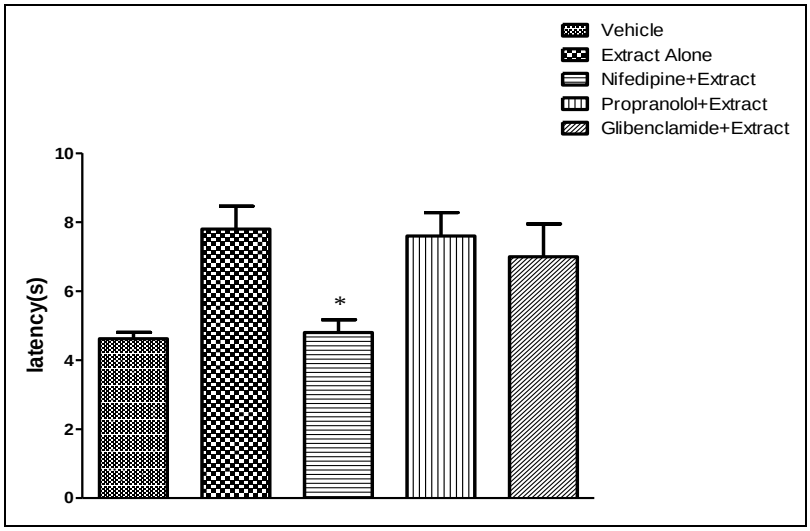

Fig 3: Effects of pretreatment with adrenergic, L type voltage gated calcium channel and ATP sensitive K^+ channel blocker in the tail flick.**p*<0.05 compared to Extract alone (Control).

Effects of pretreatment with adrenergic, L type voltage gated calcium channel and ATP sensitive K^+ channel blocker in the tail flick test

Pretreatment with propranolol and glibenclamide did not significantly affect the tail flick latency in mice. But pretreatment with nifedipine significantly reversed the anti-nociceptive effect of the extract (Figure 3).

Discussion

Using chemical and thermal models of nociception the anti-nociceptive properties and possible mechanism of action of *Eukalyptus globulus* was investigated in this present study.

In this study it was observed that the extract was relatively safe for use since there was no mortality and any sign of toxicity in the mice up to a dose of 5000 mg/kg. These suggest that the leaf extract of *E. globulus* may have a wide safety margin when administered orally [20].

The formalin induced paw licking test is a chemical model of nociception where the injury to the tissue (paw) is caused by chemical irritant formalin [21]. This model is useful in screening new agents with analgesic potentials because of its ability to examine the central and peripheral mechanisms involved in nociception [21, 22]. The formalin induced paw licking test has two phases, the early phase which is also referred to as neurogenic phase which often involves central mechanisms, while the late phase also known as inflammatory phase because of the involvements of inflammatory mediators like Substance P and Bradykinin [23]. Analgesics like morphine that act centrally have the ability to inhibit both phases while peripherally acting analgesics have more effect in the inflammatory phase by preventing the production of inflammatory mediators [24]. But, recent researches show that NSAIDs such as diclofenac can also inhibit both phases [25, 26]. The ability of the leaf extract of *E. globulus* to significantly reduce pain perception in both phases in this test may suggest the involvement of peripheral and central systems in the mechanism of action of *Eukalyptus globulus.*

Pain sensitivity is usually assessed by the tail flick test. In this test the ability of the agent tested to increase withdrawal latency when the tail is immersed in a water bath containing water of 55 ºC indicates that the agent has analgesic potentials [24]. The anti-nociceptive potential of an agent is determined by the latency. From previous studies it has been confirmed that morphine related drugs have the ability to increase the withdrawal latency [24], though currently it has been shown that NSAIDs can also increase the withdrawal latency [26]. Increased in withdrawal latency observed with antinociceptive agents is as a result of the activation of the periaqueductal gray matter (PAG) to produce endogenous peptides that goes to the spinal cord to prevent transmission of pain impulses in the dorsal horn, thereby preventing nociceptor activation [27]. The ability of the ethanol leaf extract of *E. globulus* to prevent the increase in withdrawal latency in this model of nociception may indicate that the extract exhibits its anti-nociceptive activity through central mechanisms.

Phytochemistry revealed the presence of alkaloids, flavonoids, saponins, terpenoids, anthraquinones and cardiac glycosides in the ethanol extract of *Eucalyptus globulus*. Alkaloids are known to have a wide range of pharmacological properties. These pharmacological properties include its ability to inhibit inflammation and pain [28], the presence of alkaloids in this extract may contribute to the anti-nociceptive ability exhibited by the extract in this study. The extract is very rich in flavonoid. In the plant kingdom flavonoid constitute a large group of aromatic amino acids [29]. Flavonoids are anti-oxidants and this has led to their use therapeutically [30]

Anti-inflammatory, wound healing, antimicrobial are some of the pharmacological activities associated with saponins extracted from plants [31]. Due to its ability to interact with signalling pathways in neurons, flavonoids have neuroprotective properties [32] and also can prevent processes of neuroinflammation in the brain [33]. The individual or combination of the classes of bio compounds (Alkaloids, flavonoids, saponins and terpenoids) present in *Eukalyptus globulus* may be responsible for the anti-nociceptive properties demonstrated by the extract in this study.

Blockers of pathways that are involved in nociceptive pathways were used to investigate the mechanism of its antinociceptive activity. Blockers used in this study include glibenclamide, nifedipine and propranolol. Thermal model (tail-flick test) was used for the study.

Administration of adrenoceptors blocker propranolol, glibenclamide ATP-sensitive K^+ channel did not affect the anti-nociception of *Eukalyptus globulus* leaf except for the pretreatment with L-Type Voltage Gated Calcium Channel blocker (nifedipine) which significantly abolished the anti-nociception of *Eukalyptus globulus* showing that its antinociceptive activity is non-adrenergic dependent, does not involve ATP sensitive K^+ channel. But the L-Type Voltage Gated Calcium Channel might be involved in the mechanism of action of the extract of *Eukalyptus globulus* leaf.

In conclusion, this study clearly revealed the antinociceptive activity of *Eukalyptus globulus* leaf, and showed that the mechanism of action of anti-nociception may not be due to the adrenergic system, ATP sensitive K^+ channel but the L-Type voltage gated calcium channel may be involved in the anti-nociceptive activity of *Eukalyptus globulus* leaf.

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