Arch. Bas. App. Med. 1 (2013) 103 - 106

ARCHIVES OF BASIC & APPLIED MEDICINE

Research Article

Antinociceptive Effects of Methanol Extract of *Callophyllum inophyllum*: Possible Roles of the Opioidergic, Adrenergic and Cholinergic Pathways

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Received: June 16, 2012
Revised version accepted: July 29, 2012

Abstract

The antinociceptive activities of the methanol extract of Callophyllum *inophyllum* (CI) leaves were investigated using thermal and chemical tests of nociception: tail flick, the hot plate, acetic acid-induced writhing and the formalin-induced paw licking test. In the thermal tests, the extract delivered per oral (p.o) dose dependently resulted in the prolongation of both the hot plate and the tail flick latencies at all doses. The extract dose dependently reduced the number of abdominal constrictions in the acetic acid-induced writhing test and also considerably reduced the licking times in both phases of the formalin-induced paw licking test. While naloxone had no effect on the antinociceptive activity of the extract, both atropine and prazosin produced an inhibition of its antinociception as revealed by significant reductions in the thermal latencies compared with the control. It may therefore be concluded from this study that Chlorophyllum inophyllum antinociception is mediated via the activation of the cholinergic and alpha adrenergic pathways .

Keywords: *Callophyllum inophyllum;* Antinociceptive; Opioid; Adrenergic; Cholinergic.

Introduction

Callophyllum inophyllum belongs to the family of *Clusiaceae*. It is a broad-leaved evergreen tree occurring as a litoral specie along the beach crests, although sometimes in the inter-land (Kadambi, 1957). It is a medium sized evergreen, ornamental and medicinal tree that averaged 8 to 20m in height. It is widespread along the coast of East Africa from Kenya to Mozambique. Phytochemical analysis revealed the presence of amentoflavone (Goh and Jantan, 1999), tripterpines (Kumar *et al.*, 1976), campesterol (Delaveau *et al.*, 1971) and stimasterol (Cambie and Ash, 1994).

The fresh bark of *Calophylum inophyllum* is used for the treatment of diabetes (Holdsworth and Wamoi, 1982), rheumatic pain (Clatchey, 1996) , skin infections (Khan *et al.*, 1980) and arrhythmias (Arora *et al.*, 1962). But the neuropharmacological mechanism(s) of these activities has not been explored. Our study therefore examined the neuropharmacological basis involved in *Chlorophyllum inophyllum* extract induced antinociception.

Animals: Male albino Swiss mice (20-35g) and Wistar rats (180-200g) were used for the study. They were purchased, housed and bred at the pre-clinical animal house of the college of Medicine, University of Ibadan, Nigeria where they were kept in a 12h light-dark environment at room temperature and fed with mouse cubes (Ladokun feeds, Nig Ltd) and water *ad libitum.*

Drugs and Chemicals: The following drugs were used: formaldehyde solution (Merck, Germany), acetic acid (BDH, Great Britain) , naloxone hydrochloride (Sigma-Aldrich, St. Louis, USA) , prazosin hydrochloride , and atropine (Research Biochemicals Inc., Natick, M A).

Plants materials: Fresh leaves of the plant *Callophyllum inophyllum* were obtained from the Forestry Research Institute of Nigeria, Jericho, Ibadan in July 2011. They were authenticated by Mr. A Edewo of the herbarium unit of the Institute where a voucher specimen with no CI 5452 was also deposited.

Extraction procedure: Callophyllum inophyllum leaves were fully shade-dried at room temperature and reduced to a powdery form. 500g of the powdered sample was exhaustively extracted with 2.5L of methanol (analytical grade) for 3 days. The macerated mixture was filtered through Whatman filter paper No 1 and the filtrate concentrated using Buchi rotavapor regulated at 40^{o}C under reduced pressure. . The yield of the preparation was 6.92 percent. The extract was stored in the refrigerator at 4^{o}C and dilutions made as and at when necessary using normal saline for pharmacological studies.

Antinociceptive tests

Tail flick test: The test was carried out using the original method of Eddy and Leimbach (1953) as modified by Ibironke *et al* (2000). Briefly, animals in the various groups were placed on a hot plate connected to a thermocouple, the hot plate temperature was maintained at 55±2.0^{o}C and a cut

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off time of 60s was imposed to avoid significant tissue damage.

Animals in the experimental groups (2, 3 and 4) were given 100, 200 and 400mg/kg of the extract of *Calophyllum inophyllum* (p.o) after a 12-hr fast, those in groups 1 and 5(control and reference groups) had 10ml/kg normal saline (p.o) and 10mg/kg indomethacin intraperitoneally (i.p) respectively. The rats were then placed in turn on the hot plate 30min after the administration of either drug, saline or extract and the reaction time, which is the time taken for the animal to start licking the paw or jump from the hot plate was noted as the hot plate latency (HPL). This test was carried out at 30, 60, 90 and 120 min after the administration of the various agents. The longest latency which occurred at 60min was selected and used as the reference point.

Tail immersion test: The details of the tail immersion procedure was essentially similar to those published earlier (D′ Armour and Smith, 1941). Briefly, using a circulating immersion heater (Catalogue No 13-874-170), Fischer Scientific, Pittsburg, PA) a constant temperature of 50±2ºC was maintained in the water bath in which the terminal 3cm of the animal's tail in the various groups were immersed. The animals were divided into five groups of six rats each. The control group (1) received normal saline (10ml/kg, p.o), the experimental groups (2, 3 and 4) received 100, 200 and 400mg/kg of the extract respectively, p.o and the reference group (5) had indomenthacin (10mg/kg, i.p).Thirty minutes after the administration of these agents the terminal 3cm of the tails of the animals were in turn immersed in the hot water bath. The nociceptive end point was characterized by a jerk of the tail. The time taken for the animal to withdraw or flick its tail out of water was taken as the tail flick latency (TFL).

Formalin-induced paw licking test: The details of the procedure were essentially similar to that of Hunskaar and Hole (1987). Briefly, 0.2ml of 3% formalin was injected into the dorsal surface of the left hind paw of the rats in the various groups and the animals placed in a chamber with a mirror mounted on three sides for an unobstructed view of the paws, thirty minutes after, the animals in the various groups were given various agents. The control group (1) had 10ml/kg normal saline, p.o, the experimental groups (2, 3 and 4) were given 100, 200 and 400mg/kg extract of *Calophyllum inophyllum* respectively, p.o and the reference group (5) treated with 10mg/kg indomethacin, i.p. The time in seconds the animals spent licking the injected paws for the first 5min post formalin injection (first phase) and for 10min starting at the 20th min post formalin (second phase) was noted.

Acetic acid-induced writhing test: The test was carried out using the modified method of Koster *et al*. (1959). The extract at doses of 100, 200 and 400mg/kg were given to animals in the experimental groups 2, 3 and 4 respectively, p.o. The control and reference groups (1 and 5) had 10ml/kg normal saline p.o and 10mg/kg indomethacin, i.p respectively after a 12h fast. One hour later, the mice were given an intraperitoneal injection of 0.2ml acetic acid to induce the characteristic writhing. The number of writhings occurring between 5 and 10min post injection was recorded. The percentage inhibition of writhing was calculated as follows (Witkins *et al.*, 2009):

% inhibition = (1-VT)/VC x100
VT= no of writhes in drug treated mice
VC= no of writhes in control

Mechanism of Antinociception: To investigate the mechanism of *Calophyllum inophyllum* antinociception, the hot plate test was used. The animals were divided into five groups (n=6) and treated as follows: Group 1 wars administered 400mg/kg extract of *Calophyllum inophyllum* alone, groups 2, 3, 4 and 5 were given normal saline (10ml/kg), naloxone (2mg/kg, i.p), atropine (2mg/kg i.p) and prazosin (2mg/kg i.p) respectively, followed by 400mg/kg *Calophyllum inophyllum* extract 45mins later. The animals were then subjected to the hot plate test after another latent period of 45mins.

Statistical analysis

Values are means± SEM. Statistical analysis was carried out using the unpaired Student's t-test.. Values of $p< 0.05$ were regarded as significant

Results

Acetic acid induced writhing test: The results showed that the extract of *Calophyllum inophyllum* at all doses inhibited the writhing response at different levels of significance (100mg/kg, $p<0.01$, 200mg/kg and 400mg/kg, $p<0.001$. The 200mg/kg and 400mg/kg of the extract were more potent ($p<0.001$) than the reference drug, indomethacin ($p<0.01$) when compared with the control group (Figure 1).

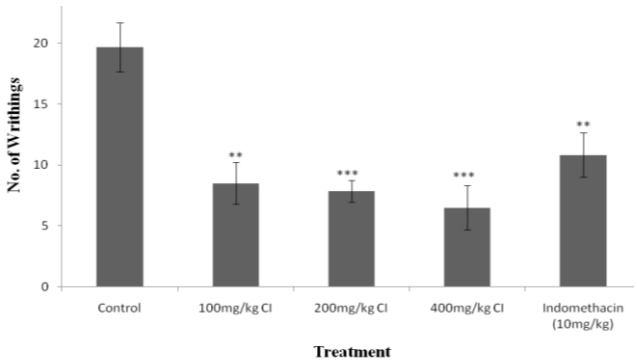

Fig 1:
Effect of graded doses of methanol extract of *Callophyllum Inophyllum* (CI) on acetic acid-induced writhings in mice. Each value represents Mean± SME, n=6, **P<0.01, ***P<0.001 compared with the control.

Formalin- induced paw licking test: The figure showed that in the late phase, all the doses of the *Calophyllum inophyllum* extract decreased the licking time significantly (100mg/kg and 200mg/kg, $p<0.05$; 400mg/kg $p<0.001$) compared with the control group. However, in the early phase, only the 400mg/kg dose produced a significant reduction in the licking time ($p<0.05$). The inhibition

produced by the reference drug, indomethacin was also significant in both the early ($p<0.05$) and the late ($p<0.01$) phases when compared with the control group (Figure 2).

The tail flick test: The results showed that all the oral doses of the extract of *Calophyllum inophyllum* caused a significant prolongation of the tail flick latency (100mg/kg and 200mg/kg, $p<0.01$, 400mg/kg, $p<0.001$) compared with the control. The inhibition produced by the 400mg/kg dose of the extract was more significant ($p<0.001$) compared with that of the reference drug ($p<0.01$) in relation to the control group (Figure 3).

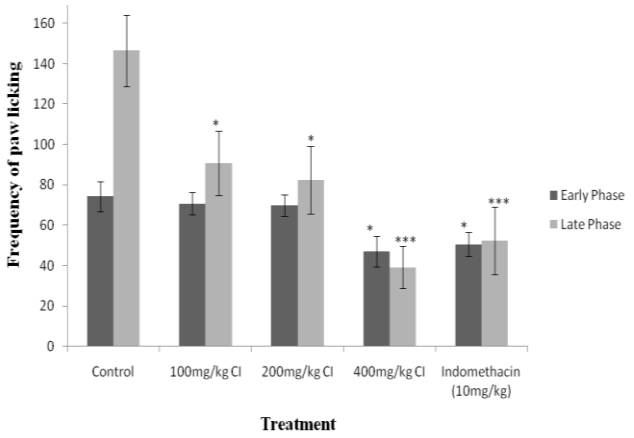

Fig 2:
Effect of graded doses of *Callophyllum Inophyllum* (CI) on formalin induced paw licking in mice. Each value represents Mean± SME, n=6, *P<0.05, ***P<0.001 compared with the control

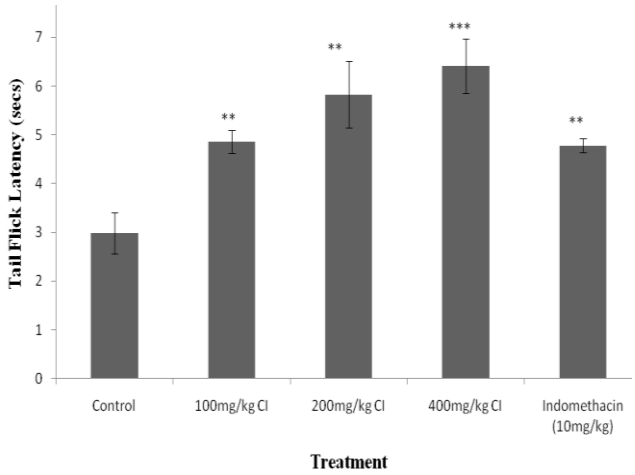

Fig 3:
Effect of graded doses of *Callophyllum Inophyllum* (CI) on tail flick latency in mice. Each value represents Mean± SME, n=6, **P<0.01, ***P<0.001 compared with the control.

The hot plate test: As shown in Figure 4, the extract of *Calophyllum inophyllum* significantly ($p<0.01$) prolonged the hot plate latency at all doses compared with the control. The reference drug, indomethacin(10mg/kg) produced a lesser inhibition ($p<0.05$) compared with that produced by the extracts at all doses ($p<0.01$)

Mechanism of antinociception using the ail flick test: Table 1 showed that administration of normal saline (10ml/kg) before *Calophyllum inophyllum* extract (400mg/kg) had no significant effect on the tail flick latency (6.82±0.56 Vs 6.85±0.97, $p>0.05$) of the extract. Similarly, administration of naloxone (2mg/kg) before the extract did not significantly affect the tail flick latency of the 400mg/kg extract compared with when given alone (6.82±0.56 Vs 6.81±0.90, $p>0.05$). However, administration of both atropine (2mg/kg) and prazosin (2mg/kg) 45 minutes before the extract significantly reduced the tail flick latencies of the extract (6.82±0.56 Vs 4.98±0.27, $p<0.01$ for atropine and 6.82 ±0.56 Vs 4.80±0.34, $p<0.05$ for prazosin) compared with when the extract was given alone.

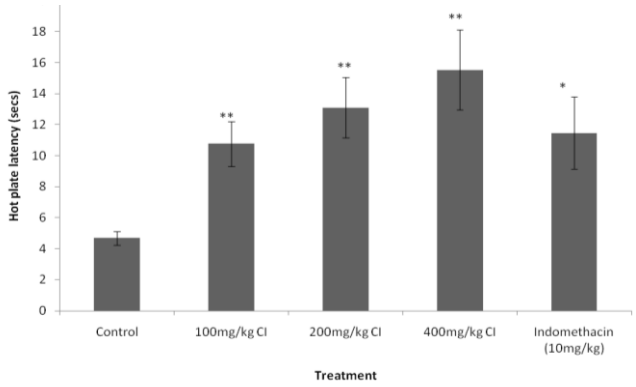

Fig 4: Effect of graded doses of *Callophyllum Inophyllum* (CI) on hot plate latency in rats. Each value represents Mean± SME, n=6, *P<0.05, **P<0.01 compared with the control

Table 1:
Effect of naloxone, atropine and prazosin on the antinociceptive effect of CI extract using tail flick test.

Groups	Doses	Tail flick latency (sec)
Extract only (Control)	400mg/kg	6.82±0.56
Saline + Extract	10ml/kg+ 400mg/kg	6.85±0.97^{NS}
Naloxone + Extract	2mg/kg 400mg/kg	6.81 ± 0.90^{NS}
Atropine + Extract	2mg/kg 400mg/kg	4.98 ± 0.27*
Prazosin + Extract	2mg/kg 400mg/kg	4.80 ± 0.34*

Each value represents mean ± SEM (n = 6), * p< 0.05 compared with control; ^{NS} not significant compared with control.

Discussion

The results obtained in this study showed the antinociceptive properties of the methanol extract of *Calophyllum inophyllum*, the non-involvement of the opioidergic mechanism and the possible participation of both the cholinergic and the alpha adrenergic pathways. There is paucity of data on the subject and so our findings could not be directly compared with any previous study, this report will therefore serve as a reference point for future research on the subject matter. The models of nociception selected for the study are such that both centrally and peripherally mediated effects were covered.

The hot plate test is commonly used because it is sensitive to strong analgesics; the test also limits tissue damage because of the imposed cut-off time, which limits the time the animal spends on the hot plate. This test is

supraspinally mediated and is therefore a test of central activity. It is an established fact in pain studies that any agent that causes a prolongation of the hot plate latency may be acting centrally (Bars *et al*., 2001), it therefore follows from our findings in this study that the extract of *Calophyllum inophyllum* might possess a central activity.

The ability of the extract to inhibit the acetic acid-induced writhing in mice (a model of visceral pain), suggests that it could be useful in the management of visceral pain, but it is not a selective pain test as it gives false positives with sedatives and muscle relaxants (Elizabetsky *et al*., 1995). Hence, the need to corroborate the findings with other tests as we have done in this study. Acetic acid is a chemical irritant that produces tissue necrosis of the peritoneal region accompanied by the release of chemical mediators, which cause pain either by activation or sensitization of nociceptors that encode tissue injury (Gene *et al.,* 1998; Pastor *et al.,* 1996; Deraedt *et al.,* 1980). The abdominal constriction is related to the sensitization of nociceptive receptors (Bose *et al.,* 2007). Diclofenac and other NSAIDs can inhibit the number of writhings in this model, by inhibiting cyclooxygenase in peripheral tissues, thus interfering with the mechanism of transduction in primary afferent nociceptors by blocking the synthesis or release of inflammatory mediators (Panthong *et al*., 2007). It is therefore possible to suggest that the extract may be acting via mechanisms similar to that of NSAIDs.

The formalin test is a valid model of nociception that is sensitive to various classes of analgesic agents. In this study, formalin exhibited a biphasic action, the first phase (neurogenic) is mediated by direct stimulation of nociceptive primary afferents and the second phase (inflammatory) is due to the release of chemical mediators that sensitize or activate nociceptors (Tjosen *et al.,* 1992). . The results of this study showed that the extract of *Chlorophyllum inophyllum* had an inhibitory effect on both phases of formalin nociception. Thus, these inhibitory effects demonstrated *by Calophyllum inophyllum* against the neurogenic and inflammatory pains may suggest an action similar to those of opioids. On the mechanism of the antinociceptive activity, the hot plate test was selected because of its supraspinal nature and is considered to be selective for centrally acting analgesic agents.

The mechanism was evaluated by prior administration of normal saline, naxolone (opioid receptor blocker), atropine (cholinergic blocker) and prazosin (alpha adrenergic blocker). While naloxone administration had no significant effect on the hot plate latency, thereby rulling out the probable participation of the opioid pathway, both atropine and prazosin caused a significant reduction ($p<0.05$) in the hot plate latency when administered prior to CI extract.

In conclusion, the present results provide evidence that *Chlorophyllum inophyllum* extract exerts a pronounced antinociceptive effect mediated via the activation of both the cholinergic and the alpha adrenergic systems

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