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Research Article

Anti-Inflammatory and Analgesic Activities of Ethanolic Leaf Extract of *Calotropis procera*

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ABSTRACT: Ethanolic extract of the leaf of *Calotropis procera* was investigated for its anti-inflammatory and analgesic activities. The extract was evaluated using formalin-induced paw lick, carragenaan-induced paw oedema in Wistar rats, acetic acid-induced writhing and tail flick tests in mice. Each experiment consisted of thirty animals randomly, but equally divided into groups of 100mg/kg, 200mg/kg or 400mg/kg body weight (b.w) of extract, Indomethacin (10 mg/kg b.w) or aspirin (15mg/kg b.w) pre-treated animals and a control group administered with distilled water (10ml/kg b.w). The administration of the extract was repeated for formalin-induced paw lick and acetic-acid induced writhing models in the presence of an opioid antagonist, naloxone. The data were analyzed using one way ANOVA and difference of means were considered significant at $p<0.05$. The ethanolic extract exhibited potent anti-inflammatory or analgesic effect in this study. Inhibition of formation of paw oedema by the extract (100mg/kg b.w) was significantly higher than for Indomethacin. Itching was significantly reduced in rats administered with extract in the early phase of formalin response, and was comparable to Indomethacin (10mg/kg b.w). 100 mg/kg body weight of the extract also inhibited the writhing movement comparably with aspirin (15mg/kg b.w). Same pattern was also observed with tail flick model in mice. The study showed that the mechanism of action of the analgesic or anti-inflammatory action of the leaf extract is mediated both centrally and peripherally. The analgesic or anti-inflammatory effect of the extract was not attenuated by opioid antagonist, naloxone, thus ruling out the involvement of opioid receptors in the central mechanism of action of the extract. It was therefore concluded that these activities are mediated via interaction with other nociceptive pathways.

Keywords: *Calotropis procera*, Extract, Anti-inflammatory, Analgesia

INTRODUCTION

Calotropis procera Ait. is a member of the Asclepiadaceae plant family (Aiton, 2010). It is commonly referred to as milkweed, giant swallow wort or apple of Sodom. The plants are native to southern Asia and Africa. The fruit of the plant is green with an ovoid shape. The flesh of the fruit contains a toxic milky sap that is extremely bitter and turns into a gluey coating resistant to soap. The milky sap contains a complex mix of chemicals, some of which are steroidal heart poisons known as cardiac aglycones. These belong to the same chemical family found in foxgloves (*Digitalis purpurea*). The glycosides found in *C. procera* are calotropin, calotoxin, calactin, uscharidin and voruscharin (Sieber *et al.*, 1982; Daniel, 2006).

The bark and latex are widely used as arrow and spear poison. The root bark is an emetic and the latex is used for treatment of skin diseases such as ringworm. The leaves of the plant were reported to have antioxidant and antibacterial activities (Yesmin *et al.*, 2008). The latex of *C. procera* were established by Kumar and Roy (2007) to protect against inflammation and oxidative stress in monoarthritis induced by Freund's complete adjuvant in rats.

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Previous studies by Smit *et al.* (1995) and Sehgal *et al.* (2006) demonstrated the potent cytotoxic activity of the flower of *C. procera* and this was comparable to the anticancer effect of cisplastin. Eghianruwa *et al.* (2006) also reported that *C. procera* reduced intestinal transit of rats. A closely related plant, *C. gigantea* has also been demonstrated to possess potent anti-inflammatory activity which was comparable to that observed for ibuprofen (Awasthi *et al.*, 2009; Bulani *et al.*, 2011). The current study was designed to evaluate the anti-inflammatory and analgesic potential of the ethanolic leaf extract of *C. Procera* using laboratory rats and mice.

MATERIALS AND METHODS:

Extraction of leaves

Fresh leaves of *Calotropis procera* were harvested from University of Abeokuta Research farm, Abeokuta. They were air dried for four weeks. The dried leaves were pulverized and soaked in 96% ethanol for 72 hours. The suspension was decanted and filtered. The solution obtained was clarified by filtration through celite on water pump and was then concentrated *in vacuo* using a rotation evaporator (Rotavapor R-210, Switzerland) at low temperatures. The remaining moisture was finally removed by placing small volumes in porcelain dishes in the oven set at low temperatures of 40 ^{0}C.

Experimental animals

Sixty Wistar rats and sixty Swiss mice of both sexes were used for this study. The animals were randomly divided into groups of five animals. The animals were housed at the experimental animal unit of the Faculty of Veterinary Medicine, University of Agriculture, Abeokuta. They were fed with pelletized rat and mouse rations, and allowed water *ad libitum*. They were kept under 12 hour light: dark conditions. Five groups of animals were used for each experiment. Three of the groups were administered with the leaf extract at 100mg/kg, 200mg/kg or 400mg/kg body weight b.w respectively. Another group was administered with Indomethacin (10mg/kg b.w) or aspirin (15mg/kg b.w) to serve as positive control while the fifth group was administered with distilled water (10mls/kg b.w) to serve as negative control.

Anti-inflammatory study

Carrageenan- induced paw oedema in rats: Animals were fasted overnight and administered with the leaf extract, Indomethacin or distilled water as mentioned above. One hour afterwards, oedema was induced in the paw of rats by injection of 0.1ml of 1% carrageenan into the aponeurosis of the right hind limb of rats as described by Winter *et al.* (1962). The linear circumference of the paw was measured at 0 minute, 60 minutes, 120 minutes and 180 minutes after injection of carrageenan. The increase in linear circumference at the different hours determined the inflammatory reaction observed as formation of oedema in paw (Calixto *et al.*, 2003). Cotton thread was wrapped around the paw and the circumference measured with a meter rule as described by Hess and Milonig, (1972) and Olajide *et al.* (2000). The inhibitory activity was then calculated as shown below.

Inhibition of oedema = $\frac{(Ct - Co)\ \text{control} - (Ct - Co)\ \text{test}}{(Ct - Co)\ \text{control}}$

Where Co = mean paw size in control group

Ct = mean paw size in treatment group

Formalin paw lick test in rats.

Following an overnight fast, the leaf extract was orally administered to rats in the three treatment groups. Simultaneously, 10mg/kg indomethacin (a non-selective cyclo-oxygenase inhibitor) was administered to animals in a group, while control animals received distilled water. Thirty minutes after treatment, 50 µl of 2.5% formalin was injected subcutaneously into the sub-plantar surface of the left hind paw of the rats. Responses were measured 0-5 minutes (early phase) and 20-30 minutes (late phase) after formalin injection. The licking of the injected paw and the duration was indicative of pain. The cumulative licking time was recorded.

Analgesic study

Acetic acid writhing response in mice: Mice were divided into five groups and were administered with extract (100, 200 or 400 mg/kg body weight), aspirin (15mg/kg b.w) or distilled water (10mls/kg b.w) respectively as earlier described. One hour after, 10ml/kg 0.6% acetic acid was injected intraperitonealy to each mouse. 5 minutes following acetic acid administration, the number of abdominal constrictions that occurred within subsequent 20 minutes were counted and recorded.

Tail flick test: This experiment was conducted according to the modified method adopted by Sanchez-Mateo *et al*. (2006) using hot water bath. Groups of five mice each were administered with the extract (100, 200 or 400 mg/kg body weight), aspirin 15mg/kg b.w or distilled water (10mls/kg b.w) respectively as earlier

mentioned. Thereafter, the terminal 2 cm of the mice tail were immersed in a water bath containing hot water maintained at 55±1°C by a circutine (Haake-Vison, Germany). Response to pain was taken as the time interval between immersion and withdrawal of the tail by the mice and these were taken at 30, 60 and 90 minutes after treatment.

Naloxone antagonism: Formalin paw lick in rats and acetic acid writhing tests in mice were repeated using two groups of animals per experiment. The first group of animals were administered with naloxone (1mg/kg s/c) 30 minutes before administering the leaf extract at 200mg/kg b.w. The second group also received naloxone in similar manner before administration of distilled water (10mls/kg b.w).

Statistical analysis

Data were analysed using one way analysis of variance (ANOVA) on GraphPad Prism 4.0 version. The result obtained were expressed as mean values ± standard error of mean (SEM). The statistical significant difference between the mean values were determined at $p<0.05$.

RESULTS

Carragenaan-induced paw oedema

Change in paw sizes of those administered with the extract at 100mg/kg b.w (0.12±0.02 cm, 0.14±0.05 cm, 0.22±0.14 cm and 0.09±0.04 cm) were significantly lower compared to those that served as control (0.28±0.02 cm, 0.66±0.12 cm, 0.62±0.08 cm, and 0.48±0.09 cm) at 0, 60, 120 and 180 minutes observation times. Rats administered with extract at 200mg/kg b.w recorded a consistent reduction in the swelling of paw (0.34±10 cm, 0.30±0.09 cm, 0.22±0.10 cm and 0.16±0.08 cm) at the observation times. Changes in the paw sizes of rats administered with the extract at 400mg/kg b.w (0.32±0.07 cm, 0.30±0.06 cm, 0.26±0.02 cm and 0.22±0.11 cm) were also lower when compared to the control rats (Table 1).

Formalin paw lick test

Rats administered with the leaf extract at 100mg/kg, 200mg/kg or 400mg/kg b.w demonstrated shorter mean itching period at the early phase (48.14±6.38 seconds, 68.16±10.46 seconds and 62.48±9.42 seconds) and late phase (14.12±2.63 seconds, 22.06±9.84 seconds and 48.30±7.08 seconds) compared to the control group rats at the early (89.46±6.79 seconds) and late phases (70.50±10.89 seconds) respectively. Rats administered with indomethacin demonstrated non-significantly ($p>0.05$) longer mean itching period at the early phase (74.00±8.56 seconds), but a shorter mean itching period at the late phase (13.58±8.03 seconds) compared to rats administered with the leaf extract. Rats administered with the extract at 200mg/kg but pre-treated with 1mg/kg naloxone demonstrated a non-significantly ($p>0.05$) shorter mean itching period at the early phase (59.44±3.34 seconds), and a longer mean itching period at the late phase (25.68±2.17 seconds) compared to those administered with the extract only. The mean itching period of rats administered with naloxone alone was also shorter at both phases (52.89±14.05 seconds and 6.75±1.13 seconds) compared with rats pre-treated with extract alone (Table 2).

Acetic acid writhing test

The mean number of abdominal writhing movements observed for the rats administered with the leaf extract at 100mg/kg, 200mg/kg or 400mg/kg (16.60±8.81, 40.40±4.09 and 23.30±9.88) were significantly lesser compared to rats in the control group (48.00±4.66), but more frequent compared to the rats administered with indomethacin (14.00±3.56). Rats administered with the extract at 200mg/kg, but pre-treated with naloxone exhibited lesser numbers of abdominal writhing (19.40±0.60) compared to those administered with the extract only, except rats administered with the extract at 100mg/kg. Rats administered with naloxone only exhibited significantly higher number of writhing movements (39.40±5.99) than for rats pre-treated with the extract (Table 3).

Table 1:
Effect of **ethanol extract of *C. procera* or Indomethacin on changes in paw size (cm) in albumen induced paw oedema test in rats.**

Treatment Groups	0 minute	60 minutes	120 minutes	180 minutes
Extract 100mg/kg	0.12±0.0.02[a]	0.14±0.05[ab]	0.22±0.14[a]	0.09±0.04[ab]
Extract 200mg/kg	0.34±0.10	0.30±0.09[a]	0.22±0.10[a]	0.16±0.08
Extract 400mg/kg	0.32±0.07	0.30±0.06[a]	0.26±0.02[a]	0.22±0.11
Indomethacin 10mg/kg	0.28±0.04	0.56±0.08[b]	0.38±0.07	0.08±0.06[b]
Control	0.28±0.02[a]	0.66±0.12[a]	0.62±0.08[a]	0.48±0.09[a]

Groups with same superscript with control and or Indomethacin in a column are statistically significant at $p<0.05$

Table 2:
The effect of ethanolic extract of *C. procera* or Indomethacin on cumulative itching period (in seconds) in Formalin paw lick test in rats

Treatment Groups	Early phase response	Late phase response
Extract (100mg/kg)	48.14±6.38[ab]	14.12±2.63[a]
Extract (200mg/kg)	68.16±10.46	22.06±9.84[a]
Extract (400mg/kg)	62.48±9.42	48.30±7.08
Indomethacin (10mg/kg)	74.00±8.56[b]	13.58±8.03
Control	89.46±6.79[a]	70.50±10.89[a]
Naloxone (1mg/kg) + Extract (200mg/kg)	59.44±3.34	25.68±2.17[a]
Naloxone 1mg/kg	52.89±14.05	6.75±1.13[a]

Groups with same superscript as control and or Indomethacin in a column are statistically significant at p<0.05.

Table 3:
The effect of ethanolic extract of *C. procera* or aspirin on abdominal writhing in mice injected with acetic acid intraperitonealy.

Treatment Groups	Mean number of writhing
Extract (100mg/kg)	16.60±8.81[a]
Extract (200mg/kg)	40.40±4.09
Extract 400mg/kg	23.30±9.88
Aspirin 50mg/kg	14.00±3.56[a]
Control	48.00±4.66[a]
Naloxone 1mg/kg + Extract 200mg/kg	19.40±0.60
Naloxone 1mg/kg	39.40±5.99

Groups with same superscript as control and or aspirin in a column are statistically significant at p<0.05

Table 4:
The effect of ethanolic extract of *C. procera* or aspirin on response (in seconds) to thermal pain induced by tail flick method in mice

Treatment Groups	30minutes	60minutes	90 minutes
Extract 100mg/kg	5.95±1.90	3.48±0.32	4.20±0.39
Extract 200mg/kg	3.87±0.45	3.65±0.75	3.30±0.65
Extract 400mg/kg	2.84±0.28	3.38±0.10	3.14±0.20
Aspirin 50mg/kg	4.36±1.14	3.76±0.67	3.78±0.51
Control	3.32±0.86	2.22±0.61	2.04±0.59

Groups with same superscript as control and or aspirin in a column are statistically significant at p<0.05

Tail flick

Response to thermal pain in the tail flick model was significantly longer in rats administered with the leaf extract at 100mg/kg (5.95±1.90 seconds, 3.48±0.32 seconds and 4.20±0.39 seconds), 200mg/kg (3.87±0.45 seconds, 3.65±0.75 seconds and 3.30±0.65 seconds) and 400mg/kg (2.84±0.28 seconds, 3.38±0.10 seconds and 3.14±0.20 seconds) at 30 minutes, 60 minutes and 90 minutes post administration compared to rats in the control group (3.32±0.86 seconds, 2.22±0.61 seconds and 2.04±0.59 seconds). Rats administered with 15mg/kg b.w of aspirin (4.36±1.14 seconds, 3.76±0.67 seconds and 3.78±0.51 seconds) exhibited shorter reaction time compared with rats administered with 100mg/kg extract, but longer reaction time compared with rats administered with extract at 200mg/kg and 400mg/kg b.w (Table 4).

DISCUSSION

Findings from this study showed that the ethanolic extract of the leaves of *Calotropis procera* has potent anti-inflammatory and analgesic properties. This was confirmed by the observations from all the models of inflammation and analgesia used in this study. The extract had a dose-dependent anti-inflammatory and analgesic effect with the lowest concentration of 100 mg/kg body weight having the most potent effects.

The anti-inflammatory effect of *C. procera* was demonstrated in the carrageenan-induced paw oedema model where carrageenan was unable to incite the expected oedema of the paw in the rats administered with the extract. The formalin paw lick test further demonstrated the probable mechanism of action as being mediated via both central and peripheral mechanisms of inflammation. Rats administered with the leaf extract licked their paws for a shorter period compared to rats administered with indomethacin at the early phase, but the rats licked for longer in the late phase. This shows that the mechanism of anti-inflammatory action is mediated more via the central than the peripheral mechanism of anti-inflammation.

The two phases of anti-inflammatory response are usually due to direct stimulation of nociceptors in the paw which culminates in centrally mediated pain with release of substance P in the neurogenic (early) phase. The late phase on the other hand is observed as a result of release of histamine, serotonin, bradykinin and prostaglandins (Zeashana *et al*., 2009). Some drugs, especially opioid analgesic agents, inhibit both phases equally while peripherally acting drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory only inhibit the late phase

(García *et al*., 2004; Zeashana *et al*., 2009). The possibility of the extract interacting with opioid receptors was ruled out by the reduced period of itching in rats pre-treated with naloxone; the specific antagonist for opioid receptors. In this study, naloxone did not inhibit the anti-inflammatory effect of the extract. The mechanism of anti-inflammatory action is both centrally and peripherally mediated as shown by inhibition of both phases of response, but the central mediation is via inhibition of another receptor(s) involved in inflammation other than opioid receptors. Interactions with histamine, serotonin, bradykinin and prostaglandins receptors (Zeashana *et al*., 2009) may also be involved in the late phase.

As for the analgesic effect, the leaf extract appear to act via the central and peripheral mechanisms of analgesia. Tail flick test is used to determine both centrally acting analgesics (Ramabadran *et al*., 1989) like morphine (Domer, 1990) and peripherally acting analgesics like NSAIDs which inhibit cycloxygenase in peripheral tissues, thereby interfering with the mechanism of transduction in primary afferent nociceptors (Fields, 1987). The acetic acid-induced writhing is a visceral pain model and widely used for the evaluation of peripheral antinociceptive activity (Du *et al*., 2007). These tests of analgesia further establish the probable mechanism of anti-inflammatory and analgesic action of *C. procera* as both centrally and peripherally mediated. The analgesic effect of the extract was not inhibited in the presence of naloxone. This can be inferred from the reduced mean number of abdominal writhing movement in mice administered with naloxone which thus rules out any possible interaction with opioid receptors. This confirms our finding in the inflammatory model and suggests that the leaf extract interacts with other receptors involved in inflammation. The anti-inflammatory and analgesic effects of *C. procera* were more potent than the effect of indomethacin or aspirin.

The anti-inflammatory and analgesic activity of the leaves of *C. procera* established by this study is an addition to existing knowledge on the latex of the plant which was reported to possess anti-inflammatory, analgesic and weak antipyretic activities (Dewan *et al*., 2000a, b; Sangraula *et al*., 2002). *C. gigantea*, a close relative of this plant, has also been reported to possess anti-inflammatory, analgesic and antipyretic activity (Chitme *et al*., 2004; Poddar *et al*., 2007). Other plants that has been established to have anti-inflammatory and analgesic effect include *Vitis trifolia* (Ahmed *et al*., 1993), *Morinda lucida* (Awe *et al*., 1998), *Bridelia ferruginea* stem bark (Olajide *et al*., 2000), *Asparagus africanus* (Hassan *et al*., 2008), *Careya arborea* (Gupta *et al*., 2006). Further research is needed to isolate and elucidate the active principle in leaf of *Calotropis procera* which hopefully may lead to development of a new anti-inflammatory and analgesic agent.

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