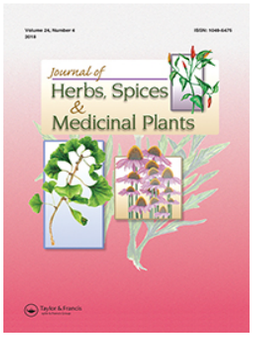

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Evaluation of antinociceptive activity of *Ritchiea longipedicellata* (Capparaceae) leaf extract in mice

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ABSTRACT

The antinociceptive effect of ethanolic extracts of *Ritchiea longipedicellata* (Capparaceae) leaves was evaluated in acetic acid, formalin, tail immersion, and hot plate models. Motor effect was assessed with rota-rod and open field tests. The effect of receptor antagonists was examined in the formalin test. The extract (50, 100, or 200 mg.kg^{-1}, p.o.) showed dose dependent inhibition of writhing with percent inhibition of 36.88%, 46.29%, and 52.48%, respectively. There was a reduction in the time spent in paw-licking in both the early and late phases of formalin test. The extract increased the withdrawal latency of the mice in tail immersion test, but failed to show an effect beyond 60 min of hotplate test. The extract impaired the mice motor performance function in the rota-rod and open field tests. The effect of the extract in formalin test was only reversed by yohimbine in the late phase. The results suggested that *R. longipedicellata* leaf extracts exerted antinociceptive effects which may partly involve α_2 adrenergic receptor mechanism.

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KEYWORDS
Antinociceptive activity; Capparaceae; mechanistic studies; *Ritchiea longipedicellata*

Introduction

Pain is regularly dealt with in daily clinical practice (24) and the available analgesic drugs exert a wide range of side effects such as gastric ulceration or liver damage (11). Several traditionally used plants exhibit pharmacological properties with great potential for therapeutic applications in the management of pain (4).

Ritchiea longipedicellata (Capparaceae) is used in Traditional African Medicine and in Nigeria small quantity of the root and leaves are chewed to relieve pain in the head and eye, and cold and upper respiratory tract infections (7). Local palm wine (sap from some palm species) and extract of *R. longipedicellata* is also used for the treatment of typhoid fever, malaria, and general illness (4,7).

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Some studies have demonstrated the anthelminthic (1), antifungal, cytotoxic (2), antiplasmodial (21), and antimicrobial activities (4) and phytochemicals reported on the plant include cleomin, a mustard oil glycoside from the root (22), and stachydrine, from the leaves (29). The present research investigated the antinociceptive activity and possible underlying mechanisms of action of the ethanolic extract from the leaves of *R. longipedicellata* using pharmacological procedures.

Materials and methods

Plant materials

The leaves of *R. longipedicellata* were collected in 2014, in Akintadu village, about 319 km to Ikire township in Osun state (7.35 latitude, 4.18 longitude), Nigeria, identified at the Department of Botany and Microbiology, University of Lagos and a voucher specimen (LUH 5648) was deposited.

Preparation of the extract

The leaves were air-dried at room temperature (25°C) and powdered in a mechanical grinder (Christy and Morris Limited, England). The powdered leaves (400 g) were macerated in 96% ethanol for 48 h. The total filtrate obtained was concentrated using the rotary evaporator (Buchi Labortechnik, CH- 9230, Flavil, Switzerland) at 40°C to obtain 32.13 g (8.03%, w/w) of ethanol extract.

Animals

Male Swiss albino mice (15–37 g) obtained from a private farm (Korede Farm Ltd, Ikeja, Lagos) were used in this study. All animal care and experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (19), and were approved by the Ethics Committee of the College of Medicine, University of Lagos (protocol number: CM/COM/08/VOL. XXV). The animals were housed in clean cages at room temperature, fed with standard laboratory diet (Vital feed, UAC PLC, Nigeria) and given clean water. However, the diet was withdrawn 12 h before dosing.

Acute toxicity test

Acute toxicity of the plant extract was determined according to the Organization for Economic Cooperation and Development guideline no. 423 (20). Four groups of five mice each were fasted for 12 h prior to experiment and a single dose of 500, 1,000, 2,000, or 5,000 mg kg^{-1} of the extract was administered orally. The mice were closely observed for toxic

symptoms and behavioral changes for the first 2 h after administration and mortality recorded within 24 h. The observation was extended up to 14 d.

Antinociceptive activity

Acetic acid-induced writhing test

Mice were divided into five groups of five animals each and pretreated as follows: Group 1, 1% tween 20 (10 mL kg^{-1}, p.o.), Groups 2, 3, and 4, extract (50, 100, and 200 mg kg^{-1}, p.o.), respectively and Group 5, acetylsalicylic acid (100 mg kg^{-1}, p.o.). Sixty minutes after treatment, acetic acid (0.6% v/v in saline, 10 mL kg^{-1}) was administered intraperitoneally. The number of writhes (characterized by constriction of abdominal wall and extension of hind limbs) was counted for 30 min (17). Percentage inhibition of writhing compared to the control group was taken as an index of analgesia and was calculated as:

$$\text{Inhibition } (\%) = \frac{\text{Number of writhes (Control)} - \text{Number of writhes (Treaments)} \times 100}{\text{Number of writhes (Control)}}$$

Formalin-induced nociception

Mice were divided into five groups of five animals each and treated as follows: Group 1: 1% Tween 20 (10 mL kg^{-1}, p.o.), Groups 2, 3, and 4, extract (50, 100, and 200 mg kg^{-1}, p.o.), respectively and Group 5 (Morphine 10 mg kg^{-1}, s.c.). Sixty minutes after administration for the oral route or 30 min for the subcutaneous route, formalin (20 µL of 1% solution) was injected subcutaneously into the right hind paw of each mouse. The time spent (s) in licking and biting the injected paw (an indicator of nociceptive behavior) was measured in the first phase (0–5 min) and second phase (15–30 min) after formalin injection (13). Percentage inhibition was calculated as:

$$\text{Inhibition } (\%) = \frac{\text{Reaction Time (Control)} - \text{Reaction Time (Treaments)} \times 100}{\text{Reaction Time (Control)}}$$

Tail immersion test

In this method, the mouse was gently handled and the tail immersed in a hot water bath maintained at temperature of 55°C ± 0.5°C (5). Each animal served as its own control. The reaction time to flick the tail from the hot water was recorded for each mouse at 60, 90, and 120 min after the administration of the extract (50, 100, or 200 mg kg^{-1}, p.o.), vehicle (10 mL kg^{-1}, p.o.), and morphine (10 mg kg^{-1}, s.c). A cut-off time of 20 s was maintained to prevent tail tissue damage in mice. Percentage inhibition was calculated using the following formula:

$$\text{Inhibition}\,(\%) = \frac{(\text{Post-treament Latency}) - (\text{Pre-treament Latency}) \times 100}{(\text{Cut-off Time} - \text{Pre-treament Latency})}$$

Hot plate test

The hot plate test was carried out at fixed temperature of 55°C ± 1°C (10). Mice were divided into five groups each consisting of five animals. Pretreatment reaction for each mouse was determined after which treatment was done as follows: Group 1: 1% tween 20 (10 mL kg^{-1}, p.o.), Group 2 (50 mg kg^{-1}, p.o.), Group 3 (100 mg kg^{-1}, p.o.), Group 4 (200 mg kg^{-1}, p.o.) crude extract, and Group 5: Morphine (10 mg kg^{-1}, s.c.). Sixty minutes after oral administration, the reaction time of animals was recorded. A post treatment cut-off time of 20 s was used.

Measurement of Motor Performance and Locomotor Activity

Rota-rod and open field tests were carried out to eliminate the interference of muscle relaxant or central depressant effect on the antinociceptive activity of *R. longipedicellata*. The rota-rod apparatus (Insight, Ribeirão Preto, Brazil) consisted of a bar with a diameter of 3 cm, subdivided into five compartments. The bar rotated at a constant speed of six revolutions per min. The animals were pretreated with the vehicle, extract (50, 100, or 200 mg kg^{-1}, p.o.) or diazepam (1 mg kg^{-1}, i.p.). Resistance to falling was measured up to 120 s. The results were expressed as the average time (s) the animals remained on the rotarod in each group (14).

Locomotor activity in an open field was determined as described (28). A wooden box (40 × 60 × 50 cm^3) with the floor divided into 12 squares was used. Male Swiss mice (n = 5), were orally treated with the vehicle, extract (50, 100, and 200 mg kg^{-1}, p.o.), or diazepam (1 mg kg^{-1}, i.p.) before being placed in the center of the box and the number of squares crossed with all paws (line-crossing) were counted for each mouse up to 120 s.

Assessment of possible antinociceptive mechanisms of R. longipedicellata

To assess the possible participation of different systems on the antinociceptive effect of the extract, groups of mice were pretreated with naloxone (1.5 mg kg^{-1}, i.p.), an antagonist of opioid receptors, metoclopramide (1.5 mg.kg^{-1}, i.p.), a dopamine (D_2) receptor antagonist and yohimbine (1 mg kg^{-1}, i.p.), an α_2 adrenoceptor antagonist 30 min before administration of the extract (200 mg kg^{-1}, p.o.). The nociceptive response was evaluated using formalin test (14).

Quantification of total phenolic, proanthocyanidin and flavonoid content

Total polyphenol content in the extract was determined (15). An aliquot of the extract was mixed with 2.5 mL Folin Ciocalteau reagent (previously diluted with water 1:10 v/v) and 2 mL of (75 g L^{-1}) of sodium carbonate. The tubes were vortexed for 15 s and allowed to stand for 30 min for color development. Absorbance was measured at 765 nm using a UV-VS spectrophotometer. The amount of total phenols was calculated as gallic acid equivalents (GAE mg g^{-1}) from a standard curve.

For the determination of total proanthocyanidin content, 0.5 mL of 50 mg mL^{-1} of extract was mixed in 3 mL of 4% vanillin methanol solution and 1.5 mL hydrochloric acid and the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm and the result explained as catechin equivalent (15). The total flavonoid content of the extract was also determined. To 0.5 mL of sample, 0.5 mL of 2% aluminum chloride ethanol solution was added, and the absorbance measured at 420 nm after keeping for 1 h at room temperature. Total flavonoid contents were calculated as quercetin equivalent from a calibration curve (15).

Statistical analysis

The results were expressed as mean ± standard error of mean (mean ± SEM) and analyzed using one-way ANOVA followed by Tukey's post hoc or a two-way ANOVA followed by Bonferroni post hoc test or Dunnett's test ($p < 0.05$) using GraphPad Prism software (ver.5.0 for Windows, GraphPad Inc., San Diego, CA).

Results

Acute toxicity study

The extract at doses up to 5,000 mg kg^{-1} did not produce symptoms of acute toxicity and mice mortality was not recorded during the observation period suggesting the low toxicity profile of the extract.

Acetic acid-induced writhing

The extract reduced the number of writhing induced by acetic acid compared to the control (Table 1). The percentage inhibition of writhing at the dose of 50, 100, and 200 mg $kg^{-1}$was 36.88%, 46.29%, and 52.48%, respectively. The activity of the reference drug, acetylsalicylic acid was higher (86.63%, inhibition) than the extract at all doses used.

Table 1. Effect of Ethanolic Leaf Extract of *Ritchiea longipedicellata* on Acetic Acid-Induced Writhing Test

Treatment	Dose (mg kg^{-1})	No of writhes	% Inhibition
Control	10 mL kg^{-1}	80.8 ± 9.18	–
Extract	50	51.0 ± 6.63*	36.88
	100	43.4 ± 4.39**	46.29
	200	38.4 ± 2.23***	52.48
Acetylsalicylic acid	100	10.8 ± 3.43***	86.63

Results represent the mean ± SEM of five animals. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the control group (One-way ANOVA followed by Tukey's test).

Formalin test

The extract showed a dose dependent reduction in score of biting and licking behavior with maximum effect of 58.92% and 75.74%, inhibition, in the early and late phase at the dose of 200 mg kg^{-1} (Figure 1). Morphine (10 mg kg^{-1}) inhibited both phases of the formalin-induced pain. The effect of the extract was lower compared to morphine (99.66% and 100.00% inhibition) at both phases, respectively.

Tail immersion test

The extract increased the reaction time at different doses (Figure 2). The highest antinociceptive effect was shown at 90 min after the administration of the extract and thereafter declined. The extract at 50, 100, and 200 mg kg^{-1} prolonged the tail withdrawal latency of the animals at 90 min by 17.16%, 24.43%, and 33.00%, respectively. Treatment of the mice with the reference drug, morphine (10 mg kg^{-1}) caused an increase in latency time that reached a maximum effect of 100%.

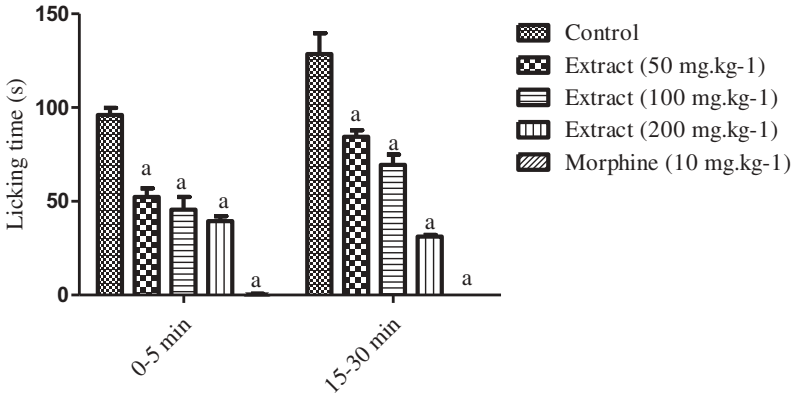

Figure 1. Effect of ethanolic leaf extract of *Ritchiea longipedicellata* on formalin-induced pain in mice. Results represent the mean ± SEM of five animals. [a]$p < 0.001$ compared to the control group (Two-way ANOVA followed by Bonferroni's test).

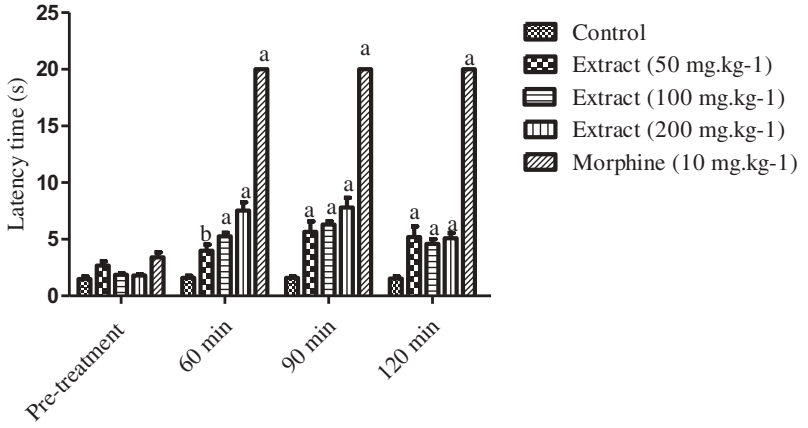

Figure 2. Effect of ethanolic leaf extract of *R. longipedicellata* on tail immersion test in mice. Results represent the mean ± SEM of six animals. $^{a}p < 0.001$, $^{b}p < 0.01$ compared to the control group (Two-way ANOVA followed by Bonferroni's test).

Hot plate test

Animals treated only with the vehicle p.o., elicited nociceptive reaction on placement on the hot plate with posttreatment latency of 3.59 ± 0.43 s compared to the pretreatment latency of 2.48 ± 0.15 s (Figure 3). The administration of the extract increased the reaction time at 60 min. The inhibition of nociception was 20.12%, 31.53%, and 32.83% at 50, 100, and 200 mg kg^{-1}, respectively. Morphine (10 mg kg^{-1}) increased the reaction time all through the observed period with 100% inhibition of nociception.

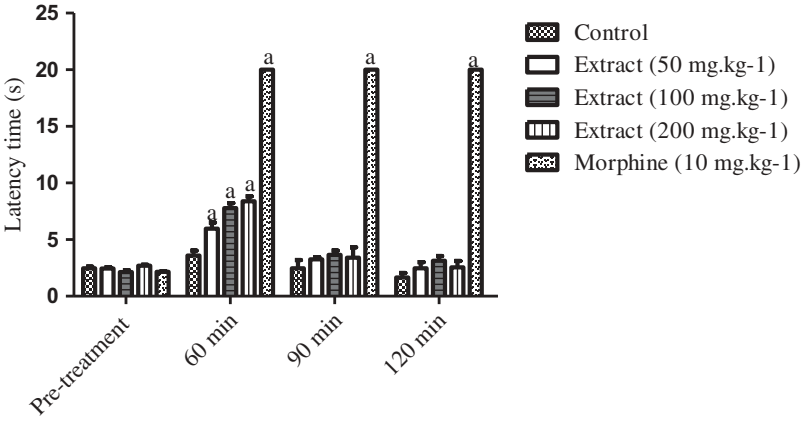

Figure 3. Effect of ethanolic leaf extract of *R. longipedicellata* on the basal reaction time of mice in hotplate test. Results represent the mean ± SEM of five animals. $^{a}p < 0.001$ compared to the control group (Two-way ANOVA followed by Bonferroni's test).

Effects of R. longipedicellata on motor function or coordination

The pretreatment of the animals with the extract at the doses of 50, 100, or 200 mg kg^{-1} caused reduction of motor activity in the rota-rod and open field tests compared to control (Figure 4). The extract decreased the time of mice on the rota-rod or the number of squares crossed in the open field test compared to control. Diazepam (1 mg kg^{-1}) also reduced behavioral response in these tests. However, the effect of the extract was less compared to diazepam-treated group.

Assessment of possible antinociceptive mechanisms of R. longipedicellata

Possible antinociceptive mechanisms of *R. longipedicellata* extract were investigated through the pretreatment of mice with several drugs that interfere in different systems (Figure 5). The result showed that none of the antagonists (naloxone, metoclopramide, and yohimbine) was able to reverse the antinociceptive effect of the extract at the early phase. However, yohimbine, modified, at least in part, the antinociceptive response elicited by the extract at the late phase of formalin-induced licking model.

Total polyphenolic content of R. longipedicellata extract

The extract contained total phenol (22.85 mg g^{-1} GAE), proanthocyanidin (25.29 mg g^{-1} catechin equivalents), and flavonoid (27.79 mg g^{-1} quercetin equivalents), respectively.

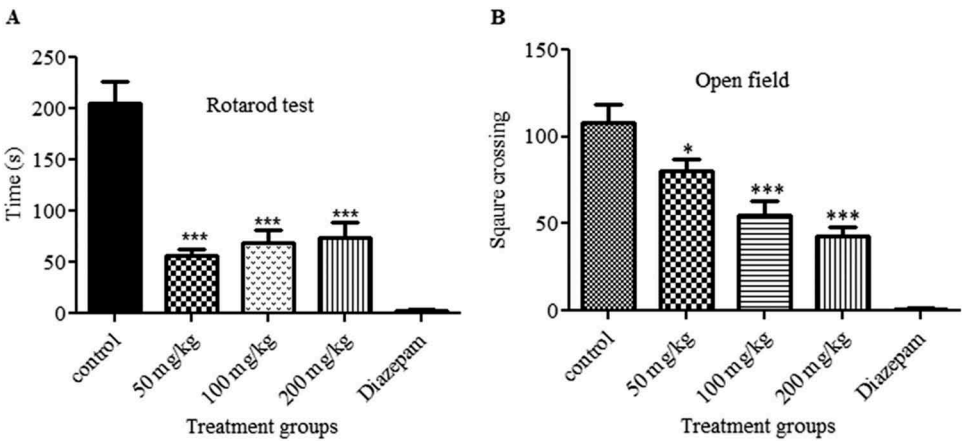

Figure 4. Effect of ethanolic leaf extract of *R. longipedicellata* on motor function in mice. Bar graphs representing (A) the run time on the rota-rod and (B) the number of square crossings in the open field test, 1 h after the oral administration of extract. Diazepam (1 mg kg^{-1}) was administered 30 min before testing. Data are mean ± SEM; $n = 5$ mice per group. Mean separation by Dunnett's test. (*$p < 0.05$, ***$p < 0.001$).

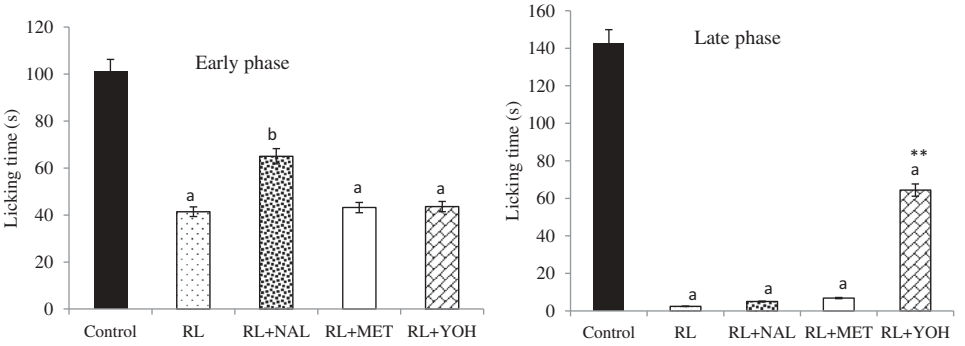

Figure 5. Effect of naloxone, metoclopramide, and yohimbine on the antinociceptive profile of ethanolic leaf extract of *R. longipedicellata* (200 mg kg^{-1}) in formalin-induced paw-licking test. RL = *R. longipedicellata* extract (200 mg kg^{-1}, p.o.), NAL = Naloxone (1.5 mg kg^{-1}, i.p.), MET = Metoclopramide (1.5 mg kg^{-1}, i.p.), YOH = Yohimbine (1 mg kg^{-1}, i.p.). Each column represents the mean ± SEM ($n = 5$). $^{**}p < 0.01$ compared to RL, ^{a} $p < 0.001$ compared to control ^{b} $p < 0.01$ compared to control (One-way ANOVA followed by Tukey's post hoc test)

Discussion

The assessment of antinociceptive activity of test articles can be done using several pharmacological tests (27). In this study, the antinociceptive responses of *R. longipedicellata* extracts were investigated in mice using acetic acid, formalin, tail immersion, and hot plate tests. Pain induced by these models occurs *via* different mechanisms which could be peripheral or central (25).

Acetic acid-induced writhing in mice represents a peripheral nociception model (9). The writhes induced by intraperitoneal injection of acetic acid originate from the sensitization of nociceptive receptors to prostaglandins (6). It has also been reported that effectiveness of drugs in the acetic acid -induced writhing response could be attributed to different mechanism, including the blockade of the effect or the release of endogenous substances (arachidonic acid metabolites) that excite pain nerve endings (27). *R. longipedicellata* reduced the writhing in mice in response to acetic acid administration, suggesting that the antinociceptive effect of the extract may be peripherally mediated *via* the inhibition of synthesis and release of prostaglandins and other endogenous substances.

The formalin test allows an assessment of antinociceptive action that is capable of distinguishing between neurogenic pain and inflammatory pain (3). The neurogenic pain in the early phase is connected with a direct stimulation of sensory nerve fibers whereas the inflammatory pain which occurs at the second phase is due to the release of inflammatory mediators such as histamine, prostaglandins, bradykinin, serotonin in the peripheral tissues, and from functional changes in the spinal dorsal horn (16). The *R. longipedicellata* leaf extract inhibited the duration of biting and licking in both phases suggesting that the extract could act through both peripheral and central mechanism.

Antinociceptive effect of the extract was further tested against thermal noxious stimuli in tail immersion and hot plate tests. Tail immersion and hot plate tests have been used for the assessment of central analgesic activity of test articles (18). An increase in the reaction time is generally considered an important parameter of central analgesic activity (23). These models are implicated in both spinal and supra spinal analgesic pathways (16). In tail immersion test, an increase in latency time was observed for the extract at the tested doses compared to the control while in the hot plate test, the extract exhibited an increase in basal reaction time at 60 min suggesting the central antinociceptive potential of the extract.

Rota-rod and open field tests were carried out to eliminate the interference of muscle relaxant or central depressant effect on the antinociceptive activity of *R. longipedicellata*. Previous studies suggested that the central nervous system depression and the nonspecific muscle relaxation effect can reduce the response of motor coordination and might invalidate the formalin test results (12). Moreover, muscle relaxants, sedatives, and central nervous system depressants are effective in tests such as the hot plate test (8). The extract interferes with locomotor activity and motor coordination of the animals, suggesting depression of the central nervous system, thus the need to investigate further the sedative or hypnotic side effects of the extract.

The mechanism of antinociceptive activity of the extract was further investigated using specific antagonists in formalin test at the dose of 200 mg kg^{-1}. This is the dose that induced the highest antinociceptive effect in all the models used. Antinociception can be mediated through different mechanisms such as opioid, cholinergic, α_2-adrenergic, and dopaminergic receptor mechanisms (26). Pretreatment of naloxone (an opioid receptor antagonist) and metoclopramide (a dopamine receptor antagonist) along with the extract could not change the antinociceptive effect of the extract, indicating the antinociception action of the extract is not mediated through opioid and dopaminergic mechanisms. On the other hand, the antinociceptive action of the extract was only blocked by yohimbine, an alpha2-adrenoceptor antagonist, at the late phase of formalin test. This observation supported partial involvement of α_2 adrenergic receptor mechanism by the extract.

Plant polyphenols, especially phenolics and flavonoids commonly found in plants are known to exhibit several biological activities including antinociceptive properties (30). Flavonoids play a role in inhibiting cyclooxygenase system, thereby interfering with arachidonic acid synthesis and, therefore, the production of prostaglandins. The quantitative phytochemical analysis of polyphenols in *R. longipedicellata* showed that the extract contained considerable amount of these group of compounds. The presence of these groups of compounds could therefore contribute to the observed antinociceptive activity of the ethanolic extract of *R. longipedicellata*. In conclusion, the ethanolic extract of *R. longipedicellata* exert antinociceptive effect in different models of nociception, which may partly involve α_2 adrenergic receptor mechanism.

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