Trop J Nat Prod Res, October 2021; 5(10):1841-1846

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Antinociceptive and Behavioural Effects of the Leaves of *Milicia excelsa* (Welw.) C. C. Berg (Moraceae)

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ARTICLE INFO

Article history:
Received 19 January 2021
Revised 21 May 2021
Accepted 19 October 2021
Published online 02 November 2021

ABSTRACT

The leaves of *Milicia excelsa* (Welw.) C. C. Berg (Moraceae) is used in the treatment of inflammatory and painful disorders and as sedative in the treatment of insanity in Nigerian traditional medicine. The antinociceptive activity of the ethanol extract (50, 100 and 200 mg/kg, p.o.) was investigated using acetic acid-induced writhing, hot plate and formalin-induced paw licking tests while the behavioural assessment was evaluated in holeboard, elevated plus maze and phenobarbitone-induced sleeping time tests. Acetylsalicylic acid, morphine or diazepam and 1% Tween 20 (10 mL/kg) were employed as positive and negative control respectively. Acute toxicity of the extract (200-3200 mg/kg, p.o.) was also determined.
The extract inhibited nociceptive response caused by acetic acid injection and increased latency time in the hot-plate test. In formalin test, the extract decreased paw licking time in both phases. The entry and the duration of time spent in the open arms of the elevated plus maze by animals treated with the extract (100 and 200 mg/kg) increased significantly compared to control. The number of head-dips and line crossed were decreased by the extract in hole board test, resembling the response of diazepam. In pentobarbital-induced sleeping time test, the extract reduced sleep latency and increased the duration of sleep compared to the negative control. No mortality was observed up to the dose of 3200 mg/kg. These results showed the antinociceptive, anxiolytic and sedative effects of the leaf extract of *M. excelsa* and provide evidence for the traditional use of the plant for pain management and insanity.

Keywords: *Milicia excelsa*, Antinociceptive, Anxiolytic, Sedative effect, Total polyphenolic contents

Introduction

Milicia excelsa Welw. C. C. Berg Syn. *Chlorophora excelsa* (Welw.) Benth. (Moraceae) is an important timber species of tropical Africa commonly named Iroko.[1] The plant is considered sacred and is used for ceremonial purposes in many local cultures in Nigeria,[2] although it has some usage in traditional remedies. The powdered stem bark is used as a carminative in the treatment of mental illness.[3] The stem produced latex which is applied on burns, wounds, sores and other skin problems. The leaves are eaten to treat insanity and the decoction or tincture form is used to treat lumbago, spleen pain, stomach pain, abdominal pain, oedema, general fatigue, rheumatism and sprains.[4]
The stem bark extract has been reported to have anti-inflammatory property[5] and preventive effect on dexamethasone-induced insulin resistance.[6] The root demonstrated antidiarrheal[7] and emmenagogue properties.[8] Regarding the leaves of *M. excelsa*, the wound healing,[9] antipsychotic and anticonvulsant properties have been studied.[10, 11]
Several compounds have been reportedly isolated from the heartwood, stembark, root and leaves extracts of the plant. Excelsaoctaphenol,[12] 5,5′-Dihydroxy-3,7,2′,4′-tetramethoxyflavone,[13] chlorophorin and 4-[(20*E*)-70-hydroxy-30,70-dimethyloct-20-enyl]-29,3,49,5-tetrahydroxy-*trans*-stilbene[14,15] were isolated from the heartwood.

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Citation: Sofidiya MO. Sofidiya MO, Akinyeke OT, Adeshakin MA, Odukoya OA. Antinociceptive and Behavioural Effects of the Leaves of *Milicia excelsa* (Welw.) C. Berg (Moraceae). Trop J Nat Prod Res. 2021; 5(10):1841-1846. doi.org/10.26538/tjnpr/v5i10.22

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Moracin M (2-(3′, 5′- dihydroxyphenyl)-6-benzofuran) was previously identified from the stem bark.[16] The isolation of 2'hydroxyatalantoflavone, atalantoflavone, neocyclomorusin, 6-geranylnorartocarpetin, cudraxanthone and betulinic acid from the root of the plant was reported by Ouete *et al*.[17] The leaves were reported to contain a benzylic diglycoside, 3,4-dimethoxybenzyl *β*-D-xylopyranosyl(1→2)-*β*-D-glucopyranoside (Excelsoside), lupeol acetate, ursolic acid, triacontyl (E)-ferulate, and 2-(3,5-dihydroxyphenyl)benzofuran-5,6-diol.[18] Our knowledge based on the available literature showed that no scientific evidence was found regarding the antinociceptive and sedative effects of the leaf extract. The present study was, therefore, carried out to evaluate the antinociceptive, anxiolytic and sedative activities of the extract of *M. excelsa.*

Materials and Methods

Plant material
Fresh leaves of *Milicia excelsa* were collected in April 2016 along Olorunshola road in Ayobo (latitude 6.5873 and longitude 3.2297), Lagos State, Nigeria. The plant sample was identified by a Taxonomist Mr Tola Oyebanji, Herbarium unit, Department of Botany, University of Lagos, Nigeria and a voucher specimen (LUH 7566) was deposited at the Herbarium for future reference.

Extraction
Air-dried and finely powdered leaves (1.330 kg) were macerated in absolute ethanol (15 L) for 48 h at room temperature (26-28°C). This was followed by filtration using a Whatman filter paper and concentration under reduced pressure (Hedolph rotary evaporator) at 40°C to yield 63 g (4.74%, w/w) of the crude extract.

Quantitative determination of total polyphenolic content
The total phenolic content was analyzed using the Folin-Ciocalteu colorimetric method.[19] The amount of total phenols was calculated as gallic acid equivalent (GAE mg/g) from the calibration curve y = 8.77x + 0.1893, R^2 = 0.997. The $AlCl_3$ method was used for the determination of total flavonoid content [20] and calculated as quercetin equivalent (QE) in mg/g; y = 8.68x + 0.5544, R^2 = 0.9985. Proanthocyanidin content was based on the procedure reported by Sun *et al.*,[21] the absorbance was measured at 500 nm and the result calculated as catechin equivalent (CE) in mg/g; y = 2.04 + 0.0534, R^2 =0.9996.

Animals
The study was approved by College of Medicine, University of Lagos Health Research Ethics Committee with approval number CM/COM/08/VOL.XXV. Thereafter, male Wistar rats (140-150 g) and albino mice (18-25 g) were purchased from a private vendor, KOMAD Farm Limited, Lagos, Nigeria.
The animals were housed in a well-ventilated room at a controlled temperature and light/dark cycle (25±2°C, 12 h light/dark cycle). They were given standard pellet diet and water *ad libitum*. The United States National Institute of Health Guide for the Care and Use of Laboratory Animals in Biomedical Research was followed.[22]

Acute toxicity
Five groups of 6 albino mice were fasted for 12 h prior to the experiment. The extract (200- 3200 mg/kg, p.o.) was administered and mice were closely observed for toxic symptoms and mortality for 24 h and every day for 7 days.[23]

Antinociceptive assays
Writhing test
The test was carried out as reported by Ishola *et al*.[24] Mice were randomly divided into five groups of six animals each. The vehicle (1% Tween 20), ethanol extract of *M. excelsa* (50, 100 and 200 mg/kg), and acetylsalicylic acid (100 mg/kg) were administered orally 60 min before the intraperitoneal injection of 1 ml of 0.6% acetic acid. The number of abdominal writhes (characterized by contraction of abdominal musculature and extension of the hind limbs) showed by each mouse was counted for 30 min. The antinociceptive activity was expressed as the percentage of inhibition of constrictions compared with the negative control group.

Formalin-induced paw licking test
Experiments were carried out according to the previously described method of Hunskaar and Hole [25] and as reported by Mbagwu *et al*. [26] Mice fasted overnight were divided into five groups of six animals each. Groups 1-III were treated orally with the extract (50, 100 and 200 mg/kg), the control group received 1% Tween 20 (10 mL/kg) and the reference group received morphine (10 mg/kg, s.c.). Sixty minutes after administration, formalin (20 µL, 1% solution) was injected s.c. into the right hind paw of each mouse. The time (in seconds) spent in licking and biting response of the injected paw, indicative of pain, was recorded for each animal. The responses of the mice were observed for 5 min (first phase) and 15-30 min (second phase) post-formalin injection.

Hot plate test
The animals were placed on a hot plate apparatus at a constant temperature of 55 ± 0.5°C and animals with a response time of up to 15 s (latency time) were selected. The selected animals were treated with vehicle (1% Tween 20, 10 mL/kg, p.o.), extract (50, 100 and 200 mg/kg, p.o.) and morphine (10 mg/kg, s.c.) as positive control. Then, 1 h after oral administration of vehicle and extract or 30 min after injection of morphine, the latency time of each mouse to jumping or licking the hind leg was taken. A maximum exposure time (cut-off time) of 30 seconds was used to prevent tissue damage to the animal's paws.[27]

Behavioural studies
Elevated plus-maze
The possible anxiolytic effect of the extract was assessed as described by Lister.[28] The plus maze consists of two open arms and two closed arms (50 × 10 × 40 cm each) elevated to a height of 50 cm.[29] One hour after administration of the extract (50, 100 and 200 mg/kg, p.o.), each mouse was placed in turn in the centre of the maze facing one of the closed arms. The exploratory behaviour was recorded for 5 min. Diazepam (1 mg/kg, s.c.) was used as standard.

Hole board test
The method previously described by File and Wardill [30] was employed. The hole board is a white painted wooden board (40 cm x 40 cm) with four equidistant holes (1 cm diameter x 2 cm depth) and divided into 4 equal sectional squares of 20 cm x 20 cm.[29] One hour after oral treatment with vehicle (1% Tween 20, 10 mL/kg), extract (50, 100 and 200 mg/kg) and diazepam (1 mg/kg, s.c.), each mouse was placed in turn at one corner of the board with the animal subsequently moving about and dipping its head into the holes. The number of head dips and sectional crossings in 5 min were recorded for individual mouse.

Phenobarbitone-induced sleeping test
The test was conducted as described by Williamson *et al*. [31] Five groups of five mice each were used. The first group (control) was administered 1% Tween 20 (10 ml/kg, p.o.), the second, third and fourth groups received the extract at the dose of 50, 100 and 200 mg/kg (p.o.) while the fifth group received Diazepam (3 mg/kg, s.c.), respectively. After 30 minutes, phenobarbitone was administered to all the animals in each group at the dose of 40 mg/kg, i.p. The latency of the loss of the righting reflex and the total sleeping time (the time between the loss and the recovery of the righting reflex) were recorded for each mouse.

Statistical analysis
Data are expressed as mean±SEM. Statistical differences were evaluated by analysis of variance (One-way ANOVA followed by Bonferroni or Dunnett's tests for comparison between each group using Graphpad prism 5 (GraphPad Software, Inc., CA). A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

In this study, we report the antinociceptive and behavioural effects of *M. excelsa*. Nociceptive tests use electrical, thermal, mechanical, or chemical stimuli.[32] Acetic acid writhing and formalin-induced licking tests was employed *as* models of chemical nociception while thermal stimulation was assessed using the hot plate test.
In the acetic acid-induced writhing test, the extract exhibited significant ($p < 0.001$) attenuation of acetic acid-induced writhing in a dose dependent manner, with percentage inhibition of 69.83%, 74.25% and 74.77% at the dose of 50, 100 and 200 mg/kg, respectively, compared to the control (Table 1). Mice pretreated with acetylsalicylic acid showed a significant ($p < 0.001$) inhibition (84.14%) compared to the control. The nociceptive pain in the acetic acid-induced writhing test occurs in the periphery via direct activation of pain-sensing nerve terminals.[33] Acetic acid acts by inducing the release of several pro-inflammatory mediators, such prostaglandin, bradykinin, substance P, prostacyclin and other cytokines which excite the nociceptors in the nerve terminals.[34] The extract and acetylsalicylic acid demonstrated significant inhibition of writhing compared to the control. This observation suggests antinociceptive effect of the extract and acetylsalicylic acid. Acetylsalicylic acid has been reported to inhibit cyclooxygenases in peripheral tissues, reducing therefore prostaglandin synthesis and interfering with the mechanism of transduction in primary afferent nociceptors.[35]
Ethanol extract of *M. excelsa* demonstrated a significant ($p < 0.001$) and dose dependent antinociceptive activity in both the early (0-5 min) and late phases (15-30 min) of formalin-induced paw licking test (Figure 1). Statistically, there was no significant difference between the dose of 100 and 200 mg/kg. Morphine (10 mg/kg) showed significant ($p < 0.001$) inhibition of nociception, with little or no paw licking at both phases.
Formalin-induced nociceptive test is a model of chronic pain. It describes nociceptive response in both neurogenic and inflammatory phases.[36] The neurogenic phase (early phase) is expressed as a direct result of stimulation in the paw and reflects centrally mediated pain with release of substance P while the inflammatory phase (late phase) is due to the release of histamine, serotonin, bradykinin and

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

prostaglandins.[37] The extract in this test, demonstrated significant antinociceptive effect in both phases suggesting effect in both neurogenic and inflammatory pain responses.

The result of the hot plate test is presented in Table 2. The extract demonstrated a significant ($p < 0.001$) and dose dependent increase in latency time to the thermal stimulus compared to the control. At 60 min, the extract (50, 100 and 200 mg/kg) produced maximum nociceptive inhibition of 39.36, 40.39 and 75.69% respectively.

In the hotplate, the sensation of pain in mice is produced by means of thermal stimulation, which is transmitted to the central nervous system through the afferent nerve.[38] This test is a model for studying supra-spinal action of compounds through central mechanism.[39] The extract produced a dose-dependent prolongation of hot plate latency suggesting a central supraspinal antinociceptive activity.

Effect of the crude extract of *M. excelsa* on behaviour of mice in elevated plus maze test is shown in Figure 2. The entry and the duration of time spent in the open arms by animals treated with the extract (100 and 200 mg/kg) increased significantly ($p < 0.001$) compared to the control. Rats treated with the extract at the dose of 100 and 200 mg/kg spent 175.3 ± 7.40 s and 187.0 ± 4.62 s in the open arms respectively, compared to control (78.14 ± 3.36 s). Diazepam (1 mg/kg) also increased the time spent (255.9. 1.08 s) in the open arms compared to control.

Behaviour in the elevated plus maze is used as a means for assessing exploratory, anxious, and motor behaviours. Rodents have a natural aversion to open space and the basic principle of this model is the conflict between exploration and aversion to open spaces.[40] An anxiolytic agent increases the frequency of entries and the time spent in open arms of the maze. In this assay, the extract increased the number of entries and time spent in the open arms compared to control. These results indicate an anxiolytic-like effect of the extract. This findings agree with a previous report by Akinpelu *et al*.[41] revealing the anti-anxiety effect of *M. excelsa* in this test.

In hole board test, head-dip is strongly linked to anxiety. A decrease in the number of movements and in head-dipping behaviour indicates a central nervous system depressant effect.[42] In addition, the decrease of spontaneous locomotion was considered sedative as it reduced excitability of the central nervous system.[43,44] The extract in a dose-dependent manner reduced the number of head-dips and line crossed with the lowest dose not significant in the number of lines crossed compared to the control. Significant ($p < 0.001$) effect in both parameters was also observed with diazepam (Figure 3). The results showed that the extract has a decreasing effect on the exploratory activity of the mice, suggesting a sedative effect.

The central depressant activity of the extract was further demonstrated by the ability of the extract to potentiate pentobarbitone-induced hypnosis. Potentiation of pentobarbitone sleeping time is an indication of central nervous system depressive activity.[45] The results showed that diazepam treated group caused a significant ($P < 0.001$) increase in the sleep duration and a significant ($P < 0.001$) decrease in the sleep latency compared to the control. The pretreatment of animals with the extract at the dose of 50, 100 and 200 mg/kg, p.o. significantly ($P < 0.05$, $P < 0.01$) shortened sleep latency (7.60±0.75, 7.20±0.66 and 7.00±0.71 min) compared to control (12.00±1.92 min) (Table 3). The extract, however, significantly increased the sleeping duration compared to control. This significant increase is dose-dependent, suggesting a depressant-like effect. The results of this study support the use of the plant in the treatment of insanity. The potentiality of the extract to modulate the CNS could be explored in the treatment of anxiety and other CNS disorders.

Table 1: Effect of ethanol extract of *Milicia excelsa* in acetic acid-induced writhing test

Treatment	Dose (mg/kg)	No of writhes	% Inhibition
Control	10 ml/kg	128.00 ± 3.19	-
Acetylsalicylic acid	100	20.33 ± 2.70***	84.14
Extract	50	38.67 ± 6.78***	69.83
	100	33.00 ± 4.34***	74.25
	200	32.33 ± 3.70***	74.77

Each value represents the mean ± SEM, n = 6. ***p < 0.001 compared with control (one-way ANOVA followed by Dunnett's test).

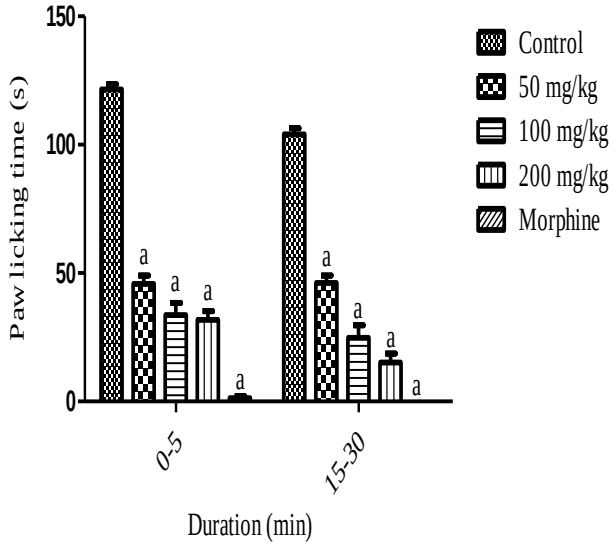

Figure 1: Effect of ethanol extract of *Milicia excelsa* in formalin-induced nociceptive test. Each value represents the mean ± SEM. (n = 6). [a]$p < 0.001$ compared with control (two-way ANOVA followed by Bonferroni's post-test.

Table 2: Effect of ethanol extract of *Milicia excelsa* in hot plate test

Treatment	Dose (mg/kg)	Pre-treatment reaction latency (s)	Post-treatment reaction latency (s)	Inhibition (%)
Control	10 ml/kg	3.1 ± 0.42	3.73 ± 0.35	-
Morphine	10	4.05 ± 0.41	30.00 ± 0***	100
M. excelsa	50	5.04 ± 0.63	14.87 ± 1.35***	39.36
	100	5.27 ± 0.37	15.31 ± 0.70***	40.39
	200	5.95 ± 0.72	24.5 ± 2.32***	75.69

All values were expressed as mean ± SEM, (n = 6). ***$p < 0.001$ compared with control (one-way ANOVA followed by post-hoc Dunnett's test).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

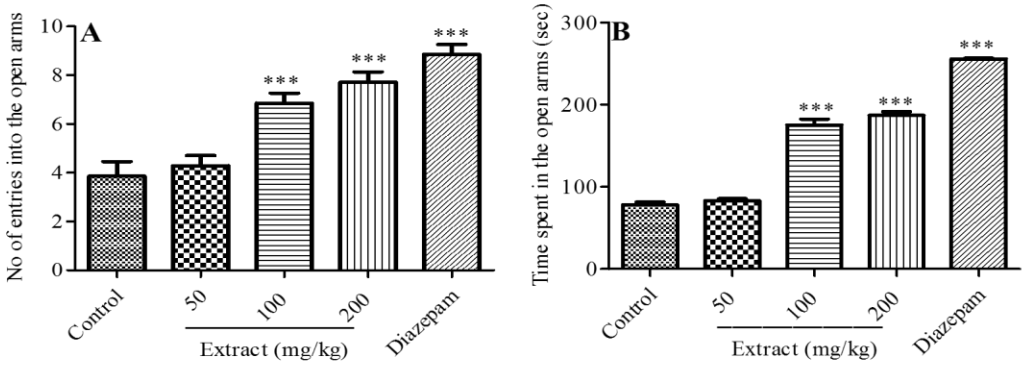

Figure 2: Anxiolytic effect of ethanol extract of *Milicia excelsa* in elevated plus-maze test in mice. All values were expressed as mean ± SEM, n = 7; ***$p < 0.001$ compared with control. A- No of entries into the open arms; B- Time spent in the open arms (sec)

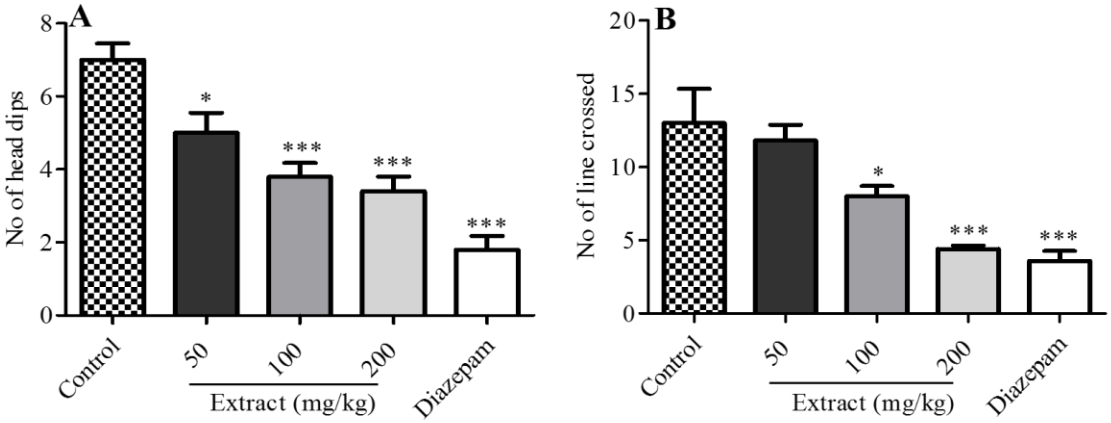

Figure 3: Effect of ethanol extract of *Milicia excelsa* on hole-board test. All values were expressed as mean ± SEM, n = 5. *p < 0.05, ***p < 0.001 compared with control (one-way ANOVA followed by post-hoc Dunnett's test). A- No of head dips; B- No of line crossed.

Table 3: Effect of ethanol extract of *Milicia excelsa* on phenobarbitone-induced sleeping time

Treatment	Dose (mg/kg)	Mean onset of sleep (min)	Mean duration of sleep (min)
Control	10 ml/kg	12.00 ± 1.92	219.60 ± 0.51
Extract	50	7.60 ± 0.75*	277.20 ± 0.97***
	100	7.20 ± 0.66*	291.80 ± 1.46***
	200	7.00 ± 0.71**	319.40 ± 1.50***
Diazepam	3	5.60 ± 0.25***	453.00 ± 7.77***

All values were expressed as mean ± SEM, n = 5. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control (one-way ANOVA followed by post-hoc Tukey's test).

Milicia excelsa presented no mortality up to the dose of 3200 mg/kg, p.o. and LD_{50} could not be determined, suggesting low toxicity.[46] The content total phenols, proanthocyanidins and flavonoids present in the crude extract was calculated as 95 mg gallic acid equivalent/ g, 85 mg catechin equivalent/ g and 69.6 mg quercetin equivalent/ g respectively. Phytochemical studies reported by Ouete *et al*.[18] support the presence of polyphenols and triterpenes in the leaf extract of this plant, as the authors reported the isolation of 3,4-dimethoxybenzyl *β*-D-xylopyranosyl(1→2)-*β*-D-glucopyranoside and 2-(3,5-dihydroxyphenyl) benzofuran-5,6-diol, lupeol acetate, ursolic acid and triacontyl (E)- ferulate). Among the compounds reported in *M. excelsa*, the triterpenes, ursolic acid and lupeol acetate have been reported to demonstrate antinociceptive and antidepressant- like effects in mice.[47,48] The presence of these constituents could play important role in the observed antinociceptive and behavioural effects of the extract.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Conclusion

The study demonstrated that the crude leaf extract of *M. excelsa* exhibited significant antinociceptive activity. In addition, the behavioural study of the extract suggests anti-anxiety like and sedative effects. These observations provide evidence for the use of the plant in traditional medicine for painful conditions and insanity.

Conflicts of interest

The authors declare no conflict of interests.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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