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Analgesic, anxiolytic and sedative-like activities of leaves of *Alpinia calcarata* Roscoe in mice

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The objectives of the study were to evaluate the analgesic, anxiolytic and sedative-like activities of methanol extract of leaves of *Alpinia calcarata* Roscoe in mice model. Analgesic activity was investigated using the acetic acid-induced writhing test and formalin-induced paw licking test. *In vivo* neuropharmacological effects, including anxiolytic and sedative effects were examined by open field, light-dark, elevated plus maze, thiopental sodium-induced sleeping time and hole cross tests behaviors in mice. The extract produced significant ($p<0.001$) reduction in writhing and licking response in acetic acid-induced writhing and formalin-induced paw licking tests, respectively. Administration at a dose of 400 mg/kg.bw of leaves extract significantly ($p<0.001$) attenuated anxiety-like behavior in mice by decreasing movement in open field, increasing the time spent and number of entries in the open arms of elevated plus maze, and a significant increase in the time spent in the illuminated compartment in the light box in the light-dark test. The extract significantly ($p<0.01$) potentiated thiopental sodium-induced sleep and reduced the number of sectional crossings relative to the control group, indicating sedative effects. Based on the results obtained from *in vivo* activities, the leaves of *A. calcarata* was found to be a potential source of new analgesic, anxiolytic and sedative compounds.

Key words: *Alpinia calcarata*, Swiss albino mice, analgesic, anxiolytic, sedative

INTRODUCTION

The genus *Alpinia* belongs to the family Zingiberaceae, and has long been used for many decades for medicinal and non-medicinal purposes. Plants of this genus have extensively been reported by several research studies for their potential biological activities. For example, antioxidant, antibacterial, larvicidal, cytotoxic and vasodilator activities of *Alpinia purpurata* were reported (Chan and Wong, 2015); the principle phytoconstituents responsible for antibacterial effect are kumatakenin and two steroidal glycosides and were isolated from leaves extract (Villaflores et al., 2010). Fruit extract of *Alpinia oxyphylla* showed the presence of yakuchinone-A (Oonmetta-aree et al., 2006) and norcardinane (Muraoka et al., 2001) possessing cardiotonic effect; kernel of the plant contains protocatechuic acid having neuroprotective effect (An et al., 2006) and diarylheptanoids produce anti-inflammatory effect (Chun et al., 2002). The plant also showed antidiarrheal, antidiuretic, antineoplastic,

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antioxidant, anti-inflammatory, anti-allergic, viscera protective and antidiabetic activities (Abubakar et al., 2018). Remarkable bioactivities such as anti-inflammatory, cytotoxicity, homeostasis, lipid regulation, antioxidant, antiviral, antimicrobial, antiosteoporosis of *Alpinia officinarum* were reported (Abubakar et al., 2018). A. officinarum showed the presence of two flavonoids, four diarylheptanoids, one sterol and one heptanone- all of these compounds possess antiemetic effect (Shin et al., 2002) and also cause inhibition of prostaglandin biosynthesis (Kiuchi et al., 1982). Alpinia zerumbet showed the presence of labdane-type diterpenes such as zerumin A and zurumin B (Xu et al., 1996). These essential oils are believed to have antinociceptive effect (De Araújo Pinho et al., 2005); flavonoids with antihypertensive effect (de Moura et al., 2005; Mpalantinos et al., 1998) were also found in this species. Chemical constituents of aerial parts of Alpinia katsumadai include stilbenes, monoterpenes, diarylheptanoids, labdanes and chalcones (Ngo and Brown, 1998) and showed antioxidant activity (Lee et al., 2003). Rhizome of Alpinia galanga showed the presence of an antifungal compound named acetoxychavicol acetate (Janssen and Scheffer, 1985; Oonmetta-aree et al., 2006); the compound also exhibited antitumor activity (Itokawa et al., 1987). *Alpinia calcarata* Roscoe is widely distributed in different regions of Bangladesh. It is cultivated as ornamental plants in Bangladesh, India, Sri Lanka, and Malaysia. This rhizomatous plant is commonly used as systemic medicinal sources in Sri Lanka. Traditionally, *A. calcarata* has been used by various indigenous communities as a remedy for bronchitis, cough, respiratory ailments, diabetics, asthma and arthritis (Perera et al., 2014; Ramanayake, 1994). Anecdotal use of this plant against pain and inflammation was scientifically proven by the findings of potent antinociceptive effect in mice model; also, neurological effects were simultaneously performed and observed dose-dependent *effect* (Arambewela et al., 2009a, b, 2004). Researchers also showed that rhizomes of *A. calcarata* possess antifungal, antibacterial, aphrodisiac, anthelmintic, antioxidant, gastroprotective and antidiabetic activities (Arambewela and Arawwawala, 2005; Arambewela et al., 2010, 2009a, b; Ratnasooriya and Jayakody, 2006). Extensive chemical analyses of different parts of A. calcarata and diverse groups of phytochemicals were reported. Eighteen different volatile oils were found in rhizome, leaves and root; among these, rhizome and leaves mainly contain 1,8-cineol while α-fenchyl acetate is the principle essential oil in root (Rahman et al., 2013; Tewari et al., 1999). Kong et al. (2000) isolated four diterpenoids such as calcaratarins A-D, a sesquiterpenoid named shyobunone and coumarins from rhizome extract of A. calcarata (Kong et al., 2000); in another study, flavonoids were reported in the same plant part (Hema and Nair, 2013).

Pain is a common nonspecific manifestation in many diseases. Non-steroidal anti-inflammatory drugs and opiates are generally used in pain management, but many adverse reactions occur with these drugs such as gastrointestinal disturbances, renal damage, respiratory depression, and possible dependence (Pergolizzi Jr et al., 2016; Scheiman, 2016; Sevinsky et al., 2017). Everyone experiences pain at some points in life, but pain accompanied with depression and anxiety is hard to endure as well as treat. People affected with psychotic disorders generally tend to experience more severe and long-lasting pain than others (Ploghaus et al., 2001). Diseases such as fibromyalgia (Gracely et al., 2012), irritable bowel syndrome (Mudyanadzo et al., 2018), low back pain (Sagheer et al., 2013), headache (Beghi et al., 2010), nerve pain (Sieberg et al., 2018), etc. often display mixed symptoms by anxiety, depression and pain. In addition to this, there is a strong relationship between sedation and pain and procedural sedation is widely used in various painful medical conditions (Frolich et al., 2013). In patients with psychotic disorders as well as pain, various psychotherapies can be used on their own to treat pain or may be combined with drug treatment. Numerous scientific reports have been published on beneficial pharmacological effects of plant in pain and neurological disorders (Akhigbemen et al., 2019; Aouey et al., 2016; Benjumea et al., 2016; Huda et al., 2019; Khan et al., 2017; Roy et al., 2019); plants enriched with phytoconstituents effective again analgesia and neurological disorders would be boon for mankind. The therapeutic potentials of the rhizome of *A. calcarata* have been well established, and the medicinal effects of its leaves are yet to be explored. Thus, our present study aims to examine antinociceptive and neurological activities of methanolic extract of leaves of *A. calcarata*.

MATERIALS AND METHODS

Experimental animals

Swiss albino mice (20-25 g) of both sexes were obtained from International Centre for Diarrhoeal Disease Research and Jahangirnagar University, Bangladesh. Animals were housed in groups of six in cages (40 cm x 30 cm x 17 cm; made up of polypropylene base and stainless-steel net) under a standard 12 h light : 12 h dark cycle (light phase 7 a.m. – 7 p.m.) in a room maintained at 23-25ºC and at approximately 50-55% relative humidity. Food and water were allowed *ad libitum* during the study period. In all experiments, mice were divided into four groups and each group consisted of six mice. All animal procedures and experimental protocols were approved by the Departmental Research Ethics Committee of the institution.

Plant materials

The leaves of *A. calcarata* were collected from botanical garden, Dhaka, Bangladesh and were authenticated by National Herbarium, Dhaka, Bangladesh. The leaves were sorted, cleaned, dried at room temperature and finally pulverized. About 400 g powdered plant material was taken separately in a clean, flat bottomed glass

container and soaked in 1500 ml of 80% methanol at room temperature for fifteen days with occasional shaking and stirring. Then the solution was filtered using filter cloth and Whatman filter paper No. 1 and concentrated with a rotary evaporator (RE-EV311-V, LabTeck S.R.L, Italy). It rendered a gummy concentrate of greenish black color. The gummy concentrate was designated as a crude methanolic extract.

Chemicals and drugs

Methanol (Merck, Germany) was used as a solvent during extraction. 0.6% acetic acid (98% v/v) (Loba Chemie, India) aqueous solution was prepared and was used to induce writhing on mice. Formaldehyde, 37% (Loba Chemie, India) was used for the preparation of 2.5% formalin. Aspirin (dose used: 100 mg/kg.bw; Albion Laboratories Limited, Bangladesh) was used as a positive control or standard during the study (acetic acid induced writhing and formalin-induced paw licking test); diazepam (1 mg/kg.bw) for neuropharmacological tests was also collected from the same source. Distilled water was used as control or vehicle. Distilled water, aspirin or diazepam and plant extracts were administered orally.

Analgesic test

Acetic acid induced writhing test

Acetic acid induced writhing test was performed as described in the literature (Hishe et al., 2018). The test samples and both controls were administered 30 min before induction of writhing by intraperitoneal injection of acetic acid (0.6%, 0.1 ml/10 g.bw). Number of writhing shown by each mouse was counted and recorded for 30 min. Contractions of the abdomen, elongation of the body, twisting of the trunk and/or pelvis ending with the extension of the limbs was considered as writhing. Results were expressed as mean percentage inhibition of writhing (PIW) (Hishe et al., 2018):

$$\text{PIW} = \frac{\text{No. of Writhes (control)} - \text{No. of Writhes (sample)}}{\text{No. of Writhes (control)}} \; x\; 100$$

Formalin induced licking test

In the licking test, formalin (2.5%, 0.02 ml) was injected subcutaneously to plain surface of the left hind paw of mice after 1 h of administration of test samples. Licking and biting of the injected paw were considered as an indication of pain. The amount of time spent in licking was recorded in two phases: at first 0-5 min and last 20-30 min after formalin injection (Wheeler-Aceto et al., 1990). The results were expressed as percentage inhibition of licking response (PIL) (Hishe et al., 2018):

$$\text{PIL} = \frac{\text{Time spent licking for Control} - \text{Time spent licking for Sample}}{\text{Time spent licking for Control}} \text{ x } 100$$

Behavioural assays

Open field test (OFT)

Open field test was performed in a box (72 cm x 72 cm x 36 cm) made of plywood and clear Plexiglas. The floor of the box was divided and marked into sixteen (18 cm x 18 cm) squares. Controls and extracts were administered orally 30 min prior to the test. Each mouse was then put into the apparatus and the number of square blocks visited by each mouse was calculated for 5 min on 0, 30, 60, 90 and 120 min intervals (Consolini et al., 2006).

Elevated plus maze test (EPM)

The apparatus used for elevated plus maze test was comprised of two open arms (35 cm x 5 cm) across from each other and perpendicular to two closed arms (35 cm x 5 cm x 15 cm) with a center platform (5 cm x 5 cm) with minor variations in the related literature (Hritcu et al., 2011). The open arms were exposed having no wall whereas the closed arms were enclosed by walls 15 cm high. Each mouse was placed in the center area of the maze with its head directed toward a closed arm after 1 h of treatment with samples and allowed to move freely about the maze for 5 min. After each trial, all arms and the center area were cleaned with 10% ethanol.

Light-Dark test (LDT)

The apparatus used for the light/dark transition test consisted of a cage (21 cm x 42 cm x 25 cm) divided into two sections by a partition with door (7 cm x 7 cm); the light compartment is 2/3 of the box, is brightly lit and open, the dark compartment is 1/3 of the total box and is covered and dark. Experimental animals were kept for an hour in a dark testing room to adapt with new environment before subjected to light-dark test. Then, each mouse was placed at the center of the light compartment with its back to the dark compartment, and then transition behavior over 5 min was observed with the following parameters- time spent in light compartment time and number of transitions. Typically, mouse was expected to move around the periphery of the compartment until they find the door. All four paws must be placed into the opposite chamber to be considered an entry (Gong et al., 2006).

Thiopental Sodium induced sleeping time test

In this test, controls and extracts were administered to each mouse first. After 20 min, thiopental sodium (40 mg/kg.bw) was injected intraperitoneally. The animals were then observed for onset of sleep and duration of sleep (Moniruzzaman et al., 2015).

Hole cross test

Hole cross test was done in a steel cage having dimensions of 30 cm x 20 cm x 14 cm. A partition which has a hole of 3 cm diameter at a height of 7.5 cm was fixed in the middle of the cage. After administration of sample, each mouse was introduced into the cage and the number of passages from one chamber to other through the hole inside the cage was counted for 3 min on 0, 30, 60, 90 and 120 min intervals (Ali et al., 2014).

Statistical analysis

Data were presented as mean ± SEM values. One-way ANOVA with Dunnett's test was performed using GraphPad Prism (version 8.3). A probability level of 0.05 (adjusted P value according to GraphPad Prism) or less was accepted as significant; $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ vs. vehicle; $^{\alpha}p < 0.05$, $^{\beta}p < 0.01$, $^{\gamma}p < 0.001$ vs. either aspirin or diazepam.

RESULTS

Writhing response in acetic-acid induced mice

As shown in Figure 1A, aspirin significantly ($p<0.001$)

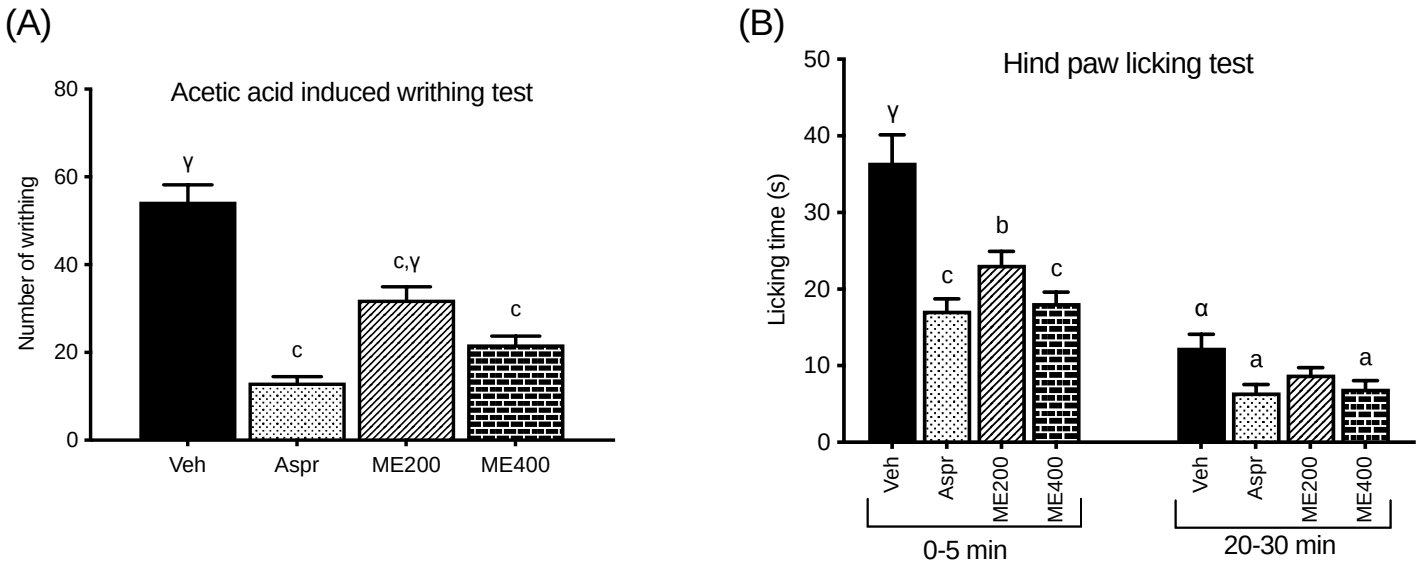

Figure 1. Effect of methanolic extract of leaves of *A. calcarata* on analgesic tests. 'Veh' stands for vehicle or control, 'Aspr' for aspirin and 'ME' for methanolic extract while 200 and 400 were the doses in mg/kg.bw. Values are mean ± SEM (n=6) and One-way ANOVA with Dunnett's test was performed.
[a]p<0.05, [b]p< 0.01, [c]p<0.001 vs. vehicle; [α]p<0.05, [β]p<0.01, [γ]p<0.001 vs. aspirin.

Table 1. Effect of *A. calcarata* leaves extract on percentage inhibition in acetic acid induced writhing and hind paw licking.

Group	Acetic acid induced writhing (% inhibition)	Hind paw licking (% inhibition)	
		Early phase	Late phase
Vehicle	-	-	-
Aspirin	75.76	52.95	47.28
ME200	41.10	36.50	28.38
ME400	59.82	50.22	43.22

decreased the number of abdominal writhes (13.1) as compared to the control group (54.33). Similar to positive control (aspirin), leaves extract at both doses (ME200 and ME400) also exhibited significant reduction in the number of abdominal writhes against acetic acid induced pain. Moreover, percentage inhibition of writhing was observed 75.76, 41.10 and 59.82 for aspirin, ME200 and ME400 respectively (Table 1).

Paw licking in formalin-induced pain in mice

Figure 1B shows that animals administered leaves extract demonstrated significantly (p<0.001 for early phase and late phase) reduction of paw-licking time compared with the control group at both phases. Likewise, in both phases, aspirin also significantly (p<0.001) reduced the paw-licking time compared with the control group. However, no significant differences were observed among the leaves extract at the dose of 200 mg/kg (ME200). Calculating the percentage inhibition of licking, aspirin demonstrated highest percentage inhibition (early phase: 52.95% and late phase: 47.28%) followed by ME200 (early phase: 36.50% and late phase: 28.38%) and ME400 (early phase: 50.22% and late phase: 43.22%) of leaves extract, respectively (Table 1).

Anxiolytic effect in open field test (OF)

In open field test, number of square travelled was recorded after 0, 30, 60, 90 and 120 min. For all samples, square crossed was highest at 0 min that gradually decreased showing lowest number at 120 min (Table 2). Number of squares travelled at 0 min was 46.5 and 33.17 (p<0.001) for vehicle and diazepam respectively; these movements were slowly decreased demonstrating 33.0 and 13.17 at 120 (p<0.001) min. In case of methanolic extract, ME200 showed 43.0 and 24.00 (p<0.01) square movements at 0 and 120 min respectively while ME400 demonstrated nearly equal movements similar to diazepam at 0 and 120 min, producing 35.83 (p<0.01)

Table 2. Effect of *A. calcarata* leaves extract on anxiolytic responses in open field test.

Group	Number of squares travelled				
	0 min	30 min	60 min	90 min	120 min
Vehicle	46.5±2.41$^{\gamma}$	44.67±2.25$^{\gamma}$	42.33±2.36$^{\gamma}$	36.33±2.94$^{\gamma}$	33.00±2.60$^{\gamma}$
Diazepam	33.17±1.14^{c}	30.67±0.99^{c}	26.00±1.39^{c}	19.5±1.96^{c}	13.17±1.52^{c}
ME200	43.00±1.61$^{\beta}$	38.5±1.12$^{\alpha}$	33.67±1.54$^{a,\alpha}$	28.33±1.52$^{a,\alpha}$	24.00±1.32$^{b,\beta}$
ME400	35.83±2.52^{b}	30.67±2.42^{c}	24.17±2.06^{c}	20.00±1.63^{c}	16.33±1.63^{c}

Values are mean±SEM (n=6) and One-way ANOVA with Dunnett's test was performed. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ vs. vehicle; $^{\alpha}p<0.05$, $^{\beta}p<0.01$, $^{\gamma}p<0.001$ vs. diazepam.

and 16.33 ($p<0.001$) respectively.

Anxiolytic effect in elevated plus maze test (EPM)

In the elevated plus maze test, the number of entry in the open arm of the apparatus for vehicle and diazepam were 9.33 and 16.17 ($p<0.01$) respectively; accordingly, time spent for the controls were 70.83 and 158.3 s ($p<0.001$) respectively (Figure 2A and 2B). Methanolic extracts produced a higher number of entry than the vehicle in the open arm showing 11.6 and 12.0 for ME200 and ME400 respectively. As a result, longer durations than vehicles were observed for both extracts showing 128.2 and 145.5 s ($p<0.001$) respectively.

Anxiolytic effect in light dark test (LDT)

In this experiment, both number of entry and time spent in the light box of the apparatus were increased after the administration of diazepam compared to vehicle (Figure 2C and 2D). For diazepam, the animals showed 21.5 appearances ($p<0.01$) staying 182.8 s ($p<0.001$) in the light chamber while the vehicle showed 10.0 entries ($p<0.01$) with total spent time of 43.5 s ($p<0.001$). ME200 and ME400 caused longer duration to stay of 142.5 s ($p<0.001$) and 173.8 s ($p<0.001$) respectively with 15.17 and 18.67 ($p<0.05$) entries.

Sedative effect in thiopental sodium induced sleeping time test

The effect of extract on induction of sleep and its duration was shown in Figure 3A and 3B. Diazepam took far less time (11.5 min, $p<0.001$) to induce sleep in mice than vehicle (42.33 min, $p<0.001$). ME200 induced sleep in slightly less time (11 min, $p<0.001$) than diazepam; onset of sleep was further decreased when the dose of extract increased to 400 mg/kg.bw (ME400) (8.33 min, $p<0.001$). Consequently, duration of sleep followed similar trend ($p<0.001$) where the animals were asleep for longer period after the administration of diazepam and extract compared to the vehicle.

Sedative effect in the hole cross test

In this experiment, number of holes crossed at 0, 30, 60, 90 and 120 min were recorded and were continued to decline over time. Animals crossed around twenty holes ($p<0.001$) for diazepam and both doses of extracts while vehicle showed 31 passages ($p<0.001$) as the experiment commenced. The movements of mice were dropped as time elapsed; diazepam and ME400 produced nearly equal numbers at 120 min showing 5.17 ($p<0.01$) and 5.5 ($p<0.001$) respectively; ME200 caused moderately higher hole crossings (7.5, $p<0.001$) than diazepam at the same time span (Table 3).

DISCUSSION

In our research work, analgesic and neuropharmacological potentials of *A. calcarata* leaves were assessed. To determine antinociceptive effect, acetic acid induced writhing test and formalin-induced hind paw licking test were performed as peripheral models of pain. Both of these methods are commonly used for the assessment of the peripheral pathway of analgesic drugs (Jain et al., 2001). The plant extract demonstrated dose-dependent analgesic effect. In acetic acid induced writhing test, significant inhibition of nocifensive behaviors were observed at highest extract dose (59.82%) as compared to aspirin (75.76%). Acetic acid causes excitation of nociceptive nerve endings resulting to production of certain prostaglandins, activation of ion channels and increased capillary permeability (Pace et al., 2017; Ribeiro et al., 2000; Sutradhar et al., 2007; Voilley, 2004). Production of prostaglandins in peripheral tissues involves the cyclooxygenase pathway, which is blocked by common NSAIDs. Reduced nocifensive behaviors by the methanolic extract of leaves in induced writhing experiment could be assumed by interference of any of these pains and inflammation induction pathways. In formalin licking test, the licking time was recorded in two phases. In both phases, nearly equivalent effects were demonstrated by extract at 400 mg/kg.bw and aspirin; extract produced 52.95% inhibition while for aspirin, it was 50.22% in early phase. Effects were slightly attenuated

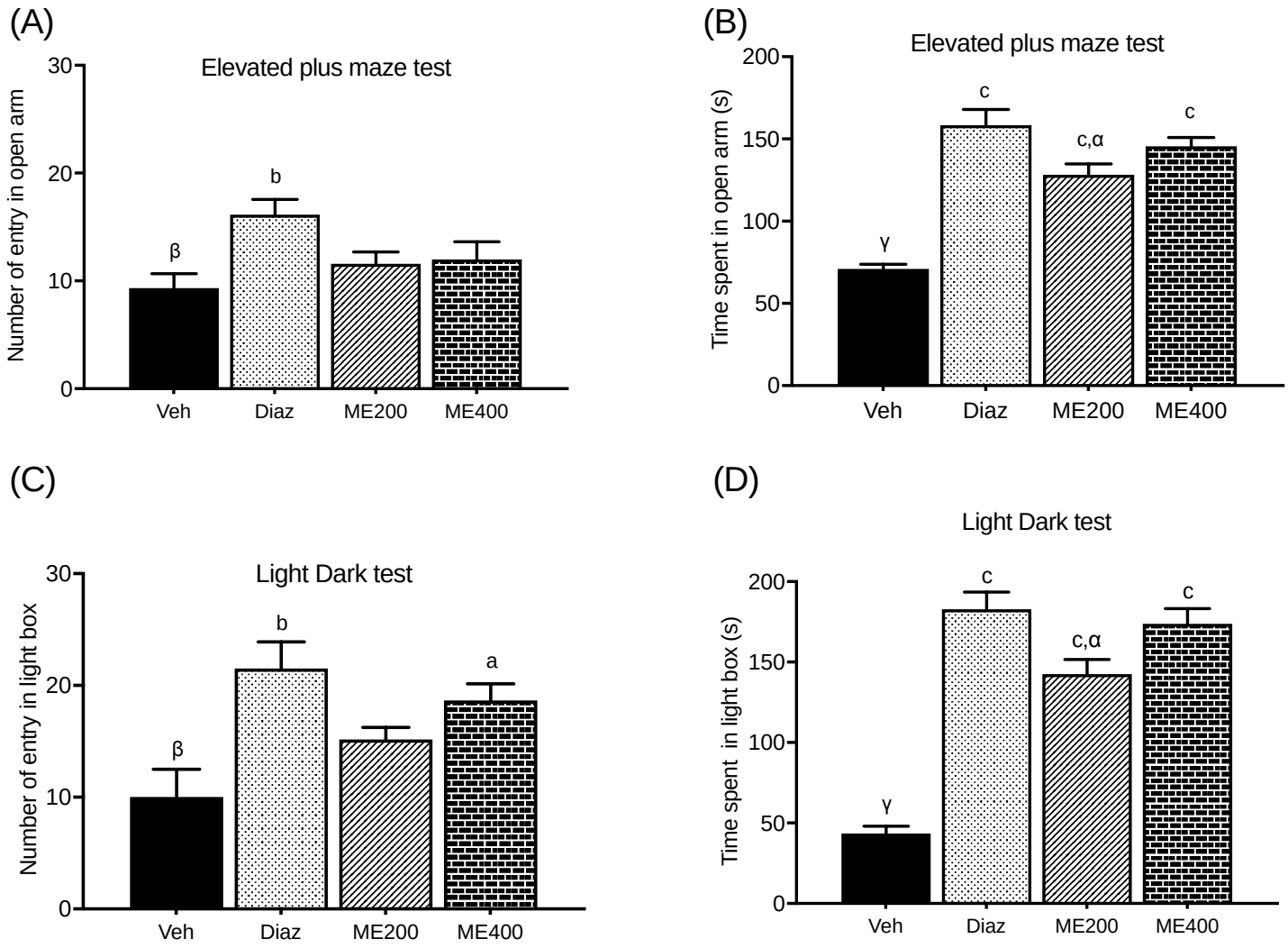

Figure 2. Effect of *A. calcarata* leaves extract on anxiolytic responses in (A and B) elevated plus maze and (C and D) light dark test. 'Veh' stands for vehicle or control, 'Diaz' for diazepam and 'ME' for methanolic extract while 200 and 400 were the doses in mg/kg.bw. Values are mean ± SEM (n=6) and One-way ANOVA with Dunnett's test was performed.
$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ vs. vehicle; $^{\alpha}p<0.05$, $^{\beta}p<0.01$, $^{\gamma}p<0.001$ vs. diazepam.

in late phase exhibiting 43.22% and 47.28% inhibition for extract and aspirin respectively. Drugs that inhibit early phase in formalin test have the ability to alleviate neurogenic pain mediated by C-fibre activation while the late phase is believed to involve inflammatory pain elicited by activation of NMDA and non-NMDA receptors and NO cascade in the peripheral tissues (Chen et al., 2016; Li et al., 2019; Raghav et al., 2018; Sorge et al., 2015). Centrally acting analgesics can inhibit equally in both phases whereas peripherally acting drugs can work only in late phase. Reduced tendency to lick by the experimental animals after administration of leaves extract indicates that *A. calcarata* could possibly block the related pathways. Alkaloids, flavonoids and sterols in extracts of other plants have been found to have analgesic effects (Both et al., 2002; Domitrovic et al., 2015; Hegazi et al., 2019; Onasanwo and Elegbe, 2006). Species from same genus such as *A. zerumbet* (De Araújo Pinho et al., 2005) and *A. oxyphylla* (Chun et al., 2002) showed antinociceptive and anti-inflammatory effects and these effects were believed to be produced by terpenoids. Phytochemical screening of *A. calcarata* showed the presence of alkaloids, phytosterols and flavonoids (Ferdous et al., 2018). Thus, any of these compounds or in combination could be responsible for the anti-nociceptive effect of extract of *A. calcarata.*

In case of neuropharmacological effects of the extract, the plant showed dose dependent activity in open field test. Standard drug- diazepam and plant extract at 400 mg/kg.bw demonstrated similar effect throughout the experimental period producing final locomotor activity of 13.17 and 16.33 respectively. Locomotion is believed to be mediated through dopaminergic pathway and other neural mechanisms (Raghav et al., 2018; Steidl et al., (2017).

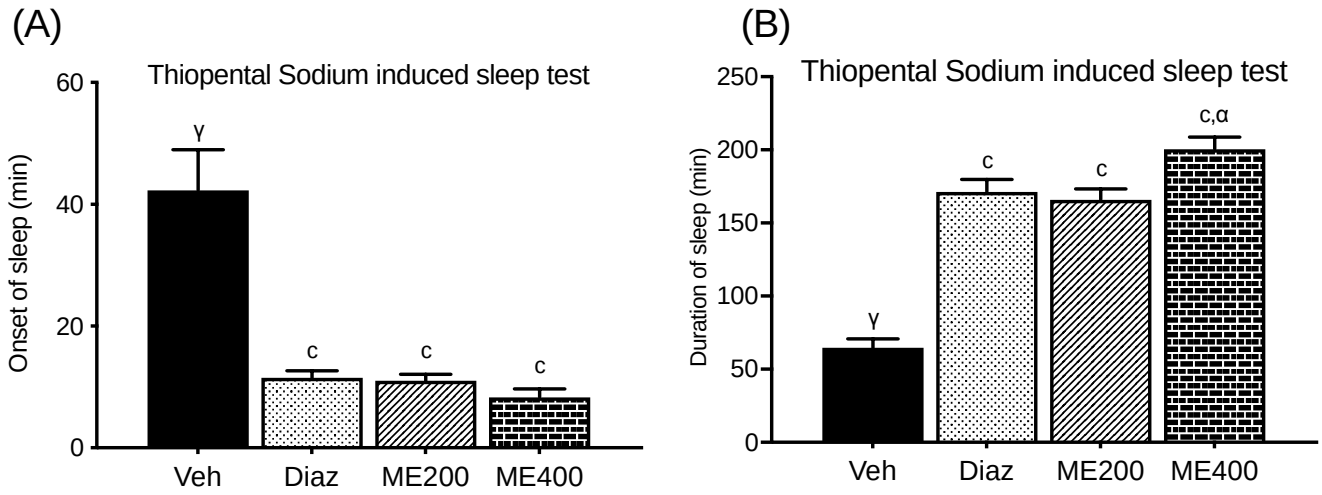

Figure 3. Effect of A. calcarata leaves extract on the (A) onset of sleep and (B) duration of sleep induced by thiopental sodium in mice. 'Veh' stands for vehicle or control, 'Diaz' for diazepam and 'ME' for methanolic extract while 200 and 400 were the doses in mg/kg. Values are mean ± SEM (n=6) and One-way ANOVA with Dunnett's test was performed. ap<0.05, bp<0.01, cp<0.001 vs. vehicle; αp<0.05, βp<0.01, γp<0.001 vs. diazepam.

Table 3. Effect of *A. calcarata* leaves extract on sedative effect in the hole cross test.

Group	Number of holes crossed				
	0 min	30 min	60 min	90 min	120 min
Vehicle	$31.0\pm0.58^{\gamma}$	$27.17\pm1.30^{\beta}$	$24.33\pm1.49^{\beta}$	$20.17\pm1.19^{\gamma}$	$17.33\pm0.95^{\gamma}$
Diazepam	20.0 ± 0.97^{c}	15.83 ± 0.31^{c}	12.0 ± 0.86^{c}	8.0 ± 0.97^{b}	5.17 ± 0.87^{b}
ME200	21.0 ± 0.45^{c}	17.17 ± 0.48^{b}	14.0 ± 0.63^{c}	10.0 ± 0.85^{a}	7.5 ± 0.43^{c}
ME400	20.17 ± 0.48^{b}	16.17 ± 0.40^{c}	13.67 ± 0.88^{c}	9.33 ± 1.14^{b}	5.5 ± 0.67^{c}

Values are mean ± SEM (n=6) and One-way ANOVA with Dunnett's test was performed.
$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ vs. vehicle; $^{\alpha}p<0.05$, $^{\beta}p<0.01$, $^{\gamma}p<0.001$ vs. diazepam.

2017). It can be suggested that inhibitory effect of methanolic extract on locomotor activity could be mediated by interference in the GABA neurotransmission of central nervous system (Liu et al., 2015). Elevated plus maze test and light dark test also showed analogous results demonstrating increased entry and time spent in the open arm or illuminated areas which correspond with similar effects as diazepam and other anxiolytic drugs (Bourin and Hascoët, 2003; Kędzierska et al., 2018; Kosari-Nasab et al., 2018). Thus, our findings suggest that leaves of *A. calcarata* could have potential phytochemicals responsible for anxiolytic effect. The classic experimental methods for the evaluation of sedative effect include thiopental sodium induced sleeping test and hole cross test. The extract showed significant reduction in sleep latency and increased thiopental sodium induced sleeping time, indicating sedative effect. Thiopental is known to enhance the inhibitory action of the GABA receptor that decreases neuronal activity (Begum et al., 2019). On the other hand, in hole cross test, the results showed dose dependent activity corroborating with the findings of thiopental sodium induced sleeping test. The prolongation of thiopental sodium induced sleep and suppression of exploratory behavior indicates sedative effect of the plant extract. Neurological effects were reported by rhizome of *A. calcarata*, and presence of terpenoids and flavonoids could result to such effect (Hema and Nair, 2013; Kong et al., 2000). Another species of *Alpinia* genus- *A. oxyphylla* showed neuroprotective effect and contains protocatechuic acid (An et al., 2006). Presence of diverse types of phytochemicals in leaves extract of *A. calcarata* certainly contains compounds that impart neurological effects.

Conclusion

The results of the present study displayed significant analgesic activity of methanolic extract of leaves of *A. calcarata* thereby scientifically confirming the traditional usage of this species for arthritis. The findings of this

study also provide the first evidence of anxiolytic and sedative like effects of the *A. calcarata* leaves extract in the CNS in mice model. Further investigations are necessary for isolation and identification of the chemical compound(s) responsible for the observed biological effects of the extracts.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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