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Research Article

Evaluation of Antinociceptive Effects of Methanolic Extract of Leucas *aspera Leaves* in Mice

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Abstract

Background: *Leucas aspera* (Family: Labiatae), commonly known as Darkolos is used for anti-pyretic, anti-rheumatic, anti-inflammatory, and anti-bacterial properties. The purpose of this study was to investigate the antinociceptive effects of methanolic extract of *L. aspera* leaves (MELA) in mice models, to validate its traditional use.

Methods: Six different pain models were performed to determine the antinociceptive activity in mice using morphine sulphate (5 mg/kg) and diclofenac sodium (10 mg/kg) as the standard drugs. MELA was administered orally at the doses of 250, 500 and 750 mg/kg. To verify the possible participation of opioid receptor in the central antinociceptive effects of MELA naloxone was used as antagonist.

Results: The results revealed that *L. aspera* possess notable antinociceptive activity in all the tested chemical and heat-induced pain models in mice. In hot plate and tail immersion tests, the extract at 500 and 750 mg/kg exhibited significant (*$p < 0.05$) antinociceptive activity. In writhing test, we observed 33.05%, 59.79% and 82.74% inhibition. In formalin test, the extract repressed 50.40%, 62.11%, and 72.35% inhibition of licking for the first phase while late phase exhibited 67.23%, 74.57%, and 84.74% inhibition of licking. The plant extract showed 24.39%, 60.05% and 80.96 % inhibition of licking in glutamate test. Similarly, cinnamaldehyde-induced nociception produced a significant (*$p < 0.05$) inhibition of 28.07%, 52.19% and 75%, respectively.

Conclusions: The antinociceptive activity of MELA is found to be exerted by the involvement of both peripheral and central mechanisms. Thus, our results provide evidence in support of the traditional use of MELA in painful disorders.

Keywords: *Leucas aspera; Antinociceptive; Pain*

Introduction

Pain is predominantly a safety mechanism connected with tissue damage, expressed by emotional experience or an unpleasant sensory organ [1]. Analgesics relieve symptomatic pain; but have no effect on the cause of pain [2]. When living tissues and cells are damaged by any harmful stimuli, injured or disturbed, it releases protons (H^+), prostaglandin E2 (PGE2), serotonin (5-HT) among others and consequently causes pain. Some pain may be minor, acute, or chronic like rheumatoid arthritis. This persistent pain associated with injury or diseases can alter the properties of peripheral nerves including damage to nerve fibers, leading to increased spontaneous alterations in conduction or neurotransmitter properties [1]. Conventional drugs possess many side effects which is making them unfavourable for therapeutic use. In this context, novel compounds are required for better pain relieving potential and minimum side effects.

In Bangladesh, *Leucas aspera* (Family: Labiatae), commonly known as Darkolos is found in Asia, Africa, and tropical countries as weed. The plant is used in the treatment of analgesic, anti-pyretic, anti-rheumatic, anti-inflammatory, and anti-bacterial. The paste is used as

topically on inflamed areas [3]; while the whole plant is used as insecticide, coughs, pain, and skin eruption [4]. The animal study with *L. aspera* revealed its anti-inflammatory activity through prostaglandin inhibition [5,6]. While the wound healing property was established in cobra venom poisoning model [7]. The leaves are effective in psoriasis and scabies, and also have anti-bacterial and anti-fungal activity [8]. Preliminary phytochemical screening revealed that the plant contain triterpenoids, oleanolic acid, ursolic acid, and α- sitosterol and β-sitosterol [9,10] along with sterols, alkaloids, reducing sugars and glucoside [11,12]. More than 25 chemical constituents are reported from the leaves including volatile u-farnesene, x-thujene, and menthol; amyl and isoamyl propionate from flower [13]; palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and ceryl alcohol from seed [14,15]; phenolics, and long-chain compounds from the shoot [16,17]; and Leuco-lactone (I) from the root [18]. The plant has been studied for anti-microbial, anti-malarial, larvicidal, anti-sporiatic, anti-plasmodial and pupicidal activities [19-23].

Earlier literature revealed that the leave of *L. aspera* possess antinociceptive property [3]. Thus, the recent study was conducted to explore the antinociceptive activities of methanolic extract of *L. aspera* in mouse models. Although, more studies are demanded to evaluate the use of *L. aspera* for the treatment of human diseases.

Materials and Methods

Drugs and chemicals

The following drugs and chemicals were used in the current study: morphine sulfate, diclofenac sodium (Square Pharmaceuticals Ltd., Bangladesh), naloxone (Hamein Pharmaceuticals GmbH), acetic acid, methanol, formalin, methylene blue, L-glutamic acid, cinnamaldehyde (Merck, Germany), glibenclemide (Square Pharmaceuticals Ltd., Bangladesh). All other chemicals and reagents were of analytical grade and high purity.

Plant material and extraction

Leucas aspera leaves were collected from Padma Garden, Rajshahi, Bangladesh and were authenticated by Principal Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. The voucher specimen ("DACB: 38390") was deposited to the herbarium for further use. The leave was dried at room temperature for 5 days. The sample was ground into the fine powder and mixed with methanol for 7 days. Then, the solvent was removed by rotary evaporator to collect the extract (9.80g extract; yield 3.92% w/w) to be used for further studies.

Animals

Swiss albino mice (20 - 25g) purchased from Pharmacology Laboratory, Jahangirnagar University, Savar, Dhaka, Bangladesh were kept under standard environmental conditions at 25 ± 2°C, 55 - 65% relative humidity with 12h light/dark cycle. The study protocol was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (SUB/IAEC/17.01) of Stamford University Bangladesh. The animal experiments were conducted following the rules for animal experiment approved by the Institutional Animal Ethical Committee.

Acute toxicity test

For acute toxicity test, 30 mice were divided into 6 groups (n = 5). The test groups received *L. aspera* leaf extract orally at doses of 100, 200, 500, 1,000, 2,000, and 4,000 mg/kg body weight; while the control group received deionized water (0.1 mL/mouse, p.o.). Then, the animals were observed continuously for the first 4h for any behavioral changes and were kept under watching up to 14 days to find out the mortality, if any [24].

Antinociceptive activity

Hot plate test

The hot-plate test was employed to measure antinociceptive activity as described by Woolfe and McDonald [25]. The response in the form of jumping, paw withdrawal, or licking was defined as hot plate latency. Each animal was placed on hot plate kept at 52 ± 0.5°C. The

reaction time within 20 s were showed on the hot plate. Control group received deionized water (0.1 ml/mouse, p.o.) and Morphine sulphate (5 mg/kg, i.p.) was used as standard drug. The test groups received extract at the doses of 250, 500 and 750 mg/kg (p.o.) gradually and the latency time was recorded at 0, 30, 60, 90, and 120 min. The analgesic activity was calculated using the following formula [26]:

% inhibition = (Drug latency - Base line latency/Base line latency) × 100.

Tail immersion test

The tail immersion test was performed by Cha., *et al*. [27] method to evaluate the antinociceptive activity of *L. aspera*. 25 mice were divided into 5 groups (n = 5). The two-thirds of animals tail was immersed in hot water at a temperature of 54 ± 1°C. The reaction time was recorded with a stopwatch at 0, 30, 60, 90 and 120 minutes of extract administration (250, 500, and 750 mg/kg, *p.o.*), control (0.1 ml/mouse, *p.o.*) or morphine sulphate (5 mg/kg, *i.p.*). To avoid tissue damage, the cut-off time was set at 20 s. The results were calculated as a percentage of the maximal possible effect (%MPE), calculated by the formula:

% MPE = [(Post drug latency−pre drug latency)/(Cut off period−pre drug latency)] × 100

Acetic acid-induced writhing test

The acetic acid-induced writhing test method was described by Zakaria., *et al* [28]. 25 mice were treated with deionized water (0.1 ml/mouse, p.o.), diclofenac sodium (10 mg/kg, i.p.), or MELA (250, 500 and 750 mg/kg body weight, p.o.). Thirty minutes later, all mice were treated with intraperitoneal injection of 0.6% acetic acid to cause a typical stretching response. Five minutes after acetic acid injection, mice were kept in individual cages and number of writhes for each group was counted for 30 min. The analgesic activity was calculated using the following formula:

% Inhibition = [Mean no of writhes(control) - mean no of writhes (test)/Mean no of writhes (control)] × 100

Formalin-induced paw licking test

The test was performed following the method described by Zakaria., *et al* [28]. About 20 µL of 2.5% formalin was injected into the sub-plantar area of the right hind paw. 25 mice were divided into five groups (n = 5), and the control animals were orally administered with deionized water (0.1 mL/mouse, p.o.), or diclofenac sodium (10 mg/kg. i.p.), while the experimental animals with MELA (250, 500 and 750 mg/kg, p.o.) 60 minutes before the formalin injection. After formalin injection, the animals were placed in an observation circle and watched for 30 minutes. The injected paws number of licking was taken in two phases. The early phase (0 - 5 minutes) was recorded during the first 5 minutes, while the late phase (15 - 30 minutes) was recorded during the last 15 minutes.

Glutamate-induced nociception test

The participation of glutamate receptors was evaluated using the method narrated by Beirith., *et al* [29]. The test animals were treated with MELA (250, 500 and 750 mg/kg, p.o.), while the control group received deionized water (0.1 ml/mouse, p.o.) and the reference group was treated by diclofenac sodium (10 mg/kg i.p.). Thirty minutes after treatments, 20 µl (10 µmol/paw) of glutamate was injected into the ventral surface of the right hind paw of mice. Mice were individually placed in an observation chamber for 15 minutes. Injected paw licking was observed as an indication of nociception.

Cinnamaldehyde-induced nociception test

Cinnamaldehyde, a TRPA1 receptor agonist, was used to investigate the antinociceptive effect of MELA in mouse paw licking test [30]. The mice were randomly separated into five groups (n = 5) and were treated with MELA at 250, 500 and 750 mg/kg oral doses, 1h before cinnamaldehyde injection. The control group received deionized water orally at 0.1 ml/mouse 30 minutes before the experiment; while the standard drug group received diclofenac sodium (10 mg/kg, i.p.). After 20 minutes, 20 µl of cinnamaldehyde (10 nmol/paw) was in-

jected intraplantar in the ventral surface of the right hind paw. Mice were monitored separately for 5 minutes and the number of licking was recorded by stopwatch [30].

Analysis of possible mechanism of action of MELA

Involvement of opioid system

To determine the role of opioid receptors in the inflection of antinociceptive activity of MELA, five groups of animals (n = 5) were pre-treated with a non-selective opioid antagonist, naloxone (2 mg/kg; i.p.) for 15 min followed by the oral administration of MELA (250, 500 and 750 mg/kg, p.o.) or morphine. Then, the animals were assessed using the hot plate test at 0, 30, 60, 90, and 120 minutes with the same cut off time of the 20s, respectively [31].

Involvement of cyclic guanosine monophosphate (cGMP) pathway

To explore the role of cyclic-guanosine monophosphate (cGMP) pathway in the antinociceptive activity of MELA, the animals (n = 5) were pre-treated with methylene blue (20 mg/kg), a non-specific inhibitor of guanylyl cyclase, intraperitonially 15 minutes before the administration of diclofenac sodium or MELA (250, 500 and 750 mg/kg, p.o.). Then, the animals were assessed using the acetic acid-induced writhing test for 30 minutes, opening from 5 minutes post injection [32,33].

Involvement of ATP-sensitive K+ channels pathway

To study the possible contribution of ATP sensitive K^+ channel in the antinociceptive effect of MELA, mice were pre-treated with glibenclamide (an ATP-sensitive K^+ channel inhibitor, 10 mg/kg), intraperitonially 15 minutes before the administration of either diclofenac sodium or MELA (250, 500 and 750 mg/kg, p.o.). Then, the animals were challenged with the acetic acid-induced writhing test for 30 minutes post-treatment [34].

Statistical analysis

Collected data were represented as mean ± Standard Error of Mean (SEM). This statistical analysis was carried out using One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test through Statistical Package for the Social Sciences (SPSS) software (version 18.00). *$p < 0.05$, vs. control was considered to be statistically significant.

Results

Acute toxicity test

In this test, the highest dose of 4,000 mg/kg did not show any mortality in the test animals. Consequently, the LD_{50} of *L. aspera* is estimated to be more than 4000 mg/kg.

Antinociceptive activity

Hot plate test

MELA appeared a significant antinociceptive effect at 500, and 750 mg/kg doses (*$p < 0.05$) as presented in table 1. Naloxone did not show any remarkable reduction of antinociceptive effect of *L. aspera*; while morphine (5 mg/kg) confirmed a significant antinociceptive effect compared to the control group (*$p < 0.05$).

Tail immersion test

In the tail immersion test, the antinociceptive effect of *L. aspera* and morphine are shown in table 2. The extract effect was more prominent after 90 min at 250, 500, and 750 mg/kg. Morphine (5 mg/kg, i.p.) showed a notable antinociceptive effect compared to control (*$p < 0.05$), as morphine was antagonized by naloxone in this test for antinociceptive activity.

Treatment		Dose (mg/kg)	Latency period (s) (%MPE) Pretreatment	30 min	60 min	90 min	120 min
Treatment without Naloxone	Control	0.1 mL/mouse	5.00 ± 0.89	7.80 ± 0.86	9.40 ± 0.74	11.00 ± 0.89	12.00 ± 1.09
	Morphine	5	9.20 ± 0.86	13.40 ± 1.20* (45.90)	15.20 ± 1.15* (54.71)	17.40 ± 1.28* (71.11)	18.80 ± 0.86* (85)
	MELA	250	5.80 ± 1.02	10.40 ± 1.20* (21.31)	13.40 ± 0.51 (37.73)	15.60 ± 1.20 (51.11)	17.20 ± 1.24* (65)
	MELA	500	7.40 ± 0.92	10.60 ± 0.67* (22.95)	13.80 ± 1.15* (41.50)	16.80 ± 1.20* (64.44)	17.80 ± 0.73* (72.50)
	MELA	750	8.40 ± 0.87	11.80 ± 1.06* (32.38)	14.20 ± 0.86* (45.28)	17.40 ± 0.51* (71.11)	18.40 ± 0.87* (80)
Treatment with Naloxone	NLX	2	8.40 ± 0.87	6.80 ± 0.86	6.20 ± 0.66	5.00 ± 0.54	3.60 ± 0.51
	NLX+ Control	2+0.1 mL/mouse	7.40 ± 0.51	7.80 ± 0.73	8.80 ± 0.86	9.40 ± 1.03	10.40 ± 1.07
	NLX+ Morphine	2+5	7.20 ± 0.58	8.00 ± 0.70 (1.63)	9.80 ± 0.73 (3.77)	11.20 ± 0.86 (2.22)	12.60 ± 1.20 (7.5)
	NLX+ MELA	2+250	5.00 ± 0.70	8.40 ± 0.51 (4.91)	9.80 ± 0.37 (3.77)	11.80 ± 0.86 (8.88)	14.00 ± 0.70 (25)
	NLX+ MELA	2+500	7.80 ± 1.15	10.00 ± 0.70 (18.03)	11.00 ± 0.70 (15.09)	13.60 ± 1.50 (28.88)	16.80 ± 1.65 (60)
	NLX+ MELA	2+750	8.20 ± 0.58	10.20 ± 0.80 (19.67)	11.40 ± 0.81 (18.86)	13.80 ± 1.06 (31.11)	16.80 ± 1.39 (60)

Table 1: *Antinociceptive effect of L. aspera extract, morphine and reversal effect of naloxone in hot plate test. Values are presented as mean ± SEM (n= 5). MELA: Methanolic extract of L. aspera; NLX: Naloxone. * p < 0.05 compared with the control group (Dunnett's test).*

Treatment		Dose (mg/kg)	Latency period (s) (%MPE) Pretreatment	30 min	60 min	90 min	120 min
Treatment without Naloxone	Control	0.1 mL/mouse	2.75 ± 0.12	3.13 ± 0.07	3.21 ± 0.05	3.33 ± 0.04	3.84 ± 0.11
	Morphine	5	3.42 ± 0.14	4.50 ± 0.06* (8.09)	5.45 ± 0.05* (13.33)	6.02 ± 0.16* (16.16)	6.95 ± 0.25* (19.25)
	MELA	250	2.57 ± 0.17	3.39 ± 0.06 (1.49)	4.20 ± 0.30* (5.86)	4.87 ± 0.21* (9.21)	5.33 ± 0.09* (9.23)
	MELA	500	3.77 ± 0.10	4.63 ± 0.18* (8.86)	5.14 ± 0.23* (11.48)	5.91 ± 0.13* (15.46)	6.26 ± 0.30* (14.94)
	MELA	750	4.57 ± 0.22	5.24 ± 0.20* (12.47)	5.83 ± 0.20* (15.58)	6.25 ± 0.17* (17.51)	6.61 ± 0.07* (17.15)
Treatment with Naloxone	NLX	2	2.11 ± 0.04	2.33 ± 0.06	3.04 ± 0.04	2.74 ± 0.11	2.79 ± 0.13
	NLX+ Control	2+0.1 mL/mouse	1.93 ± 0.05	2.16 ± 0.05	2.50 ± 0.06	2.67 ± 0.07	2.79 ± 0.09
	NLX+ Morphine	2+5	2.15 ± 0.03	2.62 ± 0.07 (-3.07)	3.46 ± 0.15 (1.47)	4.19 ± 0.04 (5.13)	5.18 ± 0.05 (8.26)
	NLX+ MELA	2+250	2.32 ± 0.04	3.14 ± 0.22 (0.04)	3.72 ± 0.12 (3.01)	4.23 ± 0.35 (5.42)	5.01 ± 0.06 (7.20)
	NLX+ MELA	2+500	3.20 ± 0.08	3.60 ± 0.07 (2.78)	4.03 ± 0.23 (4.83)	4.23 ± 0.35 (5.38)	5.04 ± 0.12 (7.41)
	NLX+ MELA	2+750	3.33 ± 0.15	4.14 ± 0.13 (5.98)	4.78 ± 0.12 (9.32)	5.06 ± 0.18 (10.40)	5.61 ± 0.10 (10.92)

Table 2: *Antinociceptive effect of L. aspera extract, morphine and reversal effect of naloxone in tail immersion test. Values are presented as mean ± SEM (n = 5). MELA: Methanolic extract of L. aspera; NLX: Naloxone. * p < 0.05 compared with the control group (Dunnett's test).*

Acetic acid induced-writhing test

The extract produced a significant inhibition (*p < 0.05) of writhing induced by acetic acid in table 3. The inhibition of antinociceptive effect was achieved at 250, 500, and 750 mg/kg at 33.05%, 59.79%, and 82.74%, respectively; while the inhibition with diclofenac sodium was 84.63 %.

Treatment	Dose (mg/kg)	Number of writhing	% of Inhibition
Control	0.1 mL/mouse	47.50 ± 1.06	-
Diclofenac sodium	10	07.30 ± 0.43*	84.63
MELA	250	31.80 ± 1.07*	33.05
MELA	500	19.10 ± 1.16*	59.79
MELA	750	08.20 ± 0.60*	82.74

Table 3: *Effects of L. aspera extract and Diclofenac sodium on acetic acid-induced writhing test. Values are presented as mean ± SEM (n = 5). MELA: Methanolic extract of L. aspera. *p < 0.05, compared with the control group (Dunnett's test).*

Formalin test

The test extract significantly reduced the licking activity in both phase of formalin-induced pain model in table 4. Similarly, diclofenac sodium (10 mg/kg, i.p.) significantly inhibited the number of licking against the early and late phases in mice (*p < 0.05).

Treatment	Dose (mg/kg)	Licking of the hind paw			
		Early phase (0-5 min)	Inhibition (%)	Late phase (15-30 min)	Inhibition (%)
Control	0.1 mL/ mouse	123.00 ± 1.38	-	35.40 ± 1.63	-
Diclofenac sodium	10	22.80 ± 1.50*	81.46	05.00 ± 0.71*	85.87
MELA	250	61.00 ± 1.52*	50.40	11.60 ± 1.69*	67.23
MELA	500	46.60 ± 1.21*	62.11	0 9.00 ± 0.71*	74.57
MELA	750	34.00 ± 1.26*	72.35	0 5.40 ± 0.51*	84.74

Table 4: *Antinociceptive effects of L. aspera extract in formalin-induced nociception. Values are presented as mean ± SEM (n = 5). MELA: Methanolic extract of L. aspera. *p < 0.05 compared with the control group (Dunnett's test).*

Glutamate-induced nociception

Oral administration of MELA at 250, 500, and 750 mg/kg produced significant inhibition of glutamate-induced pain in table 5. Similarly, diclofenac sodium (10 mg/kg, i.p.) showed 84.45% inhibition in mice showing significant antinociceptive activity compared to the control (*p < 0.05).

Treatment	Dose (mg/kg)	Glutamate-induced nociception		Cinnamaldehyde-induced nociception	
		Number of licking	Inhibition (%)	Number of licking	Inhibition (%)
Control	0.1 mL/ mouse	74.60 ± 1.44	-	45.60 ± 1.08	-
Diclofenac sodium	10	11.60 ± 0.93*	84.45	09.40 ± 0.51*	79.38
MELA	250	56.40 ± 1.89*	24.39	32.80 ± 1.02*	28.07
MELA	500	29.80 ± 1.16*	60.05	21.80 ± 1.07*	52.19
MELA	750	14.20 ± 0.86*	80.96	11.40 ± 1.03*	75.00

Table 5: *Antinociceptive effects of L. aspera extract in glutamate and cinnamaldehyde-induced nociception. Values are presented as mean ± SEM (n = 5). MELA: Methanolic extract of L. aspera. * p < 0.05 compared with the control group (Dunnett's test).*

Cinnamaldehyde-induced nociception

Oral administration of MELA at 250, 500 and 750 mg/kg produced an inhibition of the cinnamaldehyde-induced nociception with the percentage of 28.07%, 52.19% and 75%, respectively in table 5. The standard drug diclofenac sodium showed a significant antinociceptive activity of 79.38%, compared to the control group (*p < 0.05).

Involvement of cyclic guanosine monophosphate (cGMP) pathway

Methylene blue (20 mg/kg) significantly repressed acetic acid-induced writhing test in table 6. If given together, it significantly (*p < 0.05) aggravated the antinociceptive effect of MELA (500, and 750 mg/kg) compared to the control group (*p < 0.05).

Treatment	Dose (mg/kg)	Number of writhing	Inhibition (%)
Control	0.1 mL/ mouse	73.50 ± 1.73	-
Methylene Blue (MB)	20	63.60 ± 1.74*	13.47
MELA + MB	250 +20	41.90 ± 1.17*	42.99
MELA + MB	500 +20	32.10 ± 1.04*	56.33
MELA + MB	750 +20	20.40 ± 0.89*	72.24

Table 6: *Effects of L. aspera extract on involvement of cyclic guanosine monophosphate (cGMP) pathway. Values are presented as mean ± SEM (n= 5). MELA: Methanolic extract of L. aspera. * p < 0.05 compared with the control group (Dunnett's test).*

Involvement of ATP-sensitive K+ channels pathway

The writhing response of the test extract and glibenclamide on mice are presented in table 7. Single administration of glibenclamide (10 mg/kg), there was no change in writhing response as observed with the treatment of acetic acid. When *L. aspera* and glibenclamide used together, the effect of *L. aspera* was decreased at 500 and 750 mg/kg.

Treatment	Dose (mg/kg)	Number of writhing	Inhibition (%)
Control	0.1 mL/ mouse	65.60 ± 1.92	-
Glibenclamide (GB)	10	54.10 ± 1.14*	17.53
MELA + GB	250 +10	39.40 ± 1.38*	39.94
MELA + GB	500 +10	27.30 ± 1.31*	58.38
MELA + GB	750 +10	15.50 ± 1.44*	76.37

Table 7: *Effects of L. aspera extract on involvement of ATP-sensitive K^+ channel pathway. Values are presented as mean ± SEM (n = 5). MELA: Methanolic extract of L. aspera. * p < 0.05 compared with the control group (Dunnett's test).*

Discussion

The present work aimed to evaluate the antinociceptive effects of *Leucas aspera* leaves in different animal models, as the plant is reported to have activity against pain. The presently available drugs may have various side effects. For this reason, natural products are used to relieve pain and inflammation of human system. It is easily available, less costly and have little or no side effects [35].

The hot plate method is a central antinociceptive test which exerts antinociceptive effect via the central nervous system [36]. MELA appeared a significant antinociceptive effect by increasing the latency time at 500 mg/kg and 750 mg/kg doses in hot plate test (Table 1). Naloxone did not show any remarkable reduction of antinociceptive effect of *L. aspera*; while morphine (5 mg/kg) confirmed a significant

antinociceptive effect. Morphine sulphate, a well known drug, demonstrated a stronger antinociceptive effect than MELA. The tail immersion test is used as intense pain model, and the response of mice is selective for centrally acting analgesics. Additionally, the peripherally acting drugs are recognized to be inactive on heat-induced pain response [37]. In this test, the extract effect was more prominent after 90 min at 250, 500 and 750 mg/kg (Table 2). Morphine (5 mg/kg, i.p.) showed a notable antinociceptive effect. It was antagonized by naloxone in this test for antinociceptive activity. Both hot plate and tail immersion tests are used to measure the latency time of mice against thermal stimuli and here these tests are used for supraspinal and spinal reflex, respectively [38]. The tail immersion model monitors a spinal reflex involving μ_2- and δ-opioid receptors, whereas the hot plate demonstrates supraspinal reflex mediated by μ_1- and μ_2-opioid receptors [39].

Acetic acid-induced writhing test is used here to attribute visceral pain, which evaluate peripherally active analgesics [40] and it trigger localized inflammatory response by releasing free arachidonic acid from tissue phospholipid [41] connected with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products [42]. Regarding the present study, MELA produced significant antinociceptive effect (Table 3). The dose 500 mg/kg which was produced in 59.79% inhibition of antinociceptive effect. The higher dose 750 mg/kg showed the protection against the acetic acid induced writhing in 82.74%, while diclofenac sodium showed 84.63% reduction in the writhes count. The number of writhing renders antinociceptive effect sooner by inhibition of prostaglandin synthesis and follows peripheral mechanism of pain inhibition [43]. The formalin test is an efficient process for estimating antinociceptive effects. The neurogenic phase is possibly a direct result of paw stimulation and the late phase is due to the release of histamine, serotonin, bradykinin and prostaglandins [44]. Only p-cymene was capable to reduce nociceptive behavior in both phases of this test. The inflammatory phase depends on the release of excitatory amino acids, PGE2, NO, tachykinin, kinins and part of central sensitization, which is a combination of dynamic inputs from nociceptive afferents [45,46]. In formalin test, the extract repressed 50.40%, 62.11% and 72.35% inhibition of licking for the first phase while late phase exhibited 67.23%, 74.57%, and 84.74% inhibition of licking. Similarly, diclofenac sodium significantly inhibited the number of licking against the early (81.46 %) and late phases (85.87%) in mice. *L. aspera* showed an inhibition of the inflammatory pain in mice which is ascertained by an effective diminution of formalin test in second phase of nociception (Table 4).

Glutamate is deeply involved in the peripheral, spinal and supra-spinal neurotransmitter sites with glutamate receptors. It is strongly arbitrated by the activation of N-methyl-d-aspartate and α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptors [47]. The redemption of NO assist to improve the inflammatory pain reaction [48]. Oral administration of MELA at 500, and 750 mg/kg produced significant inhibition of antinociceptive activity in mice. It was 60.05 % and 80.96%. Similarly, diclofenac sodium showed 84.45 % inhibition of antinociceptive effect. It is reported that the activity of glutamate receptors contribute to the safeguarding of peripheral nociceptive process seen in the late phase of the formalin test. The current study indicated that glutamatergic system involved in the inflection of antinociceptive effects of MELA in mice (Table 5). Cinnamaldehyde is known to act as an agonist of the thermo-sensitive TRP ion channel TRPA1 [49]. It exhibits normal cold sensitivity or partial to severe deficits in cold pain sensitivity [50]. Intraplantar injection of the TRPA1 agonist 4-hydroxynonenal reduced mechanical paw withdrawal thresholds in mice and blockade of TRPA1 by systemically or locally administered antagonists reversed mechanical hyperalgesia in inflammatory and nerve injury models in mice [51,52]. The present study showed that *L. aspera* attenuated the cinnamaldehyde-induced pain model in mice (Table 5). The plant extract at 500 and 750 mg/kg exhibited significant antinociceptive activity. It was 52.19% and 75%, respectively. The standard drug diclofenac sodium showed a significant antinociceptive activity of 79.38%. This finding indicates that the test extract may interact with the TRPA1 receptor which is reducing the cinnamaldehyde-induced nociception.

The cGMP pathway depends on the synthesis of nitric oxide. It activates the guanylyl synthase enzyme [53]. As a result, cGMP plays an important role for the processing of up-down regulation of nociceptor. The action of cGMP on the ion channels depend on the activation of protein kinases and phosphodiesterases directly for the antinociceptive activity [54]. To detect the feasible involvement of cGMP in MELA induced antinociception, methylene blue was given before to inducing the nociception with the injection of acetic acid. The pre-treatment with methylene blue reduced the nociceptive action crucially which is caused by the acetic acid. Methylene blue promotes the antinoci-

ceptive activity by inhibiting peripheral nitric oxide-synthase and sGC, resulting in nitric oxide interference. When methylene blue and *L. aspera* were given together as pre-treatment, antinociceptive effect of *L. aspera* was increased in all doses of writhing test in mice (Table 6). This assessment express that the cGMP pathways may involved in the antinociceptive effects of *L. aspera* in writhing test in mice. Glibenclamide known to acts as an ATP-sensitive K^+ channel antagonist [55]. Several reports claimed that glibenclamide particularly blocked the ATP-sensitive K^+ channel, and there are no effects on another types of K^+ channel [56]. According to the test, the writhing response of the test extract and glibenclamide on mice are presented in table 7. When *L.* aspera and glibenclamide used together, the effect of *L. aspera* was decreased at 500, and 750 mg/kg. It showed significantly reversed antinociceptive activity of MELA in mice. However, our results suggest that MELA exerted its antinociceptive activity possibly through the opening of ATP-sensitive K^+ channel.

Conclusion

The antinociceptive activity of *L. aspera* is exerted by the involvement of both peripheral and central antinociceptive mechanisms. Our results provide evidence that the traditional use of MELA as analgesic in human diseases associated with pain is justified. However, further studies are needed to isolate the active principles responsible for the antinociceptive effects of *L. aspera* along with the establishment of efficacy and exact mechanism of action in suitable *ex vivo* or *in vivo* model.

Declarations

Ethics Approval and Consent to Participate

The study protocol was approved by the Institutional Animal Ethics, Medical Ethics, Biosafety, and Bio-security Committee (SUB/IAEC/17.01) of Stamford University Bangladesh. The set of rules followed for animal experiment were approved by the Institutional Animal Ethical Committee.

Competing Interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Volume 7 Issue 9 September 2019