International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 8 (4): 224-233, 2012
ISSN 1811-7775 / DOI: 10.3923/ijp.2012.224.233

Antioxidant and Analgesic Activities of *Lannea coromandelica* Linn. Bark Extract

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Abstract: Methanolic extract of *Lannea coromandelica* Linn. bark (MLCB) was subjected to evaluate its antioxidant and analgesic properties. The analgesic activity was determined for its central and peripheral pharmacological actions using hotplate as well as tail immersion method and acetic acid-induced writhing test with formalin induced pain in mice, respectively. To evaluate antioxidant potential of MLCB, total antioxidant activity, scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as well as total Reactive Oxygen Species (ROS) and assessment of reducing power were used. The extract, at the dose of 200 and 400 mg kg^{-1}, produced a significant ($p<0.05$) increase in pain threshold in hotplate and tail immersion methods whereas significantly ($p<0.05$) reduced the writhing caused by acetic acid and the number of licks induced by formalin in a dose dependent manner. In DPPH and total ROS scavenging method, MLCB showed good antioxidant potentiality with the IC_{50} value of 12.32±0.16 and 34.72±0.48 μg mL^{-1}, respectively with a good reducing power. In conclusion this study demonstrated the strong antioxidant and antinociceptive activities of methanolic extract of the bark of *L. coromandelica*. Altogether, these results suggest that the MLCB could be used as a potential antinociceptive agent along with its antioxidant potentiality.

Key words: Antioxidant, antinociceptive, *Lannea coromandelica*, free radical, formalin, ROS

INTRODUCTION

Free radical reactions are necessary for normal metabolism but can be detrimental to health as well and play a pivotal role in disease pathology (Gupta *et al*., 2007). Free radicals cause depletion of immune system antioxidants (Ouattara *et al*., 2011) change in gene expression and induce abnormal proteins and contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (El-Hela and Abdullah, 2010). Although, organisms have endogenous antioxidant defenses produce during normal cell aerobic respiration against ROS (Gill *et al*., 2011), other antioxidants are taken both from natural and synthetic origin. Synthetic antioxidants are widely used but their use is being restricted now a days because of their toxic and carcinogenic effects, leads to seek out natural antioxidants, without any undesirable effects, has increased greatly (Rechner *et al*., 2002). Phytoconstituents are the enormous source of natural antioxidants (Al-Mustafa and Al-Thunibat, 2008) and some natural antioxidant (e.g., rosemary and sage) are already exploited commercially either as antioxidant additives or a nutritional supplements but generally there is still a demand to find more information concerning the antioxidant potential of plant species. It has been mentioned the antioxidant activity of plants might be due to their phenolic, flavonoid, tannin and proanthocyanidin compounds (Frankle and Meyer, 2000; Rahman *et al*., 2011). Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Hossain *et al*., 2011). Pain can constant (chronic) or fleeting and come and go (acute). There are several types of pain, namely 'nociceptive', 'neurogenic', 'neuropathic' and 'psychogenic', which are associated with a stimulation of nociceptors, damage to neuronal tissue, dysfunction of a nerve, or psychological factors, respectively (Millan, 1999). The direct and indirect action of chemical mediators, such as arachidonic acid metabolites (prostaglandins and leukotrienes), peptides,

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serotonin, acetylcholine, cytokines, nitric oxide, among others, which can be produced or released following tissue injury or by exogenous irritants (formalin, acetic acid), are responsible for the multiplicity of events that occur during pain transmission, in both the peripheral and central nervous systems (Belfrage *et al*., 1995; Sawynok, 1998; Besson, 1999). Moreover, various free radicals as well as Reactive Oxygen Species (ROS) are also responsible for the induction of short-term algesia (Chung, 2004) and triggers some second messengers, are involved in sensitization of dorsal horn neurons that plays a fundamentally important role in neuropathic pain (Ali and Salter, 2001; Zhang *et al*., 2003).

Nature, immense source of pharmacologically active molecules, has been used for the treatment of various incurable diseases (Najiah *et al*., 2011; Nithya and Baskar, 2011). Plant extracts have been used for centuries, as popular remedies against several health disorders without any chemical modification (Nenaah and Ahmed, 2011). Medicinal plant have great value to phytochemists because of their medicinal properties (Oladosu *et al*., 2011) so that, the study of plants that have been traditionally used as pain killers should still be seen as a fruitful and logical research strategy in the search for new analgesic drugs and pain mechanisms (Calixto *et al*., 2000). Despite the severe adverse effects of morphine, steroidal or non steroidal anti-inflammatory agents are used for the treatment of pain clinically; naturally occurring agents with reduced side effects are required to substitute this chemical therapeutics. The genus Lannea belongs to the family Anacardiaceae and consists of 40 species. *Lannea coromandelica* L. is a deciduous tropical tree widely distributed in Bangladesh, India and some other tropical countries. The bark of *L. coromandelica* was used for skin disease (Franco and Narasimhan, 2009), injuries and hematochezia (Heda *et al*., 2009), hypotensive (Singh and Singh, 1996), antifungal and antibacterial (Queiroz *et al*., 2003) agent. The stem bark of this plant was used by the Garo tribes in Madhapur poorest region of Bangladesh to treat seminal weakness and excessive seminal emission (Mia *et al*., 2009). The leaf juice was orally taken to relieve ulcers and pain (Zhenga and Xing, 2009). It was also claimed to used as antidote in coma caused by narcotics, to treat dyspepsia, gout, dysentery, sore eyes, leprosy and bruises (Upadhyay *et al*., 2010). Literature reviews indicated that no studies combining the analgesic and antioxidant of the bark of *L. coromandelica* have so far been undertaken. Taking this in view and as a part of our ongoing research (Alam *et al*., 2011; Hossain *et al*., 2011) on Bangladeshi medicinal plants, the present study aimed to evaluate the analgesic activity of the methanolic bark extracts of *L. coromandelica* along with their *in vitro* antioxidant activity.

MATERIALS AND METHODS

Plant material: The bark of the plant of *Lannea coromandelica* Linn. was collected from the botanical garden of Pharmacy department, Jahangirnagar University, Bangladesh during January 2009. The plant material was taxonomically identified by the National Herbarium of Bangladesh whose voucher specimen No. JU290 is maintained in our laboratory for future reference.

Preparation of plant extract: The plant material was shade-dried with occasional shifting and then powdered with a mechanical grinder, passing through sieve #40 and stored in a tight container. The dried powder material (1 kg) was refluxed with methanol (MeOH) for three hours. The total filtrate was concentrated to dryness, *in vacuo* at 40°C to render the MeOH extract (390 g). The preliminary phytochemical analysis was performed to identify the constituents present in the extract (Kokate, 1994).

Chemicals: Ammonium molybdate, were purchased from E. Merck (Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, quercetin, and potassium ferric cyanide and 2',7'-dichlorofluorescein-diacetate (DCFH-DA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Nalbuphine, Diclofenac-Na was collected from Square Pharmaceuticals Ltd., Bangladesh. All other chemicals and reagents were of analytical grade.

In vitro antioxidant activity

Determination of total antioxidant capacity: The antioxidant activity of the MLCB was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al*. (1999). The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. Extract (0.3 mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (Shimadzu, UV-150-02) against blank after cooling to room temperature. Methanol (0.3 mL) is used as the blank experiment. The antioxidant activity is expressed as the number of equivalents of ascorbic acid using the following formula:

$$C = \frac{(c \times V)}{m}$$

where, C-total antioxidant activity, mg g^{-1} plant extract, in Ascorbic acid, c-the concentration of ascorbic acid established from the calibration curve (mg mL^{-1}); V-the volume of extract (mL); m-the weight of pure plant extract, g.

Free radical scavenging activity measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH): The free radical scavenging activity of MLCB, based on the scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca *et al.* (2001). MLCB (0.1 mL) was added to 3 mL of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated from $[(A_0-A_1)/A_0]\times100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration (µg mL) versus % inhibition.

Measurement of the inhibition of the total ROS generation: Mice kidney homogenates, prepared from the kidneys of freshly killed male Swiss albino mice, weighing 30-39 g, were mixed with or without a suspension of extracts and then incubated with 12.5 µM DCFH-DA, at 37°C for 30 min. Phosphate buffer (50 mM, pH 7.4) was used. DCFH-DA is a stable compound, which easily diffuses into cells and is hydrolyzed by intracellular esterase to yield a reduced non-fluorescent compound, DCFH, which is trapped within the cells. The ROS produced by cells oxidize the DCFH to the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF). The fluorescence intensity of the oxidized DCF was monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation and emission wavelengths of 460 and 530 nm, respectively (Label and Bondy, 1990). IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration versus % inhibition.

Reducing power activity: The reducing power of MLCB was determined according to the method previously described (Oyaizu, 1986). Extracts at different concentrations in 1 mL of 10% DMSO were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1%) and then the mixture was incubated at 50°C for 30 min. Afterwards, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of upper layer solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

***In vivo* analgesic activity**

Animal: Albino mice (25-30 g) of both sexes were used for assessing biological activity. The animals were maintained under standard laboratory conditions and had free access to food and water *ad libitum*. The animals were allowed to acclimatize to the environment for 7 days prior to experimental session. The animals were divided into different groups, each consisting of five animals which were fasted overnight prior to the experiments. Experiments on animals were performed in accordance with guidelines of the Institutional Animal Ethics Committee, Atish Dipankar University of Science and Technology, Dhaka, Bangladesh. Animal treatment and maintenance for acute toxicity and analgesic effects were conducted in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Atish Dipankar University of Science and Technology, Dhaka, Bangladesh.

Acute toxicity study: Acute oral toxicity assay was performed in healthy nulliparous and non pregnant adult female albino Swiss mice (25-30 g) divided into different groups. The test was performed using increasing oral dose of the MLCB in water (50, 100, 200, 500, 1000 mg kg^{-1} b.wt.), in 20 mL kg^{-1} volume to different test groups. Normal group received water. The mice were allowed to feed *ad libitum,* kept under regular observation for 48 h, for any mortality or behavioral changes (Sanmugapriya and Venkataraman, 2006).

Hot plate method: The animals were divided into four groups with five mice in each group. Group I animals received vehicle (1% Tween 80 in water, 10 mL kg^{-1} b.wt.), animals of Group II received nalbuphine at 10 mg kg^{-1} b.wt. while animals of Group III and Group IV were treated with 200 and 400 mg kg^{-1} b.wt. (p.o.) of the MLCB. The animals were placed on Eddy's hot plate kept at a temperature of (55±0.5)°C. A cut off period of 15 sec, was observed to avoid damage to the paw (Franzotti *et al.*, 2000). Reaction time was recorded when animals licked their fore or hind paws, or jumped prior to 0, 30, 60 and 90 min after oral administration of the samples (Eddy and Leimback, 1953; Malairajan *et al.*, 2006; Toma *et al.*, 2003).

Tail immersion test: The procedure is based on the observation that morphine like drugs selectively prolongs the reaction time of the typical tail withdrawal reflex in mice (Toma *et al.*, 2003). The animals were treated as discussed above. From 1-2 cm of the tail of mice was immersed in warm water kept constant at 55°C. The reaction time was the time taken by the mice to deflect their tails. The first reading was discarded and the reaction time was recorded as a mean of the next three readings. A latency period of 20 s was defined as complete analgesia and the measurement was then

stopped to avoid injury to mice. The latent period of the tail-flick response was determined before and 0, 30, 60 and 90 min after the administration of drugs.

Acetic acid-induced writhing test: The analgesic activity of the samples was also studied using acetic acid-induced writhing model in mice. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% v/v acetic acid but Diclofenac-Na was administered intraperitoneally 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min (Ahmed *et al*., 2004).

Formalin test: The antinociceptive activity of the drugs was determined using the formalin test described by Dubuisson and Dennis (1977). Control group received 5% formalin. 20 μL of 5% formalin was injected into the dorsal surface of the right hind paw 60 min after administration of MLCB (200 and 400 mg kg^{-1}, p.o.) and 30 min after administration of Diclofenac Na (10 mg kg^{-1}, i.p.). The mice were observed for 30 min after the injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 min post formalin injection is referred to as the early phase and the period between 15 and 30 min as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch.

Statistical analysis: All values were expressed as the Mean±SEM of three replicate experiments. The analysis was performed by using SPSS statistical package for WINDOWS (version 16.0; SPSS Inc, Chicago). Results related to the reducing power activities were statistically analyzed by applying the Student t-test and $p<0.001$ were considered to be statistically significant. All *in vivo* data are subjected to ANOVA followed by Dunnett's test and $p<0.05$ were considered to be statistically significant.

RESULTS

The qualitative chemical analysis of *Lannea coromandelica* showed the positive result for the presence of glycosides, flavonoids, triterpenoids, tannins, and steroids.

In vitro antioxidant activity

Total antioxidant capacity: Total antioxidant capacity of MLCB is expressed as the number of equivalents of ascorbic acid (Table 1). Total antioxidant capacity of MLCB was found to be 50.59±0.19 mg g^{-1} equivalent of ascorbic acid.

DPPH radical scavenging activity: The percentage (%) scavenging of DPPH radical was found to be concentration dependent with the IC_{50} value of 12.12±0.16 μg mL^{-1}, while IC_{50} value of standard ascorbic acid was found to be 12.22±0.11 μg mL^{-1} (Table 1).

Inhibition of total ROS generation: The percentage inhibition of ROS generation was illustrated in table 1 and it is observed that scavenging of ROS by the extract is also concentration dependent with the IC_{50} value of 13.72±0.48 μg mL^{-1}, while IC_{50} value of standard trolox was found to be 8.66±0.11 μg mL^{-1}.

Reducing power ability: For the measurement of the reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of MLCB and compared with standards (gallic acid, quercetin and ascorbic acid) (Fig. 1). Like the antioxidant activity, the reducing power of MLCB was found to be concentration dependent and statistically significant ($p<0.001$).

In vivo analgesic activity

Hot plate method: Result of hotplate test is shown in Fig. 2. Both doses of the extract produced a dose dependent increase in latency time when compared with the vehicle. The result was found to be statistically significant ($p<0.05$-0.001).

Tail immersion test: The tail withdrawal reflex time following administration of the MLCB was found to increase with increasing dose of the sample. The result was statistically significant ($p<0.05$-0.001) and was comparable to the reference drug nalbuphine (Fig. 3).

Acetic acid-induced writhing test: Table 2 shows the effects of the extract of on acetic acid-induced writhing in mice. The oral administration of both doses of MLCB significantly ($p<0.001$) inhibited writhing response induced by acetic acid in a dose dependent manner.

Formalin test: MLCB (200 and 400 mg kg^{-1}, p.o.) significantly ($p<0.001$) suppressed the licking activity in either phase of the formalin-induced pain in mice in a dose dependant manner (Table 3). MLCB, at the dose of

Table 1: Antioxidant activities of the MLCB on Total antioxidant capacity, DPPH and Total ROS assay methods

Sample	Total antioxidant capacity mg g^{-1} extract (in ASC)[a]	DPPH IC_{50} (μg mL^{-1})	Total ROS IC_{50} (μgmL^{-1})
MLCB	50.59±0.19	12.12±0.16	13.72±0.48
Ascorbic acid		12.22±0.11	
Trolox			8.66±0.11

[a]Ascorbic acid equivalents (ASC), (mg g^{-1} of each extract) for the total antioxidant capacity, Values are expressed as Means±SEM of triplicate experiments

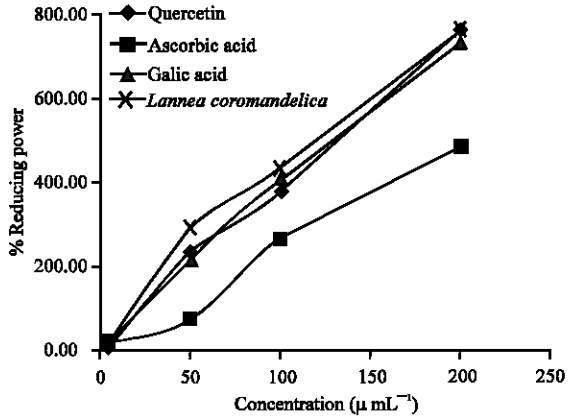

Fig. 1: Reducing power of *L. coromandelica*, quercetin, ascorbic acid and gallic acid by spectrophotometric detection of Fe^{3+} to Fe^{2+} transformation

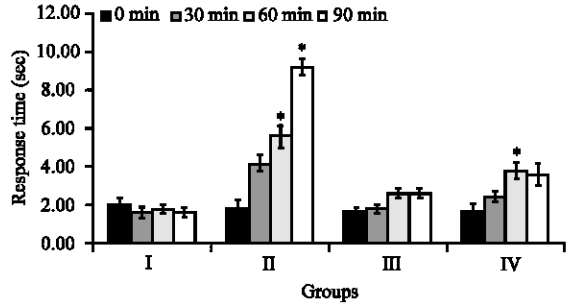

Fig. 2: Effects of the MLCB on latency to hotplate test. Values are Mean±SEM, (n = 5), *p<0.05, Dunnett test as compared to control. Group I animals received vehicle (1% Tween 80 in water), Group II received Nalbuphine 10 mg kg^{-1} b.wt., Group III and Group IV were treated with 200 and 400 mg kg^{-1} b.wt. (p.o.) of the crude extract of *L. coromandelica*

Table 2: Effects of the MLCB on acetic acid-induced writhing in mice

Groups	Dose (mg kg^{-1})	No. of writhing	% inhibition
I	Vehicle	30.8	
II	10	9.8	68.18*
III	200	24.6	20.1
IV	400	14.8	51.9*

Values are Mean±SEM, (n = 5); * p<0.05, Dunnett test as compared to vehicle control. Group I animals received vehicle (1% Tween 80 in water), Group II received Diclofenac Na 10 mg kg^{-1} b.wt., Group III and Group IV were treated with 200 and 400 mg kg^{-1} b.wt. (p.o.) of the MLCB

Table 3: Effect of MLCB in hindpaw licking in the formalin test in mice

Groups	Dose (mg kg^{-1})	Early phase (sec)	% protection	Late phase (sec)	% protection
I	Vehicle	35.67±1.38	-	46.0±1.03	-
II	10	16.0±0.90*	55.14	18.83±0.70*	59.05
III	200	27.5±0.76	22.89	21.5±0.95*	53.26
IV	400	20.33±0.65*	42.99	19.0±1.46*	58.89

Values are Mean±SEM, (n = 5), *p<0.05; Dunnett test as compared to vehicle control. Group I animals received vehicle (1% Tween 80 in water), II received Diclofenac Na 10 mg kg^{-1} b.wt., Group III and Group IV were treated with 200 and 400 mg kg^{-1} b.wt. (p.o.) of the MLCB

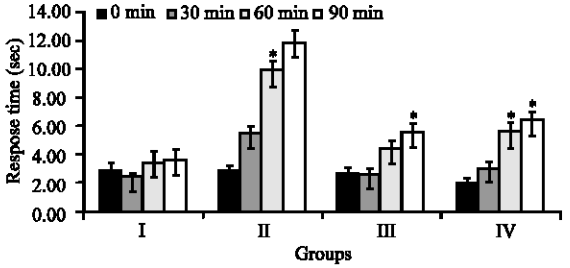

Fig. 3: Effects of the MLCB on tail withdrawal reflex of mice induced by tail immersion method. Values are Mean±EM, (n = 5); *p<0.05, Dunnett test as compared to control. Group I animals received vehicle (1% Tween 80 in water), Group II received Nalbuphine 10 mg kg^{-1} b.wt., Group III and Group IV were treated with 200 and 400 mg kg^{-1} body weight (p.o.) of the crude extract of *L. coromandelica*

400 mg kg^{-1} b.wt., showed the more licking activity against both phases of formalin-induced pain than that of the standard drug diclofenac-Na.

DISCUSSION

The upshots of oxidative stress are serious and sometimes manifested by increased activities of enzymes involved in oxygen detoxification (Lompo *et al*., 2007). Therefore, the identification of new antioxidant may reduce the risk of various chronic diseases involved in free radicals. To determine the efficacy of natural antioxidants either as pure compounds or as plant extract, a great number of *in vitro* methods have been developed in which antioxidant compounds act by several mechanisms. Determination of specific antioxidant species might be less useful than the knowledge of the total antioxidant capacity of a sample. The knowledge of total antioxidant activity can be useful in the analysis of changes in plasma antioxidant activity related to oxidative stress, or the understanding of structure–activity relationships of pure antioxidant species. Because of its simplicity and the cheap reagents it uses, the phosphomolybdenum method is an alternative to the methods already available for the evaluation of total antioxidant capacity. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the compounds having antioxidant property and is successfully used to quantify vitamin E in seeds (Prieto *et al*., 1999). $DPPH^{\bullet}$ is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Nakayama *et al*., 1993) and is usually used as a substrate to evaluate the antioxidant activity of a compound (Chang *et al*., 2002). Based on the

data obtained from this study, DPPH radical scavenging activity of MLCB (IC_{50} 12.12±0.16 μg mL^{-1}) was similar to the standard ascorbic acid (IC_{50} 12.76±0.11 μg mL^{-1}). Moreover, it was revealed that MLCB did show the proton donating ability and could serve as free radical inhibitor or scavenger. A direct correlation between antioxidant capacity and reducing power of certain plant extracts has been reported (Tanaka *et al*., 1988). The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Duh *et al*., 1999). Because a substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atom (Jayaprakasha *et al*., 2001; Khanam *et al*., 2004), the ferric reducing property of plant extracts (Fig. 1) implies that they are capable of donating hydrogen atom in a dose dependent manner. Polyphenolic compounds, like flavonoids, tannins and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity (Kahkonen *et al*., 1999). Phenolic compounds are understood to induce the cellular antioxidant system; increase approximately 50% cellular glutathione concentration. Flavonoids are important in the modulation of γ-glutamylcysteine synthase in both cellular antioxidant defenses and detoxification of xenobiotics (Muchuweti *et al*., 2007). Rahman *et al*. (2008) reported that phenolic compounds were the major phytoconstituents of *L. coromandelica* and may responsible for causing the paramount antioxidant effect.

The hotplate and tail immersion methods are commonly used for assessing central antinociceptive response. Both methods are further distinguished by their tendency to respond to the pain stimuli conducting through neuronal pathways as tail immersion mediates a spinal reflex to nociceptive stimuli, while the hot plate involves higher brain functions and is a supraspinally organized response (Chapman *et al*., 1985). Narcotic analgesics inhibit both peripheral and central mechanism of pain, while non steroidal anti-inflammatory drugs inhibit only peripheral pain (Elisabetsky *et al*., 1995; Pal *et al*., 1999). As noted, nalbuphine, the reference narcotic analgesic drug (5 mg kg^{-1}, p.o.) exhibited significant and paramount analgesic effects in both the hot plate (supra spinal) as well as the tail immersion (spinal) test; whereas, MLCB (200 and 400 mg kg^{-1}, p.o.) also produced a statistically significant but lesser in degree antinociceptive response to that of nalbuphine in both test suggesting that the plant extract may act as a narcotic analgesic. However, the mechanism(s) behind the central analgesic response of MLCB in both tested methods is not completely understood and may need further investigation.

On the other hand, acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics and represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from the tissue phospholipid (Ahmed *et al*., 2006). The response is thought to be mediated by peritoneal mast cells (Ribeiro *et al*., 2000) acid sensing ion channels (Voilley, 2004) and the prostaglandin pathways (Hossain *et al*., 2006). The organic acid has also been postulated to act indirectly by inducing the release of endogenous mediators, which stimulates the nociceptive neurons that are sensitive to NSAIDs and narcotics (Adzu *et al*., 2003). It is well known that non-steroidal anti-inflammatory and analgesic drugs mitigate the inflammatory pain by inhibiting the formation of pain mediators at the peripheral target sites where prostaglandins and bradykinin are proposed to play a significant role in the pain process (Hirose *et al*., 1984). In addition, it was suggested that non narcotic analgesics produce their action by interfering with the local reaction to peritoneal irritation thereby reducing the intensity of afferent nervous stimulation in the acetic acid induced writhing test, a model of visceral pain (Vogel and Vogel, 1997). Therefore, it is likely that MLCB might have exerted its peripheral antinociceptive action by interfering with the local reaction caused by the irritant or by inhibiting the synthesis, release and/or antagonizing the action of pain mediators at the target sites. The above findings clearly demonstrated that both central and peripheral mechanisms are involved in the antinociceptive action of MLCB. The analgesic activity of MLCB could also be linked to the mechanism of action either on central nervous system or peripheral nervous system. Interestingly, compounds like flavonoids (Kim *et al*., 2004a) and steroids, triterpenes in part, have been shown to possess anti-inflammatory, analgesic activity and the claim made by Pritam *et al*. (2011). Based on the classes of compounds detected in MLCB extract, several mechanisms of action could be used to explain the observed activities of MLCB extract.

The formalin model normally postulates the site and the mechanism of action of the analgesic (Chau, 1989). This biphasic model is represented by neurogenic (0-5 min) and inflammatory pain (15-30 min), respectively (Hunskaar and Hole, 1987). Drugs that act primarily on the central nervous system such as narcotics inhibit both as steroids and NSAIDs suppress mainly the late phase (Adzu *et al*., 2003). The suppression of neurogenic and inflammatory pains by the extract might imply that it

contains active analgesic principle that may be acting both centrally and peripherally. This is an indication that the extract can be used to manage acute as well as chronic pain. The mechanism by which formalin triggers C-fibers activation remained unknown for a relatively long time. Recently, however, McNamara *et al.* (2007) demonstrated that formalin activates primary afferent neurons through a specific and direct on TRPA1, a member of the transient receptor potential family of cation channels, expressed by a subset of C-fiber nociceptors, and this effect is accompanied by increased influx of Ca^{2+} ions. TRPA1 cation channels at primary sensory terminals were also reported to mediate noxious mechanical stimuli (Kerstein *et al.*, 2009). These experiments suggest that Ca^{2+} mobilization through TRPA1 cation channels is concomitant with noxious chemicals and mechanical stimuli as they produce their analgesic action. It is likely that the inhibitory effect of MLCB to pain response is due to inhibit the increase of the intracellular Ca^{2+} through TRPA1, presumably evoked by formalin. So, MLCB may contain substances that affect the metabolism of Ca^{2+}. Literature survey revealed that tannins, triterpenoids and flavonoid are present in *L. coromandelica* (Islam and Tahara, 2000; Islam *et al.*, 2002). Flovonoids, for example, have been found to suppress the intracellular Ca^{2+} ion elevation in a dose dependent manner, as well as the release of proinflammatory mediators such as TNFα (Kempuraj *et al.*, 2005).

Flavonoids may increase the amount of endogenous serotonin or may interact with 5-HT_2A and 5-HT_3 receptors which may be involved in the mechanism of central analgesic activity (Annegowda *et al.,* 2010). Preliminary phytochemical screening revealed the presence of flavonoids and also supported by previous researchers; for the presence of several therapeutically valued flavonoids from the *L. coromandelica* (Islam and Tahara, 2000; Islam *et al.*, 2002). Moreover, MLCB showed significant analgesic activity in the entire experimental model which may be due to its high flavonoid content as well as free radical scavenging activity, as these free radicals are involved during pain stimulation and antioxidants showed reduction in such pain (Kim *et al.*, 2004b). It is well established that sensitization dorsal horn cells in the spinal cord (central sensitization) plays a fundamentally important role in neuropathic pain. Excessive ROS affects central sensitization and triggers second messengers system that involved in sensitization of dorsal horn neurons and also activates spinal glial cells, which in turn play an important role in chronic pain (Raghavendra *et al.*, 2003). In addition, recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species form phagocytes invading the inflammation sites (Parke and Sapota, 1996). There are also reports on the role of flavonoid, a powerful antioxidant (Vinson *et al.*, 1995; Brown and Rice-Evans, 1998), in analgesic activity primarily by targeting prostaglandins (Ramesh *et al.*, 1998; Rajnarayana *et al.*, 2001). Moreover, flavonoids have the ability to inhibits eicosanoid biosynthesis. Eicosanoids, such as prostaglandins are involved in various immunological responses and are the end products of the cyclooxygenase and lipoxygenase pathways (Jothimanivannan *et al.*, 2010). Tannins are also found to have a contribution in antinociceptive activity (Ramprasath *et al.*, 2006). Again the plant extract demonstrated good antioxidant action in the tested models. So it can be assumed that cyclooxygenase (COX) inhibitory activity along with antioxidant activity may reduce the production of free arachidonic acid from phospholipid or may inhibit the enzyme system responsible for the synthesis of prostaglandins and ultimately relieve pain-sensation.

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