Original Article

Pharmacological Evaluation of Analgesic and Antivenom Potential from the Leaves of Folk Medicinal Plant *Lobelia nicotianaefolia*

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ABSTRACT

Lobelia nicotianaefolia is a medicinal plant found in the Western ghat region of Karnataka (India), with a common name [Heddumbe]. The leaves are used in traditional health care system to treat various ailments including pain and snake bites but despite this there are no proper reports supporting it. In the present study to understand its medicinal benefits, the leaf extracts were assessed for phytochemical contents and various pharmaceutical properties. The chloroform, ethanol and aqueous fractions of leaves were screened for phytochemicals and tested for acute toxicity by determining LD_{50} values in mice. Antimicrobial potency was assessed using pathogenic bacterial and fungal species. The central and peripheral analgesic activity was evaluated by hot plate and acetic acid induced writhing method respectively. Venom neutralizing ability was performed in Swiss albino mice against Russell's viper whole venom and its PLA2 fractions. The results inferred that LD_{50} values for chloroform and ethanolic leaf fractions are 0.5 g/kg and 1 g/kg respectively, whereas aqueous extract was not toxic even at 4 g/kg in mice. Among the three extracts, ethanol fraction have showed a significant anti-microbial, analgesic and anti-venom properties whereas the activity was moderate in chloroform fraction and less in aqueous fraction. Taken together this study validates the strong pharmacological properties of *Lobelia nicotianaefolia,* which was traditionally used to treat pain and snake bite.

Keywords: *Lobelia nicotianaefolia*, Analgesic; Antivenom, Russell's viper venom, PLA2.

INTRODUCTION

The use of plants as herbal medicines in curing several ailments goes parallel to the human civilization and since ancient times, different plant part extracts have been

used as traditional medicines against various diseases[1-3]. Some of the medicinal plants have also been used to treat diseases caused by microorganisms[4,5]. Drug discovery from plants involves a multidisciplinary approach combining botanical, ethnobotanical, phytochemical and biological techniques. They continue to provide us new chemical entities (lead molecules) for the development of drugs against various pharmacological targets[6-9]. This could be the reason why 80% of people in the developing countries rely on traditional plant based medicines for their initial health care needs[10-12].

Lobelia nicotianaefolia, also called Heddumbe (in Kannada) or wild tobacco is a medicinal plant, commonly found in the Western Ghats region of Karnataka, which is one of the biodiversity hot spots of the world. The plant has been recorded to contain several alkaloids. It has a long history of use as an herbal remedy to treat various ailments that include snake bite, scorpion bite, epilepsy, and number of respiratory diseases such as asthma, bronchitis, pneumonia, and cough. In India, infusion of leaves is used as antispasmodic and expectorant and even today, lobelia is suggested to help clear mucus from the respiratory tract[13,14]. The plant leaves were also used to treat sciatica, back pain and speedy recovery of wounds[15,16].

Despite its extensive use in traditional healthcare system, the plant is not much explored for its beneficial effect. With regard to its ethno medical use in treating epilepsy, a recent study have found that its antiepileptic activity is due to Lobeline an alkaloid that is present in *Lobelia nicotianaefolia*[17]. However it's potential to treat pain and snake bites have not been explored and investigated so far. To address the analgesic and antivenom potential of *Lobelia nicotianaefolia*, in the present study we have focused on evaluating the *Lobelia nicotianaefolia* Leaf (LNL) extracts for phytochemical contents with sequential organic and aqueous extract system, after which the extracts were further analysed for pharmaceutical activities following various biochemical and pharmaceutical methods. These studies have revealed that *Lobelia nicotianaefolia* leaf extracts have significant biological activity, as an analgesic, antivenom, and antimicrobial agent.

MATERIALS AND METHODS

Plant material and preparation of extract

Lobelia nicotianaefolia was identified at Shimoga, Karnataka with the help of expert taxonomist and local people help during September 2013. The collected plant leaves were shade dried and powdered, which is then subjected to sequential extraction using the solvents in the order of chloroform followed by ethanol as per the standard soxhlet extraction method[18,19]. The plant material used in the ethanol extract is dried and used for aqueous extraction. Twenty gram of powder is weighed and soaked in 50mL distilled water for 24 h and it is kept for stirring in a magnetic stirrer for another 24 h for easy extraction of the hydrophilic water soluble components. The extract obtained was filtered and distilled to concentrate the extract leaving behind the phytochemical components.

Drugs and chemicals

Nutrient agar, Saborauds Dextrose Agar (SDA), ethanol, chloroform, acetic acid streptomycin, clotrimazole and the chemicals used for phytochemical screening were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. *Daboia russelli* (Russell's viper) snake venom and partially purified PLA2 fraction was a gift sample from the Department of Studies and Research in Biochemistry, University of Mysore, Mysore. The standard drugs, Diclofenac sodium and Pentazocine, were

obtained from Novartis and Ranbaxy respectively. All other reagents used in these experiments were of analytical grade.

Phytochemical analysis of the extracts

Qualitative analysis was carried out on chloroform (C-LNL), ethanol (E-LNL), and aqueous (A-LNL) fractions of *Lobelia nicotianaefolia* leaves (LNL) to evaluate the presence of medicinally active phytochemicals. The tests were carried out to test the presence of alkaloids, saponins, tannins, glycosides, steroid or terpenoids, carbohydrates and flavonoids. The abundance of the phytochemicals were assessed and graded as +, ++ based on the methods described earlier[20].

Anti-microbial activity of the prepared extracts

The antimicrobial activity of the three LNL extracts was carried out comparatively with that of standard antibiotics by well in agar method against nine pathogenic microorganisms. It included gram positive bacteria, (*Streptococcus pyogenes, Pseudomonas aeruginosa, Staphylococcus aureus), gram* negative bacteria (*Klebsiella terrigena, Bacillus subtilis, Escherichia coli),* fungus *(Cryptococcus neoformens, Candida albicans)* and yeast (*Trichosporon).* The organisms were treated with all the three extracts. The extracts were dissolved in the control (sterile water) in the concentration of 10 mg/mL and incubated at 37°C for 24 h (for bacterial species) and 48 h (for fungal species).

Selection of animals and sample preparation

Swiss albino mice of 6-8 weeks old were used in the study. The animals had free access to food and water, and they were housed in a natural (12 h each) light-dark cycle. The animals were acclimatized for at least 5 days to the laboratory conditions before conducting experiments. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and the care of the laboratory animals was taken as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations. Animals were grouped as 4 mice per group[21]. Freshly prepared Suspensions of all the three fractions were prepared with saline at the concentration of 1 g/mL concentration as stock. The stock solutions of all the three extracts were accordingly diluted for the experiments to understand the analgesic and anti-venom activity of the extracts.

Acute toxicity test

The acute toxicity (LD_{50}) of the each extracted fraction was determined in Swiss albino mice using intra peritoneal route at the weight 25-30g by up and down / staircase method as per CPCSEA guidelines. Suspension of all three LNL extracts were diluted accordingly using saline and were administered intraperitoneally to different groups of mice at doses of 50 mg, 250 mg, 500 mg, 1000 mg and 2000 mg / kg body weight. The control was administered with 1mL of saline. Animals were observed for 48 h to study the general behaviour of animals, signs of discomfort and nervous manifestations. The death and behaviour of the animals in each group were recorded and were used for the assessment of approximate LD_{50} and acute toxicity level as well as dose fix for the further pharmacological studies[22].

Determination of Analgesic activity

Peripheral and central analgesic activity in mice was assessed by chemically as well as thermally induced pain using acetic acid induced writhing model and Eddy's hot plate assay respectively.

Peripheral analgesic activity

The peripheral analgesic activity was assessed according to the methods described by Witkin *et al*.[23], with slight modifications. Eight groups each with four mice were formed. The groups were treated as control (distilled water, *i.p*. 10 mL/kg body weight) and standard (Diclofenac sodium 5 mg/kg *i.p*.) while test groups received suspensions of chloroform, ethanol and aqueous extract from leaves of *Lobelia nicotianaefolia* (100 and 200 mg/kg *i.p*.) respectively. Acetic acid solution 0.6% v/v (10 mL/kg *i.p.*) was injected one hour after the treatment and number of writhes (i.e. index of pain reaction against chemical stimuli characterized by abdominal muscle contraction together with turning of trunk and extension of hind limbs) was counted over a period of 20 min. Analgesic activity was expressed as percentage of inhibition of writhes with respect to the control group.

Central analgesic activity

The central analgesic activity of LNL extracts were assessed in male albino mice, using Eddy's Hot plate model as described earlier[24]. In brief, Hot plate was maintained at 55 ± 1ºC. Albino mice were divided in eight groups. The animals were placed on the hot plate and the basal reaction time taken to cause a discomfort (licking of paw or jumping response whichever appeared first) was recorded at 0 min. Cut-off period 15 sec. was established to prevent damage to the paws. The treatment and groupings of mice was done in the same manner as documented in acetic acid induced writhing model except that standard group received Pentazocine (5 mg/kg *p.o.*). The reaction time in seconds was reinvestigated at 30, 60, and 120 min after the treatment. Changes in reaction time in drug treated and control were noted[25].

Antivenom activity

Venom and animal's source

The freeze-dried whole snake venom and partially purified PLA2 fraction (as powder) of *Daboia russelli* (Russell's viper) venom stored at 4°C was used to study the antivenom activity of the LNL extracts. Male inbreed Swiss albino mice 18-20 g were used for the experiment.

Venom lethal toxicity

The median lethal dose (LD_{50}) of *D. russelli* venom and PLA2 fraction was determined according to the method developed by Theakston and Reid 1983[26]. Various doses of venom and PLA2 fraction in 0.2 mL of physiological saline were injected into the tail vein of mice, using groups of 4 mice for each venom dose. The LD_{50} was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24 h of venom injection[27].

Antivenom potential of extracts

The anti-lethal potentials of *Lobelia nicotianaefolia* leaves extracts were determined against twice the LD_{50} determined for *D. russelli* venom. Various amount of *Lobelia nicotianaefolia* extracts (µL) were mixed with $2LD_{50}$ of venom sample and incubated at 37°C for 30 minutes and then injected intravenously into mice. Four mice were used at each anti-venom dose. Control mice received same amount of venom without leaf extracts. The median Effective Dose (ED_{50}) calculated from the number of deaths within 24h of injection of the venom/anti-venom mixture. ED_{50} was expressed as µl anti-venom leaf extract/mouse [26].

Statistical analysis

Data were expressed as means ± Standard Deviation (SD). The values were then subjected to one-way ANOVA

followed by Turkey's multiple comparison tests for significant difference. The level of significance was considered at $p \leq 0.05$ and $p \leq 0.01$.

RESULTS

Type of extracts and yield of phytoconstituents

Three different fractions of phytoconstituents were prepared by their solvent based extractability using sequential extraction. The extracts were dried by concentration methods as stated in methods and were measured by w/w to estimate the yields. The yields of different extraction methods were shown in Table1. The yield was slightly higher for aqueous extract compare to ethanol and chloroform extracts.

Phytochemical analysis

All the three different LNL extracts were analysed for the phytochemical constituents. The ethanol and chloroform extracts were found to contain high amount of phytochemical composition rich in glycosides, alkaloids, and carbohydrates as compared to aqueous extracts. The results were shown in Table 2.

Anti-microbial activity

The antimicrobial activity of all three LNL extract was done using different microorganisms and the experiment carried out as mentioned in the method section and the inhibition zone was recorded and compared with standard respective antimicrobial drugs. As shown in Table 3 and 4, chloroform and ethanol extract showed more antimicrobial activity when compared to aqueous extract, were the ethanol extract showed good inhibition result for *Streptococcus pyogenes* and the chloroform extract showed the highest 33 mm of inhibition for *Cryptococcus neoformens*.

Lethal dose (LD_{50}) value for the extracts

The LD_{50} values for all the three extracts obtained from *Lobelia nicotianaefolia* was evaluated in adult Swiss albino mice. The extracts were dissolved in saline and were administered intra-peritoneally as explained in the methods section. From the analysis, the LD_{50} value for chloroform extract was around 500-1000 mg/kg body weight. Ethanol extract was shown exactly around 1000 mg/kg whereas aqueous extract was identified to have LD_{50} around 2000 mg/kg body weight of mice. The dose for all the extracts was fixed around 100-200 mg/kg body weight of mice to conduct the pharmacological studies, which was determined to be about 5-10 fold lower compared to LD_{50} values.

Analgesic activity

Peripheral analgesic activity

Ethanol and chloroform extracts of LNL at 100 and 200 mg/kg *i.p.* administration to mice indicated significant analgesic activity against chemically induced pain. Chloroform extract at 100 and 200 mg/kg body weight showed significant reduction in number of writhes in 20 min at the rate of 28.82% and 48.48% respectively, whereas ethanol extract was proven to be more efficient with decreased rate of writhing of 61.73% and 62.60% and is compared to the effect shown by the positive control Diclofenac sodium (Fig 1A and 1B). The writhing found in acetic acid was taken as control and was compared with the test group.

Central analgesic activity

The mean latency time following the intraperitoneal administration of three different LNL extracts was shown in Fig 2A, 2B, and 2C. Similar to the results of acetic induced pain method, ethanol and chloroform extracts of LNL at 100 and 200 mg/kg produced a significant increase in

mean latency time throughout the observation period, i.e, at 0, 30, 60, and 120 min, compared to the control. The reference drug Pentazocine (5 mg/kg, *i.p*.) also showed a significant increase in mean latency time.

Antivenom activity of extracts

Venom and its fraction lethality

The LD_{50} dose for whole Russell's viper venom was found to be 8 µg/mice. The LD_{100} was identified as 15 µg/mice of average weight 22-26 g. However the venom fraction rich in PLA2 activity was also determined for its LD_{50} and LD_{100} values and is found to be 12 µg and 25 µg respectively. The LD_{100} values are used as control venom toxicity and the neutralizing ability of the three extracts were studied.

The neutralizing potency of the different extracts

Among the various LNL fractions, the ethanol and chloroform extracts have shown the significant neutralizing potential for the PLA2 fraction of the venom with ED_{50} (effective dose to save 50% of the mice population) value at 500 mg/kg body weight (Table 5). Whereas the aqueous extract does not show any neutralizing effect with the partially purified PLA2 fraction. None of the extracts showed significant neutralizing effect with the whole venom. However, the ethanol extract of *Lobelia nicotianaefolia* had shown a marked neutralizing potentiality at 500 mg/kg body weights which appeared to be the ED_{50} value for ethanol extract.

DISCUSSION

In order to validate the ethno-medical use of *Lobelia nicotianaefolia* (Heddumbe), in treating various ailments, our present study have investigated the LNL extracts for the potential pharmaceutical activities like analgesic, antivenom and anti-microbial properties. The study reports that among chloroform, ethanol, and water, the solvents that were employed for the extraction, the ethanolic fraction was found to possess a significant pharmaceutical activity of all the three that were mentioned above. The common pathogenic microorganisms were employed to evaluate the antimicrobial activity of LNL extracts and results have illustrated that the ethanolic and chloroform extracts of LNL have significantly inhibited the growth of Streptococcus *pyogenes* and *Cryptococcus neoformens* respectively, which shows the possibility of using *Lobelia nicotianaefolia* in treating infectious diseases caused by these organisms.

In this study the possibility of LNL extracts in alleviating pain, was investigated using peripheral and central analgesic models that employed chemical (abdominal writhing test) and thermal-induced nociception (hot plate test) respectively. Peripheral analgesic activity was evaluated by intraperitoneal injection of acetic acid which irritates the peritoneal cavity leading to stimulation of local nociceptors located at the surface of the peritoneal cavity[28]. This leads to the release of prostaglandins and other algogens with subsequent stimulation of pain nerve endings[29]. The effectiveness of LNL extracts in inducing central analgesia was studied through Hot plate test, which involves higher brain functions and is considered to be a supraspinally organized response. As evidenced from this study the LNL extracts have significantly inhibited the pain induced by Hot plate and acetic acid and this suggests that has produced both central and peripheral analgesic effect which may be due to the inhibition of chemical mediators. An assumption regarding its analgesic activity could be made which may also be probably due to the Lobeline, an alkaloid found in the *Lobelia nicotianaefolia*

that is found to increase the GABA (Gamma Amino Butryic Acid), a chief inhibitory neurotransmitter, in the brain of the mice[17], and interestingly this controls the propagation of pain signals from the periphery to higher central nervous system areas[30], thereby resulting in the inhibition of pain.

Recent evidences suggests that the snake bites have caused 94,000 deaths globally and 15,000 deaths in India and the commonest cause of fatal snake bite in southern India is found to be *Daboia russelli*, an Indian Subspecies of Russell's viper [31]. LNL extracts were subjected to antivenom studies in view of its traditional use for treating snake bites. The results from this study have implicated that ethanolic fraction of LNL showed a marked neutralization of phospholipase 2 (PLA 2) activities, an enzyme commonly found in the venom of Viperidae snakes, which are well known for their serious neurotoxic symptoms[32].

Taken together, this study reports that the LNL extracts posses a significant analgesic, antimicrobial and antivenom properties. This means that apart from alleviating the pain, it could also act as an antidote for the snake envenomation. Moreover this report validates the use of *Lobelia nicotianaefolia* in traditional health care system for treating pain and snake bite.

CONCLUSION

It is clear from the above study that the leaf extracts of *Lobelia nicotianaefolia* (Heddumbe) a folk medicinal plant, possess a strong pharmacological activity ranging from antimicrobial, analgesic, and antivenom characteristics. This is most relevant to its traditional use, during the treatment of cold, cough, mumps, stomach ache, tooth ache, scorpion bites and snakebite. However, further study is needed in order to understand the precise mechanism. In future experiments, studies with purified fractions of the extract, can be conducted for further pharmacological and toxicological characterization, such as the research on the mechanisms involved in the analgesic effect and antivenom activity.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Table 1. Percentage yield of crude fractions from the leaves of *Lobelia nicotianaefolia*

S. No.	Name of the fraction	Colour	Consistency	Percentage yield (gm)
1	Chloroform fraction (C-LNL)	Light green	Solid	30g
2	Ethanol fraction (E-LNL)	Black	Semi solid	38g
3	Aqueous fraction (A-LNL)	Brown	Sticky solid	47g

C-LNL: Chloroform fraction of *Lobelia nicotianaefolia*; E-LNL: Ethanol fraction of *Lobelia nicotianaefolia*; A-LNL: Aqueous fraction of *Lobelia nicotianaefolia.*

Table 2. Qualitative phytochemical analysis of crude fractions from the leaves of *Lobelia nicotianaefolia*

S. No.	Name of the phytoconstituent	C-LNL	E-LNL	A-LNL
1	Carbohydrates	++	+++	+
2	Amino acids and proteins	-	-	-
3	Tannins and phenols	-	-	-
4	Saponins	-	-	-
5	Terpenoids	+	+	+
6	Glycosides	++	+++	+
7	Flavonoids	-	-	-
8	Alkaloids	-	++	+

C-LNL: Chloroform fraction of *Lobelia nicotianaefolia*; E-LNL: Ethanol fraction of *Lobelia nicotianaefolia*; A-LNL: Aqueous fraction of *Lobelia nicotianaefolia.*

Table 3. Anti-bacterial activity of the *Lobelia nicotianaefolia* extracts expressed as zone of inhibition in mm

Organism	Control (Sterile water)	C-LNL (10mg/ml)	E-LNL (10mg/ml)	A-LNL (10mg/ml)	Streptomycin 5mg/ml
E. coli	-	26	33	-	24
S. pyogenes	-	23	32	-	48
K. phemoniae	-	-	-	31	27
S. aureus	-	-	-	-	30
B. subtilis	-	-	-	-	25
P. aerugenosa	-	28	-	-	22

C-LNL: Chloroform fraction of *Lobelia nicotianaefolia*; E-LNL: Ethanol fraction of *Lobelia nicotianaefolia*; A-LNL: Aqueous fraction of *Lobelia nicotianaefolia.*

Table 4. Anti-fungal activity of the *Lobelia nicotianaefolia* leaf extracts expressed as zone of inhibition in mm

Organism	Control (Sterile water)	C-LNL (10mg/ml)	E-LNL (10mg/ml)	A-LNL (10mg/ml)	Clotrimazole 5mg/ml
Trichosporon	-	11	12	-	26
C. neoformens	-	33	13	13	28
C. albicans	-	20	24	-	25

C-LNL: Chloroform fraction of *Lobelia nicotianaefolia*; E-LNL: Ethanol fraction of *Lobelia nicotianaefolia*; A-LNL: Aqueous fraction of *Lobelia nicotianaefolia.*

Table 5. Antivenom activity of different doses of *Lobelia nicotianaefolia* Leaf (LNL) fractions on Swiss albino mice

Treatment	DOSE, *i.v.* (mg/kg)	Russel's viper whole venom (15 µg)		PLA2 fraction of R. Viper venom (25 µg)	
		Mice dead	Percent survival (%)	Mice dead	Percent survival (%)
Control (Saline)	10 ml/kg	0	100	0	100
Venom fraction	-	4	0	4	0
C-LNL	100	4	0	4	0
	200	4	0	4	0
	500	3	25	2	50
E-LNL	100	4	0	4	0
	200	4	0	3	25
	500	2	50	2	50
A-LNL	100	4	0	4	0
	200	4	0	4	0
	500	4	0	4	0

C-LNL: Chloroform fraction of *Lobelia nicotianaefolia*; E-LNL: Ethanol fraction of *Lobelia nicotianaefolia*; A-LNL: Aqueous fraction of *Lobelia nicotianaefolia.*

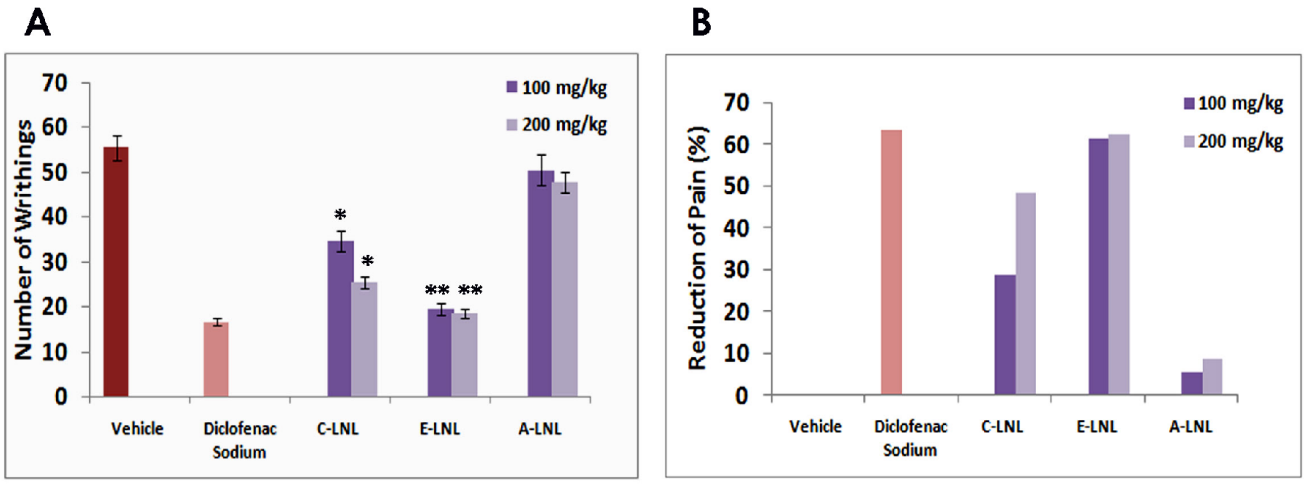

Figure 1. Peripheral Analgesic effect of different doses of *Lobelia nicotianaefolia* leaf fractions in acetic acid induced writhing in Swiss albino mice. **(A)** Ethanol and Chloroform extracts (100 and 200 mg/kg) showed significant reduction in number of writhing compared to control.**(B)** Ethanol extract have exhibited higher percentage of pain reduction comparable to the standard drug

C-LNL: Chloroform fraction of *Lobelia nicotianaefolia*; E-LNL: Ethanol fraction of *Lobelia nicotianaefolia*; A-LNL: Aqueous fraction of *Lobelia nicotianaefolia.* Values are the mean ± SD, n = 4 in each group (statistically significant values are $^{*}p < 0.05$., $^{**}p < 0.01$).

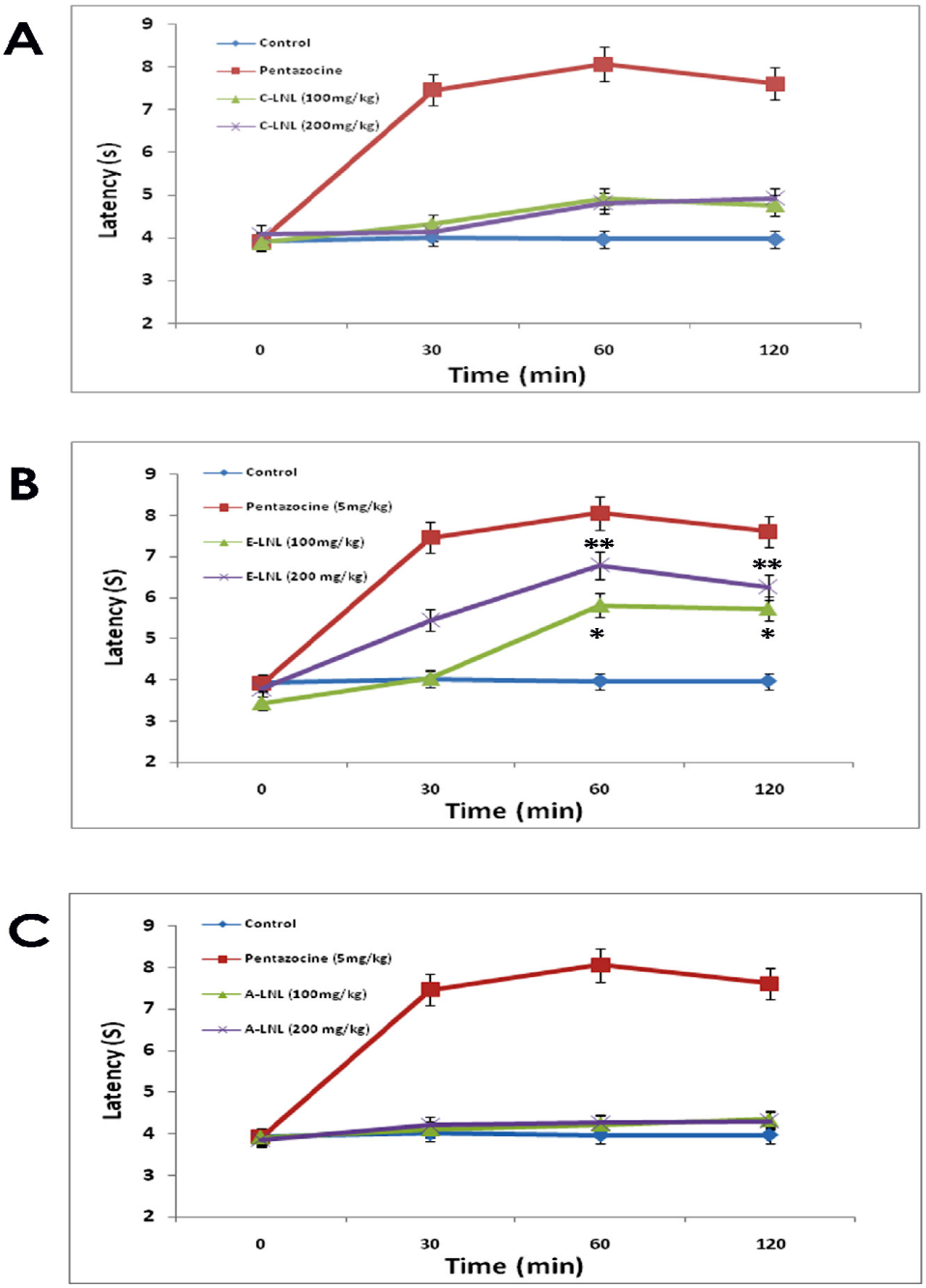

Figure 2. Central Analgesic effect of different doses of *Lobelia nicotianaefolia* leaf (LNL) fractions on hot plate test in Swiss albino mice. **(A)**C-LNL: Chloroform fraction of *Lobelia nicotianaefolia* **(B)** E-LNL: Ethanol fraction of *Lobelia nicotianaefolia*; **(C)** A-LNL: Aqueous fraction of *Lobelia nicotianaefolia.*

Values are the mean ± SD, n = 4 in each group (statistically significant values are $^{*}p < 0.05$., $^{**}p < 0.01$).