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Original article

In vitro and *in vivo* inhibitory effects of *Tabernaemontana alternifolia* against *Naja naja* venom

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ABSTRACT

Background: *Tabernaemontana alternifolia* root is traditionally used and practiced among few Indian tribes as an antidote for snakebites.
Objective: To combat and neutralize *Naja naja* venom using methanolic root extract of *Tabernaemontana alternifolia* and to explore its efficacy on venom biomarkers in search of newer herbal antidote or first-aid-point of care for therapeutics.
Materialization.
Pharmacological activities such as fibrinogenolytic, direct and indirect hemolytic activities for the neutralization of the venom were evaluated. Lethal toxicity annulation studies were performed using the murine model by pre-incubation and post-treatment protocols. Further, the neutralization of edema and myotoxicity were also evaluated.
Results: Electrophoretic analysis revealed that the complete neutralization of fibrinogen degradation was observed at 1:10 (w/w) (venom to extract). *T. alternifolia* exhibited an effective dose (ED_{50}) value of 87.20 μg/mL for venom-induced hemolysis. Venom at 2 μg concentration produced 11 mm of hemolytic radiance and was neutralized at 1:20 (w/w) venom to extract concentration. The survival time and the neurotoxic symptoms in mice were concluded to be delayed by both the methods of lethal toxicity inhibition using methanol extract. The edema ratio reduced the venom to extract ratio of 1:20 (w/w) from 173 ± 45% to 133.61% when subjected to 5 μg of venom concentration. The plant extract significantly neutralized the myotoxic activity.
Conclusion: *T. alternifolia* methanolic root extract could be a potent contributor in the effective treatment of *N. naja* venom-induced toxicity.

1. Introduction

Snakebite envenomings could result in some public health wound hazards, including anxiety, injury, paralysis, and fatality. Among the tropical diseases, snakebite is considered as one of the neglected avenues by the World Health Organization (WHO, 2009). The statistical report depicts that there are 5.5 million bites, 0.4 million amputations, and almost 0.125 million deaths globally (WHO, 2009; Warrell, 2010; Williams, 2010). Approximately 97% of death in India is in rural areas, where 45,900 deaths are estimated every year due to snakebites. The Indian Million Deaths Study (IMDS) summarizes a 30-fold increase in the number of death due to snakebites than the official record. The major impact of snakebites on rural inhabitants is the financial liability to the family (Vaiyapuri et al., 2013).

The fatality rate in South East Asia is mainly due to venom produced by the snake of the Elapidae family, which is extremely

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dangerous (Warrell, 1999). Biologically, this venom is majorly neurotoxic, cardiotoxic, and cytotoxic leading to diaphragmatic paralysis, causing death (Guieu et al., 1994). *Naja naja* (Indian cobra) is one among Indian "BIG FOUR" snakes which is responsible for several deaths (Whitaker, 1990). The only available treatment in India is the administration of polyvalent anti-dote for the neutralization of these circulating toxins. However, on the other hand, antivenom is very expensive and many times are not available to victims in case of emergency due to technical issues of ideal storage conditions, short expiry and lack of trained practitioners. Considering all the drawbacks associated with anti-venom therapy, the use of herbal treatments would contribute as a first-aid to initially neutralize the venom cocktail, which can bring a huge difference in handling snakebite victims.

Traditional healers/practitioners are well-known for treating snakebite victims from time immemorial in South Asian countries by exploring the knowledge of Ayurveda. Ethnic groups of the village tribe recommend various plants or their formulations for snakebite therapy, but with lesser scientific validation or without a precise mode of action. Pharmacologically important phytobioactives which would possess the antagonistic potential of neutralizing various snake venoms could be isolated and characterized for the better understanding of herbal therapeutics (Martz, 1992, Mors et al., 2000). Among several herbs, *Tabernaemontana* species aqueous decoction is popularly used for snakebite in Brazil. The lethal effects and myotoxicity induced by *Crotalus durissus terrificus* snake venom could be neutralized using *T. catharinensis* root bark extracts from *Apocyanaceae* family constituting lead phytocompound namely quaternary alkaloid 12-methoxy-4-methylvoachalotine (Fatima et al., 2000). Venom obtained from *Bothrops jararacussu* and its myotoxins bothropstoxin-I and II that caused myotoxic effects were neutralized by *T. catharinensis* lyophilized aqueous solution (Veronese et al., 2005). In our previous studies, we have reported the effectiveness of *Tabernaemontana alternifolia* root extracts against the neutralization of *in vitro* enzyme activities of *N. naja* and *Echis carinatus* venom (Vineetha et al., 2014). Methanol extract exhibited promising inhibition compared to other extracts studied. Therefore, in the present study, we attempt to evaluate the neutralizing potency of methanolic root extract of *T. alternifolia* against the venom produced by *N. naja* using an *in vivo* model.

2. Materials and methods

2.1. Chemicals

Chemicals such as β-mercaptoethanol, tris base, 1,2-Bis (dimethylamino)ethane (TEMED), fibrinogen, prop-2-enamide, $CaCl_2$, Folin-Denis reagent, sodium dodecyl sulfate, *N*-[(Prop-2-en oylamino)methyl]prop-2-enamide, Acid Blue 83 were purchased from HiMedia Laboratories, QF chemicals, and Sigma.

2.2. Snake venom

Lyophilized venom of *N. naja* was obtained from Irula Snake Catcher's Co-operative Society, Tamil Nadu, India and was stored at 4 °C. The obtained venom was dissolved in saline for further usage. On the day of use, the required concentration of fresh dilutions of the venom was prepared in saline. The venom was obtained following the instructions as per the Wild Life Act of India.

2.3. Preparation of plant extract

Root samples of *T. alternifolia* were collected from Kundapura Taluk, Kollur located in Udupi District, Karnataka (April 2015). The plant was authenticated as *T. alternifolia* at the National Ayurveda Dietetics Research Institute, Bangalore, Karnataka with voucher no. RRCBI-MUS/03. Fresh root sample of *T. alternifolia* was washed, air-dried and powdered. 40 g of the powder was subjected to soxhlet extraction and was concentrated by flash vacuum evaporator as described (Vineetha et al., 2014). Before extraction, the plant was immersed in saline, and the obtained solution was subjected to centrifugation for 10 min at 2000*g*. The supernatant was collected and stored for further studies.

2.4. Selection of animals

Healthy adult male Swiss Albino mice were considered with an average body weight of 25–30 g for *in vivo* studies. The procedure for handling of animals was followed according to the ordinance of Institutional Animal Ethics Committee (IAEC), Ref IAEC/NCP/92/2015.

2.5. In-vitro studies

2.5.1. Fibrinogenolytic assay

The fibrinogenolytic activity was determined using Ouyang and Teng protocol using bovine fibrinogen as the substrate (Ouyang and Teng, 1976). Bovine fibrinogen (50 μg) was incubated for 1 h with 5 μg of venom sample in 10 mM Tris Hydrochloride buffer (pH 7.4). For neutralization study 50 μg of fibrinogen was added to the methanolic extract and was incubated with *N. naja* venom for 1 h. Denaturing buffer was prepared with 4% Sodium dodecyl sulfate, 4% β-mercaptoethanol, and 1 M Carbamide. 20 μL of denaturating buffer was added for the termination of the reaction. The obtained hydrolyzed product was subjected to 12% sodium dodecyl sulfate, stained with CBB and visualized for the pattern of the protein on acrylamide slab gel (Laemmli, 1970).

2.5.2. Direct hemolytic assay

Human erythrocytes were analyzed for the inhibition of the haematolysis action induced by Cobra venom methanolic extract (Balu and Alagesaboopathi, 1995). The protocol of Murugesh *et al.* was carried out for the preparation of HRBC cell suspension (Murugesh et al., 1981). Phosphate buffer saline 0.1 M and HRBC were adjusted to pH 8 that was considered as the control whereas, phosphate buffer, venom, and HRBC were the reaction mixture (100 μg in 1 mL). *T. alternifolia* methanolic extract and venom were pre-incubated for inhibition studies at different concentrations (100, 200, 300, and 400 μg/mL). The cocktail was subjected to centrifugation for 3 min at 1000*g* and incubated in an incubator by providing the necessary conditions for 30 min at 37 °C. The obtained supernatant from centrifugation was analyzed at 540 nm using a UV–Vis spectrometer (SL 159, ELICO) to determine its absorbance. Percentage of inhibition of hematolysis was measured using the formulae:

Prohibition percent of haematolysis = Optical density of the control – Optical density of the test/Optical density of the test × 100

2.5.3. Indirect hemolytic activity (PLA_2 activity)

Agarose-erythrocyte-egg gel plate was used to measure hemolysis induced by phospholipase A_2. Agarose-erythrocyte-egg gel plate served as the phospholipid; it was prepared by adding agarose of 0.8%, RBC of 1.2%, and 1.2% of gooey golden orbs. Venom was loaded onto 1.5 mm wells on the gel plate. The hemolytic halos were measured after the plates were provided with the necessary conditions required for the reaction to take place in an incubator where the temperature was set at 37 °C overnight. Venom concentration at which hemolytic halo of 10 mm is produced is known as the minimum hemolytic halo (MHH) (Gutierrez et al., 1988).

2.6. In vivo studies

2.6.1. Evaluation of LD_{50} of the venom and neutralization of lethality

Healthy Swiss albino mice weighing 2530 g were used to determine the LD_{50} value of the Cobra venom. Venom was administered at different concentrations dissolved in 0.2 mL of saline to groups (n = 5) of Swiss albino mice through intraperitoneal (i.p.) route (Theakston and Reid, 1983). Three LD_{50} values of venom were selected as the challenging doses for venom neutralization studies. Pre-incubation of various strengths of the venom with 1:10 and 1:20 dosage along with different levels of the plant extract were used for neutralization. Venom was injected into the mice ($3LD_{50}$) subsequently with different strengths of the plant extract. The plant extract was administered at a time interval of 5 min through i.p. route. Survival time was recorded after 24 hr of injection.

2.6.2. Anti-inflammatory activity against venom-induced inflammation

Quantity of toxin essential to result in an edematous ratio of 120% is known as minimum edema dose (MED). The venom of different concentrations was introduced into groups (n = 5) mouse onto the right footpad with 20 μL of saline. Whereas, mouse administered with 20 μL of saline onto the left footpads were reserved as the control. Different concentration of the plant extract (1:10, 1:20 w/w) was incubated with MED of venom for neutralization studies. The formula to calculate the water retention ratio is:

Edema ratio = weight of induced × 100/weight of the control (Vishwanath et al., 1987).

2.6.3. Neutralization of venom-induced myotoxicity

Myotoxicity induced by the venom was measured *via* changes in lactate dehydrogenase (LDH) present in the serum. 1/2 LD_{50} venom sample in 50 μL saline was injected into groups 1 & 2 through the intramuscular route. The venom that was pre-incubated with *T. alternifolia* methanolic extract was injected into group 3 and group 4 at two different concentrations, whereas group 5 was injected with the plant mixture, respectively. Through the *retro*-orbital method, the blood was drawn by anesthetizing the mice after 3 h of injection. LDH enzyme activity was measured by allowing the blood to clot and analyzing 1:25 diluted serum (Gutierrez et al., 1990).

2.7. Statistical analysis

Prism GraphPad6 was used to determine the statistical significance between the groups through unpaired student *t*-test. P-value<0.05, 0.01, 0.001, 0.0001 was represented as a, b, c, and d, respectively. The minimum dosage of extract that is necessary to produce 50% inhibition was known as the Effective dose (ED_{50}), which is calculated using regression analysis.

3. Results

3.1. In-vitro studies

3.1.1. Neutralization of fibrinogenolytic activity

In the present study, it was found that *N. naja* venom completely degraded the α chain of fibrinogen at a concentration of 5 μg. Whereas, β and γ bands of fibrinogen did not get degraded with further increase in the venom concentration. Neutralization of *N. naja* venom at different concentrations of *T. alternifolia* methanolic extract using electrophoretic analysis revealed the complete neutralization of fibrinogen degradation at 1:10 venom:extract (w/w) concentration (Fig. 1).

3.1.2. Direct hemolytic assay

The direct hemolytic assay was performed to analyze the effect of *N. naja* venom *in vitro* through/HRBC stabilization method. *T. alternifolia* methanolic extract with various concentrations was incubated along with 100 μg/mL of *N. naja* toxin, and the percentage of neutralization on the haemolysed cells was calculated in a dosage reliant approach. The obtained results were expressed as a percent of inhibition of hemolysis when the percent of hemolysis of venom alone is 100%. *T. alternifolia* methanolic extract for the venom-induced hematolysis exhibited an ED_{50} value of 87.20 μg/mL (Fig. 2).

3.1.3. Indirect haemolytic activity (PLA_2 activity)

Agarose-erythrocyte-egg yolk gel plate was used to study the nullifying effect of hematolysis assay caused by Cobra venom through the methanolic extract of *T. alternifolia*. 1U of the hemolytic halo was formed by Cobra venom at a concentration of 2 μg. This indicates that phospholipase A_2 activity in Cobra venom and its capability to lyse human red blood corpuscles. *T. alternifolia* methanolic extract exhibited promising inhibition at 1:20 (w/w) venom:extract ratio that almost neutralized hemolysis activity (Fig. 3).

3.2. In vivo studies

3.2.1. Evaluation of LD_{50} of the venom and neutralization of lethality

LD_{50} (i.p) of 0.38 mg/kg weight of venom of *N. naja* was found to be highly lethal to Swiss albino mice (Shrikanth et al., 2019). The LD_{50} value of venom with venom survival time of 1 h and 15 min is chosen as the best dose for neutralization studies. The symptoms that are experienced by the mice include difficulty in breathing, respiratory failure, lacrimation, convulsion, and urination. Two different methods were used to compare the enhanced survival time of the control and with the mice treated with *T. alternifolia* methanolic extract. Two diverse strengths of the plant mixture were pre-incubated alongside with the toxin following the injection of the plant extract after 5 min. At venom:extract ratio 1:20 (w/w), the survival time was delayed to 05:55 h, and 06:05 h in pre-incubation and administration of the injection was terminated separately. In the case of survived mice, neurotoxic symptoms were not observed (Table 1).

3.2.2. Anti-inflammatory activity against venom-induced inflammation

Mice infused with 5 μg of Cobra toxin exhibited water retention of 173 ± 45%. At venom:extract ratio of 1:10 (w/w) and 1:20 (w/w),

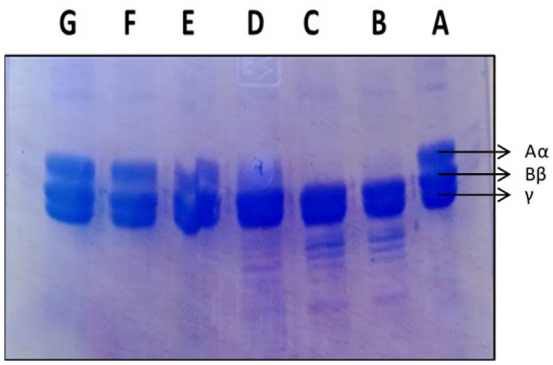

Fig. 1. Neutralization of *N. naja* induced fibrinogenolytic activity by *T. alternifolia* methanol extract. Lane A – fibrinogen control. Lane B and C – fibrinogen + 5 μg of *N. naja* venom (F + V). Lane D, E, F and G – F + V was incubated with plant extract of different concentration (venom to extract (w/w), 1:5, 1:10, 1:15, 1:20), respectively.

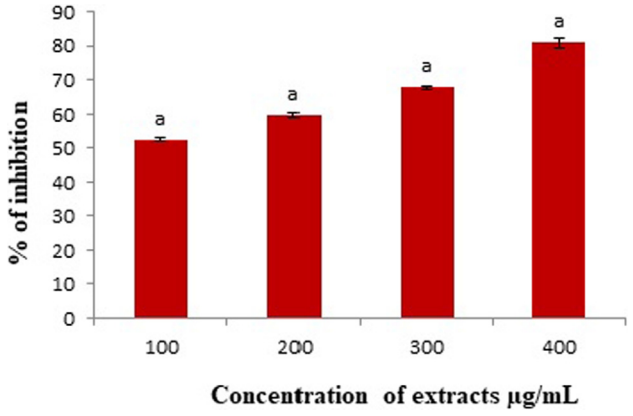

Fig. 2. Inhibition of *N. naja* venom induced hemolysis by *T. alternifolia* methanol extracts. Values represents mean ± SEM (n = 3). P < 0.05 was represented by 'a'.

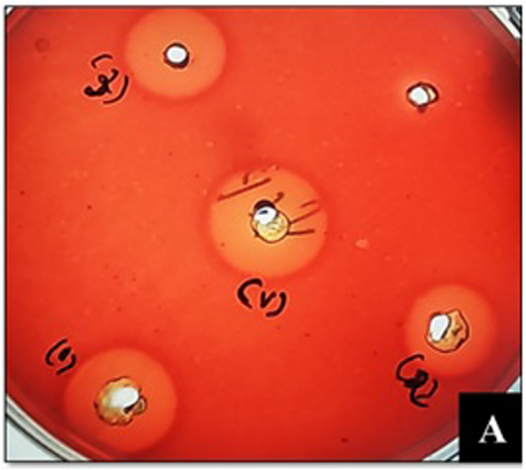

Fig. 3. Neutralization of hemolytic activity of *N. naja* venom by *T. alternifolia* methanol extract. A: (v)-1MHH venom, (1), (2), (3) and (4) – 1:5, 1:10, 1:15 and 1:20 (venom: extract w/w), respectively.

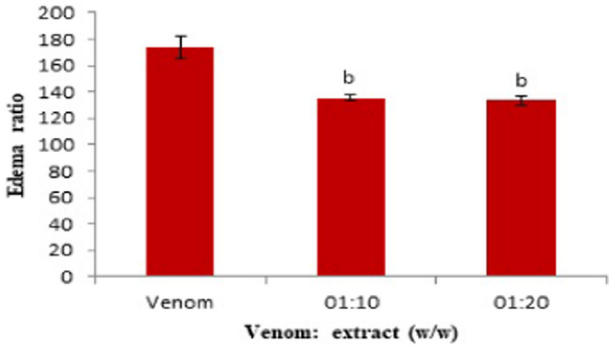

Fig. 4. Neutralization of edema induced by *N. naja* venom by methanol extract of *T. alternifolia.* 'b' represents P < 0.01.

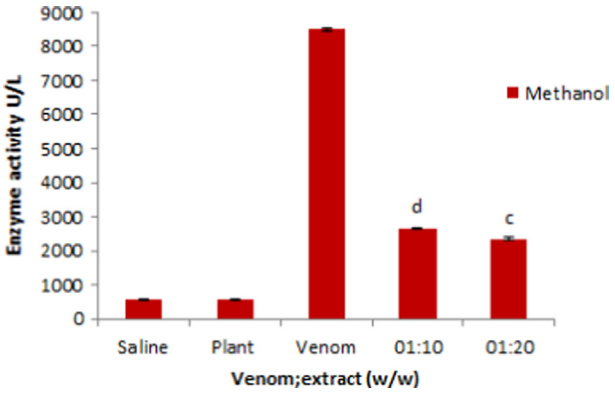

Fig. 5. Neutralization of myotoxic activity induced by *N. naja* venom by *T. alternifolia* methanol extract. 'c' represents P < 0.001 and 'd' represents P < 0.0001. LDH activity represented as U/L.

the edema ratio reduced to 135.53% and 133.61% correspondingly (Fig. 4).

3.2.3. Neutralization of venom-induced myotoxicity

Myotoxicity caused by *N. naja* venom was assessed by the LDH enzyme level to quantify skeletal muscle damage. LDH activity 8485 ± 21.21 U/L was exhibited due to the lethal dose LD_{50} value of Cobra venom, which was compared with the control mice that had a value of 568.33 ± 7.6 U/L. Methanolic extract of *T. alternifolia* in inhibition studies is found to exhibit the best myotoxic neutralizing effects on the Swiss albino mice model (Fig. 5).

4. Discussion

Cardiotoxin and post-synaptic neurotoxin are the main components of *N. naja* venom that operate in the synaptic gaps in the neuron. Cardiotoxin from the cobra regulates the cardiac cell and subsequently causes systolic arrest. Most of the elapids venom consists of a fraction that reversibly blocks the postsynaptic acetylcholine receptor (Lee and Chang, 1966, Watt et al., 1986). This neurotoxin acts as a highly specific competitor for acetylcholine receptors (Weber and Changeux, 1974). The beneficial effect of anti-serum therapy for treating the elapids bite has been questioned. According to many clinical reports, the anti-cholinesterase drug is used as an anti-venom in treating cobra and krait bites (Reid, 1964, Bawasker and Bawaskar, 2002). Phytochemicals isolated from different plants have the ability to antagonize certain toxic enzymes (Martz, 1992, Mors et al., 2000, Rajesh, et al., 2017). Therefore the use of herbal medicine for the treatment of snake bites is considered as the best method considering all the drawbacks. Serine protease and metalloproteinases are the proteinases that are attributed by the venom for fibrinogenolytic activity. *N. naja* toxin degraded α band of fibrinogen, which indicates the fibrinogenolytic activity induced by the venom. *T. alternifolia* was found to be effective at a minimum concentration of 1:10 venom to extracts concentration (w/w). The most abundant molecule that is present in snake venom is phos-

Table 1
Neutralization of challenging dose of *Naja naja* venom (3 × LD_{50}) by *T. alternofolia* methanol extracts.

	3 × LD_{50} of the venom (h)	Saline (h)	Venom: Methanol extract (w/w) 1:10 (h)	Venom: Methanol extract (w/w) 1:20 (h)
T. alternifolia (Pre-incubation)	1:15[a]	24[b]	02:30[a]	05:55[a]
T. alternifolia (Separate injection)	1:15[a]	24[b]	02:20[a]	06:05[a]

'a' –Represents all dead, **'b'** – Represents all survived.
Note: n = 5 per group and the experiment was repeated thrice independently and the average of results has been represented.

pholipases that in the body regulates myotoxicity, hypotension, hemolysis, neurotoxicity, and edema formation. In cobra venom, it was reported that it constitutes more than 50% of the cytotoxin and known to play a synergistic role with PLA_2.

Cytotoxin and PLA_2 combine together in the venom to induce the hemolytic activity, and the mechanisms of inhibition of this activity may be through the action of the Phyto molecule on the HRBC membrane and the stabilization of the protein. The plant extracts consist of flavonoids and phenolic compounds that function to inhibit the lysis of the HRBC membrane that is induced by the venom (Kumarapppan et al., 2011).

The PLA_2 activity and the hemolytic activity coincide with each other in the venom and are involved in the hydrolysis of the phospholipid. In the previous study, methanol extract of *Leucas aspera* was explored for the *N. naja* venom. 50 μg of *N. naja* venom from Southern region produced hemolytic halo and present study *N. naja* venom of 2 μg in the same geographical region produced minimum hemolytic halo of 11 mm. *Leucas aspera* neutralized all other activities induced by the *N. naja* venom but not PLA_2 activity completely. The extracts exhibited inhibition activity against the *N. naja* induced hemolytic activity with a lower concentration of plant extract compared to the earlier finding suggested that the extract is effective in neutralizing the PLA_2 activity of the venom (Gopi et al., 2014).

In the current study, neutralization of Cobra venom was carried out through the pre-incubation method, and venom injection, followed by the plant extracts injection. Only two concentrations of the plant extracts of *T. alternifolia* was explored for the neutralization of the venom. The extract exhibited promising effects against *N. naja* venom through pre-incubation and separate injection methods. The victims of snakebite were treated in the present study after envenomation, and the methanolic extract of *T. Alternifolia* contributed to the anti-venom properties against *N. naja* venom proving to be an effective antidote for snake bites.

The root extract of *Peschiera acuminata* was formerly found in viper *Crotalus durissus* toxin that causes lethal toxicity through disparate methods of antagonistic mechanisms (Fatima et al., 2000). *T. alternifolia* that also belongs to the same genus was effectual beside the elapids cobra toxin caused toxicity. It was observed that *T. alternifolia* provided better protection against *N. naja* venom compared to *C. parviflorum* extracts, which we reported in our previous studies (Shrikanth et al., 2019). The use of anti-venom for treating the neurotoxic envenomation was questioned; clinically used treatment is a ventilator along with anti-cholinesterase drugs. Anticholinesterase drug was recommended by WHO for a neurotoxic bite, and snakebite victims of neurotoxic bite recovered for the anti-cholinesterase drugs (Reid, 1964, Bawaskar and Bawaskar, 2004). *Tabernaemontana* genus is well known for its quantitatively rich alkaloid contents. Alkaloid present in the plant was extensively evaluated for anti-cholinesterase activity. 3′-R/S-Hydroxyvoacamineindole alkaloid from *T. divaricta*, *T. catharinensis,* were reported for anti-cholinesterase activity (Chaiyana et al., 2013, Nicola et al., 2013). Therefore anti-cholinesterase activities of the plant extracts may play a key role in treating the neurotoxic envenomation.

PLA_2 and metalloprotease combine together, causing edema and is involved in the release of inflammatory mediators. Anti-venom can neutralize the effects of toxins but cannot cause any effect on inflammation caused by inflammatory mediators (Felix-Silva et al., 2014). *N. naja* venom 10 μg concentration produced an edema ratio of 188 ± 9.6%, which was reduced up to 139 ± 955% by *Euphorbia hirta* plant extracts (Gopi et al., 2015). Similarly, the present study confirms the efficacy of plant extract against the venom-induced edema effects. Previously, the effect on phytobioactives on venom has been well studied by our group and is evident from the literature (Kishore et al., 2016, Kishore et al., 2015, Dhananjaya et al., 2011, Shwetha et al., 2019, Bhavya et al., 2019).

The skeletal muscles get affected by myotoxic phospholipase, and the muscles get severely damaged, which in turn elevates the level of CK and LDH in the serum. The myotoxic phospholipase is an enzyme that is responsible for acting on skeletal muscles and sternly costs the muscles ensuing change of CK and LDH released into the serum. The myotoxic effect was determined by quantitative measurement of LDH enzyme activity, which is raised as consequences of muscle damages, and we observed that LDH enzyme activity is higher in the case of *N. Naja* venom. Higher LDH activities in *N. naja* venom indicate a higher degree of myotoxic effects associated with the venom and which was significantly neutralized by the plant extract.

5. Conclusion

The current study acknowledges the neutralizing capability of *T. alternifolia* root extract against *N. naja* venom that is not reported previously. *T. alternifolia* is one of the traditionally used plants for cobra bite in India. Thus pharmacological and *in vivo* neutralization studies with potential traditional plants for snake bites could assist in better understanding of newer potent anti-venom contributors.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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