

Research Article

Studies on Antivenom Activity of *Aristolochia indica* Plant Extract Against Red Scorpion Venom by in Vivo and in Vitro Methods.

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ABSTRACT

Objective: *Mesobuthus tumulus* is the most lethal among all poisonous species of scorpion. *Aristolochia indica* is one of the plants which have long been used in traditional herbal medicine for the treatment of poisoning by animal bites. Hence the study was planned to evaluate the ethanolic extract of *Aristolochia indica* for the treatment of *Mesobuthus tumulus* envenoming. **Materials and Methods:** Calculation of LD99 of *Mesobuthus tumulus* venom was done using Turner's method. Acute toxicity and Neutralization of the lethal venom effect of *Mesobuthus tumulus* venom by plant extract at the dose of 1gm/kg and 2gm/kg in vivo was seen. **Results:** The LD99 of *Mesobuthus tumulus* venom from this study was determined to be 22.6µg/gm. In the acute toxicity and in vivo neutralization study plant extract at the dose of 1gm/kg and 2gm/Kg resulted in mean survival of 59mins and 51mins respectively. Neutralization of the lethal venom effect by plant extract at the dose of 1gm/kg and 2gm/kg by Alam and Gome's method showed mean survival of 88 mins and 75 mins respectively. **Conclusion:** Ethanolic extract of *Aristolochia indica* has protective effect against the Red Scorpion Venom and shows 50% survival benefits in mice.

Keywords: *Mesobuthus tumulus*, *Aristolochia indica*, LD99, Antivenom.

INTRODUCTION

Red scorpion (*Mesobuthus tumulus*) is the most lethal among all poisonous species of scorpion. Scorpion venom is a potent sodium channel activator and envenoming by *Mesobuthus tumulus* results insudden pouring of endogenous catecholamines into circulation due to the autonomic storm evoked by delayed inactivation of neuronal sodium channels. (Ismail M., 1995) Vomiting, profuse sweating, priapism in males and cold extremities precede the development of severe cardiovascular manifestations. Clinical manifestations depend upon dose of venom, season of sting and time elapsed between sting and hospitalization. (Bawaskar H S., 1981) Alpha-receptor stimulations play a major role in the pathogenesis of acute pulmonary oedema. About 30–50% fatality due to acute pulmonary oedema with scorpion sting has been reported from India. Early reporting of a case and immediate hospitalization to facilitate the administration of prazosin arrest the development of severe lifethreatening cardiovascular manifestations. Scorpion antivenin did not reverse and prevent the cardiovascular morbidity and mortality due to envenoming by red scorpion sting. (Bawaskar H S., 2005) There is no standard protocol for the treatment of scorpion venom poisoning. Various regimens including decongestive treatment, beta blocker, nifedipine, excessive diuretics, lytic-cocktail and insulinglucose were tried, with no benefits even the serotherapy for the scorpion envenoming is not established in India. (Bawaskar H S., 2004) *Aristolochia indica*, one of the 500 species of the family *Aristolochiaceae* is distributed

throughout the tropical, subtropical and Mediterranean countries. In Indian subcontinent, the plant is found in low hills and plains of India from Nepal and lower Bengal to Chittagong in Bangladesh and Coromondal coast. This endangered medicinal plant, locally known as Isharmul is a shrub with long twinning stem. Since the Graeco-Roman period, aristolochic acid, a constituent of *Aristolochia* species, has been used for medical purposes. The plant is used to treat cholera, fever, ulcers, leprocy, poisonous bites. It is also used as emmenagogue, antineoplastic, antiseptic, anti-inflammatory, antibacterial and phospholipase A2 inhibitor. (Dey and De 2011) The ethanolic extract of *Aristolochia indica* showed neutralization property against *Daboia russellii* venom (Meenatchisundaram et al 2009). Ethanolic extract of *Adrographis Paniculata* has some protective effect against the Red Scorpion Venom in mice but doesn't offer any survival benefit. (Brahmane R.I et. al.2010). The present study was planned to examine the safety of using the extract of one of the common medicinal plant *Aristolochia indica* in reducing the toxic effects on different cells and tissues of experimental animals injected with the venom of the most dangerous *Mesobuthus tumulus* scorpion. The study focuses on the resolving the harmful effects induced by the venom and neutralization of venom by ethanolic plant extract on experimental mice.

MATERIALS AND METHODS

Table 1. Calculation LD99 of *Mesobuthus tamulus* venom in mice receiving various doses of *Mesobuthus tamulus* venom by Turner's method (n = 2)

Venom Dose (mg/kg)	Death/Total	Death%	Corrected Death%
5	0/2	0%	0%
10	0/2	0%	0%
15	0/2	0%	0%
20	2/2	100%	87.5%

Toxicity of Mesobuthus tamulus Venom and its Neutralization by the Plant Extract

Collection of the Plant Materials: The Plant material was brought from Alibaug, Maharashtra, India. The plant was authenticated by Botanical Survey of India, Pune, Maharashtra, India.

Preparation of extract: Fresh plants were collected, cleaned under running tap water, shade dried, fine powdered and stored in airtight container until further processing. The alcoholic extract was prepared according to the procedure reported by Mahanta & Mukharjee. (Turner RA., 1965) Twenty grams of dried powder of plant was macerated in 70% of ethanol overnight. It was then packed in the tumbler of Soxhlet apparatus and was extracted using

70% ethanol refluxing at 60-80° C. The extract thus obtained was dark green to brown in colour. The stock extract thus obtained was preserved in airtight glass container and kept inside the refrigerator at 4° C.

Venom Sample: Lyophilized venom sample of *Mesobuthus tamulus* was purchased from Haffkine Institute, Parel, Mumbai, India and was stored at 2-8°C for future use, taking all the precautionary measures of handling and storage.

Experimental animals: Swiss albino mice weighing 20-30 gm were used in the study. All the animals were housed in polypropylene cages and maintained at a temperature of 25° ± 2°C. They were kept in a 12:12 hour light: dark cycle and fed on standard laboratory chow and water ad libitum. Animals were acclimatized to laboratory conditions before the test for 10 day.

Ethical clearance: The protocol was submitted and due clearance was taken from Institutional Animal Ethics Committee of National Toxicology Centre, Pune, India.

In Vivo Study: Calculation of LD99 of Red Scorpion (*Mesobuthus tamulus*) venom: Lethal dose 99 (LD99) is defined as the least amount of venom (dry weight in grams) injected intramuscularly to animals resulting in the 99% death of animals within 24 hours. The method

reported by Turner was adopted for determination of LD99. (Alam & Gomes., 2003)

The *Mesobuthus tamulus* venom was dissolved in saline and given to mice intramuscularly (i.m.) in graded doses starting with 5mcg/gm and mortality was recorded for 24 hours. Two animals were taken in each group.

Acute toxicity of *Mesobuthus tamulus* venom and its neutralization by plant extract: Animals were divided into three groups of six animals each. Each animal in the groups 1-3 was administered LD99 of *Mesobuthus tamulus* venom i.m. Animals in Group 1 received saline and this group was considered as control. Animals in Group 2 and Group 3 received plant extract orally at the dose of 1gm/kg and 2gm/kg respectively. Plant extract was given 5 minutes after the dose of *Mesobuthus tamulus* venom. In all the groups the duration of survival and the number of animals survived was recorded for 24 hours. All the groups received same volume of preparations.

Neutralization of the lethal venom effect of Red Scorpion (*Mesobuthus tamulus*) by Alam and Gomes's method: Neutralization test described by Alam and Gomes was followed. (Turner RA., 1965) Animals were divided into three groups of six animals each. LD99 of *Mesobuthus tamulus* venom was mixed in vitro with saline and plant extracts at the dose of 1gm/kg and 2gm/kg respectively for group 1, 2 and 3; then the mixture was incubated for 1 hour at 37° C and centrifuged at 2000 rpm for 10 min. The supernatant was injected i.p. into mice. The duration of survival and the number of animals survived was recorded for 24 hours after admixture injection of venom. Thus Group 1 received distilled water incubated with LD99 of *Mesobuthus tamulus* venom i.p. and served as control Group 2 received 1gm/kg plant extract incubated with LD99 of *Mesobuthus tamulus* venom i.p. and Group 3 received 2gm/kg of plant extract incubated with LD99 of *Mesobuthus tamulus* venom i.p. All the groups received same volume of preparations.

Table 2. Acute toxicity of *Mesobuthus tamulus* venom and its neutralization by plant extract

Groups(n=6)	Mean Survival Time (mins)	Total Animal Survived/ Total animals in group	Percentage Survival
Group1 LD99SV+Saline	46*±3.66 mins	0/6	0%
Group2 LD99SV+Prazocin	-	5/6	83.33%
Group3 LD99SV+PE(1g/kg)	59*±10.82mins	3/6	50%
Group4 LD99SV+PE(2g/kg)	51*±2.88 mins	2/6	33.33%

*significant ($p < 0.05$, Student's t-test)

Table 3. Neutralization of the lethal venom effect of *Mesobuthus tumulus* by Alam and Gome's method

Groups(n=6)	Survival Time (mins)	Total Animal Survived/ Total animals in group	Percentage Survival
Group1 LD ₉₉ SV+Saline	43*± 3.22 mins	0/6	0%
Group2 LD ₉₉ SV+ PE(1g/kg)	88*±28.32 mins	3/6	50%
Group3 LD ₉₉ SV+PE(2g/kg)	75*±25.86 mins	3/6	50%

*significant ($p < 0.05$, Student's t-test)

STATISTICAL ANALYSIS

The statistical analysis was done using one way analysis of variance (ANOVA) using unpaired student's t test. P value ≤ 0.05 was considered as statistically significant and ≤ 0.005 was considered to be highly significant.

In Vitro study: Phospholipase activity: Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the method described by Gutierrez et al. 1988. Increasing doses of scorpion venom (μg) was added to 3 mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% mice erythrocytes, 1.2% egg yolk as a source of lecithin and 10 mM CaCl₂. Slides were incubated at 37°C overnight and the diameters of the hemolytic halos were measured. Control wells contained 15 μL of saline. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produced a hemolytic halo of 11 mm diameter. The efficacy of *Aristolochia indica* plant extract in neutralizing the phospholipase activity was carried out by mixing constant amount of venom (μg) with different amount of plant extract (μL) and incubated for 30 min at 37°C.

Then, aliquots of 10 μL of the mixtures were added to wells in agarose-egg yolk-mice erythrocyte gels. Control samples contain venom without extracts. Plates were incubated at 37°C for 20 h. Neutralization expressed as the ratio mg antibodies/mg venom able to reduce by 50% the diameter of the hemolytic halo when compared to the effect induced by venom alone.

Procoagulant activity: The procoagulant activity was done according to the method described by Theakston and Reid, 1983 modified by Laing et al. 1992. Various amounts of venom dissolved in 100 μL PBS (pH 7.2) was added to human citrated plasma at 37°C. Coagulation time was recorded and the Minimum Coagulant Dose (MCD) was determined as the venom dose, which induced clotting of plasma within 60 sec. Plasma incubated with PBS alone served as control. In neutralization assays Constant amount of venom was mixed with various dilutions of *Aristolochia indica* plant extracts. The mixtures were incubated for 30 min at 37°C. Then 0.1 mL of mixture was added to 0.3 mL of citrated plasma and the clotting times recorded. In control tubes plasma was incubated with either venom alone or plant extracts alone. Neutralization was expressed as effective dose (ED), defined as the ratio mL antivenom (plant extracts)/mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.

RESULTS

In Vivo study: Calculation LD₉₉ of *Mesobuthus tumulus* Venom: Lethality data of *Mesobuthus tumulus* venom is shown in Table 1.

LD₉₉ was calculated by Turner's method (n = 2). The LD₉₉ of *Mesobuthus tumulus* venom from this study was determined to be 22.6 $\mu\text{g}/\text{g}$. LD₅₀ was also calculated from the same data and was found to be 11.43 $\mu\text{g}/\text{g}$.

The *Mesobuthus tumulus* venom at a dose of 22.6 $\mu\text{g}/\text{g}$ (LD₉₉) produced 100% death in mice. The ethanolic extract of the plant *Aristolochia indica* significantly increased the mean survival time and the 50% mice survival. The plant extract when used alone at the dose of 1 g/kg was found to be more effective against *Mesobuthus tumulus* venom, showing a mean survival of 59 min with 50% survival as compared to 51 min and 33.33% survival with the plant extract at the dose of 2 g/kg. Prazocin as a standard drug showed 83.33% survival of animals.

Thus by comparing the percentage survival of test plant extract with standard drug as Prazocin it can said that the *Aristolochia indica* plant extract was effective in treatment of Scorpion envenoming.

Neutralization of the Lethal Venom Effect of Red Scorpion by Alam and Gome's Method: The LD₉₉ of *Mesobuthus tumulus* venom that was mixed with saline, as control, resulted in 100% mortality of mice. However, the LD₉₉ of *Mesobuthus tumulus* venom when mixed with the ethanolic extract of plant *Aristolochia indica* resulted in a significant increase in the mean survival time and the survival of 50% of animals.

The ethanolic extract of *Aristolochia indica* plant extract is effective in neutralization of lethal venom effects as it showed 50% survival of animal when LD₉₉ with plant extract.

In Vitro study: Phospholipase activity: In phospholipase activity (PLA₂), Scorpion venom able to produce hemolytic haloes in agarose-rat erythrocytes gels. About 10 μg of red scorpion venom produced 11mm diameter hemolytic halo, which is considered to be 1U (U/10 μg). This shows that Scorpion venom have the enzymes (PLA₂) that has the ability to lyse mice RBC's. *Aristolochia indica* extracts were capable of inhibiting PLA₂ dependent hemolysis of rat RBC's induced by red scorpion venom in a dose dependent manner. We found that that 4 mg of *Aristolochia indica* plant extracts were able to completely inhibit PLA₂ dependent hemolysis of mice RBC's induced by red scorpion venom.

Procoagulant activity: The minimum coagulant dose (MCD) was determined as the venom dose inducing clotting of plasma in 60 sec. About 50 μg of red scorpion venom clotted human citrated plasma within 60 sec. In

the neutralization assay, the absence of clot formation shows the neutralizing ability of both Plant extracts. We found that that 1 mg of *Aristolochia indica* plant extracts were able to completely neutralize coagulant activity.

DISCUSSION

LD₉₉ of *Mesobuthus tamulus* venom by Turner's method was found to be 22.6 µg/g. This LD₉₉ was taken to analyze the anti-scorpion venom effect of the plant under study. LD₉₉ value was preferred as the chances of the mortality of mice with LD₉₉ dose is more than LD₅₀.

When LD₉₉ is injected in the mice, it produced 100% deaths. The ethanolic extract of plant *Aristolochia indica* significantly increased the mean survival time and the percentage survival. The best results are obtained at the dose of 1 g/kg (59 min) with 50% survival as compared to the dose of 2 g/kg (51 min) with 33.33%, which may be due to some pharmacokinetic and dynamic reasons that can further be evaluated in a separate study.

Neutralization of the lethal venom effect of red scorpion (*Mesobuthus tamulus*) when studied by Alam and Gome's method showed that when the plant extract was used at a dose of 1 g/kg, it was found to be more effective against *Mesobuthus tamulus* venom, showing a mean survival 88 min as compared to 75 min shown by the plant extract at a dose of 2 g/kg. Both of the groups showed 50% survival of animals.

The red scorpion venom showed the presence of PLA₂ enzymes by means of producing hemolytic haloes in indirect hemolytic assays. *Aristolochia indica* plant extract was capable of inhibiting PLA₂ dependent hemolysis of mice RBCs in a dose dependent manner. Procoagulant activity induced by red scorpion venom was studied using human citrated plasma and *Aristolochia indica* extract was found to be effective in the neutralization of procoagulant activity.

It was observed that the plant extract of *Aristolochia indica* provides some protection against the lethal dose of venom. Certain naturally occurring substances in *Aristolochia indica*, such as sitosterol, pentacyclic terpenes, nitro compounds (aristolochic acid), cinnamic acid derivatives, curcumimoids, polyphenolic compounds and flavonoids, are known compounds possessing protein-binding and enzyme-inhibiting properties. The leaves of *Aristolochia indica* contain Quinones, Aristolindiquinone, Lactones, Aristololide and it is claimed that the active constituent is a Sesquiterpenes and is responsible for the anti-scorpion venom property by modifying the actions of proteins and enzymes. Further studies are required to potentiate this claim.

This protective property of *Aristolochia indica* can be explored in practice where a significant amount of time is lost while shifting the patient from the Primary Health Care Centre to the Tertiary Health Care Centre.

CONCLUSION

The ethanolic extract of *Aristolochia indica* has protective effect against the red scorpion venom and shows 50% survival benefits in mice. Further clinical studies are required in humans to potentiate this claim

and discover the new treatment strategy for red scorpion envenoming.

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