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In Vitro and *In Vivo* Studies of *Argemone mexicana* Linn. Extracts Against Cobra Venom (*Naja naja)* Induced Toxicity

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Abstract

In accordance to ethnobotanical reports, the plant *Argemone Mexicana* Linn. possess anti-ophidian properties. The current study is focussed on the inhibition property of the medicinal plant extracts against selective activities (PLA_2 and hemotoxic) of Indian cobra venom. *In vitro* studies showed 100% PLA_2 inhibition (1:50) and 100% inhibition of hemolysis activity of *Naja naja* venom (1:80) by methanolic extract of *A. Mexicana. In vivo* studies were carried out to determine the ED_{50} value of the selected extracts. Histopathology analysis revealed that *A. mexicana* methanolic extract has proved its potential to inhibit the *Naja naja* venom-induced toxicity and paw edema studies revealed a reduction in the toxicity upon treatment with the methanolic extract.

Keywords: Anti-ophidian, *Naja naja*, Medicinal plants, Edema

Introduction

Snakebite is a notable health affair among the rural areas of subtropical regions. Worldwide 54,00,000 snake bites and 1,25,000 mortalities are documented annually (1). India is placed topmost among the Asian countries with 81,000 snakebites and 45,900 fatalities annually (2). Indian cobra, also known as spectacled cobra, is found throughout the country and is one of India's most poisonous snakes and is responsible for the majority of deaths caused by snakebite. *Naja naja* venom is highly neurotoxic and contains a cocktail of enzymes, carbohydrates, proteins, powerful postsynaptic neurotoxins, cardiotoxins and other small components that help venom spread into the victim's body. Neurotoxins of the cobra venom block the neuromuscular transmission across the peripheral cholinergic synapses at the neuromuscular junction by its binding to the acetylcholine receptor at the postsynaptic membrane. Postsynaptic neurotoxins are the chief lethal element in cobra venom (3). Cardiotoxins are responsible for severe myotoxicity, hemolysis and necrosis. While protease degrades the extracellular matrix, phospholipase A_2 destabilizes the membrane phospholipids releasing the arachidonic acid, a major molecule of inflammation (4).

Administration of polyclonal antivenom is the only preferred treatment against snake envenomation (5). Even though antiserum is an efficacious antidote, it has few drawbacks. Administration of antivenin presents a few sickening complications like anaphylactic reactions that are both IgE mediated and non-IgE mediated. A huge quantity of antibodies (antivenom) causes inflammation and stimulates serum sickness (6). Moreover antivenom is inaccessible to the needy and

generally marketed weakly (7). When it is accessible, the selling cost per vial overpass the income of the agricultural workers who are most affected by snake bites (8). Thus the traditional method of treating snakebite with herbs gets focused. Humans always depend on plants and their medicinal properties as it is chief and cost-effective (91). Especially, in rural areas, plants, plant extracts and decoctions are administered orally and paste made from plants are applied on the bite site. Compounds present in these traditional plants identified to counteract against numerous disorders (10). A combination of different plant extracts is also used in snake bite treatment. Investigations on *Euphorbia hirta* L. and *Leucas aspera* (Wild.) revealed potential inhibitors against *Naja naja* venom enzymes (11). Therefore the present investigation was aimed to explore the anti-ophidian activity of *Argemone Mexicana*.

Materials and Methods

Chemicals: All chemicals and solvents were purchased from SD Fine chemicals/ SISCO. *Naja naja* venom used for the study was purchased from the Irula Snake Catcher's Industrial co-operative Society Limited, Chennai, India.

Animals: Swiss Albino mice (male) weighing 20-25g maintained at proper conditions in the animal house, VIT. Permission for the experiments was pursued in advance from the Institutional Animal Ethical Committee, VIT, Vellore, India (Ethical clearance Reference No. VIT-IAEC-12).

Plant Collection: Plants samples were collected from two different areas of Vellore district, Tamil Nadu, India. Leaves of *Argemone mexicana* were collected from Satuvachari, Vellore (Tamil Nadu. India). The collected plants were authenticated by Dr. R. Siva, Professor, Vellore Institute of Technology, Vellore, Tamil Nadu, and India. Voucher specimen no: 2015/PEL/MLA1, 2015/PEL/MCA2, 2015/PEL/MPE3 and 2015/PEL/MAM4 are maintained in the laboratory.

Plant Extraction: Extraction of metabolites was performed using the method of Ramluckan *et al.* (12). In short, 500 grams of shade dried and powdered plant material was taken up for serial extraction with solvents (petroleum ether, ethanol, chloroform, methanol and water) using the soxhlet apparatus. The obtained extracts were concentrated with the help of a rotary vacuum evaporator. The final concentrate was resuspended in 10mM saline (phosphate buffered) and was used for the *in vitro* and *in vivo* studies.

Phospholipase A_2 inhibition study: PLA_2 inhibition assay was performed using by Gutierrez *et al.* (13), wherein 1% agarose plates with egg yolk as a substrate was used. Different concentrations of the plant extracts were preincubated with 50µg of *Naja naja* venom for an hour at 37°C. 1% agarose plates with 5% egg yolk and 10 mM $CaCl_2$ were prepared and wells of 3 mm diameter were made into which the preincubated samples were loaded. The zone of clearance was measured after overnight incubation.

Hemolytic Inhibition: Haemolytic inhibition study was performed according to the protocol described by Boman and Kaletta (14). Various concentrations of the plant extracts were preincubated with 100 µg of *Naja naja* venom for an hour at 37°C and then 100µl of chick erythrocyte suspension was added. After the reaction time, 1ml (ice cold) saline was added to stop the reaction. The suspension was centrifuged at 2500 rpm for 10 minutes at 4°C and the absorbance of the was taken at 540 nm to calculate the amount of hemoglobin liberated.

Neutralization of Venom Lethality: The anti-lethal potential of the plant, was studied for its neutralising potential by the dose-response manner of Gopi *et al.* (10). In detail, various concentrations of the plant extract (*A. mexicana* methanolic extract) were pre-incubated with 2 X LD_{50} dose of *Naja naja* venom in a total volume (0.2 ml PBS) and were injected intraperitoneally to the mice. The survival time of the animals

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was monitored for 48 hours, and based on the survival time, ED_{50} of the extract was calculated.

Histopathological Examination: Organs were extracted from the venom and plant extract treated animals for histopathological examination [10]. The obtained tissues were washed in 1 x PBS and fixation was achieved with 10% formalin. Tissue paraffin wax blocks were prepared and thin sections were obtained using a microtome. Sections were then stained with (hematoxylin and eosin) and was examined under a microscope.

Edema Inhibition Assay: *Naja naja* venom-induced edema inhibition activity of the plant extract (*A. mexicana* methanolic extract) was assayed as per Vishwanath *et al.*[15]. Varying in the concentrations of extract and 10µg of venom in a constant volume of 20µl PBS were incubated at 37°C for 1 hour and were injected into the right foot pads, whereas the left foot pads received saline served as control. The legs were excised at the ankle joints after 45 minutes. Edema is represented as percentage change by comparing the weight of the edematous leg (injected with venom with or without the plant extract) with that of the control leg (injected with saline).

Resulats and Discussion

Phospholipase Inhibition

The phospholipase A_2 inhibition was found to be significant (100%) in chloroform (1:60) and methanolic (1:50) extracts of *A. Mexicana* against *Naja naja* venom (Figure 1).

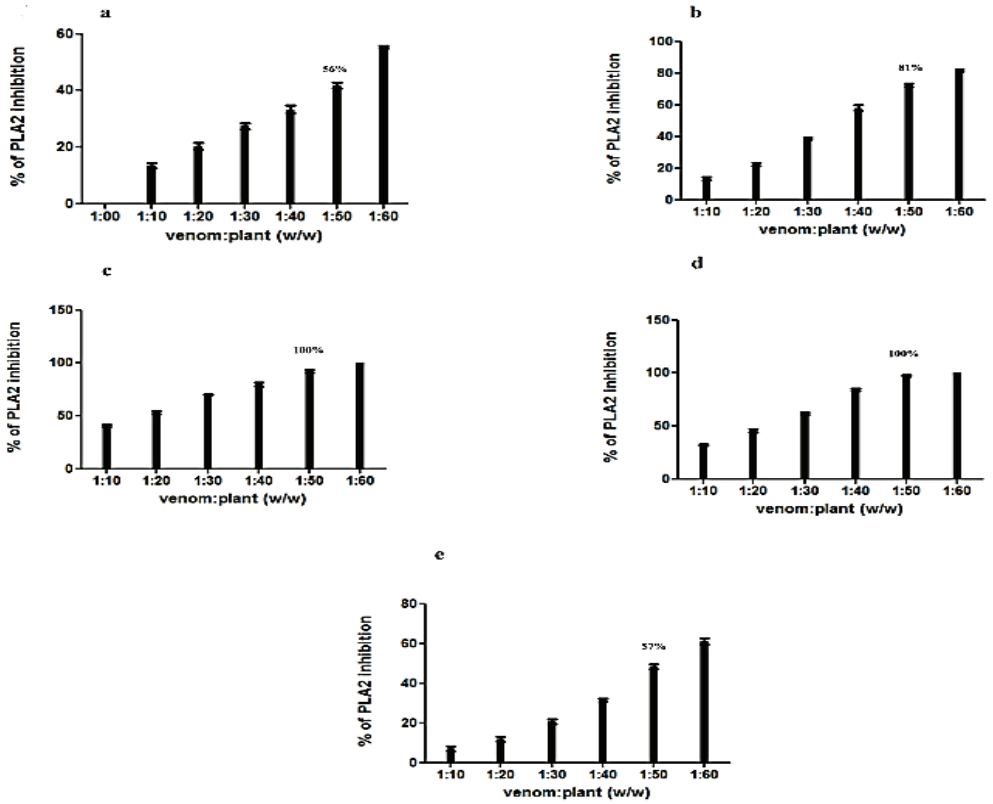

Figure 1: PLA_2 inhibition assay *A. mexicana* (a) Petroleum ether, (b) ethanol, (c) chloroform, (d) methanol and (e) aqueous.

Hemolytic Assay

Hemolytic inhibition assay showed methanolic (1:80) and ethanolic (1:100) extracts of *A. mexicana* possessed the property to inhibit the hemolytic activity of *Naja naja* venom (Figure 2).

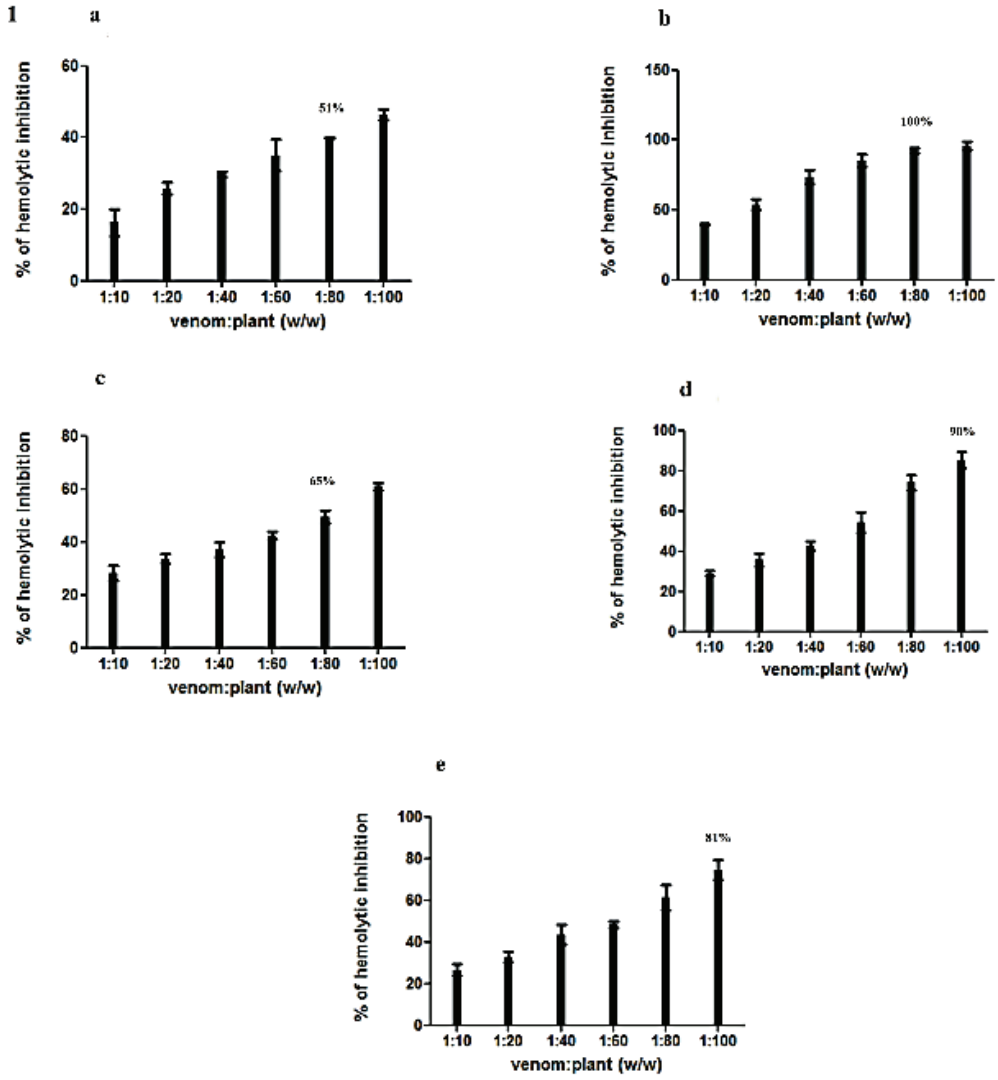

Figure 2: Hemolytic inhibition assay *A. mexicana* (a) Petroleum ether, (b) Ethanol, (c) Chloroform, (d) Methanol and (e) Aqueous.

Lethality Neutralization

Survival time of mice remarkably increased with an increase in the concentration of extract (dose-dependent manner). Mice injected with *Naja naja* venom was showed 2.3±0.2 h of survival time, while the mice treated with venom and *A. mexicana* methanolic extract showed an increase in the duration of mice survival of

38 h at 1:100 ratio and 48 h at 1:150 ratio with 100% survival in the group (Figure 3). No toxic signs were observed in the animals which were treated with saline or only extracts at its highest concentration.

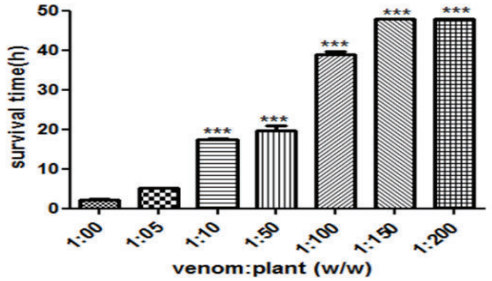

Figure 3: Graphical representation of lethality neutralization by *A. mexicana* methanolic extract

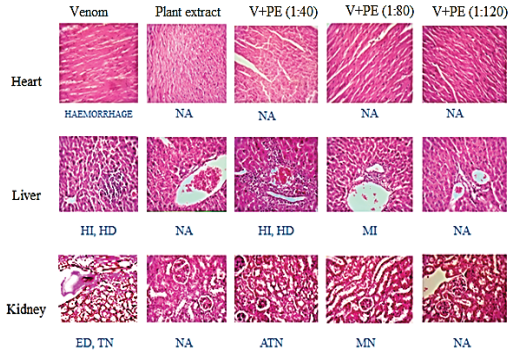

Figure 4: Histopathological examination (NA- No abnormality, HI- hepatic inflammation, HD- hepatocyte degeneration, MI-mild inflammation, ED-epithelial cell degeneration, TN- tubular necrosis, ATN- acute tubular necrosis, MN- mild necrosis, V- venom, PE- Plant extract)

Histopathological Changes

Histopathological findings of heart, liver and kidney cropped from mice treated with *Naja naja* venom in the presence /absence of *A. mexicana* methanolic extract are as follows: No abnormalities were found in the organs of mice that were treated only with saline. Tissues of the venom-injected mice showed haemorrhage in the heart; hepatic inflammation, hepatocyte degeneration, random peripheral inflammation in the liver and tubular necrosis in kidney tissues (Figure 4). Liver tissues of mice that received venom and plant extract of 40 and 80 µg/kg body weights presented random pherihepatic inflammation, hepatocyte degeneration, while tissues of mice that received venom and plant extract of 120 µg/kg body weight had no abnormalities. Similarly, kidney tissues of mice that were injected venom and 40 and 80 µg of plant extract showed acute tubular necrosis and mild necrosis, respectively. The kidney tissues of mice injected with venom and 120 µg of plant extract reported no abnormality. There were no abnormalities noted in the heart tissues of mice that were treated with venom and plant extracts of all the 3 varying concentrations. Moreover, the plant extract proves non-toxic as the tissues of all the three organs of the mice that received only plant extract showed no abnormalities.

Paw edema Inhibition

A. mexicana methanolic extract has proved its ability to decrease the edema ratio. Mice injected with *Naja naja* venom-induced edema was counter balanced by the plant extract. 10µg of venom produced an edema ratio of 178±3.2%. The edema ratio was lowered from 178±3.2% to 147±2.5% at 1:50 venom: plant extract ratio and 103±4.1% at the maximum concentration of 1:100 (Figure 5). Besides, the foot pads of mice that received only the plant extract reports no toxic response and presented an edema ratio of 101±5.1%.

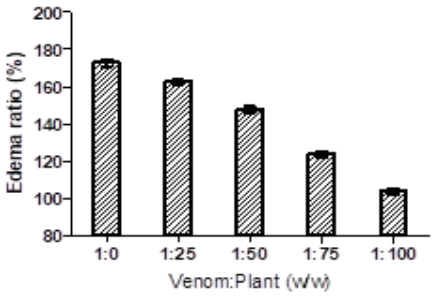

Figure 5: Venom induced edema inhibitory action of *A. mexicana* methanolic extract

Snakebite envenomation is the most 'difficult-to-treat' neglected disease throughout the world. Administration of antivenom is only encouraged and a universally approved method of treatment. Due to some drawbacks in antivenom administration, identification and utilization of complementary medicines are essential concepts in venom treatment. Very few investigations on folk medicines were studied scientifically. Further, inhibition studies of crude plant extracts and their purified compounds will lead to potential anti-ophidian substance (17). Extracts from a wide range of medicinal plants showed effectiveness against venom neutralization activity (19). Studies have exposed that extracts and their fractions of medicinal plants such as *L. aspera* employed in the traditional therapy are capable of inhibiting the enzymes of crude venom (9).

Leucas aspera belongs to *Lamiaceae* family. It is commonly used as an antipyretic and insecticide and is also validated to acquire antifungal, antioxidant, antimicrobial activities (17, 18). *Cassia alata* L. of the family *Fabacae* and the subfamily caesalpinioideae. *C. alata* is traditionally used widely in the management of ascariasis and asthma. In the treatment of snakebite, pastes made with the leaves are either administered orally or applied externally (20). *Polygala elongata is* categorized under the family *Polygalaceae*. The decoction prepared from the leaves is used for treating poisonous bites and the roots of the plant are used against snakebite (19,21). *Argemone mexicana* of the *Papaveraceae* family. Phytoconstituent screening of the plant confirms the presence of reducing sugars, saponins, flavonoids, steroids, tannins, alkaloids and glycosides. The plant exhibits antibacterial, anti-inflammatory, antidiabetic, anti-cancer, analgesic, hallucinating and sedative properties (22). Latex from the plant is used to treat cataract, reddening and itching of eyes. Root as a form of paste, used in snake bite treatment (23,24).

Venom PLA_2 is predominant in its multifunctional enzymatic activity, and therefore controlling or inhibiting this enzyme is considered the most challenging activity towards venom neutralization studies. This enzyme causes postsynaptic neurotoxicity, haemorrhage, edema and hemolysis in the snakebite victim (4). Ethnopharmacological studies revealed that medicinal plants have potential venom PLA_2 inhibitory properties due to its secondary metabolites (25,26).

The current investigation for the first time, reveals the anti-ophidian activity of extracts of *A. mexicana*. The result of the current study documents that the methanolic extract of *A. mexicana* completely inhibited the PLA_2 and also complete hemolytic inhibition of tested *Naja naja* venom.

Further, the extract was also assessed against the lethality of *Naja naja* venom in a dose-dependent manner and found to reduce the venom toxicity and increased the time of survival in the animal models. Mice treated with venom: *A. mexicana* methanolic extract ratio of 1:150 survived even after 48 hours. Comparatively, methanolic extract of *A. mexicana* exhibited a strong neutralization effect with 100% survival in the group. The possibility of engaging methanolic extract of *A. mexicana* in the treatment of snakebite is further supported by its ability to cause no toxic effects in the tissues samples of mice that were injected with only plant extract and its potency to reduce toxicity in the tissues of mice that were injected with venom and plant extract preincubated formulations. *A. mexicana* methanolic extract has proved its potential to inhibit the *Naja naja* venom-induced edema. This antivenom property of *A. mexicana*. might be possibly due to its phytoconstituents extracted by the polar solvent methanol, and the beneficiary effects of methanolic on anti-cancer reported earlier(27). Similarly, the whole methanolic plant extract of *A. mexicana* was said to be effective against vascular contraction that has been induced by noprepinephrine (28). Roots, leaves and seeds of *A. mexicana* were known for their antivenom properties (29, 30, 31). Hence, further study on secondary

metabolites can explore the potential compound responsible for venom enzyme neutralization.

Conclusion

From our investigation, we conclude that the crude methanolic extract of *A. mexicana* is active against *Naja naja* venom among all the tested extracts. The current study suggests that these extracts are a promising medicament against snake venom. Further, the isolation of active metabolites responsible for this inhibitory action will add to pharmaceutical studies' beneficial drug development.

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