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PHOSPHOLIPASES A_2: ENZYMATIC ASSAY FOR SNAKE VENOM (*NAJA NAJA KARACHIENSIS)* WITH THEIR NEUTRALIZATION BY MEDICINAL PLANTS OF PAKISTAN

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Abstract: Phospholipases A_2 (PLA_2) are the most lethal and noxious component of *Naja naja karachiensis* venom. They are engaged to induce severe toxicities after their penetration in victims. Present study was designed to highlight hydrolytic actions of PLA_2 in an egg yolk mixture and to encounter their deleterious effects *via* medicinal plants of Pakistan. PLA_2 were found to produce free fatty acids in a dose dependent manner. Venom at concentration of 0.1 mg was found to liberate 26.6 µmoles of fatty acids with a decline in pH of 0.2 owing to the presence of PLA_2 (133 Unit/mg). When quantity of venom was increased up to 8 mg, it caused to release 133 µmoles of free fatty acids with a decrease in 1.0 pH due to abundance in PLA_2 (665 Unit/mg). The rest of other doses of venom (0.3–4.0 mg) was found to liberate fatty acids between these two upper and lower limits. Twenty eight medicinal plants (0.1–0.6 mg) were tried to abort PLA_2 hydrolytic action, however, all were found useful (50–100%) against PLA_2. *Bauhinia variegate* L., *Citrus limon (L.). Burm. f, Enicostemma hyssopifolium* (Willd.) Verdoorn, *Ocimum sanctum, Psoralea corylifolia* L. and *Stenolobium stans* (L.) D. Don were found excellent in switching off 100% phospholipases A_2 at their lowest concentration (0.1 mg). Three plants extract were found useful only at lower concentration (0.1 mg), however, their higher doses were seemed to aggravate venom response. Eight medicinal plants failed to neutralize PLA_2 rather their higher doses were found effective. Standard antidote and rest of other plants extract were able to show maximum of 50% efficiencies. Therefore, it is necessary to identify and isolate bioactive constituent(s) from above cited six medicinal plants to eradicate the problem of snake bite in the future.

Keywords: phospholipases A_2, Pakistan cobra, medicinal plants, acidimetric assay, antidote

Fright from snakes is as primitive as human history. Perhaps snake biting is the most neglected tropical disease, which affects 2.5 million people and results in 100 000 deaths annually (1). There are more than two hundred species of the snakes, which are venomous on the earth. They belong to polyphyletic group of Colubroidea, however, can be classified into Cortalidae, Elapidae, Hydrophidae and Viperidae families (2, 3). Elapidae belonging to genus Naja are quite ubiquitous and constitute ten full species of Asiatic Naja. Among them, black Pakistan cobra (*Naja naja karachiensis*) is deadly poisonous and considered a sign of threat particularly in southern Punjab province of Pakistan (4, 5).

In fact, snake venoms are complex mixture of various enzymatic (hydrolytic), non-enzymatic, inorganic and organic molecules. Among hydrolytic enzymes, phospholipases A_2 are present in copious quantity, therefore their contribution towards detrimental effects cannot be waived (6). Owing to their diverse actions (mostly on the site of action) they were classified into phospholipases A_1 (hydrolyze 1-acyl group),

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phospholipases A_2 (hydrolyze central and 2-acyl group) phospholipases C and phospholipases D that hydrolyze phosphodiester linkages (7–9). Phospholipases A_2 have been responsible for many pathophysiological disorders like cardiotoxicity, neurotoxicity, edema, necrosis, hemolysis, amputation and anti-coagulation. Moreover, generation of free radicals along with reactive oxygen produced toxicities of phospholipases in the victims of snake bite (10, 11).

To neutralize deleterious effects of these phospholipases (PLA_2) numerous enzyme inhibitors have been tested previously to abstain from their undesired effects. Among them, natural antidotes (medicinal plants) have been considered the most reliable source to neutralize snakes venom. Pakistan is the hub of medicinal flora where people relay on medicinal plants for their health-related problems particularly to treat snakebite (12). It was therefore, inevitable to prove scientifically folklore claims about these medicinal plants as anti-snake venom.

To bridge this gap, present research work was designed to rationalize scientifically medicinal plants of Pakistan against *Naja naja karachiensis* phospholipases induced toxicities. It includes plants

Table1. Complete description about Pakistani medicinal plants collected for evaluation of their potentials as anti-snake venom.

No.	Tested sample (medicinal plants)	Part collected	References (anti-venom)
1	*Albizia lebbeck* (L.) Benth.	Seeds	18
2	*Allium cepa* L.	Bulb	19
3	*Allium sativum* L.	Bulb	20
4	*Althaea officinalis* L.	Roots	12
5	*Bauhinia variegate* L.	Roots	21
6	*Brassica nigra* (L. Koch)	Seeds	18
7	*Calotropis procera* (Wild.) R.Br.	Exudates Flowers	12
8	*Cedrus deodara* G. Don	Bark	18
9	*Citrus limon* (L.) Burm. f	Fruit	22
10	*Citrullus colocynthis* Schard.	Fruits	18
11	*Cuminum cyminum* L	Seeds	18
12	*Enicostemma hyssopifolium* (Willd.) Verdoorn	Full plant	23
13	*Fagonia cretica* L.	Leaves	13
14	*Leucas capitata* Desf.	Full plant	21
15	*Matthiloa incana* (L) R.Br.	Seeds	18
16	*Momordica charantia* L.	Fruit	18
17	*erium indicum* Mill.	Whole plant	12
18	*Ocimum sanctum*	Full plant	24
19	*Pinus roxburghii* Sargent	Oleoresin	18
20	*Pistacia integerrima*	Galls	18
21	*Psoralea corylifolia* L.	Seeds	18
22	*Rhazya stricta* Dcne	Leaves	12
23	*Rubia cordifolia*	Stems	8
24	*Sapindus mukorossi* Gaertn.	Fruits	24
25	*Stenolobium stans* (L) D. Don	Roots	18
26	*Terminalia arjuma* Wight and Arn	Bark	18, 24
27	*Trichodesma indicum* (L.) R.Br.	Whole plant	18
28	*Zingiber officinalis* Roscoe	Rhizome	25

Table 2. Effect of various concentration of venom on the amount of free fatty acids released in terms of change in pH of egg yolk suspension.

No.	Concentration of venom (mg/0.1 mL)	Change in pH (mean ± SEM)	Fatty acid released/min (µmole)	Enzyme activity (units/mg of crude venom)
1.	Control (saline)	(8 ± 0)	0	0
2.	0.1	(7.8 ± 0.028)	26.6	133
3.	0.3	(7.6 ± 0.028)	53.2	266
4.	0.5	(7.5 ± 0.028)	66.5	332
5.	1.0	(7.4 ± 0.028)	79.8	399
6.	2.0	(7.2 ± 0)	106.4	532
7.	4.0	(7.1 ± 0.028)	119.7	598
8.	8.0	(7.0 ± 0.028)	133	665

listed in Table 1 in comparison with reference standard (anti-sera) used in hospitals to treat snake bite.

MATERIALS AND METHODS

Collection of snakes

Naja naja karachiensis (*Naja. N. karachiensis*) were collected with the help of local charmers from Cholistan desert located in southern Punjab province of Pakistan. After collection they were dully identified by zoologist.

Snakes venom extraction

Venom from *Naja N. karachiensis* was extracted by compressing the glands below their eyes in low light atmosphere. After collection, it was lyophilized and preserved for further use in light resistant bottle in a refrigerator. Before use it was reconstituted in 0.9% saline in terms of its dry weight (13).

Plants collection

Folklore claimed twenty eight medicinal plants of Pakistan as anti snake venom (Table 1) were collected from various locations in Pakistan. After their collection, they were authenticated by renowned botanist Prof. Dr. Altaf Ahmad Dasti, Bhauddin-Zakariya University, Multan, Pakistan. Voucher specimens were deposited in the herbarium of the Botany department.

Preparation of plants extract

After washing and shade drying different plants material (1 kg) was crushed and subjected to simple maceration process. Methanol (5 L) was used as solvent for extraction and kept in extraction bottles for a period of 4 weeks. They were filtered initially by muslin cloth followed by Whatman filter paper number 41. Various plants extracts were weighed and preserved for further use after evaporation of methanol in a water bath (14).

Reference standard antidote (anti-venom)

Reference standard antidote (immunoglobulins) was purchased from local pharmacy of Nishtar Hospital, Multan, Pakistan. It was used to compare results of various plants extracts with standard anti-venom. It was manufactured by Bharat Serums and Vaccines Ltd., Ambernath (E) – 421 501, India (13).

Acidimetric enzymatic assay for snake venom phospholipases A_2

Acidimetric assay for PLA_2 enzymes was followed as described by Tan and Tan (15). Constant volumes of substrate comprising calcium chloride (18 mM), sodium deoxycholate (8.1 mM) and egg yolk were mixed and stirred for 10 min to get homogenous egg yolk suspension. By addition of sodium hydroxide (1 M) pH of the suspension was adjusted to 8.0. Snake venom (0.1–8 mg/0.1 mL) was added to the above mixture (15 mL) to initiate the process of hydrolysis and saline was used as control. A decrease in pH of the suspension was noted after two minutes with the help of a pH meter. A decline in 1.0 pH unit corresponds to the 133 µmoles of fatty acid released in the egg yolk mixture. Furthermore, enzymatic activity of phospholipases A_2 was calculated from the data obtained as micromoles of fatty acid released / minute (15).

To test anti-venom potentials of medicinal plants snake venom (0.1 mg) was pre-incubated with their extracts (0.1–0.6 mg/mL) to neutralize PLA_2 hydrolytic action. Protection offered by vari-

ous plants against phospholipases was measured and represented in terms of percentage.

RESULTS AND DISCUSSION

PLA_2 are the most significant component of cobra venom. In this acidimetric assay, they were hydrolyzed and released free fatty acids in the presence of sodium deoxycholate.

Snake venom was found to liberate free fatty acids in dose dependent manner. This was measured in terms of a decrease in pH of the egg yolk mixture. Snake venom at concentration of 0.1 mg was found to liberate 26.6 µmol of free fatty acids. However, on increasing the quantity of venom (0.3, 0.5, 1.0, 2.0 and 4.0 mg) large amount of fatty acids (53.2, 66.5, 79.8, 106.4 and 119.7 µmol/min) were liberated, respectively. Complete drop of 1.0 pH from 8 to

Table 3. Medicinal plants of Pakistan having antidotal properties against *Naja naja karachiensis* phospholipases A_2 in terms of an increase in pH of an egg yolk suspension.

Scientific names of plants	pH at various concentrations of plants extract (mg/mL)			Maximum protection
	0.1	**0.3**	**0.6**	(%)
Albizia lebbeck (L.) Benth.	7.9	7.9	7.9	50
Allium cepa L.	7.9	7.9	7.9	50
Allium sativum L.	7.9	7.9	7.9	50
Althaea officinalis L.	7.9	8.0	8.0	100
Bauhinia variegate L.	8.0	8.0	8.0	100
Brassica nigra (L. Koch)	7.9	7.9	7.9	50
Calotropis procera (Wild.) R.Br. (exudates)	7.9	7.9	7.9	50
Calotropis procera (Wild.) R.Br. (flower)	7.9	7.9	7.9	50
Cedrus deodara G. Don	7.9	7.9	7.9	50
Citrullus colocynthis	7.9	7.9	8.0	100
Citrus limon (L). Burm. f	8.0	8.0	8.0	100
Cuminum cyminum L.	7.9	7.9	8.0	100
Enicostemma hyssopifolium (Willd.) Verdoorn	8.0	8.0	8.0	100
Fogonia cretica L.	7.9	7.9	7.9	50
Leucas capitata Desf.	7.9	7.9	7.9	50
Matthiloa incana (L) R.Br.	8.0	7.9	7.9	100
Momordica charantia L.	7.8	7.8	8	100
Nerium indicum Mill.	7.9	8.0	8.0	100
Ocimum sanctum	8.0	8.0	8.0	100
Pinus roxburghii Sargent	7.9	8.0	8.0	100
Pistacia integerrima	8.0	7.9	7.9	100
Psoralea corylifolia L.	8.0	8.0	8.0	100
Rhazya stricta Dcne	7.9	7.9	7.9	50
Rubia cordifolia	7.9	7.9	7.9	50
Sapindus mukorossi Gaertn.	7.9	7.9	8.0	100
Stenolobium stans (L) D. Don	8.0	8.0	8.0	100
Terminalia arjuma Wight & Arn	7.9	7.9	8.0	100
Trichodesma indicum (L.) R.Br.	7.9	7.9	7.9	50
Zingiber officinalis Roscoe (A-rak)	8.0	7.9	7.9	100
Standard anti-sera (reference standard)	7.9	7.9	7.9	50
Saline	8.0	8.0	8.0	Control

7 (corresponds to 133 µmol of fatty acids) was observed at concentration of 8.0 mg of *Naja naja karachiensis* venom. Enzymatic activity of PLA_2 (units/mg) was found to be elevated as the quantity of venom was improved. Values of 133, 266, 332, 399, 532, 598 and 665 units/mg of PLA_2 were found in 0.1, 0.3, 0.5, 1.0, 2.0, 4.0 and 8.0 mg of crude venom, respectively. Complete data about enzymatic activity of PLA_2 are shown in Table 2.

Twenty eight medicinal plants having concentration ranging from 0.1 to 0.6 mg/mL were evaluated to neutralize venom's phospholipases activity. All medicinal plants were found to have potentials to inhibit phospholipases hydrolytic action but vary in their potency. Among them *Bauhinia variegate* L., *Citrus limon (L.). Burm. f, Enicostemma hyssopifolium* (Willd.) Verdoorn, *Ocimum sanctum, Psoralea corylifolia* L. and *Stenolobium stans* (L.) D. Don were effective at 0.1 mg/mL to inhibit complete PLA_2 hydrolytic action(s). Their lower doses were equally valuable as their higher doses to bestow 100% protection. *Althaea officinalis* L., *Citrullus colocynthis, Cuminum cyminum* L., *Momordica charantia* L., *Nerium indicum* Mill., *Pinus roxburghii* Sargent, *Sapindus mukorossi* Gaertn., *Terminalia arjuma* Wight & Arn showed their potentials at higher concentration (0.3 or 0.6 mg/mL) to inhibit completely (100%) PLA_2 enzymatic actions. Three plants (*Matthiloa incana* (L.) R.Br., *Pistacia integerrima* and *Zingiber officinalis* Roscoe) were recorded to halt 100% PLA_2 activity only at lower dose (0.1 mg/mL). Their higher concentrations were proved to aggravate venom response. Rest of all plants was found to neutralize 50% PLA_2 hydrolytic actions at all concentrations (0.1–0.6 mg/mL). Among them *Albizia lebbeck* (L.) Benth., *Allium cepa* L., *Allium sativum* L., *Brassica nigra* (L. Koch), *Calotropis procera* Wild. (flowers and exudates), *Cedrus deodara* G. Don, *Fogonia cretica* L., *Leucas capitata* Desf., *Rhazya stricta* Dcne, *Rubia cordifolia and Trichodesma indicum* (L.) R.Br. are included. Standard antidote was found 50% effective to combat poisonous effects of PLA_2. Complete detail about working efficiencies of various antidotes have been summarized in Table 3.

People living in tropical and subtropical areas of the world are awfully more prone to snake bite envenomation. Among various noxious proteins of cobra, venom PLA_2 are in abundance and play a pivotal role in emergence of toxicities. They are tiny richest molecules that cause havoc by alteration in physiological processes within victims (16).

PLA_2 were found to interact with other components particularly lipids of the cells. Both specific and non specific PLA_2 developed protein – phospholipids interaction with covalent, non covalent or disulfide interactions. PLA_2 (Asp49 variants) caused to hydrolyze phospholipids or released free fatty acids (16, 17). This was the reason when quantity of snake venom was increased it liberated greater amount of free fatty acids (in the presence of deoxycholic acid) due to abundance of PLA_2.

There are numerous protein (enzyme) binding constituents that have been reported previously to neutralize snake venom peptides. Medicinal plants are stuffed with these compounds, therefore have engrossed therapeutic priorities in application of natural inhibitors. Medicinal plants of Pakistan have primeval record to neutralize snake venom proteins due to abundance of miscellaneous secondary metabolites. Among them quinonoids, terpenoids, polyphenols, xanthenes and flavonoids were documented previously to minimize snake venom toxins (12).

CONCLUSION

On above grounds, present research work has confirmed folklore claims (as anti-snake venom) about medicinal plants to rationalize them scientifically in traditional system of medicine. Among 28 medicinal plants extracts only six were found to provide 100% protection against PLA_2 hydrolytic actions at minimum concentration of 0.1 µg/mL. They were proved more potent and effective when compared with standard antidote (reference standard). However, further study is inevitable for discovery and isolation of bioactive constituent(s) from six the most potent and effective medicinal plants to handle adroitly the problem of snake poisoning.

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