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A Remedial Approach for *Naja naja karachiensis* Envenomation: Enzymatic Assay for Alkaline Phosphatase Activity in Extracts of Local Plants of Pakistan

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Abstract.- Present study was performed to inhibit alkaline phosphatases (ALPases) present in snake venom *Naja naja karachiensis* by 28 medicinal plants of Pakistan. 2.87 U/mg, 4.75 U/mg, 6.70 U/mg and 8.6 U/mg of phosphatase activity were estimated for 0.2 mg, 0.4 mg, 0.6 mg and 0.8 mg of crude venom respectively in dose dependent manner. All plants extract (625 ng to 2.5µg) were found to inhibit (80% to 93%) snake venom (0.4 mg) ALPases. Standard antidote was found to pose 93% hindrance at 2.5 µg/ml while EDTA (specific metalloenzyme inhibitor) was observed 91% effective. Extract of *Sapindus mukorossi* Gaertn was declared the best anti-venom as reference standard however, isolation and identification of bioactive constituent(s) is necessary for complete treatment of snake bite envenomation.

Key words: Alkaline phosphatases, medicinal plants, Pakistan cobra, antisera.

INTRODUCTION

Snakes venom is complex mixture of hydrolytic enzymes, non enzymatic proteins, organic and inorganic molecules (Asad *et al*., 2013; Dhananjaya and D'Souza, 2011; Khan, 1999). Among hydrolytic enzymes proteases, phospholipases (PLA) were more screened pharmacologically whilst nucleases, nucleotidases and phosphatases (acidic as well as alkaline phosphatases) were less characterized (Matsui *et al*., 2000; Marshall, 2005). It was due to toxinologists who claimed previously their non-toxic and digestive role (Dhananjaya and D'Souza, 2011). However, recent studies revealed that ALPases are deadly poisonous owing to their hydrolytic tendency towards phosphate esters non-specifically. In the victims they act on various substrates like 3'- AMP, 5'- AMP, FMN, 5'- dAMP, deoxy-dinucleotide phosphates, ribose-3-phosphate, 5'- phosphoribose-1-pyrophosphate and ATP (preferably). ALPases act on ATP along with phosphodiesterases (PDE) caused in generation of adenosine moieties (Wuster, 1996; Feroze *et al*., 2010). Concurrently other enzymes in snake venom like phospholipases, proteases, cardiotoxin and cytotoxic peptides assist in cell necrosis lead to liberation of nucleic acids (DNA and RNA) (Alirol *et al*., 2010; Razi *et al*., 2011). Nucleic acids cleaved by nucleotideases and ALPases and resulted in large amount of adenosine. Adenosine is a multitoxin and induced miscellaneous hazardous effects like hypotention, heart block, redness, inflammation, anti-platelet aggregation, renal failure, unconsciousness, pain, analgesia via different adenosine (A_1, A_{2a}, A_{2b} and A_3) receptors (Dhananjaya and D'Souza, 2011).

Immunoglobulins are effective for snake-bites; however, recovery depends on dose, neutralization and way of inoculation (Warrell, 2010). Nevertheless, their high cost, serum sickness reactions and scarcity in rural areas have aroused the quest for new sources of venom inhibitors (Razi *et al*., 2011). Medicinal plants (natural antidotes) have been documented previously to have various constituents to inhibit snake venom enzymes. Pakistan is a hub of medicinal plants where mostly rural people depend on them for their health-related problems (Asad *et al*., 2011). To the best of our knowledge mostly medicinal plants of Pakistan have not been assessed for their antivenom potentials to neutralize alkaline phosphatases regardless of their ethnobotanical use as antisnake venom (Asad *et al*., 2011, 2014; Khan *et al*., 2009). Due to this reason venom from *Naja naja karchiensis* was evaluated

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scientifically for the first time to neutralize ALPases by local medicinal plants used in Pakistan.

MATERIALS AND METHODS

Collection of Naja naja karachiensis

Cobra snakes were collected from Cholistan desert situated in Southern Punjab province of Pakistan. They were collected with charmers and further duly identified by zoologist.

Milking of snake venom

Pakistani cobra snakes venom was milked by squeezing their glands below their eyes. Whole experiment was carried out in low light surrounding. Subsequently it was lyophilized and then refrigerated in a light resistant container. Before use lyophilized venom was reconstituted in saline in terms of its dry powder weight (Razi *et al*., 2011).

Plant collection

Medicinal plants of Pakistan were collected from various areas (Khyber Pakhtoonkhawa and Gilgit Biltistan) of the country. Different parts of the plants were collected on the basis of their ethnobotanical claims as anti-venom. Prof. Dr. Altaf Ahmad Dasti identified them and voucher specimens were deposited in the herbarium of the Institute of Pure and Applied Biology, Bahauddin-Zakariya University, Multan, Pakistan. Complete description about collection of medicinal plants of Pakistan is provided in Table I.

Extraction of plant material

Shade dried plants material were chopped and further subjected to simple maceration process. Dried powder of desired part(s) of plant material (1Kg) was soaked in methanol (5L) in the extraction bottles. Homogenates were kept at ambient temperature for a period of four weeks. Subsequently they were filtered two times, initially with an ordinary filter paper and later on by Whatman filter paper # 41. Methanol was evaporated at room temperature and different extracts were weighed and stored for further research (Hussain *et al*., 2007). Milky exudates of *Citrullus colocynthis (L.)* Schrad were collected, lyophilized and then stored for further use.

Reference standard antidote

Reference standard antiserum (antidote) was purchased from pharmacy store (Raza Pharmacy) at Nishtar Hospital, Multan, Pakistan. It was utilized to compare different plants extract for neutralization of alkaline phospahtases toxicities. Alkaline phosphatases (with enzyme commission number 3.1.3.1) was supplied by Bharat Serums and Vaccines Limited, Ambernath, India (Asad *et al*., 2012).

Enzymatic assay for alkaline phosphatases

Activity of alkaline phosphatses (ALPases) in *Naja naja karachiensis* venom was assessed with the release of *p*-nitrophenol from its substrate (*p*-nitrophenyl phosphate) by their hydrolytic action. Briefly, reaction mixture was prepared by mixing 0.5 ml of 0.5 M glycine buffer (pH=8.5), 0.5 ml of 0.01M *p*-nitrophenyl phosphate and 0.3 ml of 0.01 M $MgSO_4$. Subsequently, *Naja naja karachiensis* venom (0.2-0.8 mg/0.1ml) was added. Reaction mixture was incubated at 37 ºC for half an hour. At the end of this period 2 ml of NaOH (0.2 M) solution was added and further kept for 20 minutes at room temperature to halt the reaction and to confer stable yellow color to *p*-nitrophenol which absorbed maximally at 400 nm (Kanagasabapathy and Kumari, 2000; Sulkowski *et al*., 1963; Yap *et al*., 2011). A standard curve of known concentrations of *p*-nitrophenol was constructed and ALPase activity was expressed as micromole of product released per minute.

To evaluate anti-venom activity of various antidotes (standard antisera and medicinal plants) they were preincubated with venom (0.4 mg) at different concentration ranges from 312 ng/ml to 05 µg/ml for 30 minutes before measuring ALPase activity (Ushanandini *et al*., 2006; Dhananjaya *et al*., 2006). Specific metalloenzyme inhibitor (EDTA, 2mM) was used to monitor the inhibition of ALPases activity. Additionally venom was also evaluated upon heating (0.4 mg, 100ºC, 10 min) and then after cooling (25ºC, 45 min) for ALPases activity (Chakrabarty *et al*., 2000).

Table I.- Different medicinal plants collected from various locations in Pakistan having ethnobotanical evidences as anti-venom.

Sr. No	Family (scientific name of the plant)	Area of collection (Part collected)	Voucher number	Reference
1.	Amaryllidaceae (*Allium cepa* L).	Bahawalpur (seeds)	STW.42	Makhija and Khamar (2010)
2.	Anacardiaceae (*Pistacia integerrima* J. L. Stewart).	Murree Hills (galls)	STW.458	Baquar (1989)
3.	Apiaceae (*Cuminum cyminum* L).	Sargodha (seeds)	STW.516	Baquar (1989)
4.	Apocynaceae (*Nerium indicum* Mill)	Haripur (roots and leaves)	STW.564	Asad *et al.* (2011)
5.	Apocynaceae (*Rhazya stricta* Dcne)	Leaves (Lakki Marwat)	STW.565	Asad *et al.* (2011)
6.	Apocynaceae (*Calotropis procera (*Aiton) W.T. Aiton)	Haripur (milky latex & flower)	$STW.566_{(a)}$ $STW.566_{(b)}$	Asad *et al.* (2011)
7.	Bignoniaceae (*Stenolobium stans* (L.) Seem).	Haripur (roots)	STW.669	Baquar (1989)
8.	Boraginaceae (*Trichodesma indicum* (L.) Sm).	Sind province (complete plant)	STW.604	Baquar (1989)
9.	Fabaceae (*Bauhinia variegate* L).	Haripur (roots).	STW.374	Shinwari and Shah (2007)
10.	Brassicaceae (*Brassica nigra* (L.) W. D. J. Koch)	Manshera (seeds)	STW.302	Baquar (1989)
11.	Brassicaceae (*Matthiola incana* (L.) W. T. Aiton)	Rawalpindi (seeds)	STW.322	Baquar (1989)
12.	Cucurbitaceae (*Momordica charantia* L).	Abbottabad (fruit)	STW.706	Baquar (1989)
13.	Cucurbitaceae (*Citrullus colocynthis* (L.) Schrad).	Bahawalpur (fruits)	STW.702	Baquar (1989)
14.	Combretaceae (*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn).	Islamabad (bark)	STW.502	Baquar (1989), Prajapati *et al*. (2010)
15.	Fabaceae (*Psoralea corylifolia* L).	Peshawar (seeds)	STW.418	Baquar (1989)
16.	Gentianaceae (*Enicostema hyssopifolium* (Willd.) I. Verd).	Jhelum (fresh plant)	STW.553	Daniel (2006)
17.	Lamiaceae (*Leucas capitata* Desf)	Rawalpindi (whole plant)	STW.615	Shinwari and Shah (2007)
18.	Lamiaceae (*Ocimum sanctum* L)	Islamabad (whole plant)	STW.626	Prajapati *et al*. (2010)
19.	Amaryllidaceae (*Allium sativum* L).	Bhakkar (bulb)	STW.46	Ugulu (2011)
20.	Malvaceae (*Althaea officinalis* L).	Rawalpindi (roots)	STW.411	Asad *et al.* (2011)
21.	Fabaceae (*Albizia lebbeck* (L.) Benth)	Bahawalpur (seeds).	STW.381	Baquar (1989)
22.	Pinaceae (*Cedrus deodara* (Roxb. ex D. Don) G. Don)	Nathia Gali (bark)	STW.25	Baquar (1989)
23.	Pinaceae (*Pinus roxburghii* Sarg)	Murree Hills (oleoresin)	STW.26	Baquar (1989)
24.	Rubiaceae (*Rubia cordifolia* L)	Murree Hills (stems)	STW.689	Baquar (1989)
25.	Rutaceae (*Citrus limon* (L). *Burm.* F)	Haripur (fruit)	STW. xx	Rita *et al*. (2011)
26.	Sapindaceae (*Sapindus mukorossi* Gaertn)	Niswari Bazaar Rawalpindi (fruits)	STW.463	Prajapati *et al*. (2010)
27.	Zingiberaceae (*Zingiber officinale* Roscoe)	Lahore (rhizome)	STW.66	Duke and Ayensu (1985)
28.	Zygophyllaceae (*Fagonia cretica* L)	Lasbella (leaves and twigs)	STW.433	Razi *et al.* (2011)

Statistical analysis

All numerical values were obtained as mean ± SEM by using Microsoft Excel version 2007. Student *t*-test was applied by the guidelines and instructions published by British medical Journal. Level of significance was set at 0.05.

RESULTS AND DISCUSSION

Standard graph of *P*-NP was generated with positive correlation coefficient (r) equal to 0.999 and $y = 0.009x + 0.001$. High doses of cobra venom were found with greater enzymatic activity in a dose

dependent manner. Various activities of ALPase *i.e.*, 2.87, 4.75, 6.70 and 8.60 U/mg were found in 0.2, 0.4, 0.6 and 0.8 mg of crude venom respectively. Furthermore, heated venom resulted in cessation of phosphatase activity as shown in Table II.

Table II.- Enzymatic activity of alkaline phosphatases at various concentrations of *Naja naja karachiensis* venom in terms of *para*-nitrophenol (*P*NP) released.

Concentration of venom used for enzymatic activity	Absorbance at 400 nm (Mean ± SEM)	Enzyme activity (Unit/mg)
0.2 mg/0.1 ml	0.31±0.01	2.87
0.4 mg/0.1 ml	0.51±0.01	4.75
0.6 mg/0.1 ml	0.72±0.02	6.70
0.8 mg/0.1 ml	0.93±0.02	8.60
Effect of heat on venom	Same as sample blank	Nil
Cooling effect on heated venom	Same as sample blank	Nil

Dose of venom was fixed at 0.4 mg to monitor the neutralizing tendencies of miscellaneous antidotes. *In vitro* experimentations revealed that different medicinal plants within concentration range of 625 ng to 2.5 µg were found maximally (80% to 93%) active to cope with deleterious effects of ALPase. Standard antidote was found to pose maximum hindrance (93% at 2.5 µg) against ALPase whilst EDTA (specific metalloenzyme inhibitor) was proved to halt 91% activity. Treatment of cobra venom with standard antidote resulted in maximum neutralization of 4.4 U/mg of phosphatase activities however, 0.36 U/mg activities were outstanding. On comparison of mean absorbance of various plants extract (anti-venom) with reference standard, 21% medicinal plants *(Brassica nigra* (L.) W. D. J. Koch, *Enicostema hyssopifolium* (Willd.) I. Verd, *Pinus roxburghii* Sarg, *Rhazya stricta* Decne, *Sapindus mukorossi* Gaertn and *Stenolobium stans* (L.) Seem*)* were found anti-venom up to 93% effective at $p<0.5$. Moreover, 46% plant extracts (*Albizia lebbeck* (L.) Benth, *Allium cepa* L, *Allium sativum* L, *Bauhinia variegate L, Citrullus colocynthis* (L.) Schrad, *Citrus limon* (L.) Burm. F. *Cuminum cyminum* L. *Matthiola incana* (L.) W. T. Aiton, *Ocimum sanctum* L, *Pistacia integerrima* J. L. Stewart, *Psoralea corylifolia* L, *Rubia cordifolia* L and *Zingiber officinale* Roscoe) were observed (≤91%) supportive having $P < 0.5$. Remaining 33% plants extract (*Althaea officinalis* L, *Calotropis procera* (Aiton) W. T. Aiton (both exudates and flowers), *Cedrus deodara* (Roxb. ex D. Don) G. Don, *Fagonia cretica* L, *Leucas capitata* Desf, *Momordica charantia* L, *Nerium indicum* Mill, *Trichodesma indicum* (L.) Sm and *Terminalia arjuna* (Roxb. ex DC.) Wight & Arn) were found ≤86% valuable with $P<0.05$ to neutralize snake venom. Among various plant extracts *Sapindus mukorossi* Gaertn was found as effective as reference standard (93%, 2.5 µg) to neutralize ALPase activities.

Complete description about inhibition of ALPase activity by medicinal plants of Pakistan (at the most effective concentration) is summarized in Table III.

ALPases have been documented previously for their presence in different spiders and snakes venom. They are more copious in the venom of crotalids, elapids and viperids, therefore, present study was designed to neutralize ALPases present in cobra venom via medicinal plants extracts, as a step towards scientifically validating their efficiencies as an antidote (Rodrigues *et al*., 2006; Dhananjaya and D'Souza, 2011).

ALPases produced their toxic effects in dose depended manner however elevated temperature led to permanently cessation of ALPases activities might be due to degeneration of (locus of) this enzyme. Metalloenzyme inhibitor (EDTA) has shown inhibitory effects by sequestration of metallic ions that are necessary for normal functioning of ALPases (Chakrabarty *et al*., 2000). Neutralizing phenomenon of antidotes can be best explained by considering snake venom as antigen and antidote (antisera) as antibody. Maximum neutralization was observed when neither antigen nor antibody in excess *i.e.*, at equivalence point (Edwards, 1985). Therefore, different antidotes were exhibited maximum protection (80-93%) at lower concentration comparable to the quantity of ALPases in the cobra venom. Highest protection offered by various plant extracts at different

Table III.- List of maximum protection posed by various plant extracts at the most effective concentration to mask alkaline phosphatase activity in 0.4 mg/0.1ml of snake venom.

Sr. No.	Scientific name of anti-dote	Most effective concen-tration (µg/ml)	Activity of alkaline phosphatase		Masked enzymatic activity (U/mg)	Maximum protection offered (%)
			Before treatment (U/mg)	After treatment (U/mg)		
1.	*Albizia lebbeck* (L.) Benth	1.25	4.75	0.69	4.06	85*
2.	*Allium cepa* L	2.5	4.75	0.63	4.12	87*
3.	*Allium sativum* L	2.5	4.75	0.62	4.13	87*
4.	*Althaea officinalis* L.	0.625	4.75	0.72	4.03	85*
5.	*Bauhinia variegate* L.	1.25	4.75	0.72	4.03	85*
6.	*Brassica nigra* (L.) W. D. J. Koch	1.25	4.75	0.60	4.15	87*
7.	*Calotropis procera* (Aiton) W. T. Aiton (exudates)	0.625	4.75	0.67	4.08	86*
8.	*Calotropis procera* (Aiton) W. T. Aiton (flower)	0.625	4.75	0.89	3.86	81*
9.	*Cedrus deodara* (Roxb. ex D. Don) G. Don	1.25	4.75	0.86	3.89	82*
10.	*Citrullus colocynthis* (L.) Schrad	0.625	4.75	0.65	4.10	86*
11.	*Citrus limon* (L.) Burm. f.	1.25	4.75	0.42	4.33	91**
12.	*Cuminum cyminum* L	2.5	4.75	0.64	4.11	86*
13.	*Enicostema hyssopifolium* (Willd.) I. Verd	0.625	4.75	0.40	4.35	91**
14.	*Fagonia cretica* L	2.5	4.75	0.85	3.90	82*
15.	*Leucas capitata* Desf	1.25	4.75	0.82	3.93	83*
16.	*Matthiola incana* (L.) W. T. Aiton	2.5	4.75	0.63	4.12	87*
17.	*Momordica charantia* L	0.625	4.75	0.96	3.79	80*
18.	*Nerium indicum* Mill	0.625	4.75	0.86	3.89	82*
19.	*Ocimum sanctum* L	1.25	4.75	0.65	4.10	86*
20.	*Pinus roxburghii* Sarg	1.25	4.75	0.48	4.27	90**
21.	*Pistacia integerrima* J. L. Stewart	2.5	4.75	0.45	4.30	90**
22.	*Psoralea corylifolia* L	0.625	4.75	0.50	4.25	89*
23.	*Rhazya stricta* Decne	2.5	4.75	0.42	4.33	91**
24.	*Rubia cordifolia* L	0.625	4.75	0.44	4.31	91**
25.	*Sapindus mukorossi* Gaertn	2.5	4.75	0.33	4.42	93**
26.	*Stenolobium stans* (L.) Seem	0.625	4.75	0.64	4.11	86*
27.	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn	2.5	4.75	0.96	3.79	80*
28.	*Trichodesma indicum* (L.) Sm	0.625	4.75	0.74	4.01	84*
29.	*Zingiber officinale* Roscoe	1.25	4.75	0.77	3.98	84*

Note: * indicates values significantly different from reference value.
** indicates values non-significantly different from reference value.

concentrations depicted their relative potencies as shown in Table III**.** Medicinal plant extracts at high doses (10mg/ml) were unable to show their best neutralizing effects (data is not shown). It is due to the equivalence point which was not attained due to excess of antidotes in addition to their intrinsic

colors that have created interference in absorbance pattern led to erroneous results (Asad *et al*., 2011).

CONCLUSIONS

Among all medicinal plants, *Sapindus mukorossi* Gaertn extract proved equally effective as reference standard to inhibit snake venom ALPases. Presence of secondary metabolites like xanthenes, phenols, quinonoids, terpenoids and various flavonoids have previously been documented to inhibit snake venom enzymes. Secondary metabolites neutralize snake venom and pose hindrance in binding of various enzymes to their potential targets, thus producing neutralizing effects. However, further study is inevitable to evaluate and characterize bioactive component(s) from potential medicinal plant extracts.

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