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Compensatory effects of medicinal plants of Pakistan upon prolongation of coagulation assays induced by *Naja naja karachiensis* bite

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The present study was carried out to evaluate 28 medicinal plants of Pakistan having folklore claims to neutralize coagulopathy induced by *Naja naja karachiensis* bite in comparison with standard antidote. Venom was tested on citrated human plasma to determine its effect on prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT). Snake venom (200 µg/ml) was found to delay PT (13 ± 0.57 to 23 ± 0.57 sec), aPTT (35 ± 1.52 to 48 ± 2.0 sec) and TT (13 ± 0.57 to 33 ± 0.57 sec) within 4.5% coefficient of variance. Prolongation of PT and TT suggested the presence of thrombin-like or plasminogen activating enzymes. Methanolic plant extracts (5 µg/ml) were considered as effective standard antidote. *Enicostemma hyssopifolium* (Willd.) Verdoorn (PT = 22 ± 0.57 sec, aPTT = 36 ± 1.00 sec, TT = 19 ± 0.57 sec) and *Stenolobium stans* (L) D. Don (PT = 16 ± 0.57 sec, aPTT = 36 ± 0.57 sec, TT = 29 ± 0.57 sec) were considered the most protective (≥70%, but ≤ 92%) from the rest of the listed medicinal plants. Nevertheless, further studies are required for identification and segregation of bioactive constituent(s) as an alternate and cheap source to treat anticoagulation.

Keywords: Antidote, coagulopathy, medicinal plants, *Naja naja karachiensis*.

INCIDENCES of snake-bite poisoning are particularly frequent in tropical and subtropical areas of the world, resulting in high rate of mortality and morbidity. Hence it has received the attention of several researchers to find out the root cause of snake-bite poisoning and to pave the way for possible treatment[1]. Like other countries of the world, snake-bite envenomation is common in Pakistan, where 20,000 deaths are reported annually[2]. Among various species of Asiatic *Naja* (complex Asiatic cobras), *Naja naja karachiensis* causes serious disorders in the victims of snake bite. Severe pain, necrosis, bleeding from wounds, blood in urine, inflammation, gum bleeding and coagulopathies are some of the complications arising due to *N. n. karachiensis* bite[3–5].

Coagulopathy is one of the key after-effects of *N. n. karachiensis* envenomation. It is the clotting defect in which blood is unable to congeal contrary to normal blood. Clotting disorders have been monitored by coagulation assays in diagnostic laboratories[5]. These include PT (prothrombin time), aPTT (activated partial thromboplastin time) and TT (thrombin time). Coagulation assays are surrogate markers for various blood-clotting factors. PT has presumptive evidences about II, V, VII and X clotting factors, while aPTT possesses information about VIII, IX and XI factors. TT is linked with fibrinogen (factor I) along with its measurements[5]. Administration of antisera is an appropriate therapy to combat snake-bite envenomation. However, due to their limited supply and high cost, the rural population is unable to afford them[2].

Consequently victims have to rely on medicinal plants to treat snake bite as they have been reported in the literature to neutralize various snake venoms[6]. In the present study, various medicinal plants of Pakistan (widespread in different locations) were collected to facilitate the victims against *N. n. karachiensis* venom-induced coagulopathies. These included *Albizia lebbeck* (L.) Benth, *Allium cepa* L., *Allium sativum* L., *Althaea officinalis* L., *Bauhinia variegate* L., *Brassica nigra* (L.) W. D. J. Koch, *Calotropis procera* (Aiton) W. T. Aiton, *Cedrus deodara* (Roxb. ex D. Don) G. Don, *Citrullus colocynthis* (L.) Schrad, *Citrus limon* (L.). Burm. f, *Cuminum cyminum* L., *Enicostemma hyssopifolium* (Willd.) I. Verd, *Fogonia cretica* L., *Leucas capitata* Desf, *Matthiloa incana* (L.)

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Table 1. Medicinal plants of Pakistan used to combat with coagulapathies produced by *Naja naja karachiensis* bite

Tested sample (medicinal plant)	Family	Place of collection	Plant part used	References (anti-venom)
Albizia lebbeck (L.) Benth	Fabaceae	Bahawalpur	Seeds	14
Allium cepa L.	Amaryllidaceae	Bhakkar	Bulb	15
Allium sativum L.	Amaryllidaceae	Bhakkar	Bulb	16
Althaea officinalis L.	Malvaceae	Rawalpindi	Roots	6
Bauhinia variegata L.	Fabaceae	Haripur	Roots	17
Brassica nigra (L.) W. D. J. Koch	Brassicaceae	Manshera	Seeds	14
Calotropis procera (Aiton) W. T. Aiton	Apocynaceae	Haripur	Exudates	6
Calotropis procera (Aiton) W. T. Aiton	Apocynaceae	Haripur	Flowers	6
Cedrus deodara (Roxb. ex D. Don) G. Don	Pinaceae	Nathia Gali	Bark	14
Citrus limon (L.). Burm. f	Rutaceae	Haripur	Fruit	18
Citrullus colocynthis (L.) Schrad	Cucurbitaceae	Bahawalpur	fruits	14
Cuminum cyminum L.	Apiaceae	Sargodha	Seeds	14
Enicostemma hyssopifolium (Willd.) I. Verd.	Gentianaceae	Jhelum	Full plant	19
Fagonia cretica L.	Zygophyllaceae	Lasbella	leaves	2
Leucas capitata Desf	Lamiaceae	Rawalpindi	Full plant	17
Matthiloa incana (L.) W. T. Aiton	Brassicaceae	Rawalpindi	Seeds	14
Momordica charantia L.	Cucurbitaceae	Abbottabad	Fruit	14
Nerium indicum Mill	Apocynaceae	Haripur	Roots and leaves	6
Ocimum sanctum L.	Lamiaceae	Islamabad	Full plant	20
Pinus roxburghii Sarg	Pinaceae	Murree	Oleoresin	14
Pistacia integerrima J. L. Stewart	Anacardiaceae	Murree	Galls	14
Psoralea corylifolia L.	Fabaceae	Peshawar	Seeds	14
Rhazya stricta Dcne	Apocynaceae	Lakki Marwat	Leaves	6
Rubia cordifolia L.	Rubiaceae	Murree	Stems	14
Sapindus mukorossi Gaertn	Sapindaceae	Local market, Rawalpindi	Fruits	21
Stenolobium stans (L.) Seem	Bignoniaceae	Haripur	Roots	14
Terminalia arjuna (Roxb. ex DC.) Wight & Arn	Combretaceae	Islamabad	Bark	14, 20
Trichodesma indicum (L.) Sm	Boraginaceae	Sind	Whole plant	14
Zingiber officinale Roscoe	Zingiberaceae	Lahore	Rhizome	22

W. T. Aiton, *Momordica charantia* L., *Nerium indicum* Mill, *Ocimum sanctum* L., *Pinus roxburghii* Sarg, *Pistacia integerrima* J. L. Stewart, *Psoralea corylifolia* L., *Rhazya stricta* Dcne, *Rubia cordifolia* L., *Sapindus mukorossi* Gaertn, *Stenolobium stans* (L.) Seem, *Terminalia arjuna* (Roxb. ex DC.) Wight & Arn, *Trichodesma indicum* (L.) Sm and *Zingiber officinale* Roscoe. Their extracts were tested for use as antidote in the traditional system of medicine.

Black patternless Pakistani cobras were collected from Cholistan desert in the southern Punjab Province of Pakistan and were duly identified[2]. Venom was collected by squeezing the glands of the snakes below their eyes. The entire procedure was carried out in a dark environment. Immediately after collecting the venom, it was freeze-dried and stored in light-resistant bottle at 8°C. Before use it was reconstituted in 0.9% sodium chloride solution[2].

A variety of medicinal plants were collected from different locations in Pakistan. Various plant parts were collected on the basis of folklore evidences for snake bite. After identification by a qualified taxonomist, voucher specimens were submitted to the herbarium of the Institute of Pure and Applied Biology, Bahauddin-Zakariya-University Multan, Pakistan. Overall information about the medicinal plants is provided in Table 1. Thousand grams of chopped plant material was soaked in 5000 ml of methanol. They were kept for four weeks in extraction bottles and then subjected to filtration. Filtrates were evaporated in a water bath for acquisition of different plant extracts. They were weighed and stored for further experimentation[2].

Standard antidote (antiserum) was supplied by the main pharmacy of Nishtar Hospital Multan, Pakistan. It was used to compare neutralizing tendencies of various plant extracts with standard antisera against coagulopathies produced by *N. n. karachiensis* venom. Antiserum was manufactured by Bharat Serums and Vaccines Limited, Ambernath, India[5].

Different coagulation assays were performed on platelet-poor plasma (PPP) obtained from the blood of healthy volunteers in a tube containing K_3-EDTA. Platelet-rich plasma (PRP) was obtained after centrifugation of blood at ambient temperature for 15 min at 200 *g*. Subsequently, PRP was centrifuged again for 20 min at 2000 *g* to get PPP[4].

Fixed quantity of venom (200 µg/ml) was mixed with 100 µl of plasma for PT and aPTT assays, whereas 200 µl of plasma was used in case of TT assay. The resulting mixture was subjected to incubation at 37°C for 3 min in a water bath, except for a mixture of aPTT which was incubated after the addition of aPTT reagent (APPTest, Weiner Lab, Argentina). Stop watch was started immediately with the addition of PT reagent (200 µl, Soluplastin, Weiner Lab, Argentina), calcium chloride (100 µl,

Table 2. Reverse anti-coagulant action of medicinal plants to combat delay in coagulation time (PT, aPTT and TT) produced by snake venom in comparison with standard antidote

Sample tested (normal plasma, venom 200 µg/ml and various antidotes 5 µg/ml)	PT (sec)			aPTT (sec)			TT (sec)		
	Mean ± SD (n = 3)	CV (%)	Protection (%)	Mean ± SD (n = 3)	CV (%)	Protection (%)	Mean ± SD (n = 3)	CV (%)	Protection (%)
Healthy human plasma	13 ± 0.57	4.3	100	35 ± 1.52	4.3	100	13 ± 0.57	4.5	100
Positive control (venom)	23 ± 0.57	2.5	0	48 ± 2.00	4.1	0	33 ± 0.57	1.7	0
Albizia lebbeck (L.) Benth	18 ± 1.00**	5.5	50	38 ± 0.57*	1.5	77	24 ± 1.00**	4.1	45
Allium cepa L.	23 ± 0.57*	2.5	0	38 ± 2.00*	5.2	77	26 ± 0.57**	2.1	35
Allium sativum L.	23 ± 1*	4.3	0	39 ± 1.15**	2.9	69	27 ± 2.08**	7.6	30
Althaea officinalis L.	21 ± 0.57*	2.7	20	39 ± 0.57**	1.4	69	32 ± 0.57**	1.7	5
Bauhinia variegata L.	18 ± 0.57**	3.1	50	37 ± 1.00*	2.7	84	27 ± 1.52**	5.7	30
Brassica nigra (L.) W. D. J. Koch	22 ± 0.00*	0.0	10	38 ± 1.15*	3.0	77	33 ± 0.57**	1.7	0
Calotropis procera (Aiton) W. T. Aiton (exudates)	18 ± 0.57**	3.1	50	38 ± 0.00*	0	77	33 ± 1.00**	3.0	0
Calotropis procera (Aiton) W. T. Aiton	22 ± 0.57*	2.6	10	37 ± 0.57*	1.5	84	26 ± 2.00**	7.6	35
Cedrus deodara (Roxb. ex D. Don) G. Don	23 ± 0.57*	2.5	0	38 ± 1.00*	2.6	77	22 ± 0.57**	2.5	55
Citrullus colocynthis (L.) Schrad	19 ± 0.57**	3.0	40	40 ± 1.00**	2.5	61	21 ± 1.15*	5.5	60
Citrus limon (L). Burm. f	19 ± 0.57**	2.9	40	42 ± 1.52**	3.6	46	27 ± 0.00**	0.0	30
Cuminum cyminum L.	22 ± 1*	4.5	10	38 ± 1.00*	2.6	77	31 ± 0.57**	1.8	10
Enicostemma hyssopifolium (Willd.) I. Verd.	22 ± 0.57*	2.5	10	36 ± 1.00*	2.7	92	19 ± 0.57*	2.9	70
Fogonia cretica L.	22 ± 0.57*	2.5	10	38 ± 1.73*	4.5	77	25 ± 1.15**	4.6	40
Leucas capitata Desf	23 ± 0.57*	2.4	0	37 ± 0.57*	1.5	84	27 ± 0.57**	2.1	30
Matthiloa incana (L.) W. T. Aiton	20 ± 0.57**	2.9	30	37 ± 0.57*	1.5	84	26 ± 1.00**	3.8	35
Momordica charantia L.	20 ± 1**	5.0	30	39 ± 0.57**	1.4	69	33 ± 1.52**	4.5	0
Nerium indicum Mill	21 ± 0.57*	2.7	20	40 ± 1.15**	2.8	61	28 ± 0.57**	2.0	25
Ocimum sanctum L.	17 ± 0.57**	3.5	60	37 ± 0.57*	1.5	84	27 ± 0.57**	2.1	30
Pinus roxburghii Sarg	23 ± 0.57*	2.4	0	38 ± 1.00*	2.6	77	26 ± 1.15**	4.3	35
Pistacia integerrima J. L. Stewart	23 ± 0.57*	2.5	0	40 ± 0.57**	1.4	61	23 ± 0.57**	2.4	50
Psoralea corylifolia L.	21 ± 0.0*	0.0	20	45 ± 1.00**	2.2	23	27 ± 1.52**	5.5	30
Rhazya stricta Dcne	22 ± 1.5*	6.7	10	47 ± 1.00**	2.1	7	27 ± 0.57**	2.4	30
Rubia cordifolia L.	18 ± 1.0**	5.5	50	46 ± 0.57**	1.2	15	28 ± 0.57**	2.0	25
Sapindus mukorossi Gaertn	20 ± 0.57**	2.9	30	38 ± 1.00*	2.6	77	22 ± 1.00**	4.5	55
Stenolobium stans (L.) Seem	16 ± 0.57**	3.5	70	36 ± 0.57*	1.5	92	29 ± 0.57**	2.0	20
Terminalia arjuna (Roxb. ex DC.) Wight & Arn	18 ± 0.57**	3.1	50	37 ± 1.52*	4.1	84	28 ± 0.57**	2.0	25
Trichodesma indicum (L.) Sm	18 ± 0.0**	0.0	50	37 ± 0.57*	1.5	84	25 ± 1.00**	4.0	40
Zingiber officinale Roscoe	21 ± 1.0*	4.7	20	38 ± 0.57*	1.5	77	24 ± 1.52**	6.2	45
Standard antisera	22 ± 0.57***	2.6	10	36 ± 1.00***	2.7	92	19 ± 1.00***	5.2	70

*Indicates about medicinal plants that fall within 95% confidence limit of mean of standard antidote for PT (20.59–23.41 sec), aPTT (33.5–38.5 sec) and TT (16.5–21.5 sec) coagulation assays.
**Plants that do not fall within 95% confidence limit of mean of standard antidote for PT, aPTT and TT coagulation assays.
***Set as reference standard for respective coagulation tests.

0.025 mol/l) and TT reagent (100 µl, Human Wiesbaden, Germany) in the respective PT, aPTT and TT assay tubes. The tubes were moved back and forth (two times per second) and time was recorded in seconds until clot appeared for PT and TT assays. In case of aPTT, the timer was started when the mixture was in a water bath initially for 25 sec and then removed for recording the clotting time[5,7,8]. To neutralize prolongation in coagulation time, venom was pre-incubated with various concentrations of medicinal plant extracts (5–640 µg/ml) and again clotting time was determined.

Venom from *N. n. karachiensis* was found to cause delay in coagulation parameters such as PT, aPTT and TT. Snake venom at concentration of 200 µg/ml was found to delay PT (13 ± 0.57 to 23 ± 0.57 sec), aPTT (35 ± 1.52 to 48 ± 2.0 sec) and TT (13 ± 0.57 to 33 ± 0.57 sec) within 4.5% coefficient of variance (Table 2).

Coagulopathy is one of the most important clinical complications arising after snake-bite poisoning. It is alarming that little information is available on the exact cause of it; however, it is diagnosed by abnormal blood tests. PT, aPTT and TT are routinely performed for diagnosis of coagulopathy[9].

Anti-coagulation of blood by snake venom has been attributed to its strong or weak reverse congealing tendencies. Snake venom anti-coagulant leads to inhibition of extrinsic tenase (TF-VII_a complex) or prothrombinase complex (factor V_a and Ca^{++}) resulting in PT prolongation.

However, snake-bite poisoning-related disorders of intrinsic prothrombin activators (factors VIII, IX, XI and XII) are often detected by aPTT prolongation. Thrombin time test reveals defects in conversion of factor I towards solid fibrin threads formation or scarcity in fibrinogen level[10]. There are several manifestations for coagulation test prolongation after snake-bite envenomation. Among them activation of protein C, inhibition of factors IX or X, thrombin inhibitors and phospholipases A_2 are more conspicuous[11]. American *Agkistrodon* species particularly *Agkistrodon contortrix contortrix* venom has been reported to impede normal coagulation (degrade V_a and $VIII_a$) by protein C activation. Venom from *Deinagkistrodon acutus*, *Trimeresurus flavoviridis* and *Echis carinatus leucogaster* undergoes Ca^{++}-mediated binding to factor IX or X or both and impedes coagulation cascade (by induction of conformational changes in anticoagulant proteins). Thrombin inhibitors are rare and minimize the binding of α-thrombin to fibrin (ogen). Additionally, they bind with fibrinogen-associated exosite of α-thrombin and thus halt thrombin action(s). Phospholipases A_2 degrade phospholipids and hence inhibit the formation of prothrombinase complex. Formosan habu (crotalid snake, *Trimeresurus mucrosquamatus*), *Naja nigricollis*, *N. m. mossambica*, *N. naja* and *N. melanoleuca* venom have been documented previously[10,11] to induce anticoagulation via phospholipases A_2. Similar effects have been observed *in vitro* using *N. n. karachiensis* venom on PPP. The venom not only delays PT but also TT (does not happen usually), which leads one to assume the occurrence of thrombin-like (fibrinogenolytic) or plasminogen activating enzymes that degrade fibrin and fibrinogen[5,12].

Various enzyme-neutralizing and protein-binding constituents have shown therapeutic relevance of natural inhibitors to combat snake-bite poisoning. Medicinal plants of Pakistan inhibit different enzymes due to the presence of abundant secondary metabolites. Phenols, flavonoids, xanthenes, quinonoids and various terpenoids neutralize snake venom[6]. Secondary metabolite(s) maximally neutralize snake venom enzyme(s) when they are in equimolar concentration to the latter[13]. They create obstacles in the binding of various proteins to their potential targets and hence are responsible for minimizing anticoagulation induced by *N. n. karachiensis* venom. Among the 28 medicinal plants studied, *Stenolobium stans* (L.) Seem and *E. hyssopifolium* (Willd.) I. Verd were the most suitable plant extracts to treat anti-coagulation comparable to standard antisera (antidote) after snake-bite envenomation.

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