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ANTI-HEMOLYTIC PROPERTY OF LOCAL MEDICINAL PLANT(S) UPON PAKISTANI COBRA VENOM INDUCED HEMOLYSIS

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ABSTRACT

Present study was carried out to assess scientifically anti-hemolytic properties of medicinal plants against Pakistani cobra induced hemolysis. Venom from *Naja naja* and *Naja naja karachiensis* were found to destabilize human red blood corpuscles membrane (HRBC) however, the effect of later was found somewhat severe. Against *Naja naja karachiensis* venom twenty six medicinal plants of Pakistan were evaluated but only *Cedrus deodara* G. Don (P>0.5) was proved the most effective (72%) in stabilizing HRBC membranes. *Althaea officinalis* Linn, *Calotropis procera* (Wild.) R.Br, *Citrus limon* (L). Burm. f, *Enicostemma hyssopifolium* (Willd.) Verdoorn, *Leucas capitata* Desf and *Stenolobium stans* (L) D. Don were found anti-hemolytic (0.5>P>0.1) at various concentration range (20 to 320 µg/mL) in comparison with standard anti-sera (56%). Flowers extract of *Calotropis procera* (Wild.) R.Br and bulbs of *Allium cepa* L were found helpful to neutralize snake venom hemolysis at P<0.1. However, remaining plants extract (p<*0.05*) were found to be hemolytic and potentiated the effect of venom instead of having anti-hemolytic potentials. On scientific basis this study emphasizes to rationalize, the use of listed medicinal plants in traditional system of medicine as an anti-hemolytic. Nevertheless, further study is inevitable to identify and isolate bio-active compound(s) from above cited potential medicinal plant(s) extract.

Key words: Pakistani plants, Anti-hemolytic, Antidote, venom, *Naja naja karachiensis*, *Naja naja*.

INTRODUCTION

Snake biting is one of the serious issues of the world due to high rate of mortality and morbidity. Among all the snakes on the earth, only two hundred species are poisonous. They are grouped into Cortalidae, Viperidae, Elapidae and Hydrophidae families (Matsui *et al*., 2000; Warrell, 2010). Deadly venomous snakes were categorized into Elapidae, Viperidae and Hydrophidae families that are abundantly found in southern Asian countries like Pakistan. Due to this reason snake bite envenomation results in frequent health problems especially in Asiatic underdeveloped countries. Elapidaes belonging to genus Naja are very common and found as ten full species of Asiatic Naja. Pakistani cobra (southern black Pakistan cobra) is one of them (Wuster, 1996; Feroze *et al*., 2010) and considered a sign of threat especially in southern Punjab province of Pakistan. It has been estimated that 20,000 deaths occur annually in Pakistan due to snake bite poisoning (Gutierrez *et al*., 2006). Apart of it, victims who survive suffer different complications like bleeding from wounds (local hemorrhage), hematuria, bleeding gums, local pain, tissue necrosis, swelling and blistering (Davidson *et al*., 1995; Gutiérrez *et al*., 2009).

Local hemorrhage is one of hemostatic disorders after Viperid and Elapid (including cobra) snakes' envenomations (Gutiérrez *et al*., 2005; Razi *et al*., 2011). Hemolysis from cobra bites are not very common but has been noticed hence it is a contributing factor of snake poisoning that can't be overlooked. Equine anti-sera help in combating snake bite envenomation however does not protect local tissue damage, i.e. hemorrhage necrosis and edema (Ushanandini *et al*., 2006; Davidson *et al*., 1995). Standard anti-sera is an appropriate treatment for snake bite however high cost, hypersensitivity and shortage of supply has developed the interest to search for cheap and alternate treatment especially from natural inhibitors.

Medicinal plants are one of the cheap sources and their extracts have been used extensively to neutralize venom induced hemolysis. Among them *Andrographis paniculata* Nees, *Andrographis alata* Nees, *Andrographis lineate* Nees, *Coccinia indica*, *Crateva magna*, *Gloriosa superb*, *Hydrocotyle javanica*, were investigated previously (Kumarapppan *et al*., 2011; Balu & Alagesaboopathi, 1995; Vijayabharathi *et al*., 2005). Pakistan has long history of medicinal plants where

variety of them have been used locally especially for snakebite (Asad *et al*., 2011). It is therefore necessary to evaluate them scientifically their folklore claims as an anti snake venom (anti-hemolytic). Present research article reports for the first time medically important medicinal plants of Pakistan and to validate their potentials against Pakistani cobra venom induced hemolysis. *Albizia lebbeck* (L.) Benth, *Allium cepa* L, *Allium sativum* L, *Althaea officinalis* L, *Bauhinia variegate* L, *Brassica nigra* (L Koch), *Calotropis procera* (Wild.) R.Br, *Cedrus deodara* G. Don, *Citrus limon* (L). Burm. f, *Cuminum cyminum* L, *Enicostemma hyssopifolium* (Willd.) Verdoorn, *Leucas capitata* Desf, *Matthiloa incana* (L) R.Br, *Momordica charantia* L, *Nerium indicum* Mill, *Ocimum sanctum*, *Pinus roxburghii* Sargent, *Pistacia integerrima*, *Psoralea corylifolia* L, *Rhazya stricta* Dcne, *Rubia cordifolia*, *Sapindus mukorossi* Gaertn, *Solanum xanthocarpum* Schard & Wendle, *Stenolobium stans* (L) D. Don, *Terminalia arjuma* Wight & Arn, *Trichodesma indicum* (Linn) R.Br and *Zingiber officinale* Roscin were collected and later on their neutralizing potentials were compared with reference standard antidote.

Experimental

***Naja naja karachiensis* (patternless southern black Pakistan cobra):** Black southern pattern less form of Pakistani cobra snakes (*Naja naja karachiensis*) were collected with local charmers from Cholistan desert, Southern Punjab province of Pakistan. Venom from *Naja naja* (patterned form cobra in Pakistan) was also collected from snakes abundant in Thatta, Sind province of Pakistan. All snakes were duly identified by zoologist.

Venom extraction: Venom from non captive snakes was collected in low light condition at ambient temperature by squeezing the glands below their eyes in a container having deionized water (100 ml). This mixture was centrifuged at 10,000 rpm for 60 min. After freeze drying (lyophilization), it was stored at 4ºC in a sterilized light proof and air tight container. Before use it was reconstituted (1 mg / mL) in saline in terms of dry weight (Asad *et al*., 2012; Shafqat *et al*., 1987).

Plant materials: Ethnobotanically claimed medicinal plants of Pakistan were collected from different areas as mentioned in table 1. After collection they were authenticated by expert botanist Prof. Dr. Altaf Ahmad Dasti (Institute of Pure and Applied Biology, Bahauddin-Zakariya University, Multan, Pakistan) and voucher specimens were deposited in the Herbarium of the same department. Overall detail is shown in table 1.

Preparation of plant crude extracts: Shade dried plant materials (1kg) were chopped and then subjected to extraction after passing through sieve 22. Extracts were prepared by using methanol (5L) as solvent via simple maceration method at optimum temperature for a month. After filtration, filtrate was evaporated using water bath for a period of nearly a week at 25±3 ºC. Finally plants extracts were weighed and preserved (2-8 ºC) for further experimentation (Razi *et al*., 2011).

Snake venom antiserum: Standard snake venom antiserum (lyophilized) was purchased from the local market manufactured by Bharat Serums and Vaccines Limited, Ambernath (E) - 421 501, India.

Anti-hemolytic (anti-venom) activity by human red blood corpuscles (HRBC) membrane stabilization method: Venom induced hemolysis and its neutralization by medicinal plants of Pakistan was carried out by following the method of Vijayabharathi *et al*, (2005).

Briefly, blood was collected from healthy volunteers by vein puncture using heparin as anticoagulant. After washing thrice with saline, it was subjected to centrifugation at 3000 rpm and packed cells were separated. Venom (100 µg/mL, 1mL), Phosphate buffer (0.15 M, pH 7.4, 1mL) and HRBC (1% v/v, 1mL) were mixed and incubated at 37 ºC for 30 minutes. Subsequently mixture was centrifuged at 1000 rpm for 3 minutes. Absorbance of the supernatant (due to release of hemoglobin) was measured at 540 nm. For anti-hemolytic activity, snake venom was incubated with various concentrations of plant extracts (20-320 µg / mL) at ambient temperature for half an hour. All samples were prepared in saline and tube containing saline served as control. Hyposaline (0.25% NaCl) was served as positive control for hemolysis. Finally percentage hemolysis and protection was calculated by using the formula given below.

Percentage hemolysis = (Absorbance of treated sample / Absorbance of control) × 100 = Y
Percentage protection = 100 – Y = Z.

Statistical analysis: All numerical values were mentioned as mean. They were calculated by Microsoft Excel 2007. Paired *t*-test was applied under the guidelines and instructions published in British medical Journal. The level of significance was set at 0.05.

RESULTS

Venom from *Naja naja karachiensis* (pattern less southern Punjab black Pakistani cobra (fig. 1) was found to destabilize HRBC membrane resulted in hemolysis as seen with *Naja naja* (patterned form widespread in Sind province, as shown in fig. 1) venom. However, hemolysis was more prominent by *Naja naja karachiensis* venom as mentioned in table 2. Anti-hemolytic property of twenty six medicinal plants of Pakistan was assessed by application of their methanolic extracts at concentrations ranging from (20-320 µg/mL) by HRBC membrane stabilization method (as mentioned

in table 1). Additionally their outcomes were matched with standard antiserum used in local hospitals to diminish hemorrhage. Out of twenty six only one plant extract at concentrations of 20, 40, 80, 160 and 320 µg/ml was found to neutralize significantly ($p>0.5$) hemolysis induced by venom *Naja naja karachiensis* at 100 µg/mL. Standard antiserum was also tested at the same concentrations as tested for plant extracts. We observed different moods in percentage protection of different anti-dote against hemolysis. Protection percentage was highest 72% in case of *Enicostemma hyssopifolium* (Willd.) Verdoorn and *Cedrus deodara* G. Don against hemolysis. Percent protection offered by standard anti-sera (56%) was seemed less in comparison with these two plants. Flowers extract of *Calotropis procera* (Wild.) R.Br and bulbs of *Allium cepa* L were found useful at $P<0.1$. Standard anti-sera and *Enicostemma hyssopifolium* (Willd.) Verdoorn extract at lower concentration (20 µg/mL) was found more protective to HRBC membrane as compared to their higher concentrations (40-320 µg/mL). They were found least protective at concentration of 320 µg/mL. However, extract from *Enicostemma hyssopifolium* (Willd.) Verdoorn at every concentration was recorded more protective in stabilization of HRBC membrane. In case of *Calotropis procera* (Wild.) R.Br and *Cedrus deodara* G. Don percentage protection increases up to 160 µg/mL then descends down. Figures 2, 3 and 4 completely described clear glimpse of anti-hemolytic effects of selected medicinal plants with standard anti-sera.

Table 1. Parts of medicinal plants collected from various areas of Pakistan having folklore evidences as anti-hemolytic (anti-snake venom) along with voucher specimen number deposited at the Herbarium.

Sr. No	Scientific name of the plant, (Family).	Anti-venom part used (location in Pakistan)	Voucher Number	Folklore anti-venom reference
1.	*Albizia lebbeck* (L.) Benth, (Mimosaceae).	Seeds (Bahawalpur).	STW.381	(Baquar, 1989)
2.	*Allium cepa* L, (Alliaceae).	Bulb (Bhakkar).	STW.42	(Makhija and Khamar, 2010)
3.	*Allium sativum* L, (Liliaceae).	Bulb (Bhakkar).	STW.46	(Ugulu, 2011)
4.	*Althaea officinalis* L, (Malvaceae).	Roots (Rawalpindi).	STW.411	(Asad *et al*., 2011)
5.	*Bauhinia variegate* L, (Caesalpinaceae).	Roots (Haripur).	STW.374	(Shinwari *et al*., 2007)
6.	*Brassica nigra* (L Koch), (Cruciferae).	Seeds (Manshera).	STW.302	(Baquar, 1989)
7.	*Calotropis procera* (Wild.) R.Br, (Asclepiadaceae).	Milky latex, (Haripur).	STW.566	(Asad *et al*., 2011)
8.	*Cedrus deodara* G. Don, (Pinaceae).	Bark (Nathia Gali)	STW.25	(Baquar, 1989)
9.	*Citrus limon* (L). Burm. f, (Rutaceae).	Fruit (Haripur).	STW. xx	(Rita *et al*., 2011)
10.	*Cuminum cyminum* L, (Apiaceae).	Seeds (Sargodha)	STW.516	(Baquar, 1989)
11.	*Enicostemma hyssopifolium* (Willd.) Verdoorn, (Gentianaceae).	Fresh plant (Jhelum).	STW.553	(Daniel, 2006)
12.	*Leucas capitata* Desf, (Lamiaceae).	Whole plant (Rawalpindi).	STW.615	(Shinwari *et al*., 2007)
13.	*Matthiloa incana* (L) R.Br, (Cruciferae).	Seeds (Rawalpindi).	STW.322	(Baquar, 1989)
14.	*Momordica charantia* L, (Cucurbitaceae).	Fruit (Abbottabad).	STW.706	(Baquar, 1989)
15.	*Nerium indicum* Mill, (Apocynaceae).	Roots and leaves (Haripur).	STW.564	(Asad *et al*., 2011)
16.	*Ocimum sanctum*, (Lamiaceae).	Whole plant (Islamabad).	STW.626	(Prajapati *et al*., 2010)
17.	*Pinus roxburghii* Sargent, (Pinaceae).	Oleoresin (Murree hills)	STW.26	(Baquar, 1989)
18.	*Pistacia integerrima*, (Anacardiaceae).	Galls (Murree hills)	STW.458	(Baquar, 1989)
19.	*Psoralea corylifolia* L, (Papilionaceae).	Seeds (Peshawar)	STW.418	(Baquar, 1989)
20.	*Rhazya stricta* Dcne, (Apocynaceae).	Leaves (Lakki Marwat)	STW.565	(Asad *et al*., 2011)
21.	*Rubia cordifolia*, (Rubiaceae).	Stems (Murree Hills)	STW.689	(Baquar, 1989)
22.	*Sapindus mukorossi* Gaertn, (Sapindaceae).	Fruits (local market in Rawalpindi)	STW.463	(Parganiha *et al*., 2012)
23.	*Stenolobium stans* (L) D. Don, (Bignoniaceae).	Roots (Haripur)	STW.669	(Baquar, 1989)
24.	*Terminalia arjuma* Wight and Arn, (Combretaceae).	Bark (Islamabad)	STW.502	(Baquar, 1989; Prajapati *et al*., 2010)
25.	*Trichodesma indicum* (Linn) R.Br, (Boraginaceae).	Whole plant (Sind province)	STW.604	(Baquar, 1989)
26.	*Zingiber officinale* Rosc, (Zingiberaceae).	Rhizome (Lahore)	STW.66	(Duke and Ayensu, 1985).

Allium sativum L, *Albizia lebbeck* (L.) Benth, *Bauhinia variegata* L, *Brassica nigra* (L Koch), *Cuminum cyminum* L, *Matthiloa incana* (L) R.Br, *Momordica charantia* L, *Nerium indicum* Mill, *Ocimum sanctum* L, *Pinus roxburghii* Sargent, *Pistacia integerrima* J. L. Stewart, *Psoralea corylifolia* L, *Rhazya stricta* Dcne, *Rubia cordifolia* L, *Sapindus mukorossi* Gaertn, *Terminalia arjuna* Wight & Arn, *Trichodesma indicum* (Linn) R.Br and *Zingiber officinale* Rosc extracts at various concentrations were not found anti-hemolytic ($p<0.05$) rather they have cytotoxic and synergistic effect with venom (overall results are summarized in Table 3).

Sixty seven percent plants were unable to prove scientifically ($p<0.05$) their folklore potential as anti-hemolytic however; seven percent were active to stabilize HRBC having $p<0.1$. Twenty two percent plants were found correct with their ethnobotanical claim as anti-venom (anti-hemolytic) with $0.5>p>0.1$. However, merely single one was short listed as the most effective anti-dote ($p>0.5$) for venom induced hemolysis (fig. 5).

Table 2. Absorbance of hemoglobin release due to hemolysis of HRBC by various hemolytic agents.

Sr. No	Hemolytic agent(s)	Concentration used	Absorbance (540 nm)
1.	Hyposaline (positive control)	0.25%	4.000
2.	*Naja naja karachiensis* venom	100 µg/mL	0.151
3.	*Naja naja* venom	100 µg/mL	0.140
4.	Saline (negative control)	0.9%	0.038

Table 3. Effect of application incubated mixture of different anti-dote (plant extracts / antiserum) and *Naja naja karachiensis* venom on isolated HRBC (human red blood corpuscles) membrane stabilization.

Sr. No	Plant extracts / Standard anti-sera	HRBC membrane stabilization	Comment(s)
1.	*Calotropis procera* (Wild.) R.Br. *Cedrus deodara* G. Don. *Enicostemma hyssopifolium* (Willd.) Verdoorn.	Plant concentration (20-320 µg / mL) fully stabilize HRBC membrane.	a. Folklore claim has found correct as anti-hemolytic. (Data has shown at figure 4).
2.	*Allium cepa* L. *Althaea officinalis* Linn. *Citrus limon* (L). Burm. f. *Leucas capitata* Desf. *Stenolobium stans* (L) D. Don.	High concentrations (< 160 µg / mL) stabilized HRBC membrane.	a. Elevated concentrations seemed to be cytotoxic.
3.	*Albizia lebbeck (L.) Benth.* *Allium sativum* L. *Bauhinia variegate* L. *Brassica nigra* (L Koch). *Cuminum cyminum* L. *Momordica charantia* L. *Matthiloa incana* (L) R.Br. *Nerium indicum* Mill. *Ocimum sanctum.* *Pinus roxburghii* Sargent. *Pistacia integerrima.* *Psoralea corylifolia* L. *Rhazya stricta* Dcne. *Rubia cordifolia.* *Sapindus mukorossi* Gaertn. *Terminalia arjuma* Wight and Arn. *Trichodesma indicum* (Linn). *Zingiber officinale* Rosc.	All concentrations (20-320 µg / mL) unable to stabilized HRBC membrane.	a. Hemolytic effect evoked rather anti-hemolytic.. b. Folklore claim has not been found correct as anti-hemolytic. c. Cytotoxic effect.
4.	Standard antiserum. (Used in hospitals)	Concentration ranges rom (20-320 µg / mL) fully stabilized HRBC membrane.	a. Standard antiserum worked efficiently as anti-hemolytic. .

Figure 1. *Naja naja karachiensis* (left and middle) and Naja naja (right)

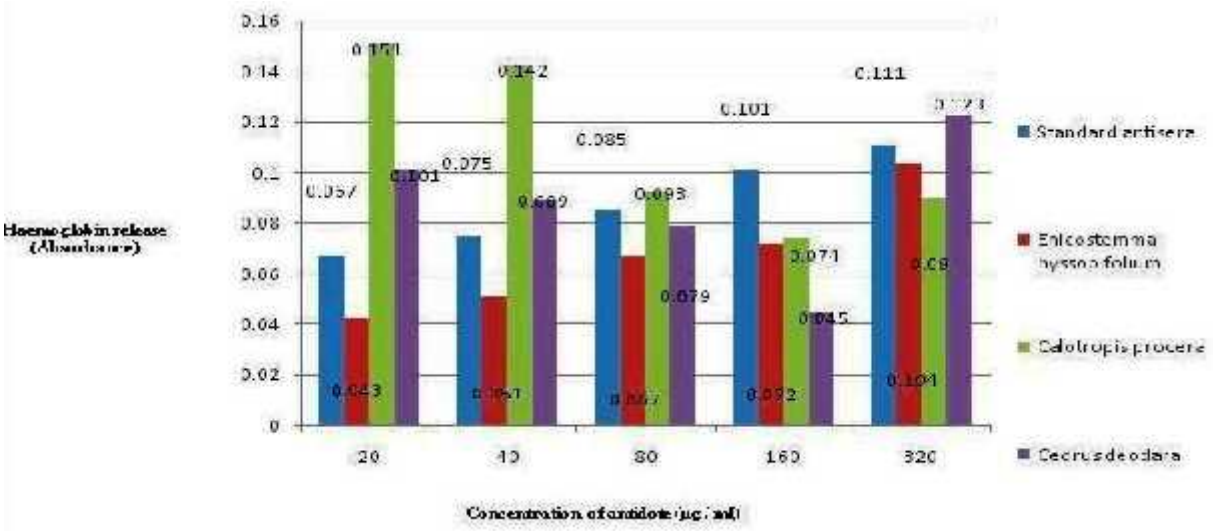

Figure 2. Antihemolytic properties of the most effective Pakistani medicinal plants in comparison with standard antidote posed by *Naja naja karachiensis* venom via the release of haemoglobin.

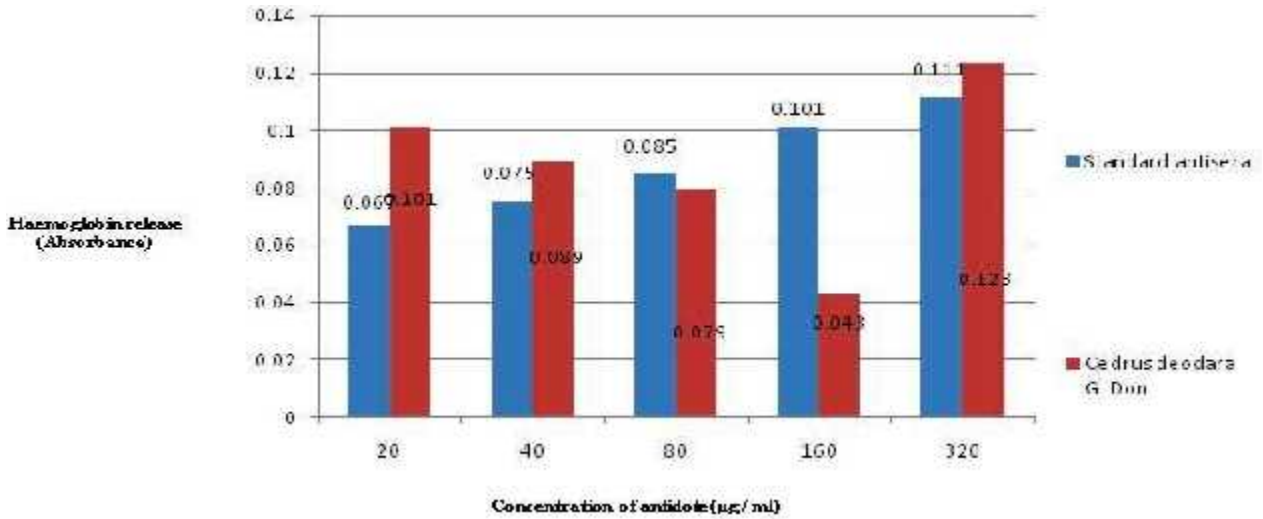

Figure 3. Comparison of antihemolytic property between *Cedrus deodara* plant extract and standard anti-sera posed by *Naja naja karachiensis* venom in terms of haemoglobin release at 540 nm.

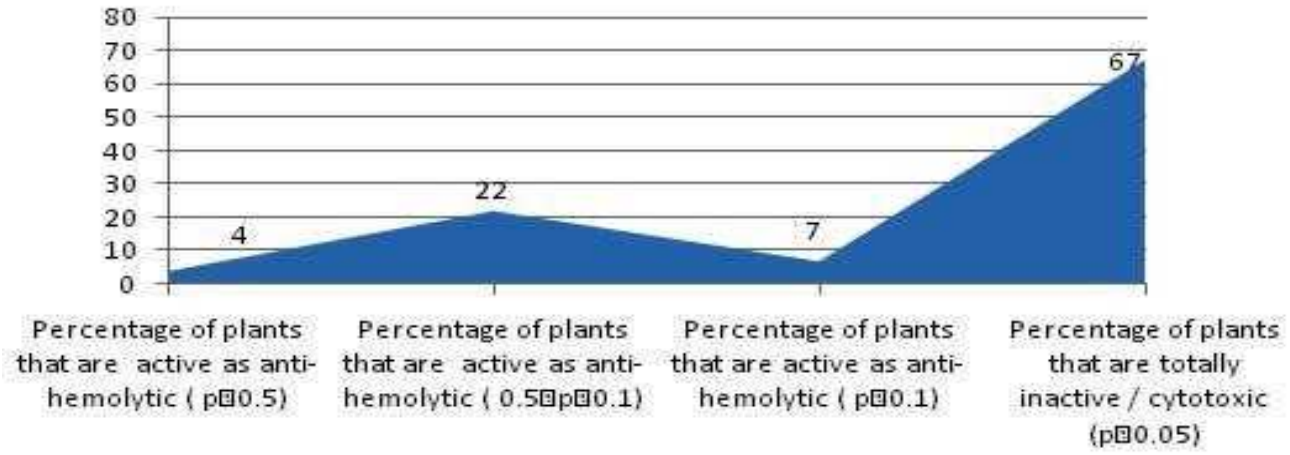

Figure 4. Comparison of effective percentage protection posed by medicinal plants of Pakistan with standard anti-sera against *Naja naja karachiensis* venom induced haemolysis in terms of statistics (probability).

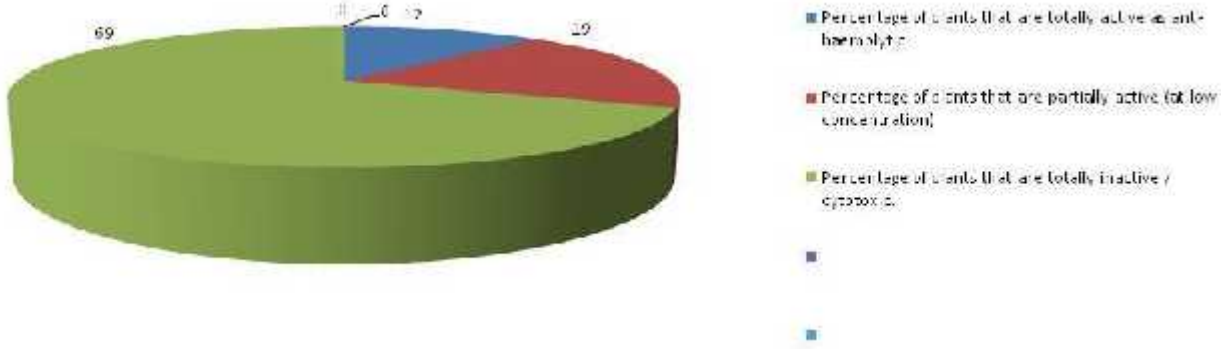

Figure 5. Breakthrough of medicinal plants of Pakistan having response against *Naja naja karachiensis* venom induced hemolysis.

DISCUSSION

Tropical and subtropical regions of the world particularly southern Asian countries have been reported enormously for snake bite envenomation. Infact snake venom is a complex mixture of proteins and peptides that alter permeability of membranes which play a pivotal role in hemolysis of RBCs. Among toxic proteins phospolipases A_2 (PLA_2) are the most abundant and one of the contributing factors towards hemolysis. Phospholipases enzymes bind with cell membrane lipids by complex formation via covalent, non covalent or disulfide bonds. After binding PLA_2 enzyme caused hydrolysis of intact phospholipids and released free fatty acids and lysophospholipids. As a result HRBC membranes destroyed and change the environment of target protein(s) therefore resulted in agonistic, antagonistic effects and additionally posed interference in binding with physiologic ligands. All these aspects are contributing factors for venom induced hemolysis of HRBC (Kini, 2003; Condrea *et al*., 1964). In contrast to snake venom phospholipases hyposaline induced hemolysis by stress in HRBC membranes. It is due to the formation of transient resealing fissures in the HRBC membranes during cell swelling process (Arias *et al*., 2010).

Several protein binding and enzyme inhibiting compounds have engrossed therapeutic importance as natural inhibitors to combat snake venom peptides. Medicinal plants of Pakistan have primeval record to deactivate such proteins and peptides. It is due to diverse secondary metabolites like polyphenols, terpenoids, flavonoids, quinonoids and xanthenes that have been reported previously to neutralize snake poison (Asad *et al*., 2011). Similarly antioxidants in plants are beneficial to minimize oxidative hemolysis resulted from degradation of biomolecules with generation of free radicals (peroxide and superoxide) during HRBC lyses. Due to these reasons present work was designed to evaluate medicinal plants of Pakistan having folklore evidences as anti snake venom to rationalize them scientifically in traditional system of medicine. Among different evaluated medicinal plnats *C. deodara*, *C. procera* and *E. hyssopifolium* were found as an alternate source of protection from venom induced hemolysis

equally with same potentials as reference standard antidote. However, in future it is essential to detect and separate bioactive constituent(s) from these shortlisted medicinal plants.

Conflict of Interest: Authors have not found any conflict of interest among them and all are committed for publication.

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