1. Introduction

Snake venom is a complex cocktail of majorly proteins (enzymatic, non-enzymatic proteins) and other components like inorganic ions, carbohydrates, lipids, amines and nucleotides found in minor quantities [1–3]. Synergistic effect of enzymatic and non-enzymatic components is responsible for clinical effects of venom.

Snake envenomation is a neglected tropical disease listed under Category A by the World Health Organization [4]. In India, 49,500 mortality occurs due to snake bite [5]. ‘BIG FOUR’ snakes of India namely, *Bungarus caeruleus* (krait), *Naja naja* (cobra), *Echis carinatus* (saw-scaled viper) and *Daboia russelii* (Russell’s viper) are medically important snakes, as they are responsible for highest mortality and morbidity.

Polyvalent anti-snake venom (PASV), sera obtained from immunized horse against BIG FOUR is the currently available treatment for snake bite in India [6]. However, factors like storage, side-effects induced, neutralization of only free circulating venom components, and decreasing efficiency of PASV over years has led to search for new alternate therapies to treat snake bite [7–9].

Medicinal plants with anti-ophidian properties used in traditional system can serve as potential antidote or help in better treatment along with PVAS. Apart from these, studies also suggest that compounds from medicinal plants have the ability in treating...
secondary damages induced by snake venom. Sarangdhar Samhita of Ayurvedic literature has emphasised importance of concept of poly-herbalism to achieve greater treatment efficiency of diseases [10]. There are scientific reports proving crude extracts to be more effective than individual herbal compounds [11].

A compound AILPA (Azadirachta indica PLAZ inhibitor) isolated from neem leaves had exhibited anti-PLAZ activity on cobra and Russell’s viper venom [12]. Tarannum et al. [13] have reported that among ethanol, methanol and aqueous bark extracts of Butea monosperma, ethanolic extracts have shown significant inhibitions on hylauronidase activity of Venom of Russell. Polyphenols obtained from the aqueous extracts of Areca catechu had shown significant inhibitory actions against venom of Naja kauthia in in vitro exhibiting its antivenom potential [14]. Srithamma et al. [15] proposed from their study that, aqueous extracts were efficient in neutralizing lethality and myotoxicity induced by N. kauthia venom in vivo. The aim of present study is to explore potential of crude aqueous ethanolic extract cocktail of medicinal plants, A. catechu, A. indica, B. monosperma, Citrus limon peel, and Clerodendrum serratum (1:1:1:1:1) for anti-ophidian properties against BIG FOUR venom through ex vivo and in vivo methods.

2. Materials and methods

2.1. Collection and storage of venom

Krait, cobra, Russell’s viper and saw-scaled viper venom was procured from Irula snake-catchers, Vadanemmel village, Kancheepuram, Tamil Nadu, India. Venoms were stored at 4 °C, required concentration was dissolved in desired volume of sterile-saline. The study was approved by the IAEC (DSCP/DST/IAEC/06/16-17).

2.2. Collection of medicinal plants and phytochemical extraction

Medicinal plants in the study were selected based on knowledge of traditional herbal healers and literature review. In vitro and pharmacological assays were performed for selected plants and further, plants showing significant inhibitions were selected to evaluate its potential as cocktail. A. catechu (RRCB1_MUS114) and C. serratum (Dept. of Botany GKVK. Plant specimen no. 44) were collected in Bengaluru. A. indica (CBGDGRI-111) and B. monosperma (CBGDGRI-114) was collected from Shimoga. C. limon fruit was procured from a local market in Bengaluru. Plant samples were identified and authenticated at Ayurvedic Research Institute for Metabolic Disorders, Bengaluru and Shri. C.B. Gutta Ayurvedic Medical College and Hospital, Dharwad.

Leaves of A. indica and C. serratum, stem bark of B. monosperma, fruit peel of lemon and seed of areca nut were shade-dried and ground into a fine powder to obtain uniform particle size. Phytochemicals were extracted in aqueous ethanol (70%) in Soxhlet extractor through continuous exhaustive extraction at 60–80 °C [16]. For areca nut, extraction was done with ethyl acetate and then with aqueous ethanol.

Extracts so obtained were concentrated using flash rotary evaporator, dried and stored in desiccator for further use. Herbal cocktail was prepared by dissolving 0.01 g of each aqueous ethanolic extract in 1 mL of saline and mixing in ratio 1:1:1:1:1.

2.3. Ex vivo studies

Ex vivo studies were performed using by modified method of Dunn and Boone [17] in seven-day old chick embryo. Candling of eggs was carried out to mark the apex; egg shell was opened to observe embryo vasculature and administer the test material.

2.3.1. Acute toxicity of the herbal cocktail

Test group (n = 6) was administered with herbal cocktail (0.2 mL) on yolk sac membrane, while the control group was administered with 0.2 mL of sterile saline only (n = 6). Embryos were then incubated at 37 °C and observed for lethality at hourly intervals for 24 h.

2.3.2. Venoms lethality determination

Lethal dose was determined using various concentrations of venom. For krait venom 1–5 μg; cobra and Russell’s viper venom 2–10 μg; and saw-scaled viper 3–15 μg/0.2 mL of saline, was taken to identify the lethal dose with n = 6 embryos for each group. Saline administered group was control. Confidence limit at 50% probability was taken to calculate LD50 by analysing death occurring within 24 h on venom administration.

2.3.3. Evaluation of antivenom activity

Challenging dose (3rdLD50) of venom was taken for neutralization studies. Control group received saline alone and the positive control group received venom alone. Test group was administered with challenging dose of venom pre-incubated with equal volume of the herbal cocktail at 37 °C for 30 min. Embryos were observed for every hour up to 6 h and number of survivals at 6 h was recorded. Cessation of heart beat and yolk sac membrane submerged into yolk was clear indication of the death of embryo.

2.3.4. Hemorrhagic activity of viperid venoms and neutralization by herbal cocktail

Different concentrations (1–5 μg/5 μL) of hemorrhagic venoms were loaded on to disc made of Whatman filter paper 1 and then, placed on veins of embryo and incubated for 3 h at 37 °C. Concentration of venom at which 2 mm hemorrhagic corona formed was considered as standard hemorrhagic dose (SHD). One SHD of venom was incubated with the herbal cocktail for half an hour at 37 °C and was loaded onto a disc and placed on veins of embryo to evaluate neutralization activity of herbal cocktail.

2.4. In vivo studies

Male Swiss albino mice (25–30 g) were used for in vivo studies. Work was carried out at In vivo Biosciences, Magadi road, Bengaluru (ethical clearance approval number DSCP/DST/IAEC/06/16–17). Mice in a group of five were housed per polyvinyl cage and maintained at standard laboratory conditions of 23–25 °C of 12 h light and dark cycles respectively. Animal care, handling and disposal were carried out according to Institutional Animal Ethics Committee (IAEC). Sosrane (over dose of commercial anesthetic) was used to euthanize animals after experiments.

2.4.1. Acute oral toxicity of herbal cocktail

Studies were performed as per Organisation for Economic Cooperation and Development (OECD) guidelines 423 in mice. Herbal cocktail was prepared to a concentration of 2000 mg/kg body weight and was administered to healthy mice (n = 5) by oral gavages. Mice were observed for a period of 14 days for any mortality, changes in behaviour and gross toxicity. Body weights were checked before administration, on 7th and 14th day after administration of cocktail.

2.4.2. Hemorrhagic activity and its neutralization

Hemorrhagic activity was determined as per method of Kondo et al. [18]. Saw-scaled and Russell’s viper venom at 10 μg and 15 μg concentration respectively was injected on mouse back intradermally and 3 h after mice were anesthetized using sosrane. Dorsal patch of skin was removed and inner surface was examined for hemorrhage.
Anti-hemorrhagic activity of herbal cocktail was evaluated by pre-incubating it with respective venoms at 37 °C for 30 min.

2.4.3. Neutralization of edema inducing activity

Edema inducing activity of venom was determined as described by Vishwanath et al. [19]. 5 μg of venom samples were prepared in saline and injected into right foot pads (n = 5). Left pads were administered with only saline that served as a control. Mice were euthanized after 1 h using somrane and foot pads were cut at ankle joints and weight of foot pads were recorded. Minimum edema dose was defined as “μg of venom required to induce an edema ratio of 120%”.

Edema ratio was calculated using the formula,

\[ \text{edema ratio} = \left( \frac{\text{weight of edematous leg}}{\text{weight of control leg}} \right) \times 100 \]

2.4.4. Myotoxicity neutralization

Myotoxicity neutralization studies were carried out as described by Gutierrez et al. [20]. 5 μg venom in 50 μL saline was injected intramuscularly (n = 3). After 3 h, mice were euthanized and thigh muscle tissue was subjected to histological examination.

2.4.5. Median lethal dose determination and neutralization potential of herbal cocktail on venom lethality

Venom lethal toxicity was determined as described by Thetakson and Reid [21]. Venom of different concentrations prepared in 0.2 mL of saline was administered i.p. for median lethal dose (LD50) determination. Animals were divided into groups (n = 5). Group receiving saline was taken as control, group receiving challenging dose of venom i.e., 3* LD50 was taken as positive control and group receiving challenging dose of venom and herbal cocktail was test group. Venom neutralizing potential of cocktail was evaluated by pre-incubating challenging dose of venom with herbal cocktail for half an hour at 37 °C. Behavioural changes and mortality were observed for every hour up to 24 h.

2.4.6. Statistical analysis

Independent student t-test was performed using IBM SPSS statistics software 20 for edema results interpretation. Regression analysis was used to calculate LD50 of venom.

3. Results

3.1. Acute toxicity studies

Rapid development, easy availability and non–invasiveness has made chick embryo an important model system for venom related research [22,23]. Embryos injected with herbal cocktail preparation survived up to 24 h with normal growth of embryo. In animals administered with herbal cocktail (2000 mg/kg), there were no behavioural changes, toxicity and/or pharmacological symptoms. Mice gained weight normally throughout 14 days of study. Thus, herbal cocktail was considered safe to be used.

3.2. Anti-hemorrhagic activity of cocktail

Vascular endothelium damage induced by venom, is due to the action of hemorrhagins leading to excessive blood loss causing imbalance in hemostasis and severe clinical conditions [24]. Hemorrhagic studies were not performed for elapid venoms as it was observed from our previous studies that, even on increasing venom concentration to 25 μg hemorrhagic activity was not observed. Hemorrhage of 2 mm was induced at 4 μg for both viper venoms in chick embryo and thus, was considered as standard hemorrhagic dose. Complete neutralization of hemorrhagic activity by herbal cocktail was observed for Russell’s viper venom and
A decrease in hemorrhagic diameter was observed for saw-scaled viper venom (Fig. 1).

In animal models, 10 mg of saw-scaled viper and 15 mg of Russell’s viper venom induced hemorrhage. Hemorrhagic activity neutralization by herbal cocktail activity was observed by for both venoms (Fig. 2).

### 3.3. Neutralization of edema inducing activity

Edema was induced in mice by injecting 5 μg of venom. Edema-inducing activity was reduced from 148 to 130% for krait venom, 162 to 143% for cobra venom and 187 to 168% for saw-scaled viper venom. Edema-inducing activity was not neutralized in case of Russell’s viper venom (Table 1).

### 3.4. Histopathological studies of myotoxicity

Myotoxic PLA2 and myotoxins (small proteins and peptides) are responsible for myotoxicity induced by snake venom [25]. Myotoxicity was induced in mice by injecting 5 μg of venom i.m. After 3 h, mice were euthanized using xosrane. Muscle section was excised, and stained. Microscopic examination revealed that krait venom had shown moderate edema between muscle fibres and normal skeletal muscle histology was observed in group treated with herbal cocktail.

Cobra venom induced severe myotoxicity; however, very mild recovery was observed in the test group. Russell’s viper venom had induced edema with minimal inflation in muscle section and herbal cocktail completely neutralized myotoxic effects of Russell’s viper venom. Mild edema was still evident in muscle section of saw-scaled viper venom treated with herbal cocktail (Fig. 3.).

### 3.5. Lethal toxicity determination

Lethal dose of every batch of venom has to be determined, as a result of inter-species variation. LD50 of krait, cobra, Russell’s and saw-scaled viper venom in chick embryo model was found to be 4.9, 3.4, 7.4 and 8.14 μg respectively.

3*LD50 of venoms was taken as challenging dose and it was observed that there was significant neutralization by herbal cocktail. In case of elapids (cobra and krait venom) groups injected with challenging dose of venom alone, embryos were found to be dead within 2 h, while in case of Russell’s viper venoms all embryos injected with challenging dose were found to be dead by 4 and 5 h respectively.

Different neutralization activity was observed by herbal cocktail for different venoms. Cobra venom was completely neutralized and all embryos survived throughout the experiment. Even, krait venom was almost completely neutralized and only one embryo died.

### Table 1
Neutralization of edema inducing activity of krait, cobra, Russell’s viper and saw-scaled viper venom by cocktail. Values represented as Mean ± SEM.

| Venom/Cocktail  | Edema ratio (%), (Mean ± SEM) |
|----------------|-------------------------------|
|                | Krait                        |
| Venom alone    | 148.7 ± 14.5                 |
| Venom: cocktail| 130.4 ± 5.3                  |
|                | Cobra                        |
| Venom alone    | 162.4 ± 5.7                  |
| Venom: cocktail| 143.9 ± 4.5                  |
|                | Russell’s viper              |
| Venom alone    | 154.3 ± 2.9                  |
| Venom: cocktail| 157.9 ± 8.8                  |
|                | Saw-scaled viper             |
| Venom alone    | 187.1 ± 6.2                  |
| Venom: cocktail| 168.2 ± 6.2                  |

### Table 2
Neutralization studies of venoms by herbal cocktail.

| Venom/Cocktail | No. of embryos survived |
|----------------|-------------------------|
|                | Krait                   |
|                | Cobra                   |
|                | Russell’s viper         |
|                | Saw-scaled viper        |
| Saline (n = 6) | 6/6                     |
| Venom (3*LD50) | 0/6                     |
| Cocktail       | 5/6                     |

Fig. 3. Myotoxicity studies of Big four venom and its neutralization by cocktail. (A) Skeletal muscle section induced with krait venom, (B) Skeletal muscle section induced with saw-scaled viper venom treated with cocktail, (C) Skeletal muscle section induced with cobra venom, (D) Skeletal muscle section induced with cobra venom treated with cocktail, (E) Skeletal muscle section induced with Russell’s viper venom, (F) Skeletal muscle section induced with Russell’s viper venom treated with cocktail, (G) Skeletal muscle section induced with saw-scaled viper venom and (H) Skeletal muscle section induced with saw-scaled viper venom treated with cocktail. Muscle tissue sections observed at 100x magnification.
was found dead in test group. In case of Russell’s viper and saw-scaled viper venom 4 and 3 embryos were alive respectively. Results are as depicted in Table 2.

Various concentrations of krait and cobra venom from 2 to 10 μg were tested to determine the lethal dose and it was found to be 192.7 μg/kg and 213.4 μg/kg (Table 3). For determining Russell’s viper venom lethal dose, concentrations 10–50 μg were tested and was found to be 1.47 mg/kg (Table 4). In case of saw-scaled viper venom, 20–100 μg concentrations were tested and lethal dose was found to be 916.6 μg/kg (Table 4).

For neutralization studies, 3xLD50 of venom was taken as challenging dose. It was observed that krait and saw-scaled viper venom was neutralized completely and all mice were alive even after 24 h. Delay in time of death by half an hour was observed in the group injected with cobra venom treated with herbal cocktail. Neutralization was not significant in case of Russell’s viper venom.

4. Discussion

Medicinal plants are generally perceived to be safe or possess minimal toxicity based on their history of use [26]. Thus, safety and toxicity evaluation has been rarely performed before human consumption and has resulted in raising concerns regarding the toxicity status [27,28]. Evaluation of safety of plant-based products thus becomes important for future use. Acute toxicity of herbal cocktail was evaluated both by ex vivo and in vivo methods and was found to be safe. Safety evaluations for polyherbal formulations like HC9, product made up of 9 medicinal plants for treatment of cancer, and Diakuy made up of 7 herbal extracts used in treatment of type II diabetes were evaluated and found to be safe [29,30]. Thus, in many instances from previous studies it can be found that either individual or combination of plant extracts are safe to use with no toxic effects. In the present study, herbal cocktail was found to be safe with no side-effects.

Hemorrhagins are zinc containing metalloprotease. They disrupt basement membrane and are known to cause fibrinogenolysis and platelet aggregation causing hemorrhage [31]. These components induce death by causing bleeding from vital organs. Neutralization of hemorrhagins in venom would thus result in minimizing or neutralizing lethal effects of venom. In this study, significant neutralizations were observed in chick embryo and animal models. Anti-hemorrhagic activity of herbal cocktail may be due to the interaction of chelators (especially in C. limon fruit peel) and polyphenols present in extracts. Chelators would have chelated zinc responsible for enzymic activity. It was reported that polyphenols obtained from A. catechu L. and Quercus infectoria Oliv had exhibited inhibition of hemorrhagic activity of Calloselasma rhodostoma and Naja naja venom [32]. Administration of polyherbal formulation (200 mg/kg) of Aristolochia bracteolata Lam.: Tylophora indica (Burm.f.) Merrill.: Leucas aspera (S.) Labiate via p.o. against Russell’s viper and cobra venom had resulted in reduction of hemorrhage [33]. Specific zinc chelating agent, N,N,N’,N’-tetrakis (2-pyridylmethyl) ethane-1,2-diamine (TPEN) at 20 mM concentration has reported to offer significant protection against saw-scaled viper venom [20].

Edema in viper bites is said to be result of potent autacoids or eicosanoids synthesized by venom components. There are several experimental reports including clinical trials, that have showed that antivenoms used to neutralize snake venom component need not necessarily stop progression of edema [34–36]. Anti-edematous activity of cocktail was observed in krait, cobra and saw-scaled viper venom. However, there was no reduction of edema observed in Russell’s viper venom. Antibodies obtained from chick embryo had shown complete neutralization of edema induced by krait and cobra venom and reduction in edematous activity by 30% for saw-scaled and Russell’s viper venom [37,38].

Polypeptides of 42–44 amino acids length like crotamine and phospholipase A2 are said to be responsible for myotoxic activity [39]. Mebs and Claus [40] have reported that distinctive specificity is exhibited among different myotoxic phospholipases. Biondo et al. [41] have stated that most antivenoms fail to neutralize myotoxic effect of venoms. Similar result was observed for cobra venom in the present study. However, myotoxic effects of other venoms were inhibited. Thus, further standardization of herbal cocktail concentration may result in proper evaluation of its efficiency.

Lethal dose determination becomes important as it varies among species and within species based on its diet and geographical locations. LD50 values were determined for all four venoms, in both chick embryo and animal models, and it was observed that viper venoms are less toxic than elapid venoms. LD50 values for venoms on six-day chick embryo from other studies was reported to be 2.5 μg for cobra, 2.6 μg for krait, 3 μg for Russell’s viper and 4.8 μg for saw-scaled viper [42–45]. Efficiency of cocktail was ascertained in the present study.

Table 3

| Group | Venom μg/25–27 g of body weight | Krait venom | Cobra venom |
|-------|--------------------------------|------------|------------|
|       | No of mice dead | % of survival | No of mice dead | % of survival |
| I     | 2                 | 1/5         | 80         | 0           | 100        |
| II    | 4                 | 2/5         | 60         | 0           | 100        |
| III   | 6                 | 2/5         | 60         | 3           | 60         |
| IV    | 8                 | 3/5         | 40         | 3           | 60         |
| V     | 10                | 5/5         | 0          | 5           | 0          |
| Saline | –                  | 0/5         | 100        | 0/5         | 100        |

Table 4

| Group | Venom μg/25–27 g of body weight | Russell’s venom | Saw-scaled venom |
|-------|--------------------------------|----------------|-----------------|
|       | No of mice dead | % of Survival | Venom μg/25–27 g of body weight | No of mice dead | % of Survival |
| I     | 10                | 80            | 20             | 20             | 60          |
| II    | 20                | 80            | 40             | 40             | 3           |
| III   | 30                | 60            | 60             | 60             | 4           |
| IV    | 40                | 60            | 80             | 80             | 5           |
| V     | 50                | 40            | 100            | 100            | 0           |
| Saline | –                  | 0/5          | 100            | 0/5            | 100         |
to neutralize snake venoms was evaluated using challenging dose of venom i.e. 3×LD50. It was observed that the herbal cocktail completely neutralized krait and saw-scaled viper venom and delayed the death time by cobra venom. Herbal cocktail did not exhibit neutralization or delay Russell’s viper venom lethality. Further studies will be required to investigate the active components acting in synergism that can help in acting on BIG FOUR venom.

Saktivel et al. [33] had reported neutralization effect by polyherbal formulation of A. bracteolata Lam., T. indica (Burm.f.) Merrill, and L. aspera S. on LD50 of cobra and Russell’s viper venom. Very few studies have reported, antidote evaluation of herbal extracts on two or more venoms.

5. Conclusion

Herbal cocktail prepared from 5 different plant extracts exhibited significant anti-edematous, anti-hemorrhagic, anti-myotoxic effects indicating, it acts in synergism against different venom components. Further purification of active components from plant extracts and standardizing the formulation and determining mode of application in in vivo models would help in development of efficient first aid formulation for victims of snake bite.

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Conflict of interest

None.

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