Journal of Ayurveda and Integrative Medicine 8 (2017) 20–26

Contents lists available at ScienceDirect

Journal of Ayurveda and Integrative Medicine

journal homepage: http://elsevier.com/locate/jaim

Original Research Article (Experimental)

Characterization of *Daboia russelii* and *Naja naja* venom neutralizing ability of an undocumented indigenous medication in Sri Lanka

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ARTICLE INFO

Article history:
Received 28 September 2016
Received in revised form
4 October 2016
Accepted 5 October 2016
Available online 13 March 2017

Keywords:
Daboia russelii
Naja naja
Venom
Neutralization
Herbal preparation

ABSTRACT

Background: Indigenous medicinal practice in Sri Lanka talks about powerful compounds extracted from native plants for treating venomous snake bites which are hardly documented in literature but are used by the indigenous doctors for thousand years.
Objective: We screened the neutralizing ability of a herbal preparation practiced in indigenous medicine of Sri Lanka, consisting of *Sansevieria cylindrica*, *Jatropha podagrica* and *Citrus aurantiifolia*, for its ability to neutralize venom toxins of *Naja naja* (Common Cobra) and *Daboia russelii* (Russell's viper).
Materials and methods: The venom toxicity was evaluated using a 5-day old chicken embryo model observing the pathophysiology and the mortality for six hours, in the presence or absence of the herbal preparation. The known toxin families to exist in snake venom, such as Phospholipase A_2, Snake venom Metalloprotease, were evaluated to understand the mechanism of venom neutralizing ability of the herbal preparation.
Results: The LD_{50} of *D. russelii* venom, as measured using the 5-day old chicken embryo model, was 4.8 ± 0.865 ug (R^2 = 84.8%, P = 0.079). The pre-incubation of venom with the herbal preparation increased the LD_{50} of *D. russelii* venom to 17.64 ± 1.35 μg (R^2 = 81.0%, P = 0.100), showing a clear neutralizing action of *D. russelii* venom toxicity by the herbal medicine. Whereas the pre-incubation of venom with the 1× venom neutralizing dose of commercially available polyvalent anti-venom serum shifted the LD_{50} venom only up to 5.5 ± 1.35 μg (R^2 = 98.8%, P = 0.069). In the presence of the herbal preparation, Phospholipase A_2 activity of *D. russelii* venom was significantly reduced from 9.2×10^{-3} mM min^{-1} to 8.0×10^{-3} mM min^{-1} and that of *N. naja* from 2.92×10^{-2} mM min^{-1} to 0.188×10^{-2} mM min^{-1}. Further, the pre-incubation of *N. naja* venom with the herbal preparation significantly reduced its Metalloprotease activity from 0.069 units min^{-1} to 0.019 units min^{-1}.
Conclusion: The herbal preparation shows a clear neutralizing action against the toxicities of *D. russelii and N. naja* venoms demonstrating the potential to be used as a plant based antidote for snake envenomation.

1. Introduction

Snake bites are proven to be a major health hazard in the tropical belt especially affecting the rural communities and agricultural sector in Asia, Africa, Oceania and Latin America. Recent studies show that the annual envenoming cases around the world is as high as 421,000–1,841,000 [1] and the deaths may be as high as 24,000–94,000 [1]. The true figurers of mortality could be even higher as a proportion of the people affected do not seek formal medical attention. The burden is mostly confined to the poorer communities and mainly is an occupational hazard in farming and agricultural communities [1,2]. Sri Lanka, a developing South Asian country, falls among the countries of highest snakebite records [1–3]. Sri Lanka is inhabited by 102 species of snakes [4]; among the mentioned snake species only 21 are considered highly venomous and five species as moderately venomous. From the highly venomous species 14 are sea snakes and 2 are terrestrial species with very low

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Peer review under responsibility of Transdisciplinary University, Bangalore.

http://dx.doi.org/10.1016/j.jaim.2016.10.001

contact with humans, and the highest weight of the morbidity and mortality are associated with snakebites of the highly venomous *Naja naja* (Common cobra) and *Daboia russelli* (Russell's viper).

The only specific treatment currently available to snake venom toxins is the hyper-immune globulins from snake venom immunized horse [5]. But the cost of anti-venom does not make it a readily accessible medication to tropical poorer regions. Therefore around the world it is an emerging trend on experimenting other possible antidotes for the snake envenomation. As a spectrum of possibilities lie within many of eastern traditional medicines extracted from plants, many of eastern herbal extracts are now under the scientific limelight.

Several studies have explored efficacy of such remedies. Extracts of *Hydrocotyle javanica* and *Gloriosa superba* gives 80–90% protection to mice treated with minimum lethal dose of venom (LD_{99}) of *Naja nigricollis* (Spitting Cobra) and has produced significant changes of membrane stabilization of human red blood cells (HRBC) exposed to hyposaline-induced haemolysis [6]. In another study, *Andrographis paniculata* and *Aristolochia indica* plant extracts were tested for neutralizing activity against *Echis carinatus* (Saw-scaled Viper) venom where both plant extracts have shown effective neutralization of venom induced lethal activity [7]. *Hibiscus aethiopicus* leaf extract completely stopped haemorrhagic activity against the venom of *Echis ocellatus* (African Carpet Viper) and *N. nigricollis* (Spitting Cobra) [8]. The methanolic root extracts of *Vitex negundo* and *Emblica officinalis ex*tracts has significantly antagonized the *D. russellii* and *Naja kaouthia* (Monocled Cobra) venom induced lethal activity in both *in vitro* and *in vivo* studies with neutralization of venom-induced haemorrhagic, coagulant, defibrinogenating and inflammatory activities [9]. In another study, snake venom neutralizing potential of *Rauvolfia serpentina* plant extract was tested by *in vitro* and *in vivo* methods against *D. russelli* venom. The *in vivo* assessment of LD_{50} in *D. russelli* venom was found to be 0.628 μg/g. *R. serpentina* plant extract effectively neutralized this venom lethality with an effective dose (ED) of 10.99 mg/3LD_{50} of venom [10].

Sri Lanka being an oriental country inheriting a great indigenous system of medicine, treating snakebites with herbal extracts is one such practice that has been widely used by indigenous doctors. Therefore, this study contributes towards the scientific evaluation of the effectiveness of a traditional herbal preparation used in Sri Lanka against *N. naja* and *D. russelli* venom toxicity and the characterization of its ethnopharmacological properties. The practice of this herbal preparation is confined to a late traditional medical practitioner from the southern coastal region of the country, who had inherited the medication through the family. The herbal preparation had been applied as a topical treatment over the bite site of the victims, who are at the early stages of envenomation by *N. naja* and *D. russelli*. The consent of the medical practitioner's family was obtained for the scientific evaluation of the herbal preparation for this study. The ethical clearance was obtained from the Institute of Biology, University of Colombo. To our knowledge, this study provides the first laboratory evidence for the venom neutralizing ability of a herbal preparation from Sri Lankan indigenous medicine. Preliminary forms of this work were presented at the 8th International Conference of General Sir John Kotelawala Defence University and at the 2nd International Conference of Traditional and Complementary Medicine [11,12].

2. Material and methods

2.1. Preparation of herbal extract

The herbal preparation was prepared by mixing together the aqueous extracts, obtained by crushing 2.5 g each of leaves of *Sansevieria cylindrica, Jatropha podagrica*, with a drop of the extract of *Citrus aurantiifolia* fruit. *S. cylindrica* is also known as the cylindrical snake plant, African spear or spear sansevieria. *J. podagrica* is known by several English common names, including Buddha belly plant, bottle plant shrub, gout plant, purging-nut, Guatemalan rhubarb, and goutystalk nettlespurge. *C. aurantiifolia* is known as the lime fruit. Fresh plant material of *S. cylindrica, J. podagrica* and *C. aurantiifolia* were collected from home gardens located off suburbs of Colombo in Kalutara district. The collected species were identified and authenticated by taxonomists from the Department of Plant Science, Faculty of Science, University of Colombo Sri Lanka. The preparation was made as a fresh aqueous extract on each day prior to testing. The volumes of the extracts individually as well as in the mixture, and the pH of the final preparation, were measured at each preparation, in order to maintain the consistency between preparations. The pH of the final preparation was 4.2.

2.2. Collection of venom samples

The venom samples of *D. russelii* and *N. naja* were collected from captive animals housed in the herpeterium of Faculty of Medicine, University of Colombo in September 2014. Samples from the two species were pooled separately and were freeze dried and were kept at −20 °C until use in the experiments.

2.3. SDS polyacrylamide gel electrophoresis (SDS PAGE) of venom

The amount of protein in the freeze dried venom samples was quantified by measuring absorbance at 280 nm wave length using Bovine serum albumin (BSA) as a standard. A 35 μg of freeze dried venom of either *D. russelii* or *N. naja*, dissolved in sample buffer was loaded on to a 12% polyacrylamide in the presence of Sodium Dodecyl Sulphate and electrophoresed at 180V for 30 min [13]. Then the gels were stained with Coomassie R-250 for 45 min followed by destaining with acetic acid and methanol, to visualize venom protein groups after 12% SDS PAGE.

2.4. Statistical analysis

Statistical analysis was carried out using Minitab 17 software. Sigmoidal dose-response curves for LD_{50} was generated using GraphPad Prism 4.03 (GraphPad Software,Inc.).

2.5. Chick embryo model for venom neutralizing activity of the herbal preparation

Freeze dried venom of *D. russelii* and *N. naja*, at varying doses, were reconstituted in PBS (7.4) to be impregnated on to 3 mm diameter of Whatman no 1 filter papers and placed over the vitelline vein on the exposed yolk sac membrane of *in vitro* cultivated 5-day old chicken embryos [14]. Pathological symptoms induced by each mentioned venom were closely monitored and recorded till death over a period of six hours. The experiment was replicated with each venom type incubated with the herbal preparation or the anti-venom (as a positive control).

2.5.1. Analysis of snake venom and treatment with herbal preparation and anti-venom

For the determination of LD_{50}, venom on 5-day old chick embryo, a gradient of 2 μg, 4 μg, 8 μg, 16 μg, 32 μg, and 48 μg of freeze dried venom dissolved in distilled water was used in the above procedure. The cut-off time for calculating number of embryo state dead/alive was six hours after treatment.

The neutralizing ability of the *D. russelii* venom by the herbal preparation was tested. It was determined by repeating the same concentration gradient of venom which have been incubated with a 2 μl of herbal preparation at 37 °C for 15 min prior to application on embryo, and comparing any shift in the LD_{50}. Commercially available Anti-venom (Snake venom anti-serum IP by VINS bioproducts limited) was used as the positive control in the 1× neutralizing dose (1167 μg of anti-venom for 1 μg of *N. naja* and *D. russelii* freeze dried venom) as per the manufacturer's recommendations. Final volume of each treatment added on the filter paper was always maintained at 4 μl. Results were statistically analyzed and the LD_{50} was calculated using the Probit values [15] in Minitab 17.

2.6. Phospholipase A_2 (PLA_2) neutralizing assay

The PLA_2 activity [16] was determined by egg yolk solution diluted 50% by mixing egg yolk and 5 mM TBS (Tris Buffered Saline) at pH 8 in 1:1 ratio. A 100 μl portion of this diluent solution was then mixed with 100 μg of freeze dried venom reconstituted with distilled water, alone or pre-incubated with 2 μl herbal preparation, at 37 °C for 15 min. The final volume of the venom was maintained 4 μl. After 30 min incubation of the reaction mixtures the samples were boiled in 100 °C water for 2 min to stop the PLA_2 activity and titrated with 12 mM NaOH in the presence of 4 μl of phenolphthalein till the color of the mixture changes to slight pink. The volume of titrated NaOH was recorded and the moles of liberated fatty acids due to venom PLA_2 action on the phosphatidylcholine in the egg yolk were calculated.

2.7. Proteolytic assay of Snake Venom Metalloprotease

A weight of 100 μg of freeze dried *N. naja* venom or the venom pre-treated with the herbal extract for 15 min at 37 °C was incubated in 1 ml of 50 mM Tris–HCl (pH 8.5) containing 1–2 mM Calcium Chloride and 1% Casein for 30 min [17]. The reaction was stopped by adding Trichloroacetic acid to a final concentration of 1%. Then the mixture was centrifuged at 18,000 rpm for 5 min, and the hydrolysis product of casein in the supernatant, produced due to venom metalloprotease activity, was determined by measuring absorbance at 280 nm.

3. Results and discussion

3.1. Visualization of the venom components using SDS PAGE

The venom proteins were separated on 12% SDS PAGE to identify the protein families in venom. At least 12 prominent protein bands, ranging from 3 to 188kD, were visible upon loading of 35 μg of total venom protein (Fig. 6). It was notable that, for same loading of venom protein (35 μg) from *D. russelii* or *N. naja*, the intensity of protein bands of *D. russelii* being significantly high. The composition of the venom varied significantly among the two species. Greater variation of the banding pattern of the two species was significant within 38 to 188kD region. Banding pattern from 3 to 28kD ranges was more likely to be shared among the two species. Based on the molecular weights on SDS-PAGE, clusters of venom protein bands could be categorized in to protein families as given in the manual on snake vernom protein components [17]. When compared with the characteristic venom protein profiles given in the above manual, these banding patterns revealed various protein families, such as PLA_2, phosphodiesterases, metalloproteases, etc., which could be recognized for the two venoms (Fig. 1).

3.2. Pathophysiological changes on the chick embryo by snake venom toxins

The pathophysiological changes on the chick embryo upon application of venom toxin, and with the treatment of the herbal preparation, were observed. Application of 2 μg *D. russelii* venom, made the external capillary network of the embryo to show a clear bleeding within 30 min. At this stage still the major blood vessels remained intact (Fig. 2A and B). In the progressive stages, the size of the blood vessels reduced and the heart beat started to drop with bleeding in the capillary bed becoming very prominent and intensified. Then death occurred within 1–2 h (Fig. 2C). Incubating the venom with herbal preparation reduced the above pathophysiological effects. Rapid retraction of blood from the vascular sac was not observed and the lack of bleeding from the capillary was significant. A slight drop in the heart rate could be observed (Fig. 2D and E). However, the embryo could be kept alive till 24 h, exceeding the 6 h experimental time (Fig. 2F). On the other hand, the prescribed neutralizing dose of anti-venom (positive control) did not show visible reduction of the effects generated by the *D. russelii* venom.

The embryos applied with *N. naja* venom showed a different pathophysiological pathway, as compared to what was observed with *D. russelii* venom. With *N. naja* venom, the generation of a localized hemorrhage near blood vessels under the disc impregnated with venom was characteristic right after the application, with no onset heart rate reduction (Fig. 3). In the presence of the herbal preparation spreading of the localized haemorrhage around the disc, impregnated with venom, was reduced with unaffected heart beat (Fig. 2). Incubation of *N. naja* venom with prescribed neutralizing dose of anti-venom did not prevent the appearance of the hemorrhage in the positive control.

3.3. The analysis of the LD_{50} of snake venom toxin on the chick embryo and the effect of the treatment with herbal preparation

In order to quantify and compare the neutralizing ability of the herbal preparation, the LD_{50} values of *D. russelii* venom, alone and after treating with the herbal extract or the anti-venom, were calculated. This was done by calculating the percentage mortality of the 5-day old chick embryo at varying doses of *D. russelii* venom (2 μg–48 μg) (Table 1).

For *D. russelii* venom, treated on the chick embryo, alone, a LD_{50} value of 4.8 ± 0.865 μg of freeze dried powder of venom, which contains a 667.2 μg of total protein amount, was obtained (Fig. 4). The LD_{50} of *D. russelii* venom in the presence of 2 μl of herbal preparation was 17.64 ± 1.35 μg (Fig. 4) showing a marked increase of nearly four times higher than the LD_{50} of the venom alone. In the presence of prescribed neutralizing dose of anti-venom, the LD_{50} of *D. russelii* venom could be increased up to 5.5 ± 1.35 μg (Fig. 4).

The LD_{50} values of the three tests showed that the neutralization capacity of *D. russelii* venom by the herbal preparation is higher than that of the commercially available anti-venom, at the recommended neutralizing dose by the manufacturer (For 1 μg of *N. naja* and *D. russelii* freeze dried venom, neutralizing dose of Anti-venom is 1167 μg). The tested commercial anti-venom is a polyvalent product raised against the Indian counterpart of *D. russelii* where differences in venom could have arisen from the geographic variations, thereby making the anti-venom not as effective against the local species. This has also been observed in clinical practice where the commercial anti-venom being not very effective against snake bites by *D. russelii* [18]; thus, stresses the need for alternative treatment options for snake bites particularly against the *D. russelii*. In this regard, the herbal preparation used in the current study is

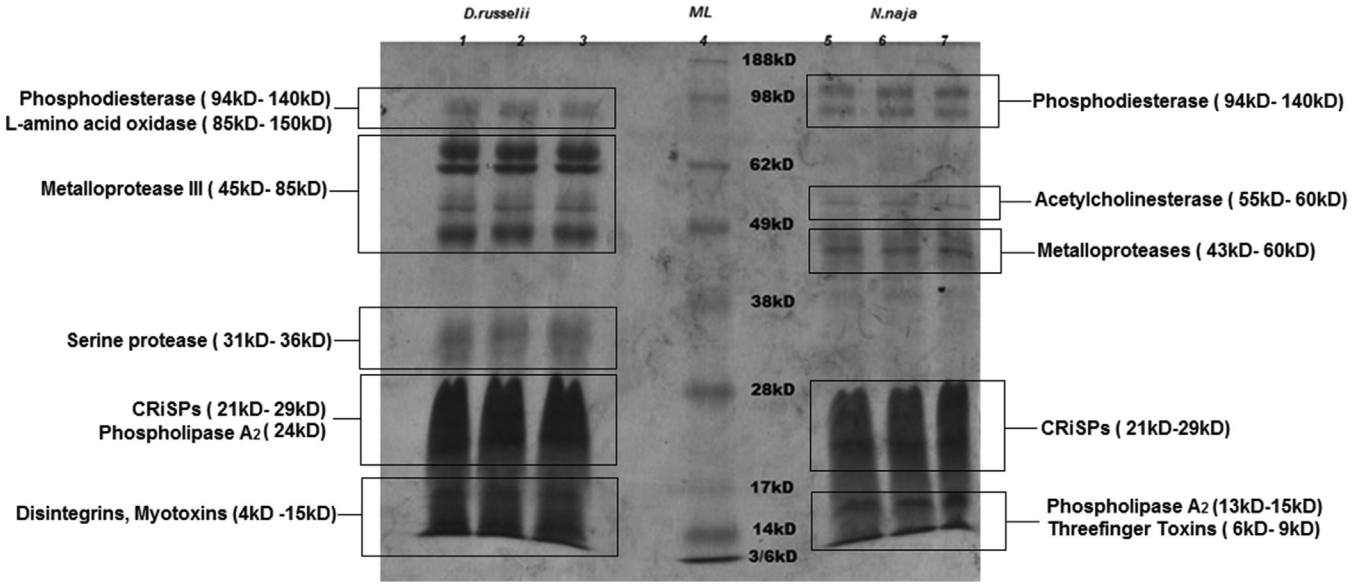

Fig. 1. SDS PAGE of *D. russelii* and *N. naja* venom components. The separated protein components are categorized in to protein families according to their molecular weights (denoted in boxes for *D. russelii* and in circles for *N. naja*). *Note that the lower molecular weight components are predominant in N. naja venom, characteristic of elapid venom.*

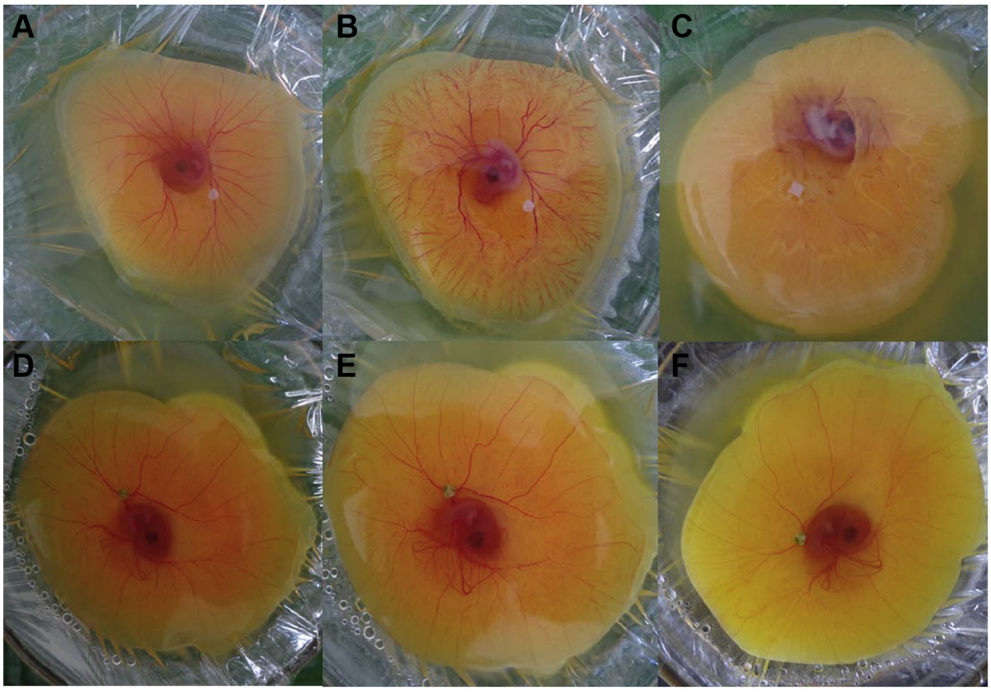

Fig. 2. Pathophysiological changes on the 5 day old chick embryo upon treating with 2 μg of *D. russelii* venom. A) At 0 min B) After 30 min (*Note: Visible signs of clear bleedings with vessels of the capillary bed appearing blurred. At this stage still the major blood vessels remained intact.*) C) After 60 min (*Note: Embryonic death with discoloration disappearance of blood vessels*). The effect of treating with herbal preparation on the venom toxicity –D) at 0 min E) In 1 h (*Note: Rapid retraction of blood from the vascular sac was not observed and the lack of the marginal hemorrhages and bleeding from the capillary was significant. slight reduction of the thickness of the blood vessels*). F) After 24 h (*Note live embryo with normal vascular network*).

promising, as it showed the clear presence of a potent neutralizing compound that could counteract the toxicity of *D. russelii* venom.

3.4. The effect of delayed treatment with the herbal preparation on its venom neutralizing ability

Having demonstrated the venom neutralizing activity by the herbal preparation, it was interesting to find out how delayed the herbal treatment can be applied effectively, after the venom toxicity is being ingested. In order to test this, the chick embryos treated with a paper disk impregnated with 16 μg of *D. russelii* venom ($\approx LD_{50}$ of *D. russelii* in the presence of the herbal preparation) were subjected to treatment of either the 2 μl of herbal preparation or 10× neutralizing dose of anti-venom, at varying time intervals. Treatment was applied on to the same disk impregnated with venom after 0 min, 5 min and 7 min.

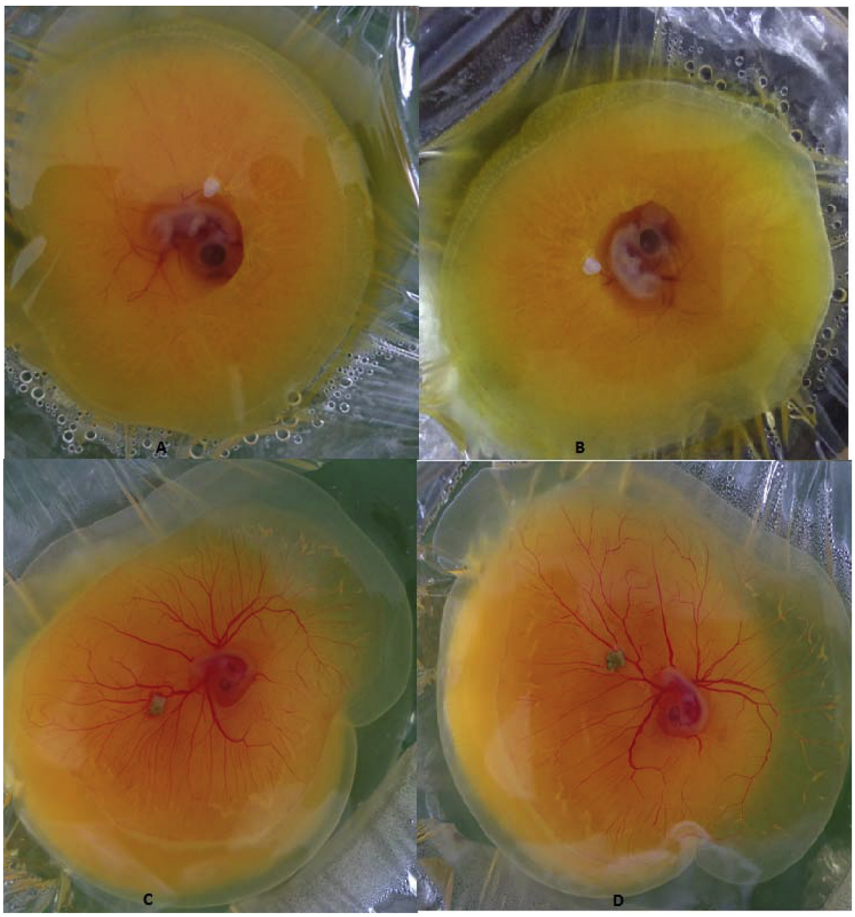

Fig. 3. Pathophysiological changes on the 5-day old chick embryo upon treating with 2 μg of *N. naja* venom. A) After 10 min, (*Note: the appearance of localized hemorrhage under the disc impregnated with venom*) B) After 25 min, (*Note: blood vessels appeared to be reducing with withdrawal of blood from the vascular sac. This was followed by death, changing the color of the embryo from healthy pink to pale white*). The effect of treating with herbal preparation on the venom toxicity C) After 1 min, (*Note: the reduction of the spread of the localized hemorrhage beyond the disc*) D) After 1 h, (*Note: unaffected heart beat and blood vessels and live embryo*).

Table 1
Percentage mortality with varying doses of *D. russelii* venom.

	Dose (μg)	Log dose	Number of affectd embrayos	% mortality	Probit value
Group 1 (*D. russelii* venom alone)					
1	2	0.30103	0/3	0	3.47
2	4	0.60206	2/3	66.66	5.44
3	8	0.90309	2/3	66.66	5.44
4	16	1.20412	3/3	100	6.39
5	20	1.301	3/3	100	6.39
6	32	1.50515	3/3	100	6.39
7	48	1.681241	2/3	100	6.39
Group 2 (*D. russelii* venom in the presence of 2 μl herbal preparation)					
1	12	1.079181	0/4	0	3.46
2	16	1.20412	0/4	0	3.46
3	20	1.30103	1/5	80	5.84
4	22	1.342423	4/4	100	6.53
5	24	1.380211	4/4	100	6.53
Group 3 (*D. russelii* venom in the presence of 1× neutralizing dose of anti-venom)					
1	4	0.602059991	1/3	33.333	4.56
2	8	0.903089987	2/3	66.66	5.44
3	16	1.204119983	3/3	100	6.73

The embryos treated with venom alone caused death in 10 min. Treatment with herbal preparation or anti-venom given after 5 min or 7 min increased the lifetime of the embryo to 25 min and 20 min respectively. Whereas, the embryos treated with the herbal preparation and anti-venom at 0 min survived for 6 h and 2 h respectively (data not shown). The observed result is highly encouraging, where it showed that the herbal extract could still be effective in treating snake bites, when the treatment is applied with a time gap after the venom toxin ingestion.

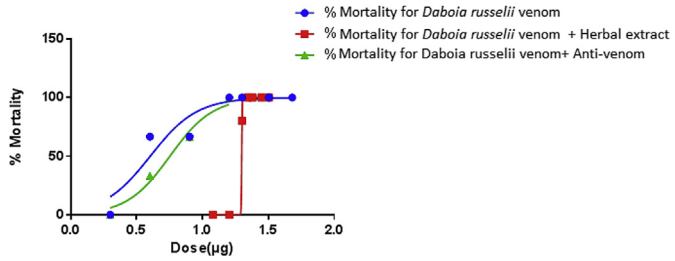

Fig. 4. Dose response curve of *D. russelii* venom for mortality of 5-day old chick embryos, following different treatments. Venom alone (blue line), Venom in the presence of herbal preparation (red line) and Venom in the presence of 1× neutralizing dose of anti-venom (green line), LD_{50} values for *D. russelii*, 4.8 ± 0.865 µg of freeze dried powder, for *D. russelii* venom with 2 µl of herbal preparation, 17.64 ± 1.35 µg, and for *D. russelii* venom with anti-venom = 5.5 ± 1.35 µg. (N = 4)

3.5. Direct analysis of PLA_2 activity inhibition

The effect on the Phospholipase activity, a known toxin in snake venoms, of *D. russelii* and *N. naja* venoms was investigated using fatty acid titration method. In this method, the fatty acids liberated from the egg yolk lecithin by the proteolytic activity of venom PLA_2 was quantified by titrating with NaOH; thereby making the amount of fatty acids liberated proportionate to the PLA_2 activity. The amount of fatty acids released by PLA_2 from *D. russelii* was 0.276 mM, and the same from *N. naja* was 0.876 mM. In the presence of the herbal preparation, fatty acids released from *D. russelii* venom dropped to 0.024 mM and from *N. naja* venom it dropped to 0.564 mM. The values show a clear decrease in the produced fatty acid amounts from the egg yolk with pre-treatment of venom with the herbal preparation, indicating an inhibition of PLA_2 enzyme activity by the herbal preparation (Fig. 5).

Therefore, one possible mechanism for the observed neutralizing activity of the herbal preparation, as was evident with the chicken embryos, could be through inhibiting PLA_2 enzyme in the venom. PLA_2 is a venom toxin, which is known to induce a vast range of pathological symptoms varying from neurotoxicity to anticoagulation toxicity [17].

3.6. Proteolytic assay of snake venom metalloprotease (SVMPs)

Metalloproteases are a very important set of proteolytic enzymes found in venom that induces hemorrhages as it can digest the proteins on the extracellular matrix. The observed hemorrhagic activity around the disk impregnated with *N. naja* venom on the chick embryo is consistent with its presence of metalloproteases in the venom. The disappearance of the hemorrhage in the presence of the herbal preparation is further consistent with the inhibition of this metalloprotease activity by the herbal treatment.

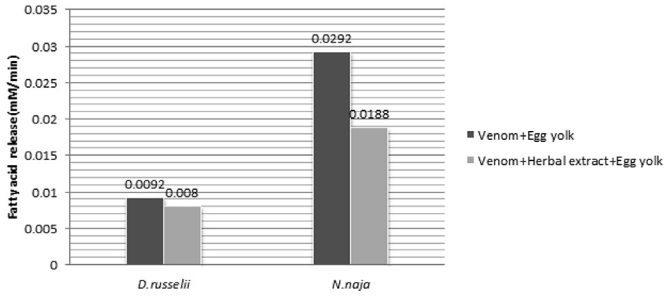

Fig. 5. Phospholipase$_2$ (PLA_2) activities of *D. russelii* and *N. naja* venom, in the presence or absence of the herbal preparation. The enzyme activity is represented as the amount of fatty acid released from egg yolk per minute. *Note the decreased fatty acid release for the herbal extract treated samples indicating inhibition of PLA_2 enzyme.* (N = 3)

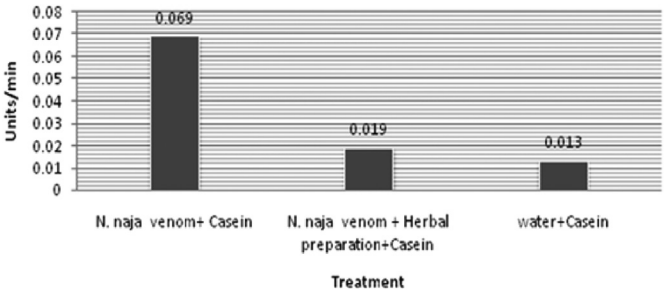

Fig. 6. SVMPs activity of *N. naja* venom in the presence and absence of herbal preparation. The enzyme activity is represented in terms of the amount of hydrolyzed product of 1% casein produced per minute. *Note the reduction in the production of the hydrolysis product of casein in the herbal extract treated samples to almost the same level as in the negative control indicating complete inhibition of the enzyme* (N = 3).

Therefore, the activity of snake venom toxin, metalloprotease, in the *N. naja* snake venom, was measured using an assay similar to the PLA_2 assay. The hydrolysis products of casein, one of the substrates of metalloproteases, were quantified as a measure of metalloprotease enzyme activity. In this assay *N. naja* snake venom alone generated an activity of 0.069 units/min. When incubated with the herbal preparation it dropped to 0.019 units/min, which was almost the same as the activity shown by the negative control test (0.013 units/min) carried only with distilled water in the absence of any venom (Fig. 6). Therefore the results showed a near complete neutralization of *N. naja* venom metalloprotease activity by the herbal preparation. Another mechanism of action for the venom neutralizing activity by the herbal preparation could therefore be through the inhibition of metalloprotease enzymes in the *N. naja* venom.

4. Conclusion

The analyzed herbal preparation of *S. cylindrica, J. podagrica* and *C. aurantiifolia* showed clear ability to neutralize the venom toxicities of both *D. russelii* venom and *N. naja*. It further inhibited the PLA_2 and metalloprotease enzyme activities of those venoms, which give rise to a wide range of pathophysiological effects. Some proteolytic activity by the herbal extract towards the venom proteins was also evident, specifically targeted on the protein families with a molecular weight in the range of 28–188kD. Therefore the neutralizing activity of the herbal preparation is suggestive to be brought through the inhibition of PLA_2 or metalloprotease activities and/or by the digestion of some of these protein toxins. This herbal preparation highlights a potential treatment option for snake venom bites as an alternative to currently practiced anti-venom treatment. The current study therefore adds to the evidence for alternative approaches for snakebite treatment using indigenous knowledge on ethnopharmacology.

Acknowledgement

The authors wish to thank the Department of Zoology, University of Colombo for providing necessary resources for the research and Faculty of Medicine, Kotelawala Defense University, Ratmalana for Laboratory facilities. The authors gratefully acknowledge Prof Ariyaranee Gnanadasan for providing snake venom and Prof Rohini Fernadupulle for providing Anti-venom samples.

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