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IN-VITRO AND *IN-VIVO* NEUTRALIZING POTENTIAL OF *RAUVOLFIA SERPENTINA* ROOT EXTRACT AGAINST *BUNGARUS CAERULEUS* VENOM

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Keywords:

Bungarus caeruleus, *Rauvolfia serpentina*, Direct haemolysis, Proteolytic Acetyl cholinesterase, ATPase, Lethal dose, Effective dose

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ABSTRACT: Snakebite is an international health hazard that needs attention in terms of effective management and better treatment to recover the health of the affected individuals and society. Tribal populations and folk therapists across the world have been using innumerable plants to treat snakebite difficulties. In the present study, the efficacy of aqueous extracts of *Rauvolfia serpentina* root for neutralizing *Bungarus caeruleus* venom by *in-vitro* and *in-vivo* methods was carried out. Various *in-vitro* neutralization tests like Direct and Indirect haemolytic activities, Acetylcholinesterase activity, Proteolytic activity, ATPase activity, and *in-vivo* assessment LD_{50} were carried out. *Rauvolfia serpentina* root extract effectively neutralizes all the venom's toxic effects. The *in-vivo* assessment of venom lethality (LD_{50}) of *Bungarus caeruleus* venom was 0.537 µg/g. *Rauvolfia serpentina* root extract effectively neutralized the venom lethality, and the effective dose (ED_{50}) was found to be10.96 mg/3LD_{50} of *Bungarus caeruleus*. All animals survived and appeared active and healthy throughout the study. The LD_{50} of *Rauvolfia serpentina* root extract was >2000mg/kg. These findings confirmed that *Rauvolfia serpentina* root extract possesses some compounds which inhibit the toxins present in *Bungarus caeruleus* venom.

INTRODUCTION: Snake envenomation is a major health problem that leads to numerous deaths, particularly in India. The majority of the snake bite incidences happened during the monsoons and during the daytime because most rural people heavily depend on cultivation [1, 2].

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About five million people worldwide are bitten by snakes annually, causing around 1, 25,000 deaths and 4, 00,000 individuals to be permanently disabled or disfigured [3].

The main families of venomous snakes in India are Elapidae which includes Common Cobra (*Najanaja*), King Cobra and Common Krait (*Bungarus caeruleus, Banded krait, Sind krait*); Viperidae includes *Daboia russelli* (Russell's viper), *Echis carinatus* (Saw-Scaled or Carpet viper), Pit viper and Hydrophiidae (Sea snakes). Neuromuscular disorders due to snake envenoming is common, including envenoming by elapid snakes

such as Kraits, Cobras, Coral snakes, Taipans, Tiger snakes, and Death adders. Snake venom-induced paralysis becomes life intimidating with progressive paralysis of the bulbar and breathing muscles which involves rapid airway provision and mechanical ventilation [4].

In tropical regions, snake bites remain an important socio-medical problem. Over one million humans were bitten worldwide every year by various snakes resulting in 70,000 deaths [5]. Elapid snake venoms are mostly neurotoxins or cytotoxins of the three-finger toxin family and phospholipases A_2 (PLA_2) [6].

Viperid venoms, in contrast, are composed mostly of PLA_2S, zinc-dependent metalloproteinases (SVMPs), and serine proteinases (SVSPs) [7]. *Rauvolfia serpentina* is commonly known as Sarpagandha, Chandrabagha, Snakeroot plant, Chotachand, Chandrika, and Harkayaetc [8]. It is used as an antidote against snake bites and bites of other toxic insects [9]. The present investigation explored *Bungarus caeruleus* venom neutralizing activity of *Rauvolfia serpentina* root extracts by *in-vitro* and *in-vivo* methods.

MATERIAL AND METHODS:

Collection and Authentication of Plant Material: *Rauvolfia serpentina* (L.) Benth. ex Kurz. (*Ophioxylon serpentinum* L.) belongs to the family Apocynaceae was collected from Anakkal region, Malampuzha, Palakkad district, Kerala after questionnaire with tribal people and from vaidyas in and around Palakkad district. Dr. Althaf Ahamed Kabeer authenticated it. Scientist 'D'. Botanical Survey of India Southern Regional Centre. Coimbatore (Herbarium voucher specimen number 1160).

Preparation of Extract: 20g of powdered sample of the herb was extracted by soaking in 180ml of distilled water in a beaker, stirred for about 6min, and left-over night. After that, the solution was filtered using filter paper (Whatman No.1), and the extracts were evaporated to dryness under reduced pressure in 40°C. The plant extracts were expressed in terms of dry weight.

Extraction yields (%) = (weight of freeze-dried extract *100 / weight of original sample)

Extraction yields of *Rauvolfia serpentina* is 2.5%

Snake Venom: The freeze-dried snake venom powders of *Bungarus caeruleus* were obtained from Irula's Snake Catchers Industrial Co-operative Society Limited Chennai and were stored at 4° C. Stock solution was prepared by dissolving 1mg of lyophilized venom in 1ml of physiological saline (1mg/ml). (Ethics committee approval number: JSSCP/IAE/PH.D/PH.COLOGY/02/2014-15).

Acute Oral Toxicity: Acute oral toxicity of all the selected plant extracts was performed as per OECD guidelines 423. A limit test of 2000 mg/kg body weight of the extracts was administered. Briefly, two thousand milligrams of the test substance per kilogram of body weight were administered to3 healthy mice by oral gavages. The animals were observed for mortality, signs of gross toxicity, and behavioural changes at least once daily for 14 days. Body weights were recorded before administration and again on Days 7 and 14 (day of termination). Necropsies were performed on all animals at terminal sacrifice.

***In-vitro* Assessment of Venom Toxicity and Neutralization Assays:**

Direct Haemolysis Assay: The hemolytic action of *Bungarus caeruleus* venom and plant extracts was studied *in vitro* by using RBC. Briefly, 5ml of citrated blood was centrifuged for 10minutes at 900rpm. The supernatant was poured off, and the pellet was washed twice with a physiological salt solution. 5ml of physiological saline and 0.5ml of RBC mixture served as a control. 5ml of distilled water with 0.5ml of washed RBC was used for 100% hemolysis. 5ml of venom/extract and 0.5ml of washed RBC served as the experimental sample. The tubes were put in a thermostat for 1hr at 37°C and centrifuged at 2000rpm for 20mts. The supernatant fluid was poured off to separate tubes from measuring the optical density using a spectrophotometer at a wavelength of 540nm against water.

Indirect Haemolysis Assay (PLA_2 activity): Phospholipase A_2 activity was measured using an indirect haemolytic assay on an agarose erythrocyte egg yolk gel plate by the method described by [10]. Increasing concentrations of *Bungarus caeruleus* venom (μg) were added to 3mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep

erythrocytes, 1.2% egg yolk as a source of lecithin and 10mM $CaCl_2$. Slides were incubated at 37°C overnight, and the diameters of the haemolytic halos were measured. Control wells contained 15µl of saline. The minimum indirect haemolytic dose (MIHD) corresponds to a concentration of venom, which produces a haemolytic halo of 11mm in diameter.

The efficacy of *Rauvolfia serpentina* root extracts in neutralizing the phospholipase activity was estimated by mixing a constant amount of venom (µg) with different amount of plant extracts (µl) and incubating for 30 min at 37°C. Then, aliquots of 10 µl of the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contain venom without plant extract. Plates were incubated at 37°C for 20 h. Neutralization is expressed as the ratio mg plant extract/mg venom able to reduce by 50% the diameter of the haemolytic halo compared to the effect induced by venom alone.

Acetyl Cholinesterase Activity: Acetylcholinesterase inhibition assay was carried out according to the modified method of [11] 200 µg of venom (1 mg/ml) was pre-incubated (1 h) with different concentrations of plant extract. The supernatant was added to the assay mixture, which consisted of 100 µl of 75mM acetylcholine iodate in 1 ml of phosphate buffer. The activity was measured by taking the absorbance at 412 nm. Venom without plant extracts was considered as control or 100% activity

Inhibition % = control-test/control ×100

Proteolytic Activity: Proteolytic activity was determined according to method [12]. Using 2%casein as substrate in 0.02 M Tris-HCl buffer (pH 8.5). Venom 200 µg (1 mg/ml) and different dilutions of plant extract were pre-incubated with 1 ml of substrate for 2 h at 37 °C.

The undigested casein was precipitated by adding 1.5 ml of 0.44 M trichloroacetic acid (TCA) and centrifuged. The digested casein in the supernatant was determined using Folinciocalteu's reagent. Venom without plant extracts was considered as a control or 100% activity.

Inhibition % = control-test / control ×100

ATPase Activity: ATPase activity was measured according to the modified method of [13]. *Bungarus caeruleus* venom 200 µg (1 mg/ml) was pre-incubated with different concentrations of plant extract of *Rauvolfia serpentina* root for 30 min. To the reaction, 1 ml of assay mixture (750 µl of 0.1 M Tris pH 7.5,100 µl of 0.1$MgCl_2$, 50 µl of 0.1 M ATP, and 100 µl of BSA) was added with gentle shaking at 37 °C and stopped at a certain time (1 h) by adding 1 ml of SDS solution.

The inorganic phosphate formed was determined by phosphate determination method by taking 400 µl of sample along 600 µl of TCA and incubating separately for 10 min at 37 °C followed by centrifugation at 1500 rpm for 10 min. About 500 µl of supernatant was added together with 500 µl of ferrous sulfate-ammonium molybdate reagent, and the absorbance was measured at 820 nm within 2 h for every 10 minutes of intervals. Reaction mixture without plant sample was referred to as control or 100 % activity. Inhibition reaction was calculated in terms of percentage (100%). Na, K-ATPase was mainly used.

Inhibition % = control-test / control × 100

***In-vivo* Assessment of Venom Toxicity and Anti-venom Effect of Plant Extracts Lethal Toxicity:** The median lethal dose (LD_{50}) of *Bungarus caeruleus* venom was determined according to the method of [14]. Various doses of venom in 0.2 ml of physiological saline were injected into the tail vein of mice, using groups of 3-5 mice for each venom dose.

The LD_{50} was calculated with a confidence limit of 50% probability by analyzing deaths occurring within 24 h of venom injection. The anti-lethal potentials for plant extract were determined against $2LD_{50}$ of *Bungarus caeruleus* venom. Various plant extracts (µl) were mixed with $2LD_{50}$ of venom sample and incubated at 37°C for 30 min, and then injected intravenously into mice. 3–5 mice were used at each antivenom dose. Control mice received same amount of venom without antivenom (plant extracts). The median Effective Dose (ED_{50}) calculated from the number of deaths within 24h of injection of the venom/antivenom mixture. ED_{50} was expressed as µl antivenom/mouse and calculated by probit analysis [15].

RESULTS:

TABLE 1: DIRECT HEMOLYSIS ACTIVITY – VENOM

Sample	OD of hemolysis	OD of Control (RBC + PBS)	OD of D. Water (100% Hemolysis)	% of Hemolysis
Bungarus caeruleus venom	1.06	0.04	1.08	94.44%

TABLE 2: DIRECT HEMOLYSIS ACTIVITY – NEUTRALIZATION ASSAY

Sample	OD of hemolysis	OD of Control (RBC + PBS)	OD of D.Water (100% Hemolysis)	% of Hemolysis
Bungarus caeruleus venom +Rauvolfia serpentina	0. 38	0.04	1.08	32%

Inhibition of Acetyl Cholinesterase Activity: The aqueous extract of the plant was taken in different dilutions starting from 200 µl to 300 µl with triplicate experiments. The maximum of Acetylcholinesterase inhibition (54.41%) occurred at 300 µl concentration of venom and aqueous extract of plant, respectively. The activity was calculated in terms of the percentage of inhibition compared to venom pre-incubated with different amounts of plant extract and venom with the substrate. The enzyme reaction was observed for every 10 min intervals at 412 nm. Acetylcholinesterase activity of the venom was considered as 100%.

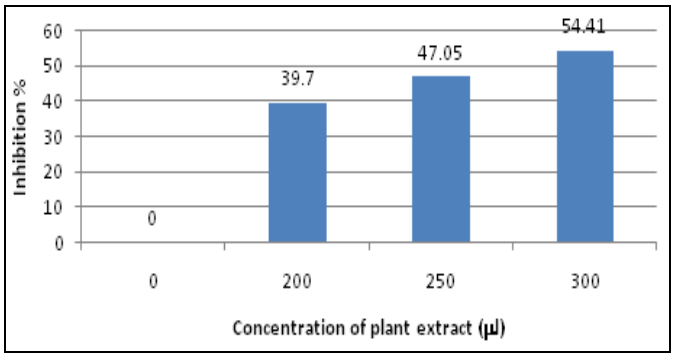

FIG. 1: ACETYLCHOLINESTERASE INHIBITION ASSAY

Inhibition of Protease Activity: To assess the in vitro antagonism of protease, the venom degrade the substrate (casein) into peptide precipitation could be observed at 600 nm.

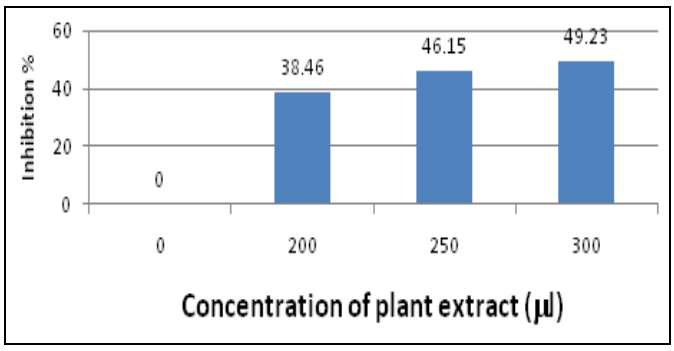

FIG. 2: PROTEASE INHIBITION ASSAY

Maximum of protease inhibition (49.23%) occurred at 300 µl concentration of venom and aqueous extract of plant, respectively. From the results, it was observed that increased plant extract could increase the inhibition of protease of *Bungarus caeruleus* activity.

ATPase Inhibition Activity: ATPase inhibition was calibrated by liberation of inorganic phosphate with of positive control of venom (200 µl) and substrate as ATP (10 µm). Different concentrations of venom and substrate were used for this reaction. The Same concentration of venom 200 µl, with different amounts of active aqueous extract of the plant (200 µl to 300 µl) was pre-incubated for the reaction. Maximum inhibition up to 53.60% has been noted at the highest amount of plant concentration.

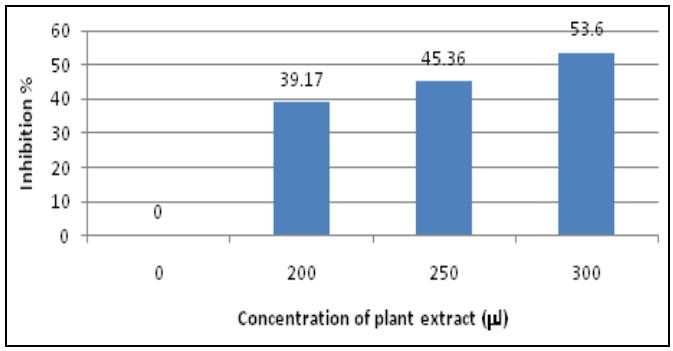

FIG. 3: ATPASE INHIBITION ASSAY

***In-vivo* Methods:** *In-vivo* assessment of venom toxicity (LD_{50}) of *Bungarus caeruleus* venom was assessed by LD_{50} range-finding test and the median lethal dose (LD_{50}) assay using mice (18-20g). LD_{50} of *Bungarus caeruleus* venom was calculated by Miller and Tainter method and was found to be 0.537 µg/g. **Table 4 and Fig. 4**. Venom neutralizing potency test (ED_{50}) using *Rauvolfia serpentina* root extract was carried out by pre incubating constant amount of venom ($3LD_{50}$) with various dilutions of *Rauvolfia serpentina* root extract prior to injection. Calculation of ED_{50} of

Rauvolfia serpentina root against $3LD_{50}$ of venom was done by Miller and Tainter method and was found to be 10.96 mg against *Bungarus caeruleus* venom. **Table 5 and Fig. 5**. All animals survived and appeared active and healthy in acute oral toxicity throughout the study. There were no signs of gross toxicity, adverse pharmacological effects or abnormal behavior. Based on the above findings, the LD_{50} of *Rauvolfia serpentina* root extract was>2000 mg/kg.

TABLE 3: CALCULATION OF LD_{50} OF *BUNGARUS CAERULEUS* VENOM IN MICE RECEIVING VARIOUS DOSES OF *BUNGARUS CAERULEUS* VENOM BY MILLER AND TAINTER METHOD (N=5)

Dose (µg/g)	Adjusted (Dose×100)	Log dose	Death/Total	Dead %	Corrected formula %	Probit values
0.025	*2.5*	0.4	0/5	0	5	3.36
0.05	*5*	0.7	1/5	0	5	4.16
0.1	*10*	1	1/5	20	20	4.16
0.25	25	1.4	2/5	40	40	4.75
0.5	50	1.7	2/5	40	40	4.75
1.0	100	2.0	3/5	60	60	5.25
2.5	250	2.4	3/5	60	60	5.25
5.0	500	2.7	5/5	100	95	6.64

Corrected formula: For the 0% dead: 100(0.25/n) = 100(0.25/5) = 5. For the 100% dead: 100[(n-0.25)/n] = 100[(5-0.25)/5] = 95, n is the number of animals in the group.

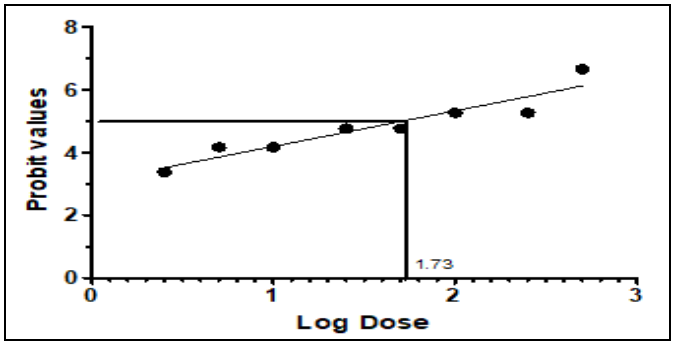

FIG. 4: CALCULATION OF LETHAL DOSE LD_{50} OF *BUNGARUS CAERULEUS* VENOM. LD_{50} of *Bungarus caeruleus* = antilog (log dose)/100 = antilog 1.73/100 = 53.70/100, 0.537 µg/g.

TABLE 4: CALCULATION OF ED_{50} OF *RAUVOLFIA SERPENTINA* AGAINST *BUNGARUS CAERULEUS VENOM* IN MICE BY MILLER AND TAINTER METHOD (N=5)

Dose (µg/g)	Adjusted (Dose×100)	Log dose	Survival/Total	Dead %	Corrected formula %	Probit values
1	*100*	2	0/5	0	5	3.36
2	*200*	2.3	0/5	0	5	3.36
4	400	2.6	1/5	20	20	4.16
8	800	2.9	2/5	40	40	4.75
16	1600	3.2	3/5	60	60	5.25
32	3200	3.5	4/5	80	80	5.84
64	6400	3.8	5/5	100	95	6.64

Corrected formula: For the 0% dead: 100(0.25/n) = 100(0.25/5) = 5. For the 100% dead: 100[(n-0.25)/n] = 100[(5-0.25)/5] = 95, n is the number of animals in the group.

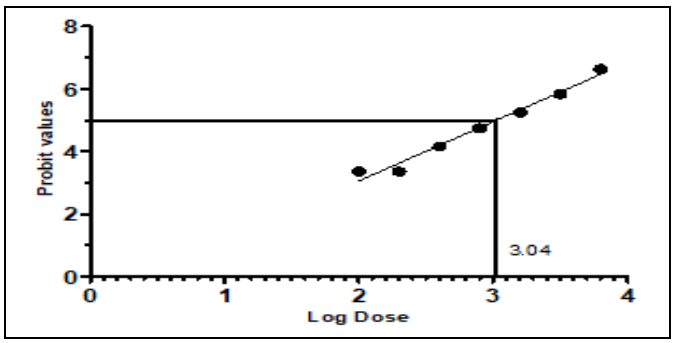

FIG 5: ED_{50} OF *RAUVOLFIA SERPENTINA* AGAINST *BUNGARUS CAERULEUS*. = antilog (log dose)/100 = antilog 3.04/100 = 1096/100= 10.96 mg.

DISCUSSION: Snakebite is constantly considered a chief health threat, leading to a high humanity rate worldwide [16, 17]. Polyvalent antiserum prepared from sheep and horses are effective for systemic envenoming after a bite. They have their own restrictions, such as indulgence and scarcity of accessibility for the rural patients [18]. Restrictions connected to anti-venom serum therapy have made people search for alternative medicines, especially medicinal plants in the past few years. Apart from Indian traditional medicines, Chinese, Greeks, and Egyptians are also identified in the usage of folk and traditional medicinal plants in snake bite treatments [19].

In recent years a number of reports on the use of plants in traditional healing by either tribal people or indigenous communities of India was increasing [20]. However, over the years, the use of antivenom has faced many constraints, notably allergic reactions, high prices, and lack of accessibility, making it challenging for people living in rural areas to access it [21]. Antivenin activities of a few medicinal plants such as *Andrographis paniculata, Andrographis lineata, Andrographis bracteolata, Clerodendrum viscosum,* and *Mimosa pudica* against the crude venom of Indian cobra have been reported using animal models in the previous literature [22, 23, 24, 25].

Elapidaevenoms have higher concentrations of acetylcholinesterase which exert an effect on nervous system [26]. *Thulasi et al* (2017) [27] reported that an aqueous extract of *Cyclea peltata* root is used for treating snakebite. In our present investigation, we checked the antitoxin activity of *Rauvolfia serpentina* root extracts against snake venom *Bungarus caeruleus*. The plant extracts were found to be effective in neutralizing the activity in an effective manner. In one of the reports, 28 *Azimatetra cantha* Lam extract effectively inhibited snake toxic enzymes like phosphomonoesterase, phosphodiesterase, acetyl-cholinesterase, hyaluronidase L-amino oxidase enzymes. In *Rauvolfia serpentina* aqueous rootextract (300µl) against *Bungarus caeruleus* venommaximum, ATPase inhibition was recorded as 53.60% for *Bungarus caeruleus* venom. Shwetha Vasudev [29] reported crude aqueous ethanolic extract cocktail of medicinal plants, *Areca catechu, Azadirachta indica, Butea monosperma, Citrus limon peel,* and *Clerodendrum serratum* for anti-ophidian properties against BIG FOUR venom through *ex-vivo* and *in-vivo* methods. Direct hemolysis using sheep RBC's was studied for *Bungarus caeruleus* venom and we found that snake venom was able to lyse the RBC's. *Bungarus caeruleus* venom showed 94.44% hemolysis. Plant extracts were able to neutralize the venom-induced hemolysis and the hemolysis was reduced below 40%. In phospholipase activity (PLA_2) 15µg of *Bungarus caeruleus* venom were able to produce 11mm diameter hemolytic halo, which is considered to be 1Unit. Plant extracts were capable of inhibiting PLA_2-dependent hemolysis of sheep RBC's induced by snake venoms in a dose-dependent manner.

In-vivo venom toxicity (LD_{50}) of *Naja naja* and *Bungarus caeruleus* venoms were assessed by LD_{50} range-finding test and the median lethal dose (LD_{50}) assay using mice (18-20 g). LD_{50} of *Bungarus caeruleus* was found to be 0.537 µg/g. Raphael *et al*., 2014 [30]reported that *Rauvolfia serpentina* root extract was used in neutralizing cobra and krait venom. In the previous report on Rajasree*et al*., 2013 [31] the ethanol extracts of *Rauvolfia serpentina* plant were tested for antivenom activity against *Najanaja* venom.

About 0.14 mg of *Rauvolfia serpentina* plant extract was able to neutralize the lethal activity of 2LD50 of Najanaja venom completely. In the previous work of Thulasi *et al*., 2017 [32] *Terminalia arjuna* bark extract was able to neutralize lethal activity of $2LD_{50}$ of *Naga naja* venom. In another study of *Thushara et al*., 2013 [33] of the *in-vivo* and *in-vitro* neutralizing potential of *Rauvolfia serpentina* plantextract against *Daboia russelli venom, Rauvolfia serpentina* plant extract was *e*ffectively neutralized by the venom lethality, and effective dose (ED) was found to be 10.99 mg/ 3LD of venom. In the previous work of Thulasi*et al*., 2020 [34] about 0.14 mg of *Rauvolfia serpentina* plant extract was able to completely neutralize the lethal activity of 2 LD_{50} of *Najanaja* venom. Timothy *et al.* [35] reported that a literature survey done in multidisciplinary databases revealed that 77 plant species belonging to 65 genera and 42 families are used to treat snakebites in Uganda. The majority of these species belong to the family Fabaceae (31%), Euphorbiaceae (14%), Asteraceae

(12%), Amaryllidaceae (10%), and Solanaceae (10%). These *in-vivo* neutralization assays suggest that plant extracts effectively neutralized the toxins present in both snake venoms. In acute oral toxicity, all animals persisted and seemed energetic and vigorous throughout the study. There were no signs of unfavorable pharmacological effects or unusual activities. Based on the above findings, the LD_{50} of all selected plant extracts was>2000 mg/kg.

CONCLUSION: The result from the *in-vitro* and *in-vivo* analysis indicates that *Rauvolfia serpentina* root extract possesses significant compounds that neutralizes the toxins present in *Bungarus caeruleus* venom. Further investigations are needed to identify and purify the active components involved in the neutralization of the snake venom.

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CONFLICTS OF INTEREST: Nil

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