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Preclinical Studies of a Multi-Component Herbal Therapy (*Rogeria adenophylla* J.Gay ex Delile, *Bombax costatum* Pellegr. & Vuillet, *Diospyros mespiliformis* Hochst. Ex A.DC. and *Entada africana* Guill. & Perr.) for the Management of Snake Envenomation

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ABSTRACT

In Northwestern Nigeria, *Rogeria adenophylla*, *Bombax costatum*, *Diospyros mespiliformis*, and *Entada africana* are traditionally used to treat snakebite envenomation. This study evaluated their antivenom properties against *Naja pallida* and *Echis ocellatus* venoms. Toxicity was assessed using Lorke's method, while venom lethality followed Theakston and Reid's protocol. Antivenom efficacy was tested *in vivo*, *ex vivo*, and *in vitro*. *In vivo*, individual extracts provided protection ranging from 20-60% protection against *E. ocellatus* venom, while the combination (COMBO) achieved 40%; no protection was observed against *N. pallida* venom. *Ex vivo*, both individual extracts and COMBO showed potent activity, achieving 100% survival against both venoms. In vitro, the extracts and COMBO inhibited haemolysis, fibrinolysis, and phospholipase A_2 activity in a dose-dependent manner. Toxicity studies revealed LD_{50} values of 2154.07 mg/kg for *B. costatum* and *D. mespiliformis*, and 1131.37 and 1264.91 mg/kg for R. adenophylla and E. africana, indicating moderate toxicity. The venoms' LD_{99} values were 0.58 mg/kg (*N. pallida*) and 9.77 mg/kg (*E. ocellatus*). These findings highlight the antivenom potential of these plants and their COMBO, supporting further exploration for developing a safe, effective multi-component herbal therapy for snakebite envenomation.

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1. Introduction

Snake envenomation represents a significant public health challenge, particularly affecting rural populations in tropical and subtropical regions. Neoteric estimates suggest that approximately 5.4 million people worldwide are impacted annually, resulting in at least 137,880 fatalities and more than triple that number suffering amputations and other long-term disabilities [1]. The current gold standard for treatment involves prompt intravenous administration of anti-snake venom (ASV) following a snakebite incident. In Nigeria, as in many developing nations, the absence of domestic ASV production necessitates complete reliance on imported products. This dependency has contributed to an ongoing crisis in antivenom supply across sub-Saharan Africa over the past three decades. Factors exacerbating this crisis include economic instability, logistical challenges in distribution, demanding storage conditions, prohibitive costs, and a loss of faith in antivenom efficacy due to the circulation of substandard imported products [2,3]. The profound public health implications, rising mortality rates, increasing incidence of disabilities, and the associated socio-economic burden on communities, especially in underdeveloped sub-Saharan countries, emphasize the urgent need for a paradigm shift in snake envenomation treatment approaches [3]. Nigeria's snake fauna includes three primary venomous species: the carpet viper (*Echis ocellatus*), black-necked spitting cobra (*Naja nigricollis*), and puff adder (*Bitis arientans*). These species can induce a range of severe clinical manifestations, including systemic hemorrhage, shock, paralysis, irreversible renal failure, and extensive tissue necrosis potentially leading to amputation [4-5].

The utilization of plant-based remedies for snakebite management has a long-standing tradition, particularly among communities in snake-endemic regions of Southeast Asia and sub-Saharan Africa, including Nigeria [6-7]. The widespread reliance on these botanical interventions stems from their accessibility, cultural acceptability, and affordability. Scientific investigations have corroborated the venom-neutralizing properties of various plants, including *Andrographis paniculate* (Burm.f.) Nees, *Guiera senegalensis* J.F.Gmel., *Moringa oleifera* Lam., *Allium cepa* L., and *Azadirachta indica* A.Juss. [8].

In Northwestern Nigeria, local communities commonly employ *R. adenophylla*, *B. costatum*, *D. mespiliformis*, and *E. africana* in snakebite management [Personal Communication]. However, the antivenom potential of these specific plants remains unexplored. Previous research has demonstrated that individual plants can neutralize toxins from multiple snake species, and synergistic venom neutralization effects have been observed both in vitro and in vivo [3]. This study aimed to evaluate the antivenin efficacy of these four medicinal plants, both individually and in combination, against the venoms of two prevalent snake species in Nigeria: the carpet viper (*Echis ocellatus*) and the red spitting cobra (*Naja pallida*), using animal and enzymatic models.

MATERIALS AND METHODS

Study area

All research was carried out in the Laboratories of the Department of Pharmaceutical and Medicinal Chemistry and the Department of Pharmacology and Toxicology, both within the Faculty of Pharmaceutical Sciences at Usmanu Danfodiyo University, located in Sokoto, Nigeria.

Collection and identification of plant materials

The whole plant materials of *R. adenophylla*, *B. costatum*, *D. mespiliformis* and *E. africana* were collected from Dundaye, Wamakko Local Government area of Sokoto State, Nigeria in the month of December, 2020. They were identified and

authenticated by Mal. Musa Magaji at the Herbarium unit, Department of Pharmacognosy and Ethnopharmacy, Usmanu Danfodiyo University, Sokoto by comparing with voucher specimen numbers viz: PCG/UDUS/Peda/0003, PCG/UDUS/Malv/0002, PCG/UDUS/Eben/0001 and PCG/UDUS/Faba/0014 for *R. adenophylla*, *B. costatum*, *D. mespiliformis* and *E. africana,* respectively. The plant names have been checked with "World Flora Online" (www.worldfloraonline.org), Accessed date on: 17th June, 2024.

Preparation of plant materials

The plant materials (stem bark each) were air dried, pulverized and preserved according to the method described in the African Pharmacopoeia (1985). The pulverized plant materials (500 g each) were extracted successively with 1.5 L of 90 % methanol using maceration method for 3 days with constant agitation and the extract obtained was filtered and evaporated in-vacuo using rotary evaporator at 40 °C to yield a residue referred to as the methanol extract for each plant as follows; MER for *R. adenophylla*, MEB for *B. costatum*, MED for *D. mespiliformis* and MEE for *E. africana*. The extracts were stored in a refrigerator for subsequent use.

Qualitative phytochemical screening

The presence of various chemical constituents such as tannins, alkaloids, and flavonoids were determined by conducting various chemical tests on the methanol extracts. according to standard operating procedures [9].

Experimental animals

Locally bred adult Swiss albino mice of either sex (21 – 30 g body weight) were acquired from Animal House Facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria. The mice were provided with a standard laboratory diet and had access to water. They were kept in clean, well-maintained cages, following a regular schedule of 12 hours of light and 12 hours of darkness. Every step taken during the experiments followed the global standards for the ethical treatment and care of laboratory animals, as outlined in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and were approved by the Usmanu Danfodiyo University Health Research and Ethics Committee with the approval number NHREC/UDUHREC/25/06/23.

Snake venom

Venom extraction from adult *Naja pallida* and *Echis ocellatus* specimens was performed by utilizing the Markfalane [10] milking technique. After collection, the venom samples underwent lyophilization and were subsequently stored at a temperature of 4°C for future use.

Standard antisnake venom

For this research, a commercially available polyvalent antivenom specific to African snake venoms was employed as a positive control. This lyophilized antiserum (ASV) was sourced from VINS BIOPRODUCTS LIMITED, a manufacturer based in Telangana, India. The production facility is located at Survey No. 117, Thimmapur Village, Kothur Mandal, in the Mahaboobnagar District. The antivenom batch used had a manufacturing date of January 2020 and was set to expire on December 31, 2023.

Acute toxicity studies

Lorke's method [11] was employed to determine the median lethal dose (LD_{50}) of methanol extracts obtained from *B. costatum, R. adenophylla, D. mespiliformis, E. africana*. The experiment was conducted in two stages. The initial phase involved nine mice, equally distributed into three groups of three. These groups were administered *i.p.* with 10, 100, and 1000 mg/kg of each extract individually. The outcomes of this preliminary stage informed the

dosage selection for the subsequent phase, where each experimental group received the following doses in the second phase as in indicated in the Lorke's Table;

Treatment	Doses (mg/kg)
B. costatum	600, 1000, 1600, 2900
R. adenophylla	200, 400, 800, 1600
D. mespiliformis	1600, 2900, 5000
E. africana	200, 400, 800, 1600

Antisnake venom studies

Lethality assay of the venom

To assess the lethality (LD_{99}) of *N. pallida* and *E. ocellatus* venoms, the protocol outlined by Theakston and Reid [12] was adopted. The LD_{99} value was calculated using probit analysis as described by Finney [13], based on mortality rates observed within 24 hours post-venom administration.

In vivo snake venom detoxifying effect

The experimental protocol followed the methodology established by Theakston and Reid [12]. A total of 25 mice were divided into five equal groups (n=5 per group). The control group received an intraperitoneal (*i.p.*) injection of normal saline at 10 mL/kg. Three test groups (2, 3 and 4) were administered the test sample(s) *i.p.* at doses of 100, 200, and 400 mg/kg, respectively. The fifth group was given standard lyophilized ASV at a dose of 1 mL per 0.6 mg. Thirty minutes post-administration of test substances, all animals except the control group were injected with 0.2 mL of reconstituted venom. Subsequently, all groups were monitored for 24 hours to observe and record any signs of toxicity or mortality.

Ex vivo snake venom detoxifying effect

A detoxification assay was conducted using five groups of mice (n=5 per group). The control group was administered intraperitoneal (*i.p.*) with a pre-determined venom dose. The remaining four groups (treatment groups) received a mixture containing the minimal lethal dose (MLD) of venom combined with either the test sample(s) at 100, 200, or 400 mg/kg, or Lyophilized ASV. These venom-sample mixtures were incubated at 37 °C for 10 minutes prior to administration. Each mouse in the treatment groups was then injected intraperitoneally (*i.p.*) with 0.2 mL of the incubated mixture. Mortality was monitored and recorded over a 24-hour period post-injection, following the method described by Abubakar et al. [14].

In vitro detoxification of the venoms
Phospholipase A_2 (PLA_2) assay

The phospholipase A_2 (PLA_2) activity was evaluated using an acidimetric assay following the method of Tan and Tan [15]. The substrate was prepared by combining equal volumes of 18 mM calcium chloride, 8.1 mM sodium deoxycholate, and egg yolk; then the mixture was homogenized for 10 minutes by stirring. The pH of the substrate was then adjusted to 8.0 using 1 M sodium hydroxide. To initiate hydrolysis, snake venom (0.6 – 0.1 mg/mL) was introduced to 15 mL of this substrate, with saline serving as a control. pH changes were recorded after two minutes using a pH meter. A unit decrease in pH equated to the release of 133 µmoles of fatty acid in the substrate. The enzymatic activity was quantified as micromoles of fatty acid released per minute. To assess the antivenin potential of the plant extracts, both individually and in combination, snake venoms (0.1 mg each) were pre-incubated with varying concentrations of the extracts (0.5, 0.25, 0.125, and 0.063 mg/mL). The extent of PLA_2 hydrolytic action neutralization was then measured. The protective effect of the extracts against phospholipases was expressed as a percentage, in line with the method reported by Yusuf et al. [16].

Haemolytic effect

The hemolytic activity was assessed using a modified version of the method described by Gomes and

Pallabi [17], utilizing bovine erythrocytes. Bovine blood (20 mL) was obtained from an abattoir using sodium citrate as an anticoagulant. The sample underwent centrifugation at 2400 rpm for 10 minutes, after which the plasma was removed. The remaining packed cells were washed with 5 mL of normal saline and re-centrifuged under the same conditions. This washing process was repeated 10 times to ensure plasma-free packed cells. Plant extracts were prepared at various dilutions (final concentrations: 0.5, 0.25, 0.125, and 0.063 mg/mL) and mixed with 100 µL of snake venom (0.6 mg/mL). This mixture was then added to 1 mL of a 1% red blood cell suspension in saline. The resulting solution was incubated at 37°C ± 1°C for 60 minutes. The reaction was terminated by adding 3 mL of chilled phosphate-buffered saline (PBS). After centrifugation at 2000 rpm for 10 minutes, the absorbance of the supernatant was measured spectrophotometrically at 540 nm. The supernatant from samples treated with snake venom alone was considered to represent 100% lysis, as per Theakston and Reid [12], and Gomes and Pallabi [17]. The hemolytic activity (HA) and the percentage inhibitory activity of the plant extracts were then calculated using a specific formula below;

$$Haemolyttic\ activity = \frac{Transference\ (\%) - Blank}{Wavelength}$$

$$\%\ Inhibitory\ actvity = \frac{HA\ of\ the\ venom\ control - HA\ of\ venom\ and\ test\ samples}{HA\ of\ the\ venom\ control}\ X\ 100$$

Fibrinolytic effect

Bovine blood was collected using 3.8% sodium citrate as an anticoagulant (9:1 v/v). The sample was centrifuged at 2500 rpm and then refrigerated at 4 °C for 15 minutes to obtain platelet-poor plasma. Various dilutions of crude venoms were prepared for the assay. The fibrinolytic activity was evaluated using the method described by Theakston and Reid [12]. Bovine citrated plasma (2.0 mg/mL) was used as the substrate. The assay involved adding 50 µL of venom to 0.2 mL of the citrated plasma solution. Just before introducing the venom solution, 50 µL of $CaCl_2$ (25 mM) was added to the mixture. The clotting time was recorded at 37 °C. To assess the inhibitory capacity of the plant extracts, serial dilutions were prepared and added to the plasma-venom mixture, resulting in final concentrations of 0.5, 0.25, 0.125, and 0.063 mg/mL. Following Theakston and Reid's [12] protocol, all fibrinolytic activities (FA) were expressed as the inverse of the recorded clotting times in minutes. The percentage inhibitory activity was then calculated using the formula below;

$$Inhibitory\ activity = \frac{FA\ of\ the\ venom\ control - FA\ of\ the\ venom\ test\ sample}{FA\ of\ the\ venom\ control}\ X\ 100$$

Statistical analysis

Data were presented as mean values accompanied by their standard error of mean (SEM). The statistical analysis of both control and experimental data employed one-way ANOVA. To compare the means, Dunnett's post hoc test was utilized. Statistical significance was established at $p<0.05$.

RESULTS

Percentage yield

Extraction yields for *B. costatum*, *R. adenophylla*, *D. mespiliformis*, and *E. africana* are summarized in Table 1.

Table 1: Percentage Yield of plant samples

Extract	Weight (g)	% Yield
B. costatum	60.56	12.00
R. adenophylla	102.00	20.00
D. mespiliformis	52.30	10.50
E. africana	100.02	20.00

Key: MEB = Methanol extract of *B. costatum*; MER = Methanol extract of *R. adenophylla*; MED = Methanol extract of *D. mespiliformis*; MEE = Methanol extract of *E. africana*

Notably, *R. adenophylla* and *E. africana* demonstrated superior extraction efficiencies compared to *B. costatum* and *D. mespiliformis*.

Qualitative phytochemical screening

Phytochemical analysis of the extracts revealed a diverse array of compounds, including saponins, carbohydrates, tannins, flavonoids, cardiac glycosides, alkaloids, steroids, and triterpenes, with minor variations among species. Notably, *R. adenophylla* lacked tannins, while anthraquinones were exclusively detected in *D. mespiliformis*. A comprehensive breakdown of these findings is presented in Table 2.

Table 2: Phytochemical Screening of MEB, MER, MED and MEE

Phytoconstituent	Test	MEB	MER	MED	MEE
Saponins	Frothing	++	++	+	++
Carbohydrates	Molisch's	++	++	++	++
	Fehling's	++	++	++	++
Tannins	Ferric chloride	++	-	++	++
	Lead acetate	++	-	++	++
Steroids/Triterpenes	Liebermann-Burchard's	++	++	++	++
	Salkowski's	++	++	++	++
Flavonoids	Shinoda	++	++	++	++
	Alkaline	++	++	++	++
	Ferric chloride	++	++	++	++
Cardiac glycosides	Keller-Kiliani	++	++	+	++
Anthraquinones	Bontrager's	-	-	+	-
Alkaloids	Mayer's	++	++	N.D	+
	Wagner's	+	++	+	++
	Dragendorff's	++	++	++	++

Key: - = absent; + = present; ++ = moderate; MEB = Methanol extract of *B. costatum*; MER = Methanol extract of *R. adenophylla*; MED = Methanol extract of *D. mespiliformis*; MEE = Methanol extract of *E. africana*, N.D = not detected

Acute toxicity studies

The median lethal dose (LD_{50}) values varied among the plant extracts; *B. costatum* and *D. mespiliformis* exhibited similar LD_{50} values of 2154.07 mg/kg. In contrast, *R. adenophylla* and *E. africana* showed lower LD_{50} values of 1131.37 and 1264.91 mg/kg, respectively (Table 3).

Table 3: Median lethal dose (LD_{50}) of extracts

Extract	LD_{50} value (mg/kg)
B. costatum	2154.07
R. adenophylla	1131.37
D. mespiliformis	2154.07
E. africana	1264.91

Key: MEB = Methanol extract of *B. costatum*; MER = Methanol extract of *R. adenophylla*; MED = Methanol extract of *D. mespiliformis*; MEE = Methanol extract of *E. africana*

Minimal lethal dose (LD_{99})

The LD_{99} values of the *N. pallida* and *E. oceallatus* were determined to be 0.58 and 9.77 mg/kg, respectively (Table 4).

Table 4: Minimum lethal dose (LD_{99}) of the venoms

Venom	LD_{99} value (mg/kg)
E. ocellatus	9.77
N. pallida	0.58

In vivo antivenin activity

The methanol extracts from *B. costatum*, *R. adenophylla*, *D. mespiliformis*, and *E. africana* demonstrated significant ($p<0.05$) *in vivo* antivenom activity against *E. ocellatus* venom. *R. adenophylla* extract provided the highest protection, with a 60% survival rate at 400 mg/kg. In contrast, *D. mespiliformis* extract offered 20% protection at the same concentration. A combination of all four extracts (COMBO) achieved 40% protection at both 200 and 400 mg/kg, comparable to the standard antivenom serum (ASV). These results are illustrated in Table 5 and Figure S1.

Table 5: *In vivo* detoxifying effect of the extracts against *E. ocellatus* venom

	% Survival (within 24 h)						
Treatment (mg/kg)	*B. costatum*	*R. adenophylla*	*D. mespiliformis*	*E. africana*	COMBO	SV	ASV
LD_{99}+ 100	60	20	0*	40	20	-	-
LD_{99}+ 200	0*	40	20	40	40	-	-
LD_{99}+ 400	40	60	20	0*	40	-	-
LD_{99} + DW	-	-	-	-	-	0*	-
LD_{99} + ASV	-	-	-	-	-		40

Key: COMBO = Combination of the four medicinal plants; DW = Distilled water; ASV = Antisnake venom. **One-way ANOVA (post hoc) at $p < 0.05$ shows that there was significantly lower survival compared to control (standard ASV), others have comparable survival rate with the control.*

Notably, neither the individual extracts nor the ASV provided protection against *N. pallida* venom, as shown in Table 6.

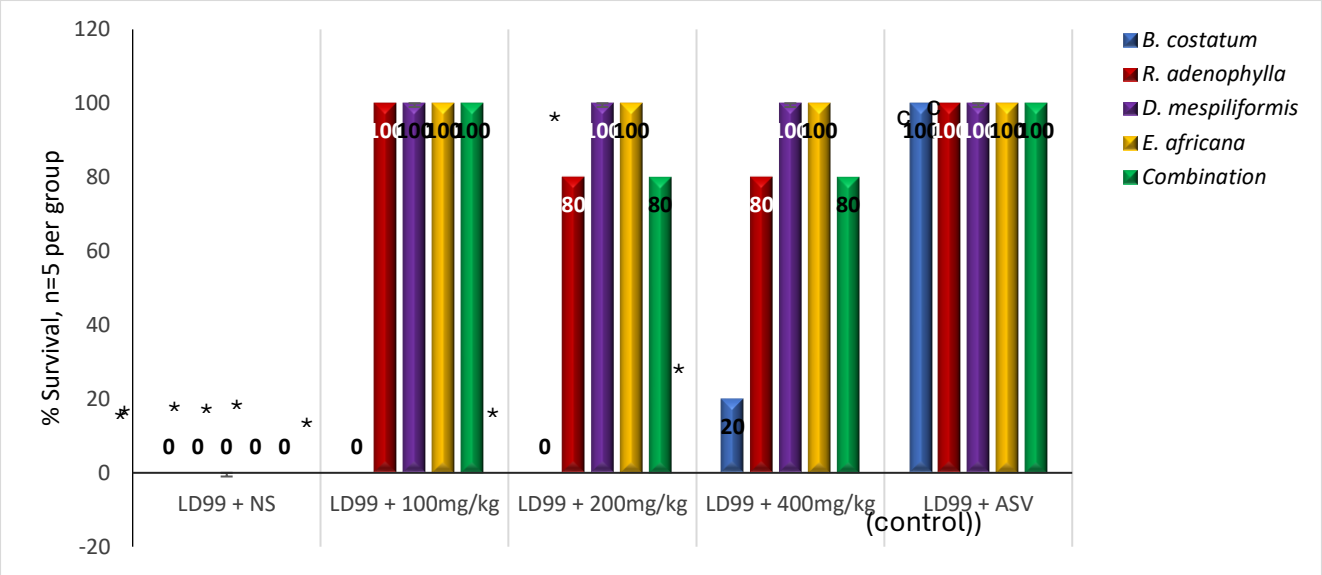
Table 6: *In vivo* detoxifying effect of the extracts against *N. pallida* venom

	% Survival (within 24 h)						
Treatment (mg/kg)	*B. costatum*	*R. adenophylla*	*D. mespiliformis*	*E. africana*	COMBO	SV	ASV
LD_{99}+ 100	0	0	0	0	0	-	-
LD_{99}+ 200	0	0	0	0	0	-	-
LD_{99}+ 400	0	0	0	0	0	-	-
LD_{99} + DW	-	-	-	-	-	0	-
LD_{99} + ASV	-	-	-	-	-		0

Key: COMBO = Combination of the four medicinal plants; DW = Distilled water; ASV = Antisnake venom. ** One-way ANOVA (post hoc) at $p < 0.05$ shows that there was significantly lower survival compared to control (standard ASV), others have comparable survival rate with the control.*

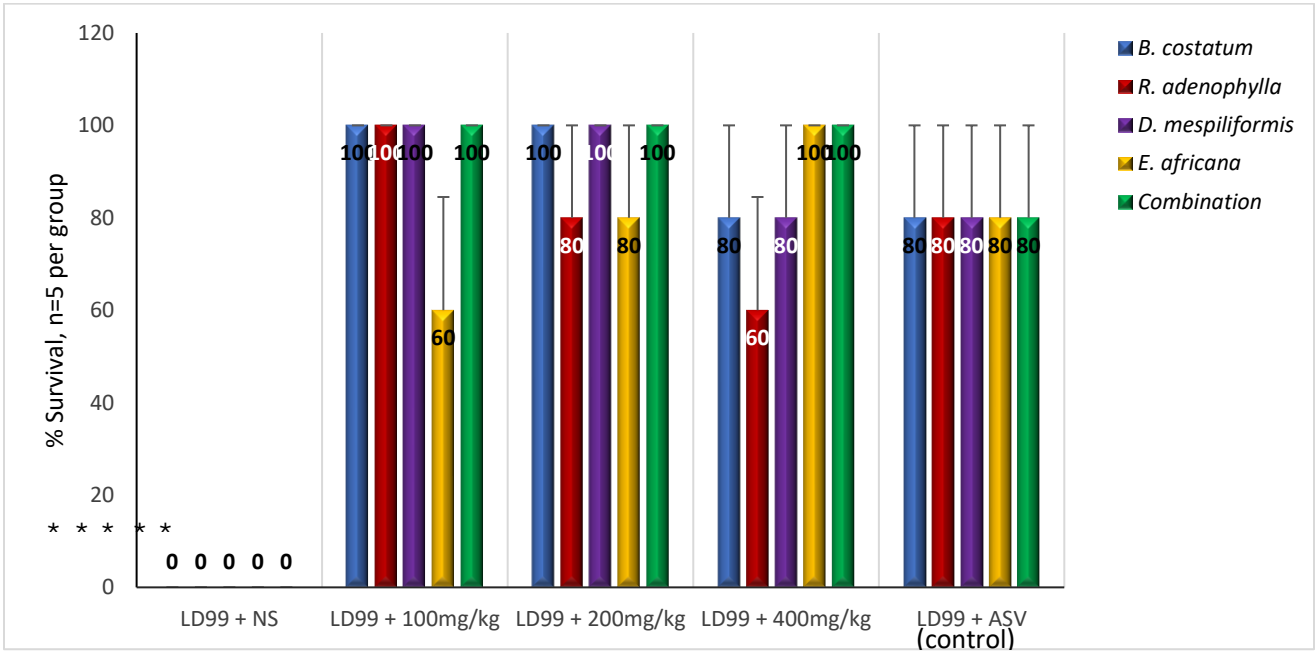
Ex vivo antivenin activity

The four selected medicinal plants (*B. costatum*, *R. adenophylla*, *D. mespiliformis* and *E. africana*) indicated potent antivenom activity against the venoms of *Naja pallida* and *Echis oceallatus* at the graded doses (100, 200 and 400 mg/kg) as shown in Figures 1 – 2.



Key: NS= Normal saline, ASV= Antisnake venom. **One-way ANOVA (post hoc) at p < 0.05 shows that there was significantly lower survival compared to control (standard ASV), others have comparable survival rate with the control.*

Figure 1: *Ex vivo* detoxifying effect of the different plants and their COMBO against *N. pallida* venom



Key: NS= Normal saline, ASV= Antisnake venom. **One-way ANOVA (post hoc) at p < 0.05 shows that there was significantly lower survival compared to control (standard ASV), others have comparable survival rate with the control.*

Figure 2: *Ex vivo* detoxifying effect of the different plants and their COMBO against *E. ocellatus* venom

E. africana and *D. mespiliformis* were able to significantly ($p<0.05$) inhibit the lethal actions of *N. pallida* venom with 100 % survival rate at the graded doses (100, 200 and 400 mg/kg) which was similar to that of the standard ASV. *B. costatum* exhibited little antivenin effect with maximum survival rate of 20 % against the venom of *N. pallida* at the highest dose (400 mg/kg) while the COMBO showed maximum protection (100 %) at the lowest dose (100 mg/kg) (Figure 1).

The four extracts combinations of the plants (COMBO) gave maximum protection (100 %) to mice against the *E. ocellatus* venom at the graded doses (100, 200 and 400 mg/kg). *B. costatum* and *D. mespiliformis* showed similar effect with 100, 100 and 80 % survival rate at 100, 200 and 400 mg/kg, respectively. *E. africana* had 60, 80 and 100 % at 100, 200 and 400 mg/kg, respectively while the standard ASV had 80 % survival (Figure 2).

In vitro detoxification

Haemolytic effect

In vitro haemolytic effect of the extracts singly and in combination against the two snake venoms (*E. ocellatus* and *N. pallida*) are presented in Figures 3 – 4 and Tables S1 – S2.

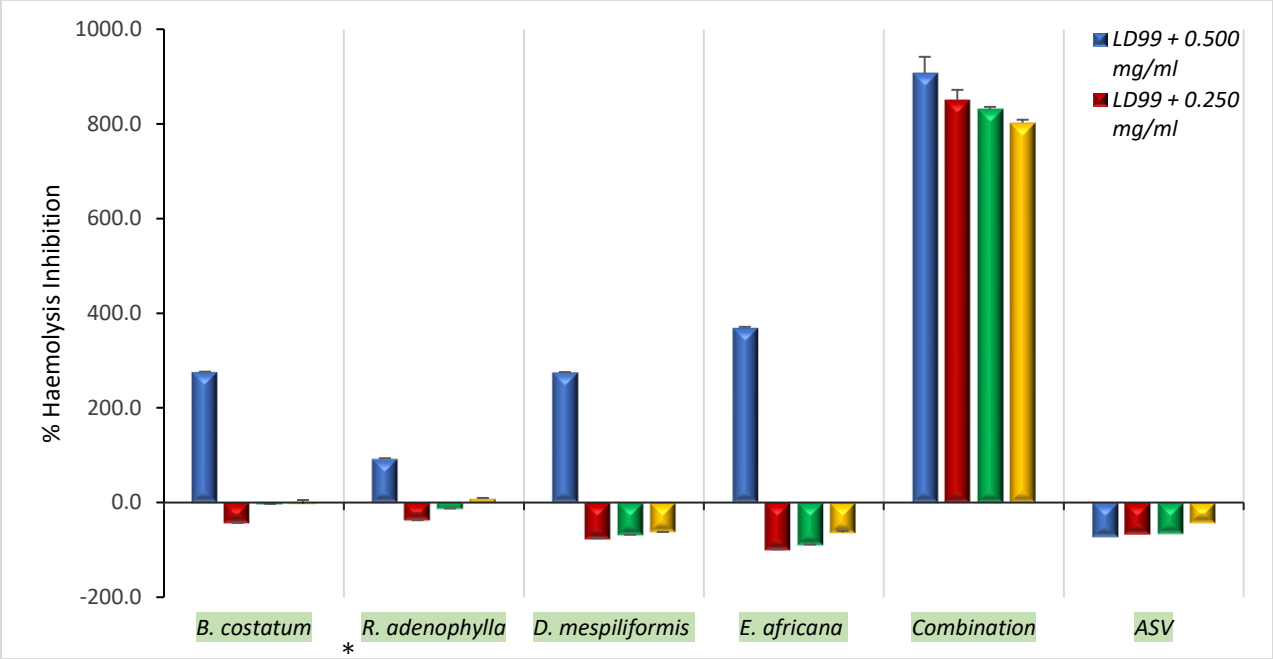


Figure 3: *In vitro* Haemolytic Effect against *E. ocellatus* venom

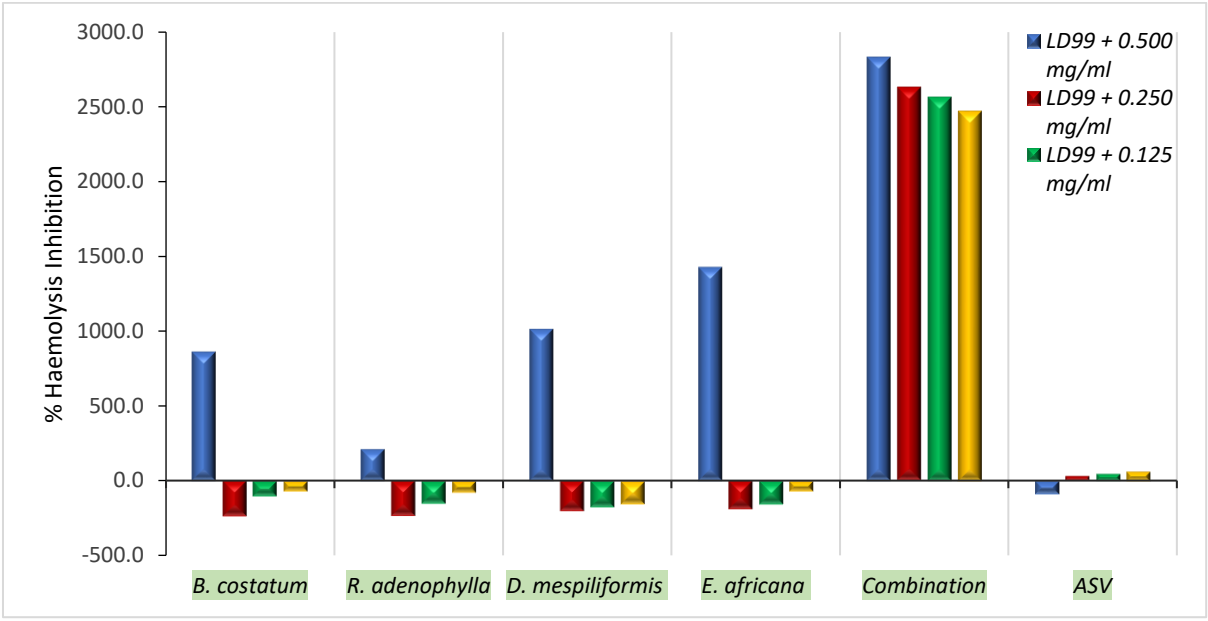


Figure 4: *In vitro* Haemolytic Effect against *N. pallida* venom

The extracts and their COMBO exhibited high percentage inhibition of haemolysis in a concentration-dependent manner against both snake venoms at the highest concentration (0.5 mg/mL).

Fibrinolytic effect

The percentage inhibition of fibrinolytic activity of the extracts and their COMBO against *E. ocellatus* and *N. pallida* venom are indicated in Figures 5 – 6 and Tables S3 – S4.

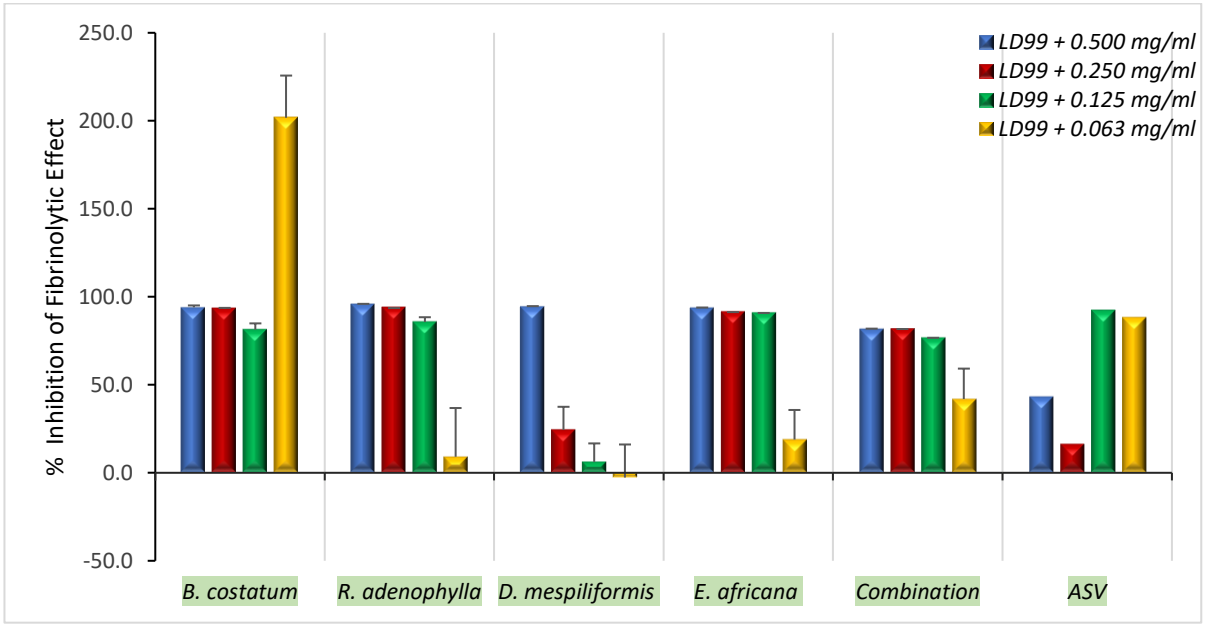


Figure 5: *In vitro* Fibrinolytic Effect against *E. ocellatus* venom

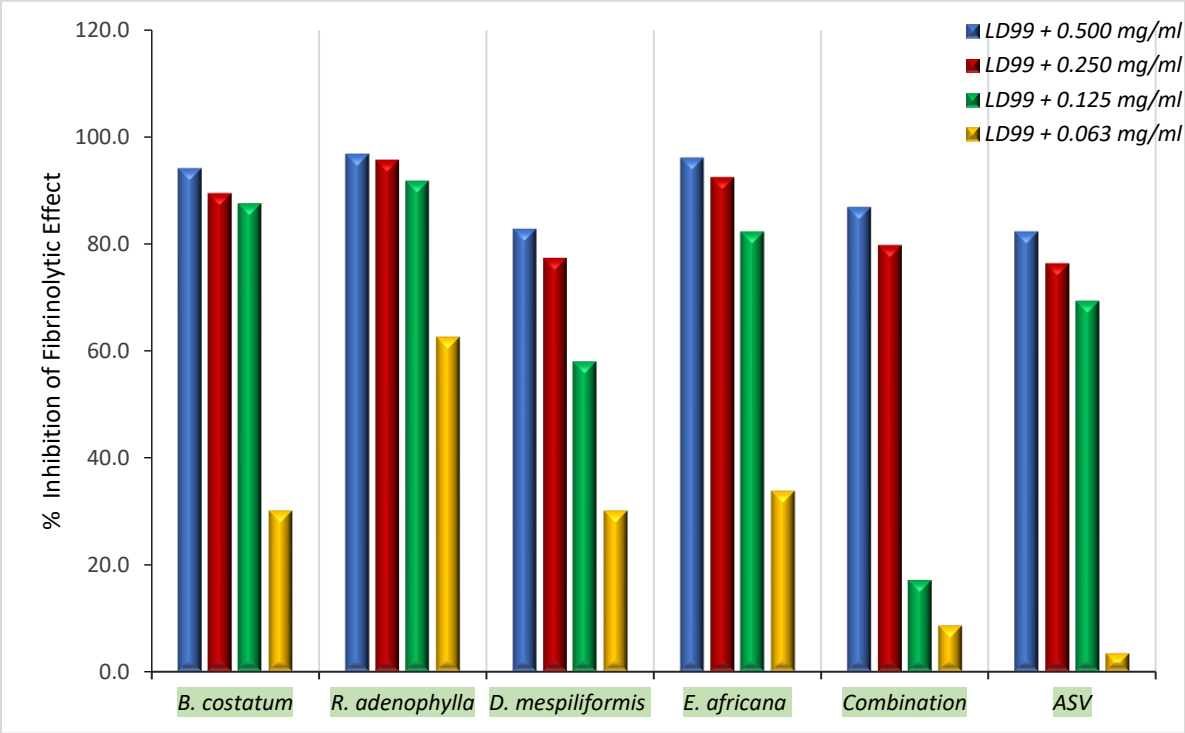


Figure 6: *In vitro* Fibrinolytic Effect against *N. pallida* venom

Venom-induced fibrinolytic effect was significantly antagonized by the methanol extracts of the four different plants and their COMBO at the different concentrations (0.5 – 0.063 mg/mL). *D. mespiliformis* and ASV exhibited lesser effect compared to the other treatment groups. Similar trend was observed against *N. pallida* venom.

Phospholipase A_2 assay

The four different plants and their COMBO exhibited significant inhibition of phospholipase A_2 enzyme activity against *E. ocellatus* and *N. pallida0* venoms and the effect was in a concentration dependent manner as indicated in Figures 7 – 8 and Table S5 – S6. The lethal actions of PLA_2 was significantly inhibited by the extracts and their COMBO, though, the effect was lower compared to the standard ASV (at 0.5 mg/mL). However, the COMBO exhibited percentage inhibition comparable to that of the standard.

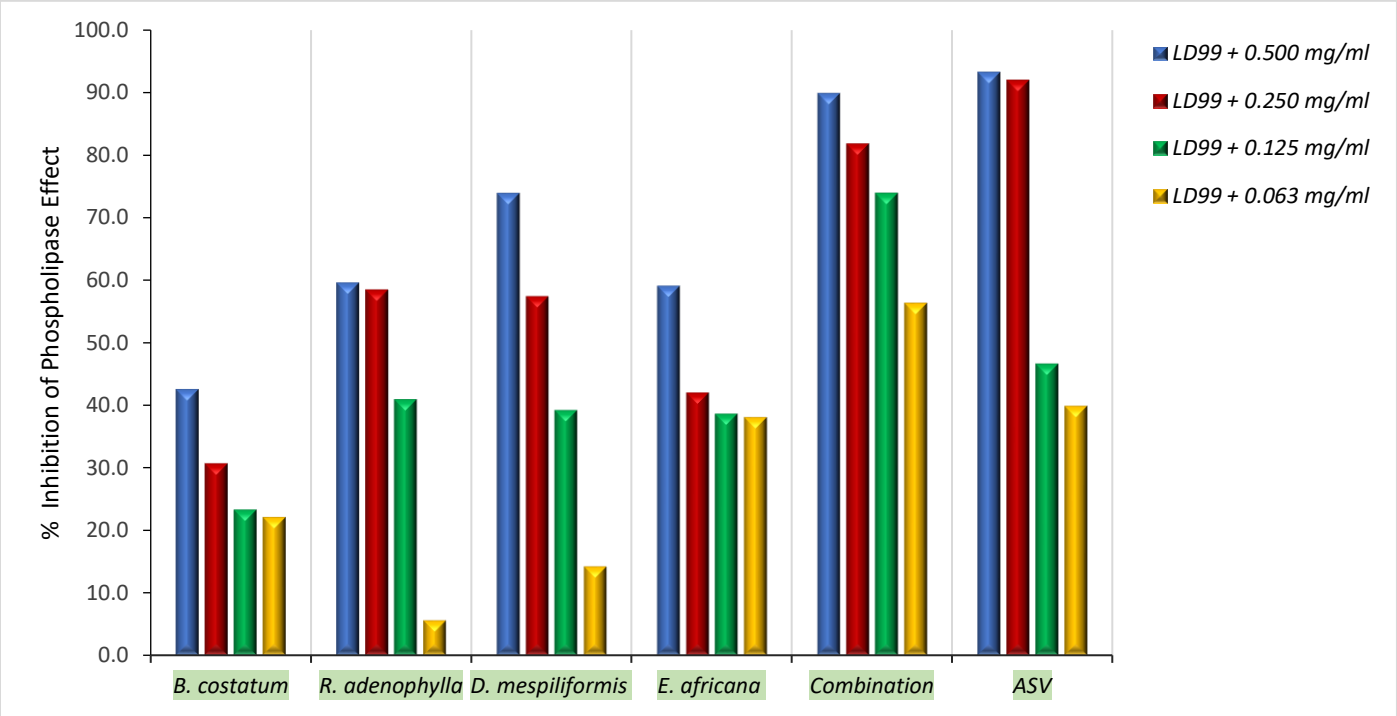


Figure 7: Effect of extracts and COMBO on *E. oceallatus* Phospholipase A_2 enzyme activity

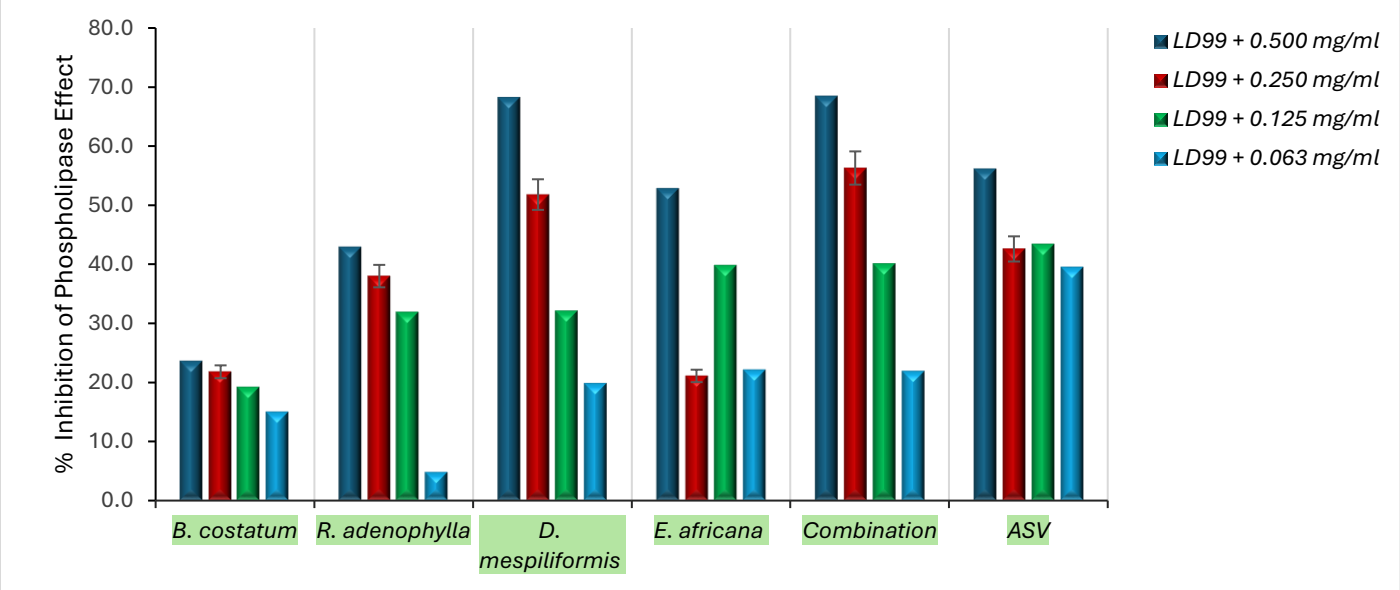


Figure 8: Effect of extracts and COMBO on *N. pallida* Phospholipase A_2 enzyme from *N. pallida*

DISCUSSION

Acute toxicity test to evaluate adverse effects that may occur following administration of a single or multiple dose of the methanol extracts of *B. costatum*, *R. adenophylla*, *D. mespiliformis* and *E. africana* given within 24 h was conducted using Lorke's method. Mortality was recorded after administration of the individual extracts at a higher dose (1000 mg/kg) within 24 h. Thus, animals indicated some signs of toxicity after administration of the extracts viz; writhing, erection of fur, muscle spasm, arching, rolling, itchy mouth and in some cases difficulty in breathing and gasping were also recorded [11,18]. Based on the estimated LD_{50} values, all the extracts were moderately toxic. Speaking about venom, it is important to carefully define its toxic and lethal properties [19]. Venom toxicity within the same

snake species varies both locally and from region to region and thus, making their standardization very difficult [20]. The MLD (LD_{99}) of the crude venoms of *N. pallida* and *E. ocellatus* in albino mice from dose response studies determined using probit analysis were estimated to be 0.58 and 9.77 mg/kg, respectively. Variation in venom toxicity is well documented; for example, Ode and Asuzu [21] reported a different concentration (6 mg/kg) for *E. ocellatus* and higher value for other *Naja* species. Harrison *et al*. [22] and Hasson *et al*. [23] also reported a different value for *E. ocellatus*.

Natural products especially from plant sources have been a source of many commercially available drugs and researches in that area towards possible discovery of new drugs and novel compounds have increased [20,24]. Furthermore, Considering the high costs of the only definitive treatment of snakebite victims (ASVs), and other adverse reactions in some patients [3,25], there is need to intensify the investigation of plant-based remedies for the treatment of snakebite. The survival rates of animals treated with the extracts showed significant variations depending on the dose and type of venom. In the case of *E. ocellatus* venom (Table 5), the extracts from *B. costatum*, *R. adenophylla*, *D. mespiliformis*, and *E. africana*, as well as the combination (COMBO), demonstrated notable survival benefits at higher doses (200 and 400 mg/kg). However, survival rates were significantly lower compared to the standard ASV control, particularly at certain doses (100 mg/kg). Conversely, in the case of *N. pallida* venom (Table 6), none of the [28]; these enzymes are abundant in snake venoms and they are responsible for the disruption of cell membranes which in turn can lead to haemolysis, alongside other proteins such as hemorrhagins and proteases [29-30]. The findings of this study revealed that, the individual extracts were able to cause stability towards the red blood cell membrane; however, the four extract combinations (COMBO) exhibited a remarkable activity by preventing rupture and/or haemolysis induced by both *N. pallida* and *E. ocellatus* venoms in a concentration-dependent extracts, nor the combination, showed any survival benefits, highlighting a lack of efficacy against this particular venom. These findings provide important insights into the varying effectiveness of the herbal extracts, warranting further investigation into their potential role in snakebite therapy. The *in vitro* detoxifying effects of four medicinal plants (*B. costatum*, *R. adenophylla*, *D. mespiliformis* and *E. africana*) and their COMBO were evaluated against the venoms of *N. pallida* and *E. ocellatus* in experimental animals. The antivenin effect of the plants and their COMBO was deduced by observing the survival rate of mice after they were injected with the pre-incubated mixture of an equivalent of the LD_{99} of each venom separately and the extracts [26]. The results indicated that *B. costatum* and *E. africana* were able to significantly ($p<0.05$) and maximally protect mice against lethality from *N. pallida* venom with 100 % survival rate at the graded doses while the COMBO exhibited 100 % survival rate against *E. ocellatus* venom; thus, this indicates that the extracts have synergistic effect which might favor the formulation of potentially potent extract. The effect observed for all the extracts were in a dose-dependent manner

Most venoms exhibit haemolytic activity which is known to induced by snake venom metalloproteases, phospholipase A_2 among other toxins [27]. Haemolysis is one of the clinical effects of snake bite which is directly associated to the action of PLA_2 enzyme on red blood cells membrane

manner. Individually, *E. africana* and *D. mespiliformis* demonstrated strong inhibition of haemolysis against both venoms.

Fibrinolysis refers to the process by which the snake venom enzymes break down fibrin, which is a protein involved in blood clothing [31]. In the quest for developing effective treatments for snakebites, it is important to understand fibrinolysis, because most venoms contain different components that disrupt the normal clothing process which in turn can lead to

excessive bleeding or cloth formation [32-33]. The ability of the individual extracts and COMBO to inhibit the process of fibrinolysis or blood clot degradation induced by the venoms was also assessed and the findings reports that all the extracts and their COMBO effectively inhibited venom-induced fibrinolysis maximally in a concentration-dependent manner. In this context, the efficacy of the individual plants and their COMBO revealed significant fibrinolytic effect against *E. oceallatus* and *N. pallida* venoms. *B. costatum* and *R. adenophylla* showed a relatively consistent fibrinolytic activity against both venoms, however, *R. adenophylla* showed a significant drop at lower concentration (0.063 mg/mL). *D. mespiliformis* demonstrated a drastic decrease in fibrinolytic activity as the concentration of the extract decreases indicating a negative value at 0.063 mg/mL; *E. africana* maintained a consistent activity at higher concentrations (0.5, 0.25 and 0.125 mg/mL), but a notable drop at lower concentration was observed. Overall, the COMBO exhibited effective fibrinolytic activity against both venoms with aa slightly moderate decrease at the lowest concentration (0.063 mg/mL); suggesting a potential synergistic effect that may enhance the overall fibrinolytic effect.

Phospholipase A_2 (PLA_2) enzyme is majorly the most toxic component of snake venoms which assist in catalyzing the hydrolysis of glycerophospholipids at the sn-2 position of the glycerol backbone there by freeing lysophospholipids and fatty acids pro-inflammatory mediators which can lead to a number of pharmacological effects such as neurotoxicity, myotoxicity, bleeding, oedema, cytotoxicity, and cardiotoxicity among others [30,34]. The PLA_2 inhibitory potential of the individual plants was lower compared to the COMBO, which indicated a remarkable inhibition of the enzyme from both snake venoms. Though the effect slightly lower compared to the standard antisnake venom. The ability of an extract and/or substance to neutralize the lethal actions of this enzyme can be said to have antisnake venom activity [3].

The components of the plant extract(s) responsible for the antivenin effect observed in this study have not been identified; but the qualitative phytochemical screening of the extracts revealed the presence of different secondary metabolites that were identified to possess antivenin effect [3,35]. Although the mechanism through which the extracts act is unclear, we presume that the various constituents in the mixture may act in synergy to elicit the antivenin effect thereby neutralizing the lethal actions of the venoms and other associated snakebite envenomation symptoms [36]. In addition, they might act via different mechanisms such as enzyme inhibition, antioxidant effects, modulation of fibrinolytic system, anti-inflammatory effects, metal-ion chelation, possible synergistic interactions of their bioactive compounds among others [36-38].

These plant metabolites constituents detected in the plants understudied contained structurally diverse chemical compounds that have proven to be effective in neutralizing the lethal effects of most poisonous snake venoms and they have been linked to most pharmacological actions of medicinal plants [3,39-40]. More so, plant extracts have been thought to act by inhibiting the toxic effects of enzymes such as phospholipase A_2 among others [41-42]; free radical formation system has been postulated by Alam and Gomes [37] to be one of the mechanisms through which plant extracts elicit their antivenom activity.

Conclusion

The four selected medicinal plants (*Rogeria adenophylla*, *Bombax costatum*, *Diospyros mespiliformis*, and *Entada africana*) exhibited slightly toxic LD_{50} values and demonstrated potent in vitro antivenom activity against the venoms of *Naja pallida* and *Echis ocellatus* at graded doses. In vivo results further supported these findings, with the combination of the four plant extracts (COMBO) providing significant protection to mice against *E. ocellatus* venom at higher doses, although survival rates were still lower compared to the standard ASV.

However, none of the extracts showed efficacy against *N. pallida* venom in vivo. These results suggest that the extracts, particularly in combination, exhibit a synergistic effect, which could contribute to the formulation of a potentially potent therapeutic product. This research provides a solid foundation for future studies aimed at developing a herbal product with antivenin activity for clinical use.

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Authors contributions

Musa Ismail Abdullahi: Conceptualization, supervision, project administration, funding acquisition, writing—review and editing. **Amina Jega Yusuf**: Conceptualization, methodology, investigation, data curation, formal analysis, writing—original draft preparation. **Nasir Ibrahim and Aliyu Muhammad Musa**: Validation, resources, visualization, writing—review and editing. **Aminu Ahmed Biambo**: Software, formal analysis, data curation, writing—review and editing. **Abubakar Alhaji Muhammad and Mustapha Salihu**: Methodology, investigation, formal analysis, writing—review and editing.

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Conflict of interests

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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