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Research Article

ANTIVENIN STUDIES OF LEAF EXTRACTS OF S. VIROSA FROM ZARIA, NOTHERN NIGERIA

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ABSTRACT

Background: Snakebite is becoming a troublesome disease in rural communities of developing countries. The traditional management of snakebite envenomation includes the use of plants administered orally of topically. Securinega virosa is one of the great African medicinal plants of which all parts are used as medicine for diseases with snake envenomation inclusive. **Objective:** To provide the bases for the use of leaf extracts of Securinega virosa in traditional medicine to treat snake envenomation. **Methods:** Swiss Albino mice of both sexes where grouped into five groups of four mice each. Pre-incubated 0.2ml at 37oC of normal saline, LD99 of Naja nigricollis snake venom alone and LD99 of its' mixture with three various concentrations of the extract were given intra peritoneally to group 1,2,3,4 and 5.respectively. The outcome of the experiment was analysed using one way analysis of variance. The methanol, ethylacetate and n-hexane extracts of Securinega virosa leaf was tested. **Results:** N-Hexane extract produced the best protection against lethal dose of Naja nigricollis venom which is significant at 20mg/kg at p-value < 0.05. **Conclusion:** Securinega virosa leaf extract possessed antivenin potential activity which supports the use of the plant in the traditional management of snake envenomation.

Key words: traditional medicine, medicinal plant, antivenin, snake envenomation.

INTRODUCTION

Major snakebite envenomings and fatalities occur in tropical regions such as; South Asia, Southeast Asia and Sub-Saharan Africa. Snakebite is a common medical emergency in developing countries [1]. The burden of envenomings in Sub Saharan Africa per year was estimated at over 314,000, of which 95% occurred in rural areas, fatalities of up to 7,300 and amputations in the range of approximately 6,000 to 15,000. The burden is believed to be underestimated since the values represent mostly the number of patients that attends conventional health facilities. It also reflects the treatment seeking approach of which more than 70% of the victims seek traditional care first [2]. And, only 8.5% of them in Nigeria attended hospitals and most vulnerable: farmers and Cattle rearers depend on traditional healers. [3-5] Several literatures have stated the unacceptable and underestimated level of snakebite burden in Nigeria.[4,6] The only validated anti-dote to snake envenomation is snake antivenom, which is linked to difficulties of accessibility and possible run out of the most active Fav Afrique anti-venom stockpile by June 2016 [7]. But there are several mentioned of traditional plants used by locals for the management of snakebite mostly in rural settings [8-10] Plants from the family Euphorbiaceae such as; *Drypetes assamica. Emblica officinalis. Euphorbia hirta* [11] were mentioned to have been used traditionally to manage snake bite. The present study intends to validate the traditional claim for the use of *Securinega virosa* in the traditional management of snakebite by carrying out some aspects of standardization and providing scientific basis for the use through the application of scientific methods.

MATERIALS AND METHODS

Collection and identification of Plant material

The fresh leaves of *Securinega virosa* used in these studies were collected on the month of February, 2016 from a wild field in Kakiyayi town, Zaria local Government area of Kaduna State, Nigeria.

The plant was identified in the Herbarium unit of the Department of Botany, Ahmadu Bello University Zaria. Voucher number was obtained (No 2520) and was deposited for future reference.

Extraction of plant material

The fresh leaves were washed with water, dried under shade, size reduced and sieved to obtain the powdered leaves. Leaves powder (300g) of *S. virosa* was extracted in continuous extraction process successively in 1 litre each of hexane, ethyl acetate and methanol using soxhlet extraction apparatus at moderate tempreture (55^{o}c) until no significant change in colour of solvent was observed for each solvent. The extracts were concentrated under reduced pressure and stored in a desicator for further experiments. The yields for n-hexane, ethylacetate and methanol extracts were 2.67g, 3.74g and15.55g respectively. Thin Layer Chromatography (TLC) phytochemical screening of the extracts shows that Hexane extract is rich in alkaloids, phenols, saponins and triterpenes/steroids, the Ethylacetate extract in phenols, saponins and triterpenes/steroids while the Methanol in alkaloids, phenols, saponins and triterpenes/steroids. [12] Solution of methanol extract and suspensions of the n-hexane and ethylacetate extracts in 2% Tween 80 were freshly prepared for this work.

Animals

A total of apparently 20 Swiss Albino mice of both sex and weights 15-30g were purchase from animal house Department of Pharmacology and therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria. Approval from ethical committee of the university was sorted out before the animal experiment. Mice were acclimatized to the environment for 7 days, feeding on normal chow pellet (from Vital Feed), and water given ad libitum and ambient temperature maintained at normal. They were divided into four groups of four mice each for the *in vivo* venom neutralization studies.

Source of venom and preparation

The venom was obtained from *Naija nigracolis* through milking by a professional snake charmer at Ahmadu Bello University Veterinary Teaching Hospital Zaria. The snake was held captive by the charmer, mouth opened and fangs placed on the edge of a glass container covered with polythene. The milking was enhanced by the pressing on and off of the snake tail. The venom drops gradually at intervals into the container. [13]

Experimental procedure

Acute toxicity study

Nine (9) mice were divided into 3 groups of 3 mice each. Mice in groups 1, 2 and 3 were given 1000, 100 and 10mg/kg of extract of *S. virosa* intra peritoneally (IP) each respectively. They were observed for signs of toxicity in addition to mortality rate over a 24 hours period. While in the second phase four mice labeled 1, 2, 3 and 4 were given IP variable doses of extract of *S. virosa* each based on the result of the first phase respectively. Then, the LD_{50} was calculated as the square root of minimum lethal dose multiplied by maximum tolerated dose. [14] The whole process was conducted each for n-hexane and ethylacetate extracts of *S. virosa*. But for methanol extract of S. virosa, the LD_{50} of 1265mg/kg was adopted. [15-17]

Assessment of in vivo venom neutralization by leaf extracts of *S. virosa*

Twenty (20) mice were divided into five groups of four mice each groups. First group was given normal saline 0.2ml IP. Group 1 received 0.2ml of LD_{99} alone (control group) while groups 1, 2 and 3 (treatment groups) were given a mixture of an equivalent of LD_{99} containing 5, 10 and 20mg/ml of extract incubated at 37^{o}C for 10min to each mice in a group respectively. All the doses were given through intra-peritonial (IP) route. A sign of neurotoxicity and number of death per group was recorded over 24 hours after the injection. Also, the LD_{99} of 9.55mg/kg was adopted. [18] The result was presented as percentage death and mean time of death ± standard error of mean (SEM). The procedure was conducted for n-hexane, ethylacetate and methanol extracts of *S. virosa* leaf respectively.

Histopathology

The brain, heart, lungs, kidney and liver tissues were collected from the animal and placed immediately into the fixative (10% normal saline).after proper fixing for about 48 hours, the tissues were dehydrated through ascending grades of alcohol from 70% alcohol to 90 % alcohol and absolute (100%) alcohol for 16 hours. The tissues were then cleared in toluene for 2 hours after which they were impregnated in molten paraffin hours for four hours. The tissues were then embedded in paraffin wax, sectioned using a rotary microtome at 5 micron thickness, the sections were then stained using the heamatoxylin and eosin staining technique. The stained sections were examined using a light microscope and relevant photomicrographs were taken using a digital camera for microscope.

Ethical approval by the Ahmadu Bello University ethical committee was soughed out prior to the commencement of the experiment.

Statistical analysis

The results were analysed using one way analysis of variance (i.e. one-way ANOVA). A P-value of < 0.05 was considered significant. This was followed by post-hoc test using dunnett with the aid of Statistical package of social sciences (spss) version twenty.

RESULTS AND DISCUSSION

Results

Toxicity Study

The LD_{50} of N-hexane extract of *S. virosa* was found to be 774.60mg/kg while that of Ethylacetate extract it is practically non-toxic in mice at the dose of 5000mg/kg IP.

In vivo* venom neutralization by leaf extracts of *S. virosa

Figure 1: Shows the effect of the leaf extract on retarding the lethal effect of venom of *Naja nigricollis*. It is found that the N-hexane extract offered better protection significantly, followed by Methanol extract. The tables (1-4) under shows that N-hexane and Methanol extract effects are statistically significant at 20mg/kg dose in a mixture with half of LD_{99} of the venom given I.P. to mice.

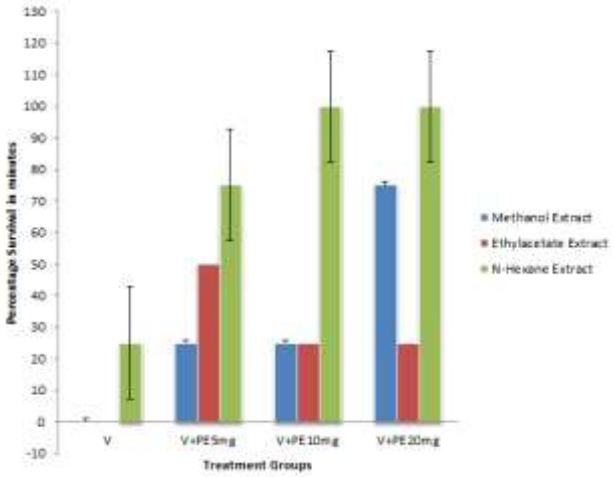


Fig. 1: Percentage survival of the leaf extracts of *S. virosa.*

Table 1: *In vivo* response to administration (I.P.) of incubated venom alone and venom in combination with *S. virosa* methanol extract in mice

Treatment	No of death/no of mice per group	survival %/2hours	Survival %/24hours
LD99	0/4	0	0
LD99 + M5mg	1/4	25	0
LD99 + M10mg	1/4	25	0
LD99+ M20mg	3/4	75*	0

* **= significant at p < 0.05**

Table 2: *In vivo* response to administration (I.P.) of incubated venom alone and venom in combination with *S. virosa* Ethylacetate extract in mice

Treatment	No of death/no of mice per group	survival %/2hours	Survival %/24hours
LD99	0/4	0	0
LD99 + E5mg	2/4	25	0
LD99 + E10mg	1/4	25	0
LD99+ E20mg	1/4	25	25

* = significant at p < 0.05

Table 3: *In vivo* response to administration (I.P.) of incubated venom alone and venom in combination with *S. virosa* N-Hexane extract in mice

Treatment	No of death/no of mice per group	survival %/2hours	Survival %/24hours
LD99	1/4	25	25
LD99+E5mg	3/4	75	0
LD99+E10mg	4/4	100	25
LD99+E20mg	4/4	100*	50

* **= significant at p < 0.05**

Table 4: Survival time on administration of venom and mixture of venom with *S.virosa* extracts in mice.

Group	Group Survival time of mice on treatment with *S. virosa* extracts		
	Methanol	**Ethylacetate**	**N-Hexane**
V	54.50± 15.80	81.00±25.78	17.50±13.46
V+PE5mg	107.75±36.45	74.25±57.87	211.75±105.04
V+PE10mg	196.00±253.07	91.25±47.29	401.50±150.87
V+PE20mg	147.25±35.78*	205.50±263.80	458.50±150.87

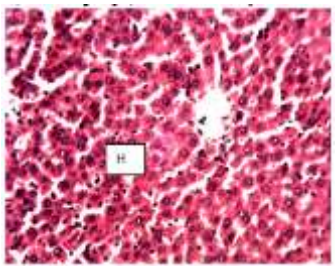
Results are presented as mean ± standard deviation, * = significant at $p < 0.05$

Histopathology

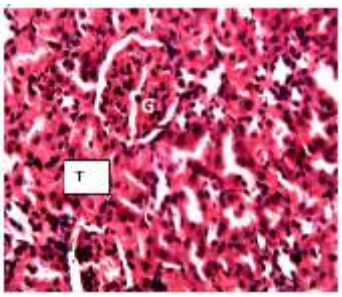
The histopathology of some organs namely; brain, heart, spleen, lungs, kidney and liver for N-hexane shows almost normal architecture of the above organs. This indicates a greater protection to the organ damage by venom of *Naja nigricollis.*

A shows normal liver, heart, spleen, kidney, lungs and brain. Examination of ''B'' shows normal heart (v) and brain (iv) tissues, but, slight lymphocyte hyperplasia (LH) and alveoli congestion (AC) were noted in kidney (ii) and lung (vi) tissues respectively. A moderate LH and vascular congestion (VC) were observed spleen (ii) and liver (i). An examination of ''D'' shows normal heart (iv). Slight vacoulation in brain (v), slight vacoulation and necrosis in the liver (i) and slight LH in spleen (iii) were observed. However, moderate alveoli congestion in the lungs (vi) was noted. A mixture of venom-n hexane *S. virosa* leaf extract treated mice ''E'' shows normal heart (vi), brain (iv) and lungs (v). Slight necrosis and LH were observed in kidney (ii) and liver (i) respectively and moderate LH in spleen (iii).

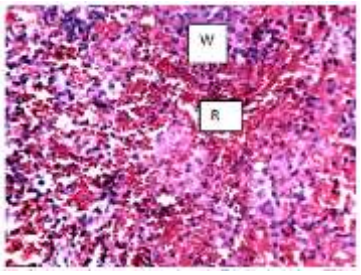
A) Control group (normal saline only treated mice)



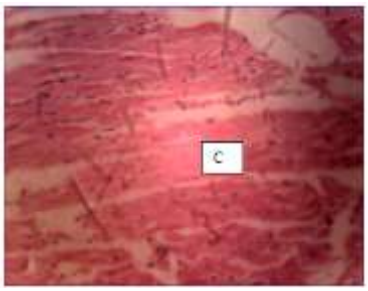
i. Liver shows normal hepatocyte (H)



ii Kidney shows normal glomerulus (G) and tubules (T).

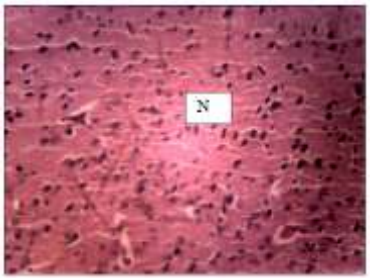


iii. Spleen shows normal red (R) and white (W)

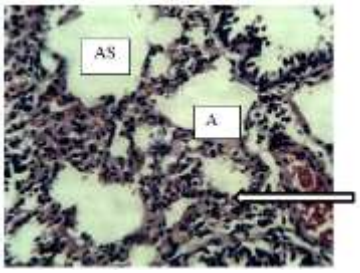


iv. Heart shows normal cardiac Tissue

Pulp tissues.

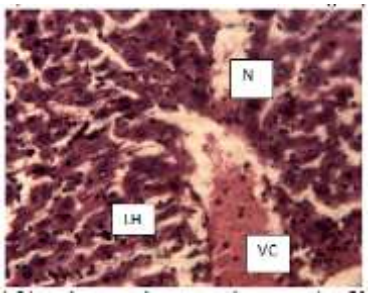


v. Brain shows normal nervous tissue(N)

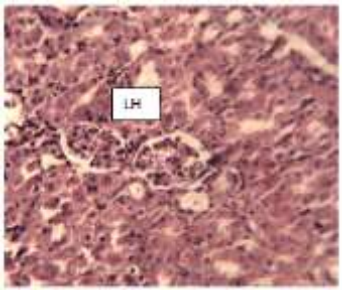


vi. Lungs show normal alveoli (A),

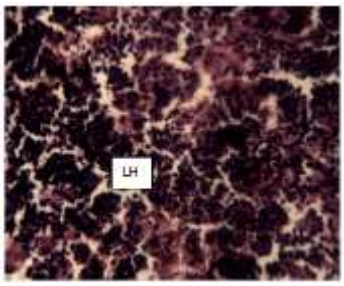
B) *S. virosa* methanol extract treated mice group



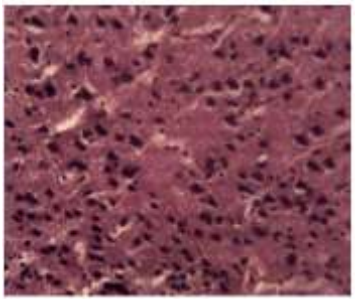
i. Liver shows moderate vascular congestion (VC) slight necrosis(N) and slight lymphocyte hyperplasia(LH).



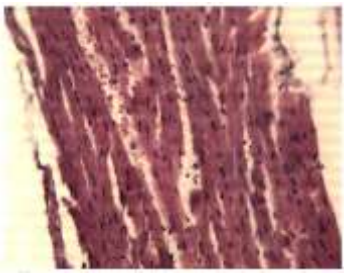
ii. Kidney shows slight lymphocyte hyperplasia (LH)



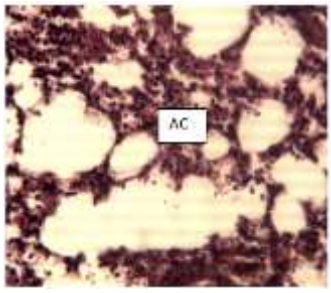
iii. Spleen shows moderate lymphocyte hyperplasia (LH)



iv. Brain shows normal nervous tissue

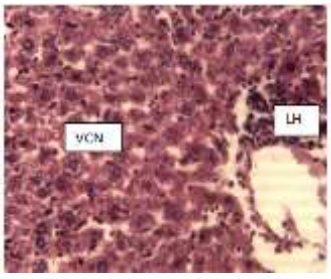


v. Heart shows normal features alveoli congestion (AC)

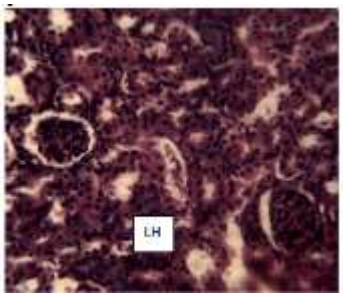


vi. Lungs show slight alveoli congestion (AC)

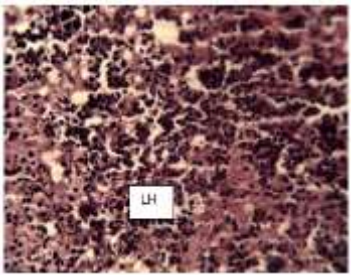
C) *S. virosa* ethylacetate extract treated mice group



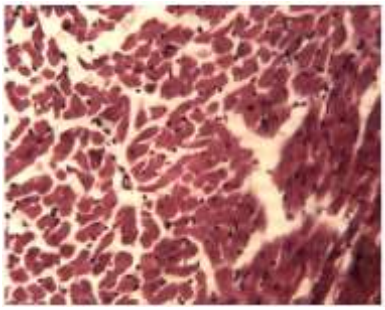
i. Liver shows slight vacoulation and necrosis (VCN) and lymphocyte hyperplasia (LH).



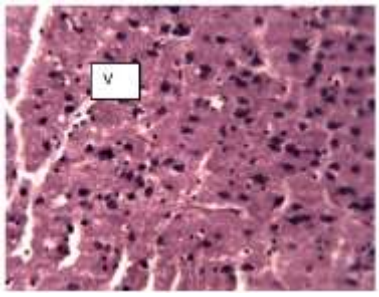
ii. Kidney shows moderate lymphocyte hyperplasia (LH).



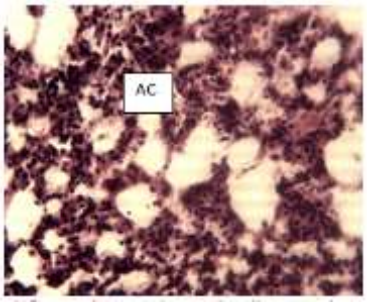
iii. Spleen shows slight lymphocyte hyperplasia



iv. Heart shows normal features (LH)

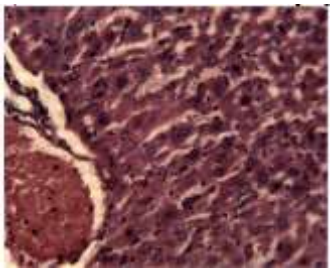


v. Brain shows slight vacoulation (V)

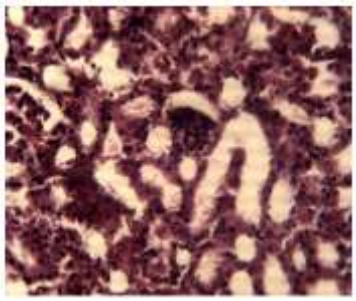


vi. Lungs show moderate alveoli congestion (AC)

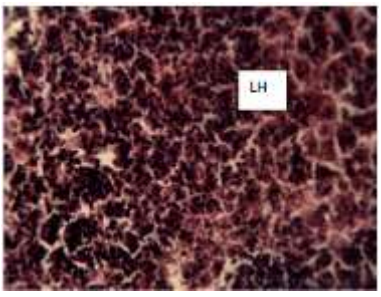
D) *S. virosa* n-hexane extract treated mice group



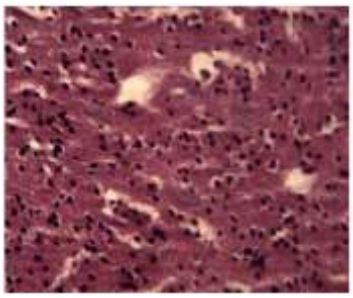
i. Liver shows vascular congestion (VC), kupfer cell hyperplasia (KH) and slight lymphocyte hyperplasia(LH)



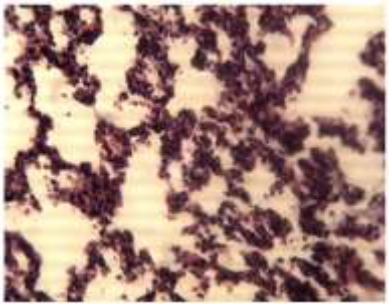
ii. Kidney shows slight tubular necrosis (TN) slight glomerular necrosis(GN) and slight lymphocyte hyperplasia (LH).



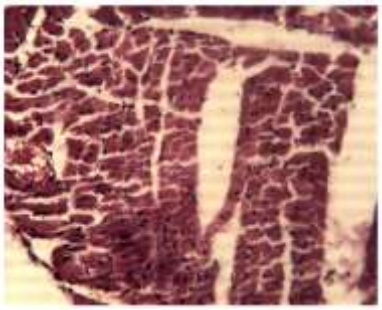
iii.Spleen shows moderate lymphocyte hyperplasia (LH)



iv. Brain shows normal feature



v. Lungs show normal feature



vi. heart shows normal feature

Plate XXXIV: effect of *S. virosa* extracts on various organs of mice (treated and control).

DISCUSSION

In order to determine the safety margin of drugs and plant products for human use, toxicological evaluation was carried out in experimental animals using Lorke's method to predict toxicity and to provide guidelines for selecting a "safe" dose in animals and also used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs. [19-21] The LD_{50} of N-hexane extract of *S. virosa* was found to be 774.60mg/kg while that of Ethylacetate extract it is practically non-toxic in mice at the dose of 5000mg/kg. This is based on the toxicity classification which states that substances with LD_{50} values of 5000 to 15,000 mg/kg body weight are practically non-toxic (Loomis and Hayes, 1996). On the other hand, the LD_{50} of methanol extract of the leaf of *S. virosa* was already determined by earlier researchers' mentioned on the same plant collected in this same study location which both determined it to be 1265mg/kg. [15-17]

Elapid such as *Naja nigricollis* envenoming is characterized by a progressive, descending neuromuscular paralysis, leading to respiratory failure and death. [22] The efficacy of antivenom against particular venom is due to the ability of antivenom molecules to bind with toxins in the venom. The most widely used method for assessing antivenom efficacy is rodent lethality testing. [23] In this study, the effect of leaf extracts of *S. virosa* on retarding the lethal effect of venom of *Naja nigricollis* was that the N-hexane extract offered better protection significantly, followed by Methanol extract at $p < 0.05$. The result also shows that N-hexane and Methanol extract effects are statistically significant at 20mg/kg dose in a mixture with half of LD_{99} of the venom given I.P. to the mice. This result slightly differ on the N-hexane extract been better protector to venom toxicity in mice but similar on the methanol extract of *C. africana* which indicate that the stem-bark crude methanol extract of *C. africana* exhibits a dose dependent in vitro detoxifying action against the crude venom of *Naja nigricollis* in a study conducted in Zaria. [24] Shekins, [25] also mentioned that there was significant difference between the times of death in the *Mucuna pruriens* Leaves extract treated group and those treated with *Naja hannah* venom only, indicating that the plant extract had effect on the activity of the venom in a study conducted in Nassarawa state. The superior activity of n-hexane extract bioactive constituents has been reported where a triterpene; Friedelin isolated from *Albizia chevalieri* shows a significant anti-venin activity of 64% protection against *Naja nigricollis* venom alone treated mice. [26] It also, shows ethylacetate extract to be the least effective antivenin similar to the earlier mentioned finding in this present study. The seeming alleviation of toxic symptoms and survival of laboratory animals (within a short time frame) after being challenged with lethal doses of venom is in good agreement with the earlier findings [26-29] where different classes of plant constituents have shown to possess *in vivo* activity against snakes venom.

The histopathology of some organs namely; brain, heart, spleen, lungs, kidney and liver for N-hexane shows almost normal architecture of the above organs. This indicates a greater protection to the organ damage by venom of *Naja nigricollis.* Studies on *Fluggea virosa* (*S. virosa* synonym) have resulted in the isolation of a C-glucoside; bergenin (one of the major active principle in the plant) which have proved to be a potent hepatoprotective agent. [30]

CONCLUSION

The findings in this work have indicated that the extract of *S. virosa* contains compounds which can neutralize *N. nigricollis* venom both in vitro and in vivo. Results obtained from this study may explain the basis for using this plant in traditional medicine to treat snake envenomation in Nigeria. It is interesting to note that a positive correlation exists between traditional use of medicinal plants and their pharmacological investigations. [11]

Acknowledgements

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Conflict of interest

There has been no any conflict of interest between authors regarding this manuscript.

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