Polyvalent Antivenom Potential of *Cnestis ferruginea* Root Extracts: Chromatographic Analysis and *In Vivo* Efficacy Against Snake Venoms

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Abstract. Snakebite is a major challenge in the world. Venoms of different snakes exert haemotoxic, neurotoxic and cytotoxic effects on the victims. Despite the successful use of serum therapy, antivenom has limitations especially in neutralizing tissue damage and being unavailable for use when needed. In this study, *Cnestis ferruginea* root extracts were investigated for antisnake venom properties against *Naja melanoleuca*, *Naja haje*, *Naja kateinsis* and *Echis ocellatus* venoms in albino rats. Concentrations used were found to have neutralized almost all toxic effects induced by the venoms. *In vivo* assessment of venom lethality (LD_{50}) for all four venoms was determined using probit analysis and found to be 0.455 and 0.465 mg/kg. The extracts neutralized the lethality of the venoms with effective dose at 50% survival (ED_{50}) found to be 1.4 mg/kg. Haematology and histopathology of liver and kidney of test rats were conducted to show the venom toxic effect and the ameliorative effect of the extract on envenomation. Phytochemical analysis of *C. ferruginea* root extract showed that it contains biochanin A, formononetin, genistein, anethole, myristicin, quercetin and chlorogenic acid which have antivenom properties. The root extracts have inhibitory action against hemorrhagic, inflammatory, and myotoxic activities of the venoms; and can be considered as polyvalent antivenom.

Keywords: Polyvalent, Antivenom, *Naja* spp., *Echis ocellatus*, *Cnestis ferruginea*, Snake bite

INTRODUCTION

Snakebites are injuries caused through bites from snakes, particularly venomous snakes, as a result of accidental provocation and are regarded as "neglected public health issue" worldwide. This prompted the World Health Organization, in 2017, to add snakebite envenoming to its list of highest priority neglected tropical diseases (NTDs).[1] World Health Organization estimated that over five million people are bitten annually, and approximately 45% develop one or more clinical illnesses. Additionally, the death count of such clinical illness ranges between 80,000 and 130,000 people annually.[2] Physiological process of snake envenoming is complex and includes the combined action of several toxins such as snake venom metalloproteinases (SVMPs), snake venom

serine proteinases (SVSPs), phospholipases A2 (PLA2), hyaluronidases, bradykinin-releasing enzymes, lectins, L-amino oxidases, and pharmacological mediators.[3]

In Nigeria, a survey estimated that snakebites occur at 497 cases per 100,000 people; and out of this figure, 12% are killed while some have one arm or leg amputated to save their lives. Although two families of venomous snakes (Viperidae and Elapidae) have been reported to be associated with envenoming in Nigeria, the survey showed that the West African carpet viper (*Echis ocellatus*) is the species responsible for most bites (90%) and 66% of deaths.[4]

Echis cellatus are characterized by their bulging eyes and short snouts, a characteristic of the *Echis genus*. Its record length is between 30-50 cm.[5] Its venom contains procoagulants, anticoagulants, haemorrhagins, nephrotoxins and necrotoxins. Its envenoming rate is estimated to be 80% and lethality not less than 20%.[6] The vipers are a family of snakes found in various parts of the world except Antarctica, Australia, Hawaii, Madagascar, and some other isolated islands. They are oviparous but most are ovoviviparous. Their venom contains proteases (protein-degrading enzymes) that cause pain, disruption of the blood-clothing system, swelling and blood loss.

Naja melanoleuca, *Naja haje* and *Naja katiensis* are of the Elapid family, commonly known as cobras. They are oviparous, venomous, and characterized by their permanently erect fangs. Their venoms are mainly neurotoxic containing phospholipases A2 and three-finger toxins.[5] Venom of *Naja melanoleuca* has been reported to have fifty-two different proteins, with three-finger toxins being the most abundant (57.1 wt.%) and 12.9 wt.% of phospholipases A2.[7]

Antivenom serum therapy administered intravenously has been the only specific treatment for snakebites. This antivenom, however, has some limitations such as excessive cost of purchase and unavailability, difficulty in storage, hypersensitivity reactions, exact dosage and close expiration which regulates its usage.[8] Administration of the antivenom may prevent death but does not prevent local tissue damage and resultant disabilities. Due to the delay in receiving antivenom serum or its low efficacy, there is low inhibition of local effects which is the leading cause of amputations, that can lead to serious social, economic, and health negative impacts, given that most victims live in rural areas.[9] Due to these challenges, the search for new complementary therapies to treat snakebites gained importance. The use of medicinal plants for the treatment of envenomation is a longstanding practice, particularly in rural communities of Africa, Asia, and South America.[9] *Cnestis ferruginea* is among the medicinal plants employed locally for the management of snakebites in the South-South region of Nigeria. This study was designed to assess the effectiveness of *Cnestis ferruginea* root as an inhibitor of snake venom toxins, aiming to validate or challenge its traditional use in snakebite management.

Cnestis ferruginea belonging to Connaraceae family is a secondary shrub with densely and rusty brown branches, with pear-shaped orange-red fruits. It is native to Africa and known to possess various antimicrobial properties.[10] This plant is used in traditional medicine for a variety of purposes; as an aphrodisiac, enema for gynecological disorders, dysentery, urethral discharge and in the management of psychiatric disorders.[11] It is used against bronchitis; as laxative taken by the Yorubas in decoction. In Côte d'Ivoire, in cases of migraine & sinusitis (root); and conjunctivitis (fruit juice). For treatment of pyorrhea (powdered bark); against headache, as red dye by the Mende of Sierra Leone for dyeing clothes. It is used against toothache (rootbark paste) by Igbos of South-eastern Nigeria and Ghana. The stem is used by the Igbos in Southeastern Nigeria to make bows.[12]

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This research explored the neutralizing activity of *Cnestis ferruginea* root extracts against cobras (*Naja melanoleuca, Naja haje and Naja* katiensis) and viper (*Echis ocellatus*) venoms using *in vivo* methods and antivenom compounds were identified using thin layer chromatography.

MATERIALS AND METHODS

Collection and Authentication of Plant Material

Fresh roots of *Cnestis ferruginea* were obtained by 10:30 hours on the 25th of May 2021 from Ipo forest in Ikwerre Local Government Area, Rivers State, Nigeria. The roots were identified and authenticated by Dr. M. G. Ajuru at the herbarium in the Department of Plant Science and Biotechnology, Rivers State University, with voucher number RSU Pb094. The Voucher specimen was deposited at the herbarium.

Preparation of Cnestis Ferruginea Root Extracts

The roots were rinsed of debris, cut into smaller sizes, air-dried, covered with muslin cloth to prevent dust from resting on them and left for two weeks at room temperature (27 °C) until completely dehydrated (air-dried). The dried roots were ground into powder to create a larger surface area for maceration and subjected to sequential extraction using dichloromethane (DCM) and ethanol for 48 hours each. Each extract was decanted, filtered, and concentrated using a rotary evaporator at 40 °C to give the crude extracts. Distilled water was used to prepare the concentrations of extracts for administration.

Snake Venoms

Naja melanoleuca, Naja haje, *Naja katensis* and *Echis ocellatus* venoms were milked from each adult snake at 9:30 hours of 20th June 2021, with the help of Mr. S. Abdullahi, a snake charmer at Bioresources Development Centre (BIODEC), Odi, Bayelsa State, Nigeria, according to the method of Goswani *et al*.[13] All four venoms were colourless except for *Echis ocellatus* that was golden-yellow. Stock solution (3.2 mg/kg) was prepared by dissolving each of the venoms in normal saline and serially diluted to 0.1, 0.2, 0.4 and 1.6 mg/kg. The venoms were preserved in a refrigerator (at 4 °C).

Experimental Animals

Albino rats weighing between 170 - 200 g were used for this experiment. They were purchased from the animal house of the Department of Pharmacy, University of Port Harcourt, Rivers State, Nigeria and housed in plastic cages of 40 x 25 x 20 cm dimensions under standard conditions. They were provided with grower pelletized feed and clean water; and acclimatized for two weeks prior to the experiment.

Acute Toxicity Check

The method of OECD/OCDE 425 was adopted. This method is called “an up-and-down procedure for acute toxicity testing”.[14] Twenty-one rats were used; six groups of three rats each were orally administered with 5000, 4000 and 2000 mg/kg body weight doses of DCM and ethanol extracts, respectively, while the seventh group, which served as the control, was given an equivalent volume of distilled water to serve as control. The rats were fasted overnight before administration and were observed for 24 hours after administration for toxic signs like excitability, dullness, diarrhea, oedema, and death.

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Venom Median Lethal Dose Determination

The LD_{50} (lethal dose at 50% death) of the venoms were determined using the method of Theakston and Reid.[15] Sixty-three rats weighing between 170 - 200 g each were randomly distributed into twenty-one groups of three rats each. Group one (positive control) was given food and water only. Groups 2-6, 7-11, 12-16 and 17-21 were administered with varying concentrations (3.2, 1.6, 0.4, 0.2 and 0.1 mg/kg) of *Naja melanoleuca, Naja haje*, *Naja katensis* and *Echis ocellatus,* respectively. Mortality was recorded within 24 hours of venom administration and the LD_{50} estimated using probit analysis. Some of the rats were dissected, organs and blood samples were collected and preserved for analysis.

Antivenom Assay

Experimental rats were divided into eleven groups of three rats each. Groups 1-11 were injected with 0.1 ml LD_{50} of *N. melanoleuca* venom (0.455 mg/ml). While group one served as the negative control (envenomed group), groups 2-6 were intravenously injected with 400, 200, 100, 50, 25 mg/kg DCM extract, while 7-11 were injected with 400, 200, 100, 50, 25 mg/kg of the ethanol extract. The same analysis was conducted but with oral administration of extracts because of the outcome of the intravenous treatment. The rats fasted overnight prior to envenomation and treatment; observation was done for 24 hours and results were recorded.

Subsequently, extracts were further serially diluted to 12.5, 6.25 and 3.125 mg/kg. A new set of rats were divided into eight groups of three rats each. Group one served as the positive control (food and water only); groups 2-8 were injected with 0.1 ml LD_{50} (0.455 mg/ml) of *N. melanoleuca* venom. Group 2 served as the negative control (envenomed group). Groups 3-5 were orally administered/treated with 12.5, 6.25 and 3.125 mg/kg DCM extract, while groups 6-8 were orally administered with 12.5, 6.25 and 3.125 mg/kg of ethanol extract, respectively. The lowest concentration of *C. ferruginea* extract with zero death (25 mg/kg) was taken as the ED_{100} (effective dose at 100% survival).

Extended work was done to include the other three venoms using their various LD_{50} (0.455 mg/ml and 0.465 mg/ml) and ED_{100} of *C. ferruginea* extract (25 mg/kg). A separate set of rats were divided into thirteen groups of three rats each. Groups 1-3 were injected with 0.1 ml LD_{50} (0.455 mg/ml) of *N. melanoleuca* venom, while groups 4-6, 7-9 and 10-12 were injected with 0.1 ml LD_{50} of *N. haje*, *N. katensis* and *E. ocellatus* venoms, respectively. Groups 1, 4, 7, and 10 served as negative control (envenomed group). Groups 2, 5, 8 and 11 were orally administered (treated) with 25 mg/kg DCM extract; while groups 3, 6, 9 and 12 were orally administered with 25 mg/kg ethanol extract. Group 13 was given food and water only (positive control). They were observed for 24 hours and results were recorded. Some of the rats were dissected, organs and blood samples (by jugular puncture) were collected and preserved for analysis.

Effective dose at 50% survival (ED_{50}), according to Spearman & Karber method, was calculated using Equation 1.[16]

$$ED_{50} = \log X_{100} - \log \frac{FD}{n} \left(\sum t - n/2\right) \quad (1)$$

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Where: ED_{50} = the 50% effective dose; log X_{100} = log dose giving 100% survival (25 mg/kg); log FD = the log dilution factor (2); n = number of rats used at each dose level (3); Σ = the sum of rats surviving at every dose level; t = number of rats alive at each dose level (14)

$ED_{50} = \log 25 - \log\frac{2}{3}\sum(14 - 3/2)$

$ED_{50}=1.2979 – 0.1003\ (12.5) = 0.144$

$ED_{50} = 10^{0.144}$ (Antilog) = 1.393 ≈ 1.4 mg/kg

In order to assay the calculated ED_{50}, a new set of rats were divided into twelve groups of four rats each. Groups 1 - 4 served as the negative control (envenomed groups). Two groups each were consecutively injected with LD_{50} of *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus* venoms. One group each out of the two injected with each of the venoms were orally administered with 1.4 mg/kg (ED_{50}) of DCM extract, while the others were orally administered with 1.4 mg/kg of ethanol extract. They were observed for 24 hours and results were recorded. Some of the rats were dissected, organs and blood samples were collected and preserved for analysis.

Statistical Analysis

LD_{50} of venoms were determined using probit analysis. Data generated from haematology of test rats were presented as mean±SD and subjected to one-way analysis of variance (ANOVA). Statistical difference between the means were compared using Student's T-test with the aid of a statistical package (IBM SPSS Statistics 20).

Thin Layer Chromatography and Phytochemical Analysis

Freshly prepared extracts were subjected to standard phytochemical analysis for various constituents such as isoflavone, phenylpropenes, glycosides, acidic compounds, anthraquinones, steroids, terpenoids.[17, 18] Thin layer chromatography of the root extracts was conducted using standard methods.[19, 20]

RESULTS

Extract Yield

DCM and ethanol extracts of *Cnestis ferruginea* yielded 16.60 g (0.33%) and 40.46 g (0.79%), respectively. DCM extract was greenish-brown sticky residue while the ethanol extract was reddish-rusty brown, like the colour of the fresh root. Both extracts had a fruity sweet aroma.

Toxicity of Extracts

Toxicity assay of DCM and ethanol extracts administered intravenously gave over 30% death rate for DCM and ethanol extracts (Figure 1). No death was recorded for oral administration except for DCM extract with 30% death rate for 5000 mg/kg body weight of rat (Figure 2) and no death for controls (administered food and water only). Both extracts significantly increased movement and smartness in animals (after oral administration) till the end of the study.

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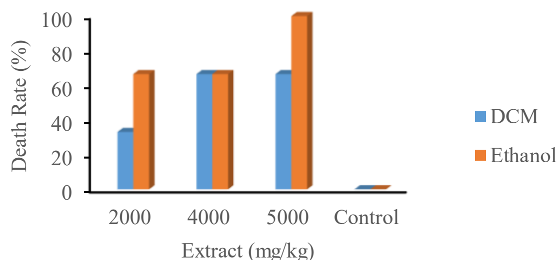

Figure 1. Dose response for acute toxicity of extracts showing death rates for DCM and ethanol extracts administered intravenously

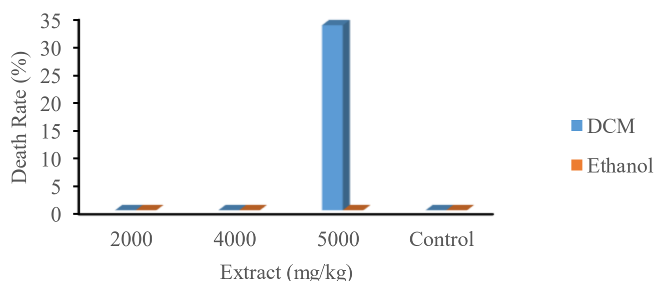

Figure 2. Dose response for acute toxicity of extracts showing death rates for DCM and ethanol extracts administered orally

Lethality and LD_{50} of Venoms

After 24 hours post envenomation, the LD_{50} for *N. melanoleuca* and *N. katiensis* was estimated to be 0.455 mg/kg while that of *N. haje* and *E. ocellatus* was 0.465mg/kg. Toxic signs noted in the rats when injected with the venom were respiratory problems with sneezing, stiffening of neck, weakness and then death within four-hour interval. Figure 3 shows death rate for each venom and control group (administered with food and water only).

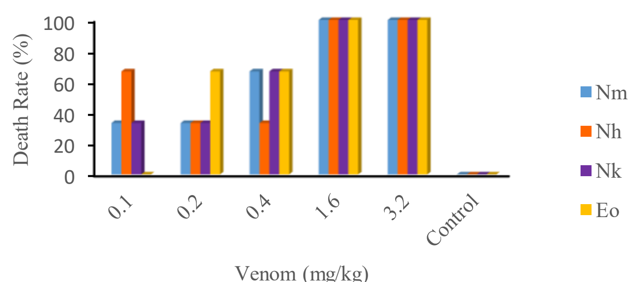

Figure 3. Lethality rate of venoms showing death rates for intravenous injection of *N. melanoleuca* (Nm), *N. haje* (Nh), *N. katiensis* (Nk) and *E. ocellatus* (Eo) venoms

Antivenom Activities of Cnestis ferruginea Extracts

Treatment for *N. melanoleuca* envenomation through intravenous injection of 25, 50, 100, 200 and 400 mg/kg DCM and ethanol extracts resulted to high death rate amongst the test group of organisms (Figure 4) but reduced mortality for oral administration (Figure 5). Death was first recorded for groups treated with ethanol extract, then for DCM extract after several hours. The least concentration that gave zero death for both extracts (oral administration) was 25 mg/kg; this was considered as ED_{100} (Figure 5). Oral administration of ED_{100} (25 mg/kg) to groups envenomed by all four venoms, resulted in the reduction of death rate to less than 30% except for groups

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injected with *N. katiensis* venom (Figure 6). ED_{50} (1.4 mg/kg), when administered orally to envenomed animals, gave less than 40% death rate except for the group envenomed by *N. haje* (Figure 7). All positive control groups recorded zero death and all negative control groups recorded 100% death.

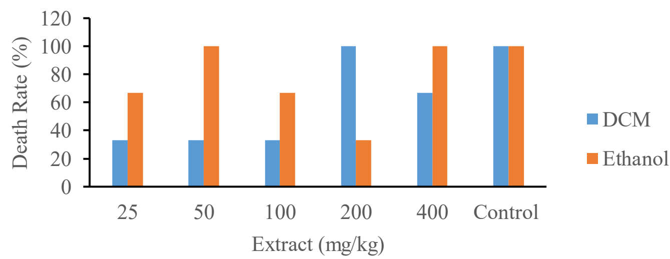

Figure 4. Dose-dependent response to intravenous treatment of *N. melanoleuca* envenomed rats with DCM and ethanol extracts at different concentrations

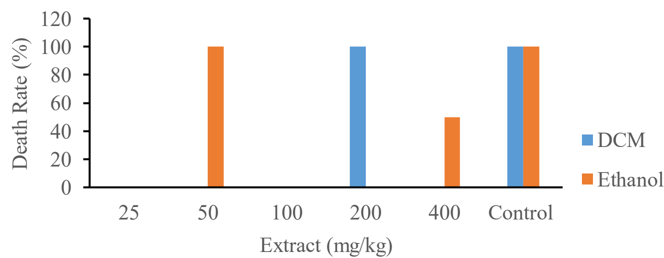

Figure 5. Dose-dependent response to oral treatment of *N. melanoleuca* envenomed rats with DCM and ethanol extracts at different concentrations

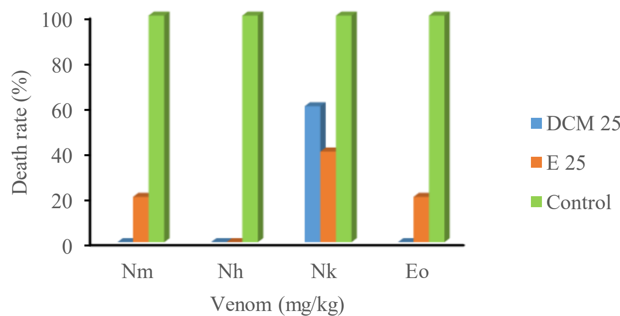

Figure 6. Treatment response of envenomed (by *N. melanoleuca* (Nm), *N. haje* (Nh), *N. katiensis* (Nk) and *E. ocellatus* (Eo) venoms) rats to ED_{100} of DCM (DCM 25) and ethanol (E 25) extracts

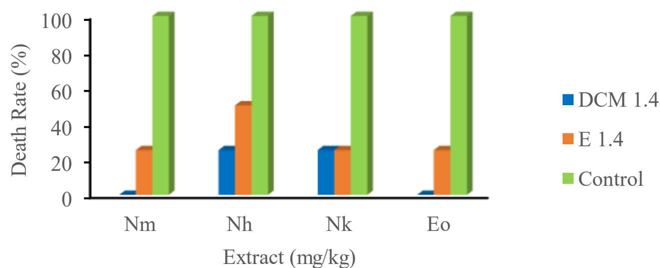

Figure 7. Treatment response of envenomed (by *N. melanoleuca* (Nm), *N. haje* (Nh),

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N. katiensis (Nk) and *E. ocellatus* (Eo) venoms) rats to ED_{50} of DCM (DCM 1.4) and ethanol (E 1.4) extracts

Haematological Profile of Test Animals

Table 1 shows the results of haematological analyses of collected blood samples. The results show variations in parameters for positive control, negative controls and groups treated with DCM and ethanol extracts after envenomation. AST, T.B and ALB for the normal group (NØ) and treated groups (C1, C2, G3) were within limit compared to the envenomed groups (Nm, Nh, Nk and Eo). ALT and ALP values showed no significant differences between normal, treated, and envenomed groups except for ALT in Eo group. The electrolyte panel test (K^+, Cl^- and Na^+) showed significant differences ($p<0.005$) between the normal and treated groups compared to envenomed groups (Nm, Nh, Nk and Eo). Urea (Ur), PCV and RBC were higher in control (NØ) and treated groups (C1, C2, G3) than in envenomed groups (Nm, Nh, Nk and Eo). White blood cells (WBC) were lower in normal (NØ) and treated groups (C1, C2, G3) than in envenomed groups (Nm, Nh, Nk and Eo).

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Table 1. Haematological profile of control group (NØ), envenomed groups (Nk, Nm, Nh, and Eo), and extract treated groups (C1, C2 and G3)

Sample ID	AST (U/L)	ALT (U/L)	ALP (U/L)	T.B (µmol/l)	ALB (g/l)	K^+ (mmol/l)	Na^+ (mmol/l)	Cl^- (mmol/l)	PCV (%)	RBC ($x10^{12}$/ l)	WBC ($x10^9$/ l)	Ur (mmol/l)
Limit	0-35	3-15	44-147	1.71-20	35-53	3.6-5.2	135-145	96-106	40-53	4.2-5.4	4.5-11	2.1-8.5
NØ	26±1.73	6.6±0.36	91±4.04	5.7±0.46	38±1.00	3.5±0.12	136±1.73	63±7.21	49±2.31	4.10±0.62	7.1±0.62	4.6±0.32
C1	25±1.73	6.1±0.15	80±2.08	5.5±0.56	38±0.58	3.1±0.35	146±2.65	63±1.73	45±2.65	4.9±0.26	7.4±0.55	3.7±0.26
C2	29±6.03	6.8±0.30	110±2.65	6.3±0.44	35±1.73	3.2±0.26	140±4.36	69±2.65	43±3.61	5.5±0.36	7.7±0.36	3.5±0.52
G3	23±4.36	7.5±0.36	98±4.58	4.9±0.31	38±1.00	3.5±0.10	137±2.65	62±3.61	46±2.08	5.9±0.44	7.8±0.26	3.3±0.36
Nk	39±3.61	9.1±0.30	85±3.61	14.2±0.36	32±1.53	2.5±0.46	115±3.61	65±3.61	34±2.65	3.9±0.36	11.6±0.62	6.5±0.17
Nm	40±2.65	9.3±0.26	92±4.93	14.5±0.52	32±2.65	2.8±0.36	112±3,61	81±3.51	38±2.08	3.7±0.42	11.9±0.46	6.2±0.40
Nh	38±1.53	10.8±0.17	100±8.62	14.3±0.46	33±1.15	2.7±0.17	129±1.73	72±4.73	34±2.65	3.7±0.17	12.5±0.26	8.3±0.26
Eo	49±5.29	16.8±0.30	83±4.73	18.5±0.44	30±2.65	2.2±0.56	110±0.58	87.7±2.52	28±2.65	3.4±0.26	13.3±0.44	8.8±0.10

Mean ± standard deviation differs from each other with 95% confidence interval. NØ = positive/ control group; C1 = *Echis ocellatus* venom + 1.4 mg/kg DCM group; C2 = *Echis ocellatus* venom + 1.4 mg/kg ethanol group; G3 = *Echis ocellatus venom* + 25 mg/kg DCM group; ALP = Alkaline Phosphatase; ALT = Alanine Aminotransferase; AST = Aspartate AminoTransferase; AST = Aspartate AminoTransferase; µmol/l = micro moles per liter; mmol/l = millimoles per litter; PCV = Packed Cell Volume; RBC = Red Blood Cell; WBC = White Blood Cell; Nm = *Naja melanoleuca* envenomed group; Nh = *Naja haje* envenomed group; Nk = *Naja katiensis* envenomed group; Eo = *Echis ocellatus* venom group; TB = Total Bilirubin; U/L = units per liter; Na^+ = Sodium; Cl^- = Chloride; Ur = Urea; g/l = gram per liter; K^+ = Potassium; ALB = Albumin.

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Histopathological Profile of Liver

Results of the effects of *C. ferruginea* DCM and ethanol extracts on cellular necrosis induced by *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus* venoms on liver compared to normal group show pathological differences in the effect of the venom and extract treatment. The micrograph of liver for positive control group (Figure 8) shows a healthy non-distorted liver but those of the envenomed groups (Figure 9) show distorted cells due to the effects of the venoms. Micrographs of envenomed liver treated with ED_{100} of the extract shows cells healed from distortion caused by venom (Figure 10); while those of groups treated with ED_{50} of the extracts (Figure 11) show that the distorted cells have been healed from the distortion caused by the venom.

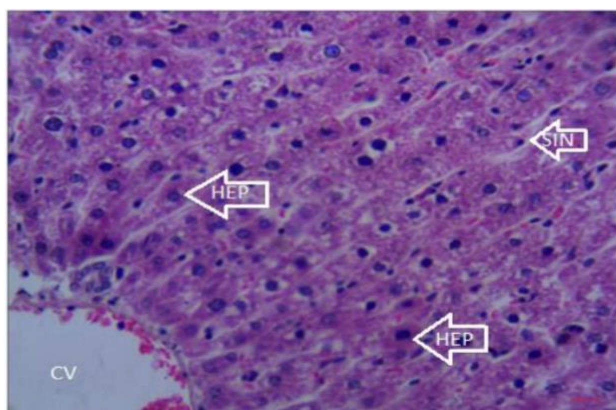

Figure 8. Liver of positive control group (NQL) showing histologically normal liver with patent central vein (CV), intact hepatocytes (HEP), and sinusoid (SIN) containing capillaries and Kupffer cells

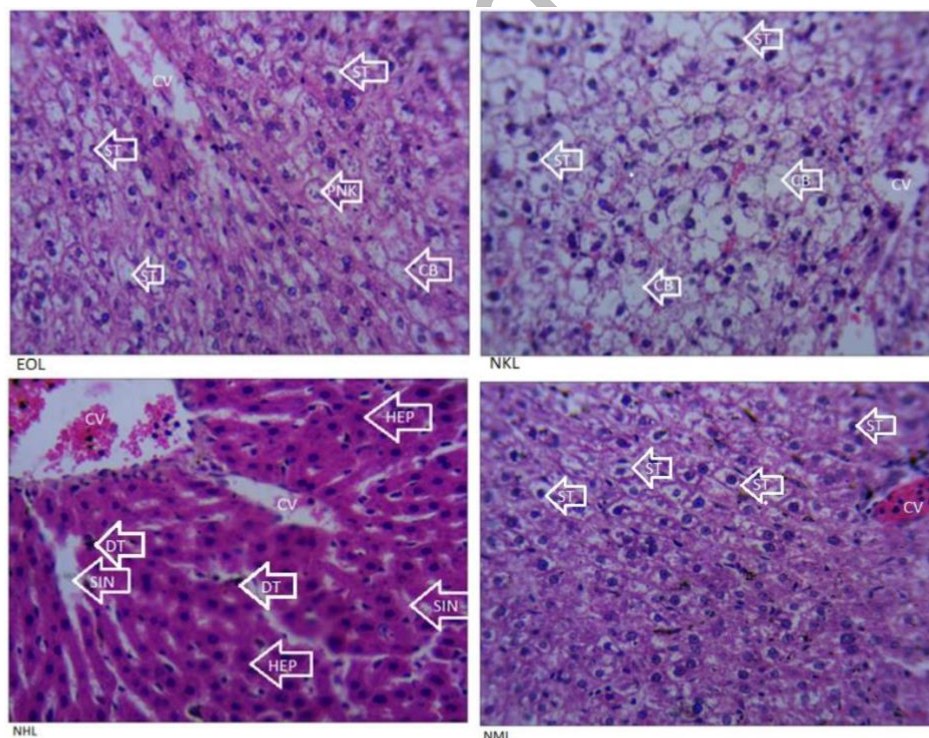

Figure 9. Liver of envenomed groups (EOL = *E. ocellatus* envenomed liver; NKL = *N. katiensis* envenomed liver; NHL = *N. haje* envenomed liver; NML = *N. melanoleuca* envenomed liver) showing histologically distorted tissues (DT), with various levels of fatty changes (ST = steatosis) and Councilman's body (CB), patent

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central vein (CV), sparsely distributed normal hepatocytes (HEP), and nuclear disintegration (PNK = Pyknosis)

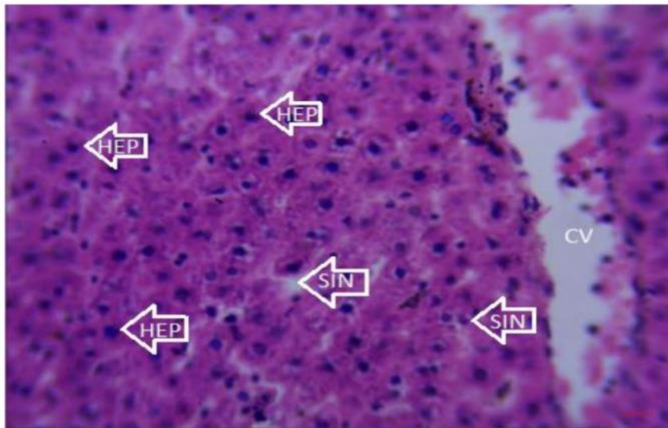
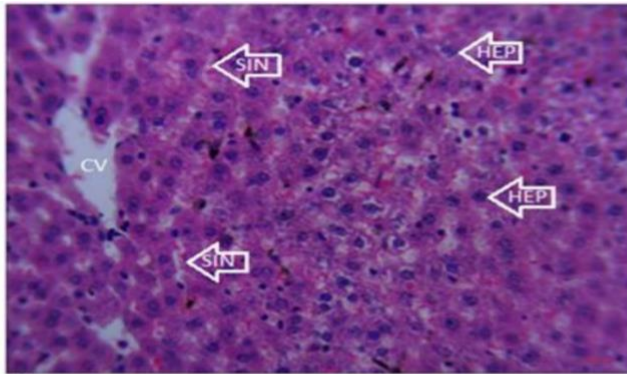


Figure 10. Liver of group treated with ED_{100} of DCM extract (G3L) showing normal patent central vein (CV), intact hepatocyte (HEP), sinusoid (SIN) containing capillaries and Kupffer cells



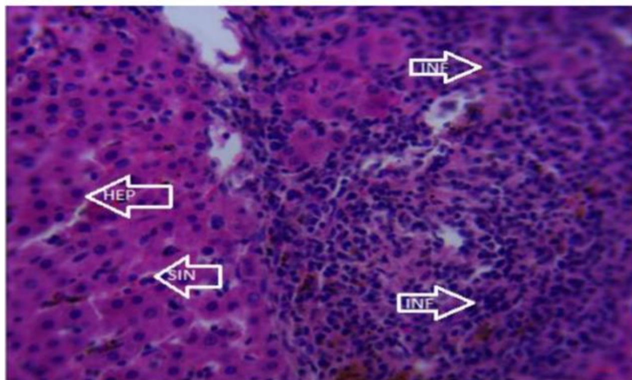


Figure 11. Liver of groups treated with ED_{50} of DCM (C1L) and ethanol (C2L) extracts showing normal and sparsely conjugated patent central vein (CV), intact hepatocyte (HEP), inflammation (INF) gradually tending to normal, and sinusoid (SIN) containing capillaries and Kupffer cells

Histopathological Profile of Kidney

Results of *C. ferruginea* DCM and ethanol extracts on envenomation induced by *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus* venoms on kidney in comparison with positive control group (normal group) and extract treated

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groups showed pathological differences in venom toxicity and extract treatment. The micrograph of kidney for positive control group (normal group) (Figure 12) shows a healthy non-distorted kidney but those of the envenomed groups show distorted cells due to the effects of the venoms (Figure 13). Micrograph of envenomed kidney treated with ED_{100} of DCM extract shows cells healed from distortion caused by venom (Figure 14). Groups treated with ED_{50} of DCM extract (Figure 15) and ethanol extract (Figure 16) showed the distorted cells being healed from distortion caused by venom.

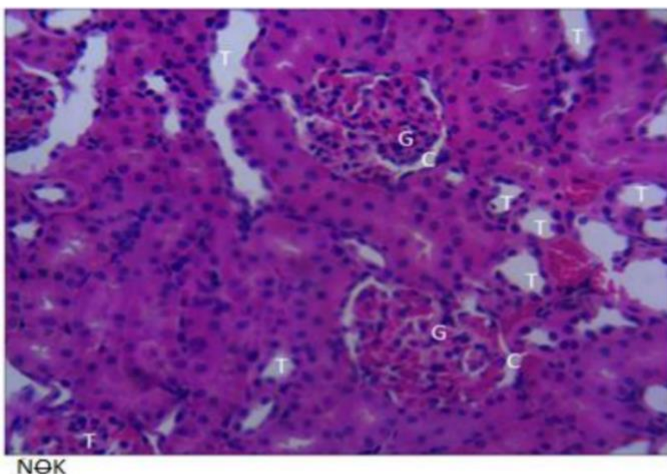

Figure 12. Kidney of positive control group showing histologically normal Kidney with glomeruli (G) surrounded by patent Bowman's Capsular (C) spaces and Tubules (T) lined by simple epithelia.

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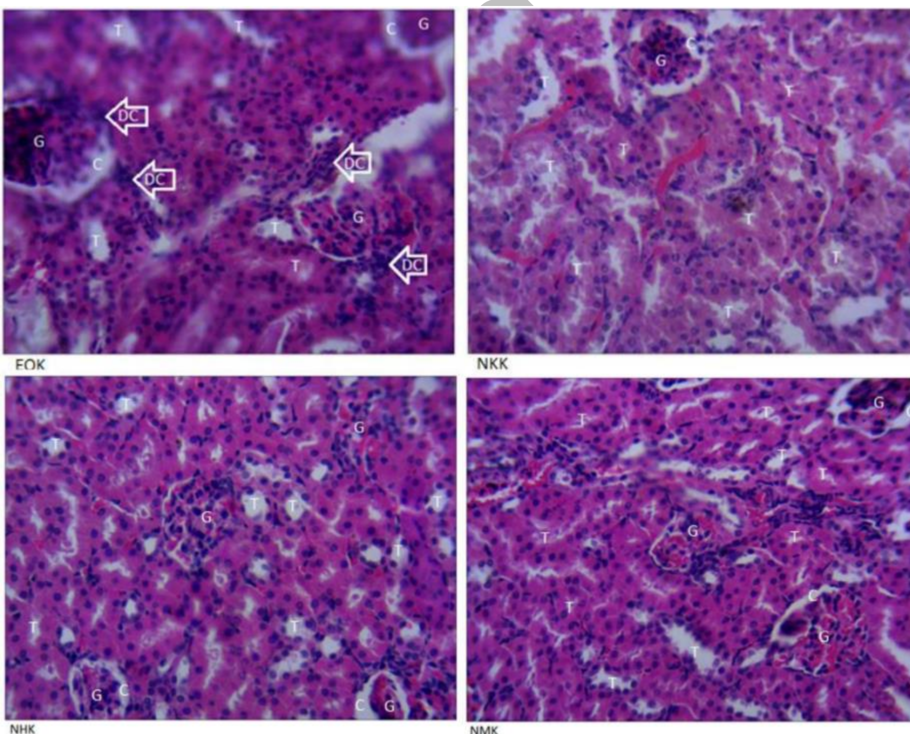

Figure 13. Kidney of envenomed groups (EOK = *E. ocellatus* envenomed kidney; NKK = *N. katiensis* envenomed kidney; NHK = *N. haje* envenomed kidney; NMK = *N. melanoleuca* envenomed kidney) showing distorted cells (DC), glomeruli (G) surrounded by patent Bowman's capsula (C) spaces, tubules lined by simple epithelia

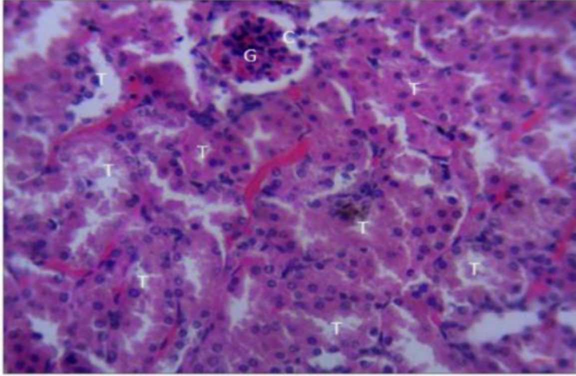

Figure 14. Kidney of group treated with ED_{100} of DCM extract (G3K) showing normal healthier glomeruli (G) surrounded by patent Bowman's Capsular (C) spaces and Tubules (T)

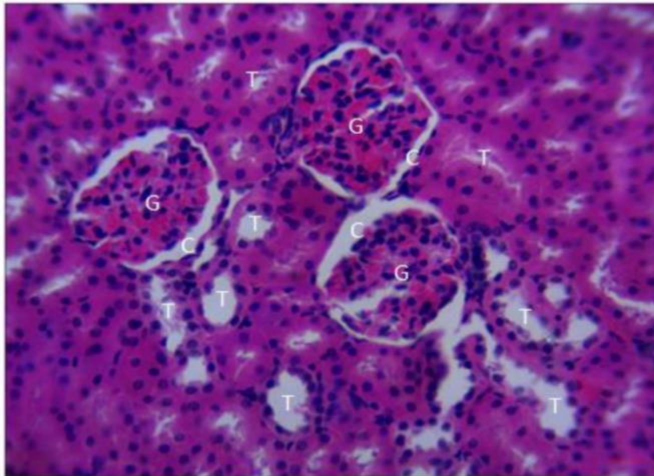

Figure 15. Kidney of group treated with ED_{50} of DCM extract (C1K) showing healthy normal kidney containing glomeruli (G) surrounded by patent Bowman's Capsular (C) spaces and Tubules (T)

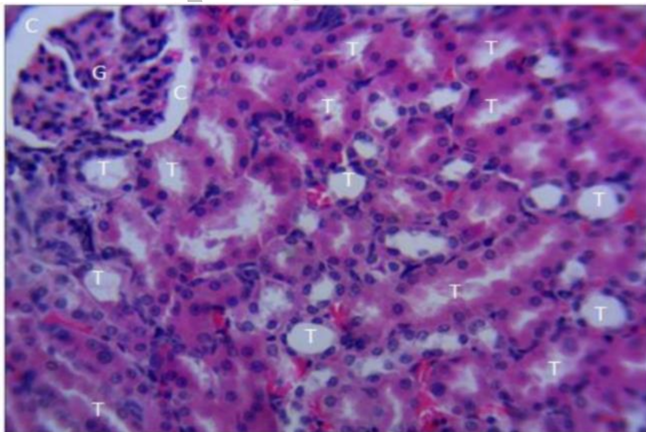

Figure 16. Kidney treated with ED_{50} of ethanol extract (C2K) showing healthy normal kidney containing glomeruli (G) surrounded by patent Bowman's

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capsular (C) spaces and Tubules (T) because of successful extract treatment

Phytochemicals with antivenom activity identified in Cnestis ferruginea Root Extract

Seven compounds made up of isoflavones, phenylpropenes, terpenoids and polyphenolic esters were identified in the root extract of *C. ferruginea* via thin layer chromatography. Biological activities of the identified compounds against snake venoms are as shown in Table 2.

Table 2. Activities of compounds identified from the extract of *Cnestis ferruginea* root

S/No.	Phytochemical	Phytochemical class	R_f value (experimental)	R_f value (literature)	Activity against snake venom
1	Biochanin A	Isoflavones	0.88	0.88	Anti-inflammatory, induces relaxation of the coronary artery, neuroprotective, L-amino-acid oxidase inhibitor, phosphodiesterase inhibitor.[19]
2	Formononetin	Isoflavones	0.90	0.85	Anti-inflammatory, phosphodiesterase inhibitor, neuroprotective, antioxidant, osteogenic action.[19]
3	Genistein	Isoflavones	0.80	0.83	Anti-inflammatory, inhibition of the nicotinic acetylcholine receptor.[19]
4	Anethole	Phenylpropenes	0.68	0.69	Inhibits phosphodiesterase, anti-inflammatory, inhibits ATPase, inhibits monoamine oxidase.[20]
5	Myristicin	Phenylpropenes	0.55	0.50	Inhibits ATPase, inhibits monoamine oxidase, anti-inflammatory, hepatoprotective, inhibits phospholipase A2.[20]
6	Quercetin	Terpenoids	0.45	0.43	Antidote, inhibits snake venom phospholipase A2, inhibits 5′-nucleotidase, inhibits ATPase, phosphodiesterase inhibitor.[21]
7	Chlorogenic acid	Polyphenol esters	0.65	0.64	Anti-inflammatory, ATPase inhibitor, inhibits 5′-nucleotidase, phospholipase inhibitor, antidote.[21]

DISCUSSION

Antiophidic ability of *Cnestis ferruginea* root extracts was investigated, in order to propose alternative options for the management of envenomation, by using a plant that is known locally for treatment of snake-related bites in southern Nigeria. Acute therapeutic mode of treatment was adopted since most treatments occur after a bite. This study and many others confirm the ability of *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus* venoms injection to induce muscle damage, edematogenic activity, hyperalgesia, and motor function impairment. Several metalloproteinases and phospholipases A2 (PLA2) are among the important components of venoms that present cytotoxic and inflammatory properties.[22]

Extraction of *C. ferruginea* root using DCM and ethanol yielded 16.6 g (0.79%) and 40.6 g (0.33%), respectively, though Sivaraman *et al*. reported a 2.5% yield from 20 g powdered root sample.[23]

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DCM and ethanol extracts of *C. ferruginea* root increased movement and smartness in the animals when used alone. For intraperitoneal injection, the acute toxicity result with 2000, 4000 & 5000 mg/kg showed mortality rate of over 50% in all the groups except 2000 mg/kg DCM extract group with less than 40% death rate (Figure 1), but for oral administration, no mortality was observed within the experimental groups after 24 hours post administration of the same concentrations of both extracts (2000, 4000 & 5000 mg/kg) except for 5000 mg/kg DCM extract (Figure 2).The animals remained active and survived for up to 28 days, indicating that the administered doses of the extract were insufficient to induce toxicity. Notably, the rats exhibited increased movement and activity compared to their behavior prior to receiving the extract. Consistent with these findings, it can be concluded that *C. ferruginea* root extracts are nontoxic when administered orally. Therefore, the extract may be considered safe for use as a potential medication. This result is in line with the acute toxicity work done by Enenebeaku *et al*.[24] Ishola *et al*. also stated that no death was recorded at doses of 1 and 2 g/kg but animals were calm, hypoactive, and passed watery stools.[11] It was also noted that intraperitoneal administration proved to be more toxic compared to oral administration. This increased toxicity may be attributed to a possible infection at the site of administration. Additionally, intraperitoneal administration allows the substance to enter the bloodstream more rapidly and directly, bypassing the liver. In contrast, oral administration requires the substance to pass through the liver first, where it is often metabolized and weakened before reaching systemic circulation.

Lethality at 50% death using 0.1, 0.2, 0.4, 1.6 and 3.2 mg/kg body weight gave LD_{50} of 0.455 mg/ml for *N. melanoleuca* and *N. katiensis;* 0.465 mg/ml for *N. haje* and *E. ocellatus* using probit analysis. These lethal doses correspond with LD_{50} of *N. nigricollis* (cobra) and *E. carinatus* (viper) as reported by Ernst and Zug.[25] Phospholipase A2 (PLA2) is the most toxic component of the venom and responsible for wide range of pharmacological problems.[13] Venom toxicity signs noted in the rats were respiratory problems with sneezing, stiffening of neck, weakness and then death within four-hour interval. This result tallies with the result of Ranawaka *et al*. that elapids (cobra) are mainly neurotoxic.[26] Death rate is as shown in Figure 3.

Effectiveness of the extracts against the venoms are described in terms of comparison of prevention of deaths and improvement in health of extract-treated groups with that of envenomed groups (negative controls). Extract intravenous treatment of envenomation, for groups treated with 25, 50, 100, 200 and 400 mg/kg of DCM and ethanol extracts, respectively, after envenomation with *N. melanoleuca*, gave over 50% death for most of the groups (Figure 4), which may be as a result of arterial or blood vessels hit and/or site infection at the site of injection but none for oral administration except for DCM 200 mg/kg, ethanol 50 and 400 mg/kg groups (Figure 5).[27]

Death rate for groups orally treated with ED_{100} (25 mg/kg) of DCM and ethanol extracts, after envenomation (using all four venoms: *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus*), showed less than 25% death rate except for *N. katiensis* that indicated above 30% death rate for both extracts (Figure 6). The percentage survival (<50%) of envenomed rats administered with *C. ferruginea* appeared to be better with ED_{100} (25 mg/kg) compared to ED_{50} of 50 and 300 mg/kg reported by Iful for aqueous extracts of *Paullinia pinnata* and *Detarium microcarpum,* respectively.[17]

Result of administration of ED_{50} (1.4 mg/kg) for each of DCM and ethanol extracts indicated less than 40% mortality rate except for *N. haje* envenomed group, which showed over 40% mortality for ethanol extract, after 24 hours

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(Figure 7). All envenomed groups treated with ethanol extract had higher mortality rate than the DCM extract-treated groups. This result showed better antivenom effectiveness than the ED_{50} of 477 mg/kg reported by Aguilar *et al*.[16]

Results of oral treatments showed that the effect of the extracts on envenomed rats were better than intravenous administration. This is in line with the observation of Iful, who posited that oral treatment has better therapeutic effect than intravenous administration.[17] Antivenom property of the extracts are in accordance with the findings of Fattepur and Gawade.[28]

Improved health of the envenomed test rats after administration of the extracts showed the antivenom properties of the extracts as can be seen in their haematological (Table I) and histological results.

Significant reduction ($p<0.005$) in ALT levels of extract treated groups (C1, C2, G3) as against envenomed groups were observed. Extract treated groups showed a much more reduced levels of ALT. Abnormal (high) levels of ALT were as a result of damaged or inflamed cells and are associated with conditions, such as inflammation (hepatitis) and scarring (cirrhosis), because it is most concentrated in and affects the liver.[29]

Levels of AST in both DCM and ethanol treated groups reduced significantly ($p<0.005$) compared to envenomed groups (Table 1). This study shows that the venoms increased capillary permeability in the rats, which was reversed by *C. ferruginea* root extracts.

ALP concentration in the bloodstream is used by diagnosticians as a biomarker in determining hepatitis or osteomalacia.[30] Abnormal levels of alkaline phosphatase in the blood of envenomed rat indicated issues relating to the liver, gall bladder or bones. There was more stable ALP levels in the groups treated with the extracts.

Total bilirubin (TB) was higher in envenomed groups, especially group induced with viper (Eo) compared to treated groups (C1, C2, G3) (Table 1). A high amount of bilirubin is expected of sickled or inflamed cells. The result shows that the extracts gave ameliorative response to the effect of the venom on the bilirubin level in the rats. Low albumin levels indicate a problem with the kidney, liver, or other health challenges. Liver panel includes measurements of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, and bilirubin (Table 1).[29]

Increase in packed cell volume (PCV) in *C. ferruginea* treated rats compared to envenomed groups (Tables 1), may be because of dehydration due to diuretic outcome of the extract.[31] Leukocytosis (high white blood cell counts) occurred in envenomed rat groups, possibly due to tissue necrosis and inflammatory responses by the venom. High white blood cell count is the initial finding that leads to diagnosis of primary haematological disorder, like leukemia, in animals. The extracts eliminated these effects partly due to anti-inflammatory effects of the extract as described by Ishola *et al*.[11] The overall result indicated that the extracts of *C. ferruginea* root were protective of the blood cells against adverse effects of the snake venoms.

Electrolyte panel test levels (potassium, sodium, and chloride) were within range in the normal and treated groups compared to the significant difference in envenomed groups. Lowest value was observed in viper envenomed group (Eo). Potassium levels are used to monitor health conditions related to kidney disease. Potassium (K^+) levels were higher in envenomed groups compared to extract-treated groups. Too much or too little levels of potassium in the blood indicates serious medical problems. Low sodium (Na^+) levels in envenomed groups (Nk, Nm, Nh, and Eo) are

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indications of kidney failure, as against the levels in extract-treated groups. Elevated levels of chloride as seen in envenomed groups are signs of dehydration and kidney disease, though there is a lower level in Nk envenomed group (Table 1).

Red blood cells or erythrocytes were lower in envenomed groups compared to extract-treated groups (Table 1). This is a result of an infected bone marrow, as red blood cells are produced there. The level of blood urea was within range for all groups except Eo group. Envenomed groups have higher urea values tending towards uremia, a dangerous medical condition that causes urea to accumulate in the blood because kidney could not filter it as waste, but it then enters the bloodstream. This shows the negative effect the venom has on the kidney; it also shows that the extracts ameliorated this effect in the rats and agrees with the work of Okafor *et al*.[32]

Histopathological examination of liver sections in the positive control group (Figure 8) showed a patent normal appearance of central vein (CV) and intact hepatocytes and sinusoids (SIN) containing capillaries and Kupffer cells with no visible lesion compared to liver of envenomed groups (NML, NHL, NKL and EOL of Figure 9) with visible haemorrhages, distorted tissues, congested central vein, hepatocytes with different fatty changes (steatosis), councilman body and nuclear disintegrated pyknosis which are evidence of distortion. This coincides with the work of Adeyi *et al*.[33]

The group treated with ED_{100} (25 mg/kg) of DCM extract (Figure 10) showed histologically normal liver with patent central vein. Groups separately treated with ED_{50} (1.4 mg/kg) of DCM and ethanol extract (Figure 11) also gave comparable results with that of the normal group. These are clear indications of a healthy liver due to treatment with the extracts.

The kidneys are a pair of bean-shaped organs on either side of the spine that filter blood, remove waste, control the body's fluid balance, and keep the right levels of electrolytes. Studies of the kidney sections of rats treated with ED_{100} (Figure 14) and ED_{50} (Figures 15 & 16) of both extracts showed no significant microscopic changes compared to those of the positive control (Figure 12). For these treated groups, kidney sections showed normal glomerulus (G) containing mesangial cells, mesangial matrix, and capillaries; normal Bowman's capsule spaces (C), renal tubules (T) lined with simple epithets. Compared to these are the envenomed groups (Figure 13), especially the viper venom, EoK, with distorted kidney showing damaged cells (DC). This shows that the Glomeruli matrix; which contains the mesangial cells (blue/black), mesangial matrix/fluid (purple) and the capillaries (reddish) had been negatively affected by the presence of the venom; an indication of an unhealthy system. This agrees with the findings of Chaiyabutr *et al.* that viper venoms affect the histopathology of kidney.[34] Extracts of *C. ferruginea* roots reversed the negative effects of the venom on the kidneys. This agrees with the conclusion made by Adeyi *et al*. that plants contain phytochemicals active against the negative effects of snake venoms.[33]

Phytochemical analysis of *C. ferruginea* root extracts identified the presence of terpenoids, acidic compounds, polyphenol esters (alkaloidal), isoflavones and phenylpropenes (Table 2). Phytochemicals are usually involved in protection of various diseases and in curing. Functions of alkaloids are mostly related to protection. They are widely used as an analgesic, inhibitor of acetylcholinesterase.[35]

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The result of phytochemical analysis of *C. ferruginea* root extracts, revealed the presence of polyphenolic compounds (terpenoids, isoflavones and polyphenol esters), which are responsible for the neutralizing effect of plants that are in popular use against the action of snake venoms. This also suggests that *C. ferruginea root* extract have anti-snake venom potential since polyphenols possess protein-binding and enzyme inhibitory properties which inhibit snake venom phospholipase A2 (an enzyme present in cobra venoms) activities.[36, 37] Phytochemicals counter venom-induced Phospholipase A2 (PLA2) activity primarily by binding to the enzyme's active site, blocking its ability to hydrolyze cell membrane phospholipids, and neutralizing its inflammatory effects. These plant-derived compounds can inhibit PLA2 enzymes, reducing venom-induced hemolysis, edema, and anticoagulation.

Phytochemical analysis of *C. ferruginea* carried out by Akharaiyi *et al.* showed that it contains tannins, alkaloids, saponins, phlobotannins, flavonoids, cardiac glycosides, anthraquinones and terpenoids; though Khalid *et al*. reported that aristolochic acid, a gymnemic acid, wedelolactone, cabenegrin-i, stigmasterol, sitosterol, ar-turmerone, rutin, hesperidin, iridin, caffeic acid, 2-hydroxy-4-methoxy benzoic acid are some phytochemicals that are active against snake envenomation.[10, 38]

These results suggest that the inhibitory effect of the extracts against snake venom-induced pathological symptoms could be, partly, due to the active components of the phytochemical compounds and the likelihood of the extracts acting through a systematic intervention rather than through physical interface with the venom, which is the likely means of action of many polyphenolic compounds found in plant extracts.[39]

Table 2 shows the activities of some phytochemicals present in the extracts. Isoflavones (biochanin A, formononetin and genistein) with R_f values of 0.88, 0.90 and 0.80 had similar value with that obtained by Wang.[19] These isoflavones also exert various activities against snake venoms by inhibiting snake venom enzymes (phosphodiesterase, ATPase). Biochanin A is an O-methylated isoflavone recognized for its capability to counteract the toxicity of snake venom. Its protective effects are attributed to its chemical structure, which features both hydroxy and methoxy groups characteristic of phenolic compounds. The primary mechanism by which biochanin A mitigates snake venom toxicity involves the direct inhibition of venom enzymatic activity. Biochanin A acts as a small-molecule inhibitor, binding to the active sites of venom proteins. This interaction prevents the venom enzymes from engaging with target tissues and exerting their harmful effects. In addition to enzymatic inhibition, biochanin A contributes to the structural stabilization of venom proteins. By binding to these proteins, it helps maintain their structure, reducing their ability to interact deleteriously with biological tissues and further minimizing the toxic impact of the venom.[40] Phenylpropenes (myristicin and anethole) also showed similar R_f values (0.55 and 0.68, respectively) compared to that reported in literature and research showed they exert some activity against the effect of snake venom enzymes.[20] Myristicin is recognized in literature for its ability to inhibit phospholipase A2 (PLA2) enzymatic activity. This inhibition typically occurs through the binding of myristicin to the enzyme, which alters its structural integrity and interferes with its function.[41] The terpenoids (quercetin and chlorogenic acid) similar to that obtained by Ćetković have been proven to be antidotes for snake bite (vipers and cobras).[21] The terpenoids (quercetin and chlorogenic acid) similar to that obtained by Ćetković have been proven to be antidotes for snake bite (vipers and cobras).[21]

CONCLUSIONS

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This study investigated the effects of snake venom on albino rats and the protective role of *Cnestis ferruginea* root extracts. Envenomed groups exhibited significant disturbances in electrolyte balance, red blood cell count, and kidney and liver structure, indicating organ damage. However, treatment with *Cnestis ferruginea* root extracts ameliorated these effects, resulting in near-normal test results and tissue health. Chromatographic analysis revealed the presence of various bioactive compounds such as terpenoids, isoflavones, and polyphenols, which are proposed to contribute to the anti-venom properties by inhibiting venom enzymes. The findings suggest that *Cnestis ferruginea* root extracts have potential therapeutic value against snakebite-induced damage. The study not only demonstrates the therapeutic promise of *Cnestis ferruginea* root extracts in mitigating snakebite-induced organ damage but also contributes compelling evidence for the continued exploration and development of plant-based antidotes. Such therapeutic agents could play a crucial role in regions where snakebite envenomation remains a persistent medical challenge and access to conventional antivenoms is limited.

ETHICAL APPROVAL

Experiments were approved by the University of Port Harcourt Research Ethics Committee with assigned number UI-ACUREC: 18/0108, in accordance with the guidelines of the National Code for Health Research Ethics (NCHRE). All animal experiments were performed according to ethical standards.

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