World Journal of Advanced Pharmaceutical and Life Sciences

Journal homepage: https://zealjournals.com/wjapls/

ISSN: 2799-0222 (Online)

(RESEARCH ARTICLE)

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Assessment of antibacterial efficacy and toxicological implications of emperor scorpion (*Pandinus imperator*) venom using animal model

Ayodeji Adams DAHUNSI [1,*] and Bukola Catherine AKIN-OSANAIYE [2]

[1] *Department of Microbiology, Faculty of Science, University of Abuja, Nigeria.*
[2] *Department of Biochemistry, Faculty of Science, University of Abuja, Nigeria.*

World Journal of Advanced Pharmaceutical and Life Sciences, 2024, 06(01), 001–011

Publication history: Received on 05 November 2023; revised on 09 January 2024; accepted on 11 January 2024

Article DOI: https://doi.org/10.53346/wjapls.2024.6.1.0082

Abstract

This research work was carried out by collecting 50 *Pandinus imperator* Scorpions and ascetically extracting venom locally from them and testing the venom on some selected bacteria. The extracted venom used was both in the lyophilized and crude (non-lyophilized) state to determine their different efficacy and was passed through a membrane filter to ensure sterility. Bacterial (*Pseudomonas aeruginosa, Escherichia coli, and Acetinibacter baumannii*) were isolated from drinking water sources of the University of Abuja after which it was preserved in slant for further use. Serotyped bacteria were also obtained and used in this study. Appropriate differential and selective agar were used to culture and sub-culture the isolates and serotyped while Analytical Profile Index (API) kits were used to confirm the isolated bacteria. A sensitivity test was carried out on each bacterium with the venom at concentrations of 25%, 50%, 75%, and 100% for crude venom while 10, 20, 30, and 40 mg/ml for the lyophilized venom, minimum inhibitory concentration, and minimum bactericidal concentration were also determined. The result shows lowest zone for crude venom against isolated bacteria was 3mm at 25% against *Acinetobacter baumanni* while the highest was 11.5 mm at 100% against *Pseudomonas aeruginosa*, the lowest for crude venom against serotyped was 4 mm at 25% against *Acinetobacter baumanni* and highest was 12.2 mm at 100% against *Pseudomonas aeruginosa*, lowest zone for lyophilized venom against isolated bacteria was 2.8 mm at 10 mg/ml against *Acinetobacter baumanni* while highest was 10.8 mm at 40 mg/ml against *Pseudomonas aeruginosa*, also the lowest for lyophilized venom serotyped was 3.8 mm at 10 mg/ml against *Acinetobacter baumanni* and highest was 11.5 mm at 40 mg/ml against *Pseudomonas aeruginosa*. The lowest MIC was 25% and 20 mg/ml while the highest was 50% and 30 mg/ml, the lowest MBC was 50% and 30 mg/ml while the lowest was 75% and 40 mg/ml. The toxicity of the venom was carried out by testing the venom on mice which shows no harmful effect on them. ANOVA statistical analysis carried out showed no significant difference in the activities of both venom states.

Keywords: Animal model; Antimicrobial Peptides; Antibacterial Activity; Emperor Scorpion; *Pandinus imperator*; Scorpion Venom; Toxicity

1. Introduction

Antimicrobial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi, or protozoan, as well as killing viruses. The discovery of antimicrobials like penicillin and tetracycline paved the way for better health for millions around the world. Before 1941, the year when penicillin was discovered, there was no true cure for some diseases such as *gonorrhoea* (a venereal disease involving inflammatory discharge from the urethra or vagina), strep throat (a painful infection in the throat caused by group *A streptococcus*), or pneumonia (a lung infection in which the air sacs are filled with fluid) (Cosgrove *et al*., 2003).

* Corresponding author: DAHUNSI Ayodeji Adams

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Resistance to antibiotics is a rising concern among healthcare professionals, driving them to search for peptides with antimicrobial activities in plants, bacteria, and animals (Meylears *et al*., 2002; Shaini *et al*., 2010). Antimicrobial peptides (AMPs) are key effectors of the innate immune response of animals and show antimicrobial therapy potency (Bulet *et al*., 2004; Vancompernolle *et al*., 2005; Dhople *et al*., 2006; Yibao *et al*., 2009; Ramamoorthy, 2009). Most AMPs that display hydrophobic and cationic properties have a molecular mass below 25 to 30 kDa (Marsh *et al*., 2009; Reddy *et al*., 2004; Meylears *et al*., 2002). So far, more than 2000 natural AMPs have been isolated, sequenced, and submitted until now. Some natural antimicrobial peptides are being developed for use either as antibiotics for topical use in healthcare or as preservatives in the food industry. Apart from the extreme diversity in their primary and secondary structures, AMPs have *in vitro* effects on a wide spectrum of bacteria (Gram-negative and Gram-positive) and cells, including parasites, tumor cells, fungi, and viruses (Bulet *et al*., 2004). AMPs have non-specific processes involved in the antimicrobial action. The unique property of an antimicrobial peptide is its ability to interact selectively with bacterial lipid membranes and cause disruption. This property could decrease the likelihood of microbes acquiring resistance to AMPs. This could give AMPs a major advantage over conventional antibiotics, and in response, these peptides have been extensively investigated as potential antimicrobial agents (Harris *et al*., 2009; Thennarasu *et al*., 2010a, b; Ramamoorthy *et al*., 2010; Thennarasu *et al*., 2005).

Scorpions are particularly resistant to bacterial aggressions, and it is of interest to analyze the molecules responsible for this resistance (Sabatier *et al*., 1996). The scorpion venoms have three different groups of ingredients and peptides, based on their molecular mass with enzyme activity such as hyaluronidases, phospholipases, and sphingomyelinase (Chao *et al*., 2008). The second group contains mainly a peptide fraction with molecular masses around and lower than 10 kDa such as toxins and cytolytic compounds. The third group is composed of components such as ions, free amino acids, biogenic amines, neurotransmitters, acylpolyamines, heterocyclic compounds, and alkaloids (Ma *et al*., 2010; Kuhn-Nentwig, 2003). Different antimicrobial peptides such as micropores have been identified in plants and animals (He *et al*., 2008; Zhao *et al*., 2009), BmKb1 and BmKn2 (Zeng *et al*., 2004), Hadrurin (Torres-Larios *et al*., 2000) and Mucroporin (Chao *et al*., 2008).

Caerin is an antimicrobial peptide that was first found in amphibian, Australian green tree frogs (Wong *et al*., 1997). Later different variants were isolated from several Australian frogs of the Litoria genus (Brian *et al*., 2000; Steinborner *et al*., 1998) and African frog (Ramamoorthy *et al*., 2010). In recent years, caerin and caerin-like antimicrobial peptides were been found in scorpions such as *Mesobuthus lupus* and *Mesobuthus martensii* (Luo *et al*., 2005). Antibacterial activity of caerin on Gram-positive and negative bacteria has been shown with only small variation in their minimum inhibitory concentration (MIC) values (Boland and Separovic, 2006; Chia *et al*., 2011).

1.1. Justification

The venom or antimicrobial peptides found in scorpions have shown a broad-spectrum activity against a wide range of pathogenic microorganisms. Viral, bacterial, fungal, and parasitic infections ranging from malaria, hepatitis C, meningitis, tetanus, and many nosocomial infections, etc have been treated successfully with scorpion venom. Scorpion venom is also used for the treatment of non-communicable and neurological diseases such as stroke, rheumatism, diabetes, cancer, brain tumor, human neuroblastoma cells, etc (Attarde and Pandit, 2016; El-Bitar *et al*., 2015; Petricerich *et al*., 2013). The emperor scorpion (*Pandinus imperator*) and several additional venom peptides have been characterized as antibacterial peptides with a broad spectrum of bacterial targets against even antibiotic-resistant strains (Baradaran *et al*., 2012).

The purpose of this study was to determine the possibility of using The Emperor Scorpion (*Pandinus imperator*) venom as an alternative to conventional antibacterial agents in the future.

1.2. Statement of the problem

Deepti *et al*. (2014), indicated a free chlorine concentration of up to 4 ppm was not effective in killing multidrug-resistant *A. baumannii* isolates. These observations indicate the inefficiency of currently used chlorine concentrations in killing *A. baumannii* in water, thereby warranting additional research and corrective measures. Water and soil are considered a major habitat for *A. baumannii* although the pathogen has been isolated from other sources, including foods, arthropods, animals, and humans (Baumaan, 1998; Berlau *et al*., 1999; Fournier and Richet, 2006; Munoz-Price and Weinstein, 2008; Mussi *et al*., 2005). Moreover, protozoans such as *Acanthamoeba* have been reported to support the growth of *A. baumannii*, and act as its reservoir in water (Cateau *et al*., 2011). Several studies have indicated the potential presence of *A. baumannii* in water systems from many parts of the world (Ferreira *et al*., 2011; Zhang *et al*., 2013). The ability of *A. baumannii* to thrive in water may result in fatal infections in all age groups (Kempt and Rolain, 2012).

Objectives

The objectives of this study are to:

- Extract venom from Scorpion (*Pandinus imperator*);
- Assess the antibacterial activity of *Pandinus imperator* venom against selected bacterial isolates;
- Reveal the Minimum inhibitory concentration and minimum bactericidal concentration of the venom against the selected organisms; and
- Determine the safety of venom using an animal model

2. Material and methods

2.1. Study area

Gwagwalada is one of the six Local Government Area Councils of the Federal Capital Territory of Nigeria, together with Abaji, Kuje, Bwari, Kwali; and the Abuja Municipal Area Council (AMAC). Gwagwalada is also the name of the main town in the Local Government Area, which has an area of 1,043 km^2 and a population of 157,770 at the 2006 census. It is located at an elevated of 210 meters above sea level. Its coordinates are $8^0$56'29'' N and $7^0$5'31'' E in DMS (Degree Minutes Seconds) or 8.94139 and 7.09194 (in decimal degrees). Its UTM position is KQ98 and it is Joint Operation Graphics reference is NC32-13 (Wikipedia, 2016).

2.2. Media and preparation

All media used were prepared according to the manufacturer's standard of preparation.

2.3. Sample collection

2.3.1. Method of venom collection

50 numbers of Emperor Scorpion (*Pandinus imperator*) which were identified in Zoology Department, Faculty of Science, University of Abuja, venoms were collected by the electrical stimulation method described by Ozkan and Filazi, 2004. A series of regular currents were applied to shock the scorpion until the venom was ejected. For that purpose, the body of the scorpion was immersed in a saline solution for better electrical conduction and gave a shock with the electrode by using a simple 12-volt battery. The venom droplet was recovered in a sterile test tube after which part of the venom in the test tube was placed in a container containing ice and used within 30 minutes while another part was kept frozen until use. Venom was recovered using distilled water and centrifuged (10,000 g). The supernatant was lyophilized (freeze-dried) and then kept at 20 °C until use. All venom collected was passed through the membrane filter.

2.3.2. Collection of Test organisms

20 ml of drinking water samples were from sources of drinking water supply in the University of Abuja main site, mini campus, and staff quarters for the isolation of selected test bacteria. All the samples were taken to the Microbiology Laboratory University of Abuja for culturing, isolation, and identification of the isolates.

2.4. Preparation of samples

2.4.1. Preparation of The Emperor Scorpion (Pandinus imperator) venom

The venom of *Pandinus imperator* was collected and measured in percentage since venom is in liquid state. Raw venom represents 100% while 50%, 25%, and 12.5% were diluted with 50%, 75%, and 87.5% of appropriate neutral diluents (sterile water) respectively, and also 10, 20, 30, and 40 mg/ml for the lyophilized.

2.4.2. Preparation of test organism

The samples collected were taken to the laboratory in a sterile plastic container. Using the serial dilution method, they were cultured on an enrichment medium, incubated, plated on, and incubated on MacConkey agar at 37°C for 24 hours. The suspected colonies were further sub-cultured on selective medium (CHROM agar, Eosin methylene blue (EMB), and Cetrimide agar) on which the colonies were red, metallic green sheen, and blue-green/yellow-green color, then the single colony was picked, screened and identified based on Analytical Profile Index (API). The serotyped organism was sub-cultured on selective media and confirmed based on the normal standard biochemical techniques (Cheesbrough, 1998).

2.4.3. Antibacterial activity of The Emperor Scorpion (Pandinus imperator) VENOM (Peptides)

Antimicrobial susceptibility test of the isolates was done following the standard agar well Diffusion method (Bauer *et al.,* 1996) at different concentration (12.5%, 25%, 50%, and 100%) and (10, 20, 30, and 40 mg/ml) of the venom and the concentration(s) was tested on the positive isolates at different concentration to determine the antibacterial activity by zones of inhibitions was measured and recorded.

2.4.4. Determination of minimum inhibitory concentration (MIC)

The lowest concentration of scorpion venom without turbidity is observed in the test tube. The MIC values of the extracts were determined by using the dilution method of the venom. Various concentrations (12.5%, 25%, 50%, and 100%) and (10, 20, 30 and 40 mg/ml) of the venom was prepared.

Test tubes were set up; 2ml of nutrient broth was pipetted into each sterile test tube that was already labeled properly. Control tubes were also set up. Venom control tube: Tube A (test tubes containing venom and broth),

- Organism control: Tube B (test tube containing Broth and each inoculum) and
- Negative control: Tube C (test tube containing the Broth only). Using sterile Pasteur pipettes 0.2 ml suspension of the test organisms was introduced into the test tube according to their labels. Also, 2-3 drops of the different stock solutions were introduced using sterile Pasteur pipettes into the test tubes containing both Broth and inoculums. The preparation was incubated at 37ºC for 24 hours after which the test tube was observed for turbidity or clearness. The least concentration where no turbidity was observed was determined and noted as the minimal inhibitory concentration (MIC) value.

2.5. Determination of minimum bactericidal concentration (MBC)

The lowest concentrations of scorpion venom that has an antibacterial effect on the organism are determined as the minimum bactericidal concentration (MBC). This will be determined from the broth dilution resulting from MIC tubes by sub-culturing to the surface of freshly prepared nutrient agar plates by using a sterile inoculating loop to streak the plate as described by Vollekova *et al*., (2001) and Usman *et al*., (2007). All the test tubes that showed no microbial growth after 24 hours of incubation i.e. the test tubes will be clear, will be sub-cultured to the surface of freshly prepared nutrient agar plates and incubated at 37ºc for 24 hours, after which the plates will be observed for growth.

The plates with the lowest concentration of the venom showing no bacterial growth after incubation were noted and recorded as the MBC.

2.5.1. Comparing the effects of emperor scorpion venom with commercial antibiotic

The result was compared with standard antibiotics. Inhibition effects were measured in terms of the zone of inhibition.

2.5.2. Toxicity testing using animal model

Toxicity testing using animal models involves assessing the potential harmful effects of a substance, such as a drug or venom, on living organisms. The purpose of these tests is to evaluate the safety of the substance and understand its impact on various physiological systems.

2.5.3. Animal selection

Animals were chosen based on factors such as size, lifespan, genetic similarities to humans, and relevance to the study's objectives. Used animals was mice.

2.5.4. Dosing

The mice were exposed to different doses of the substance being tested. The doses varied to simulate different levels of exposure, including low, moderate, and high concentrations

2.5.5. Administration of dose

The venom was administered to the animals orally and intravenously**,** this was aim **to** mimic how humans might be exposed to the venom.

2.5.6. Observation/Monitoring

The mice were closely monitored for any signs of toxicity, such as changes in behavior, appearance, weight, or physiological parameters. Regular observations were made to detect acute or chronic effects.

2.5.7. Sample collection

Brain, kidney, liver, and heart were collected at specific time points to assess the venom's impact on the organs.

All these were done at the Veterinary Teaching Hospital (VTH), Ahmadu Bello University Zaria, Nigeria.

2.6. Statistical analysis

Descriptive statistics were used to analyze the data into simple percentages and standard deviation of the mean, Microsoft Excel and statistical significance determined by ANOVA were used to know if there was any statistically significant difference between the venoms and isolates. Differences in survival were considered significant when $P \leq 0.05$.

3. Results

Table 1 Physical characteristics of *Pandinus Imperator* Venom

Color	odour	Form	PH	Room Temperature (28°C)
Colourless	Odourless	Transparent Liquid	4.5	Dried up easily

The result of the Physio-chemical characteristic of *Pandinum Imperator* Venom is shown in table 1. the venom was observed to be colourless, odourless and transparent liquid which dries easily at room temperature.

Table 2 Analytical Profile Index (API-20E)

Bacteria Identification Using Analytical Profile Index (API)	
Preference	**Identity**
Permanent site	*Escherichia coli; Pseudomonas aeruginosa; Acetinibacter baumannii*
Mini Campus	*Escherichia coli; Pseudomonas aeruginosa; Acetinibacter baumannii*
Staff Quarters	*Escherichia coli; Pseudomonas aeruginosa; Acetinibacter baumannii*

The result of the Analytical profile index of the bacterial identification of isolates shows the different bacteria isolated from the three water sources studied were *E.coli, and P. aeruginosa and A. baumannii (*Table 2)

Table 3 Zone Diameter of Inhibition (mm) of *Pandinus imperator* Venom against organisms isolated from University of Abuja Drinking water

Microorganisms	Zone Diameter of Inhibition(mm)			
	Concentration (%)			
	25	50	75	100
Escherichia coli	6.1±0.0	7.2±0.0	9.0±0.0	10±0.0
Pseudomonas aeruginosa	5.7±0.0	7.0±0.0	9.0±0.0	11.5±0.0
Acinetobacter baumannii	3.0±0.0	4.7±0.0	6.0±0.0	8.2±0.0

Key: Values are Zone Diameter of Inhibition ± Standard error of mean

The result for the mean ± SD inhibition zone of diameter of *Pandinus imperator* Venom against organisms isolated from University of Abuja Drinking water at different concentration of 25%, 50%, 75% and 100% respectively. For the purpose of this findings, to interpret the results of the zones diameter of inhibition, the antibacterial and inhibitory effect of the venom was considered at the concentration of 30mg/ml as compared to that of chloramphenicol (30µg), a standard antibiotic. When interpreted using CLSI, 2004 guide, zone of chloramphenicol (30µg) ≥18 is sensitive, ≤12 is resistant, while 13-17 is intermediate

Table 4 Zone Diameter of Inhibition (mm) of Ciprofloxacin against isolated organisms

Microorganisms	**Zone Diameter of Inhibition(mm)**			
	Concentration(mg/ml)			
	62.5	**125**	**250**	**500**
Escherichia coli	26	31	36	38
Pseudomonas aeruginosa	26	27	34	35
Acinetobacter baumannii	23	25	28	31

Key: Values are Zone Diameter of Inhibition ± Standard error of mean

The mean SD of inhibition zone of ciprofloxacin against isolate organism at different concentrations of 62.5mg/ml, 125mg/ml, 250mg/ml and 500mg/ml

Table 5 Minimum inhibitory concentration (MIC) of Pandinus *imperators* venom on isolated test organisms

Microorganisms	**Concentration (%)**			
	25	50	75	100
Escherichia coli	+MIC	+	+	+
Pseudomonas aeruginosa	+MIC	+	+	+
Acinetobacter baumannii	-	+MIC	+	+

This is the lowest concentration of the venom required to inhibit the growth of the organisms. Key: + = Clear (No growth) - = Turbid (Growth)

Table 6 Minimum Bactericidal concentration (MBC) of *Pandinus imperators* venom on isolated test organisms

Microorganisms	**Concentration (%)**			
	25	50	75	100
Escherichia coli	+	-MBC	-	-
Pseudomonas aeruginosa	+	-MBC	-	-
Acinetobacter baumannii	+	+	-MBC	-

This is the lowest concentration of the venom required to kill the organisms.; Key: + = Bacterial growth - = No bacterial growth

The minimum bactericidal concentration of *pandinus imperators* venom at different concentration 25%, 50%. 75% and 100% on isolated organism. The MBC obtained in the study was 75% which indicates that the venom was effective against the isolated test organisms.

Histology of the brain of mice treated with *Pandinus imperator* venom, there was no lesion found, this shows there was no damage done to the brain tissues by the venom.

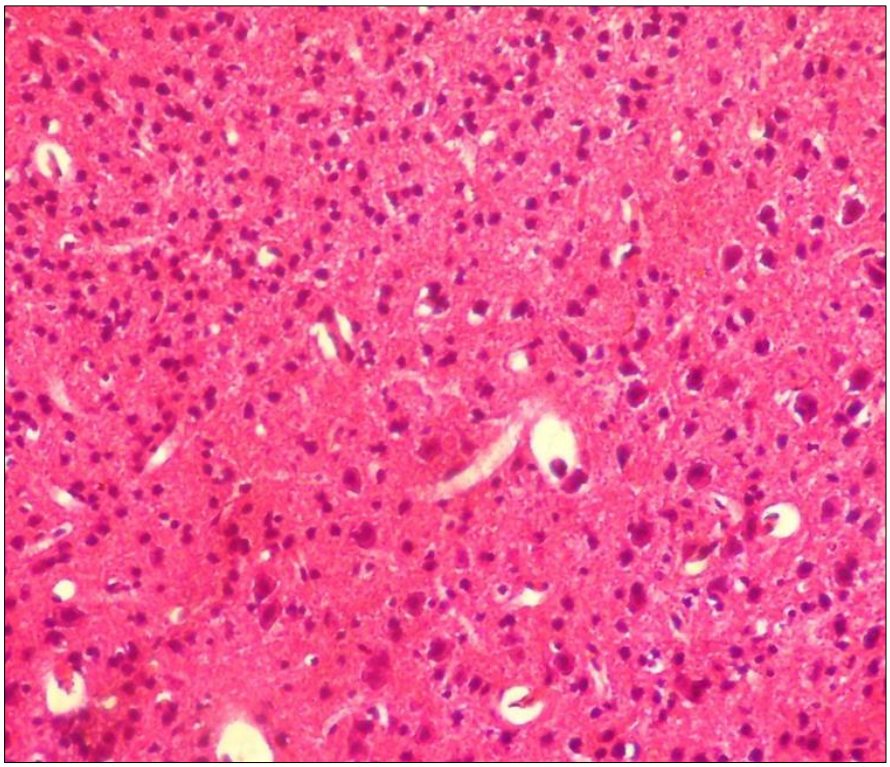

Figure 1 Photomicrograph of the section of the brain from mice treated with *P*. *imperator* venom with no lesion. H & E x 400

Histology of kidney of mice treated with *Pandinus imperator* venom shows that there was no lesion, this shows there was no damage to the kidney by the venom.

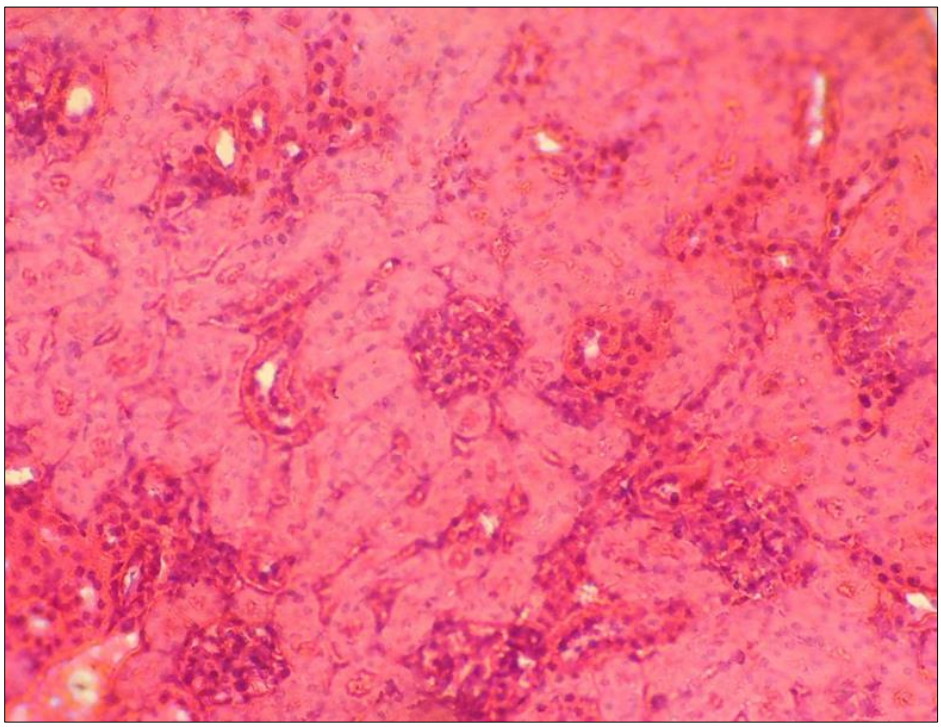

Figure 2 Photomicrograph section of kidney from mice treated with *P.imperator* venom with no lesion. H & E x 200

Histology of liver of mice treated with *Pandinus imperator* venom, there was no lesion, this shows there were no abnormal cells observe nor any damage done to the liver by the venom.

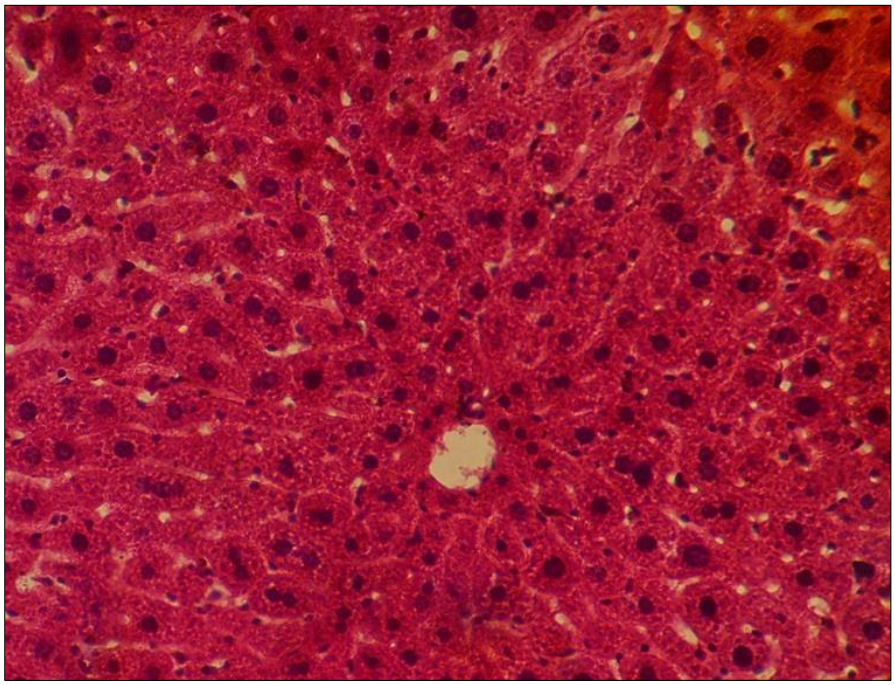

Figure 3 Photomicrograph section of liver from P.imperators venom with no lesion. H & E x 400

Histology of heart of mice treated with *Pandinus imperator* venom, there was intact muscle fiber, this shows there was no abnormality or any damage done to the heart by the venom.

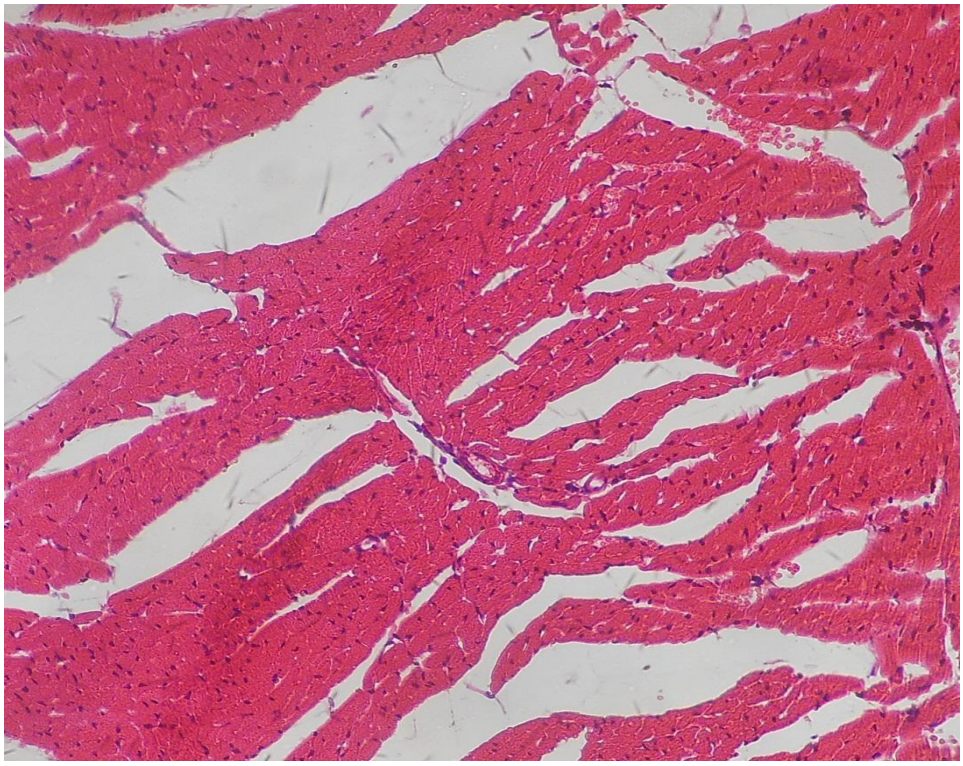

Figure 4 Photomicrograph section of heart from P.imperators venom with intact muscle fibers. H & E x 200

4. Conclusion

From this research work, it can be concluded or deduced that Venom of Pandinus imperator called Emperor Scorpion found in West Africa is an antibacterial agent that is non-toxic to animals, the scorpion venom either in the lyophilized or crude state has same potency and can both be safely used as a therapeutic agent.

Compliance with ethical standards

Acknowledgments

I want to thank my supervisor Prof. B.C. Akin-Osanaiye for her motherly role throughout my research work even when I defaulted, her calls and her chats just to mention but a few, in short, she pampered me like her own biological child.

To my H.O.D of Microbiology Department Dr. L.O. Okwute, I say God bless you ma, for all your encouragement. To my postgraduate coordinator Dr S.C. Ugoh and family, Daddy, you are too wonderful, you have always been a blessing to my family, and God will never forget your labor of love.

To the lecturers and staff of microbiology department, Prof. N.R. Isu, Prof. O.O. Agarry, Dr. S.S. Machunga-Mambulla, Mr. B.O. Akanbi who always calls me, Mr. Mathew Idoko a long-time friend, Mr. Giwa, Mallam Jubril, Mrs. Adelabu, Mallam Bukar and Mrs. Esther.

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

Ethical approval for the study was obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), Ahmadu Bello University Zaria, Nigeria.

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