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Research Paper

Investigating the Effects of Methanolic Leaf Extract of Annona Muricata (Sour Sop) On Snake Venom-Induced Toxicity In Female Mice: Considering Hepatic Function, Neutralizing Effect, Hemorrhagic Response And Liver Histology.

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ABSTRACT
Snakebite is an important cause of morbidity and mortality and is one of the major health problems in Nigeria. The study aims to determine the neutralizing effect of methanolic extract of Annona muricata leaves on snake venom induced toxicity, considering, venom haemorrhagic effect, and hepatotoxicity of the relative liver weight and body weight in wistar mice. Forty-seven female mice were used in the study and divided into six groups. The mice were acclamatized for 14 days. The extract administration lasted for 21 days. Group A served as the positive control. Group 2 served as the negative control and received 600mg/kg of Naja nigricollis venom only. Group 3 received 600mg/kg of Naja nigicollis venom and 100mg/kg of annona muricata extract. Group 4 received 600mg of najanigricollis venom and 200mg/kg of annona muricata extract. Group 5 received 600mg/kg of najanigricollis venom and 600mg/kg of aanona muricata leaves extract. Group 6 received 600mg/kg of najanigricollisvenon and 800mg/kg of annona muricata leaves extract. The haemorrhagic lesion was estimated after 24 hours. Values were considered significant p<0.05 and not significant: p>0.05). The result demonstrated a significant (p=0.004) increase in the AST level in-group A compared to B, groups C, D, and F had significant decrease and group E had a non-significant decrease compared to group B. The ALT results showed a non-significant (p=0.267) increase in-group A compared to B, groups C, D, and E had a non-significant decrease and group F had a significant (p=0.018) compared to group B. The ALP findings showed a non-significant

(p=0.513) increase in-group A compared to B, groups C, E, and F had a non-significant decrease and group D had a non-significant increase (p=0.588) compared to B. The result revealed a significant (p=0.007) increase in the relative liver weight in group A compared to B, groups C, D, E, and F had significant decrease compared to group B. The bodyweight result had a non-significant (p=0.836) decrease in group A compared to B, groups C, D, E, and F had a non-significant compared to group B. The result also revealed a significant (p=0.000) increase in the hemorrhagic diameter in group A compared to B, groups C, D, E, and F had significant decrease compared to group B. Histological findings of the liver reavealed that the hepotocytes and central vein are normal with mild congestion of blood vessels and inflammatory background.

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I. INTRODUCTION

Snakebite is an important cause of morbidity and mortality and is one of the major health problems in Nigeria (Faruq *et al*., 2002). It is a neglected public health problem in most of the countries in Africa (Razi *et al*., 2011). Rural populations are frequent victims of snake bites as they go about their daily food production and animal rearing activities and as they reside in the comfort of their homes. Unfortunately, many of these snakebite cases go unreported and thus do not appear in official epidemiological statistics. Health workers often have little or no formal training in the management of snakebite, and appropriate antivenom is rarely available (WHO, 2010).

According to world health organization (WHO, 2017), about 5.4 million snakebites occur each year, resulting in 1.8 to 2.7 million cases of poisonous envenomation. There are between 81,410 and 137,880 snakebite related deaths and around three times as many amputations and other permanent disabilities each year (Yemane *et al*., 2021). Most of these occur in Africa, Asia and Latin America. In Asia up to 2 million people are envenomed by snakebites each year, while in Africa an estimated 435,000 to 580,000 snakebites occur annually that need treatment. Envenoming affects women, children and farmers in poor rural communities in low- and middle-income countries. The highest burden occurs in countries where health systems are weakest and medical resources sparse (Williams *et al*., 2010).

Snake venom is a highly toxic saliva containing zootoxins that facilitates the immobilization and digestion of its prey which also provides defense against threats. Snake venom is injected by unique fangs during a bite, although some species are also able to spit venom (Bauchot, 2006).

The glands that secrete these zootoxins are a modification of the parotid salivary glands found in other vertebrates and are usually located on each side of the head, below and behind the eye, and enclosed in a muscular sheath. This venom is stored in large glands called alveoli in which it is stored before being moved by a duct to the base of a channeled or tubular fangs through which it is ejected (Halliday and Kraig, 2002; Bottrall*et al*., 2010).

Venom contains more than 20 different compounds, which are mostly proteins and polypeptides (Halliday and Kraig, 2002). The complex mixture of proteins, enzymes, and various other substances has toxic and lethal properties (Bauchot, 2006). Venom serves to immobilize prey (Mattison, 2007). Enzymes in venom play an important role in the digestion of prey (Bottrall*et al*., 2010) and various other substances are responsible for important but non-lethal biological effects (Bauchot, 2006).

Presently, antivenom immunotherapy is the only treatment available against snake envenomation. The side effects of antivenom include anaphylactic shock, pyrogen reaction and serum sickness. These symptoms are possibly outcomes of the action of non-immunoglobulin proteins present in higher concentrations in antivenom (Devi *et al*., 2002).

Medicinal plants are plants that possess therapeutic properties or exert beneficial pharmacological effect in human or animal body. Medicinal plants, also called medicinal herbs, have been discovered and used in traditional medicine practices since prehistoric times. Plants synthesize hundreds of chemical compounds for functions which includes, defense, and protection against insects, fungi, diseases, and herbivorous mammals (Gershenzon and Ullah, 2022).

Annona muricata, also known as soursop, graviola and guanabana, is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world. In Nigeria it is commonly called 'shawa shop'. Annona muricata linn is a species of the Annonaceae family that has been widely studied in the last decades due to its therapeutic potential. The medicinal uses of the Annonaceae family were reported long time ago by Billón in 1869, and since then, this species has attracted the attention due to its bioactivity and toxicity (Shaniba*et al*., 2020).

The crushed seeds are believed to have anthelmintic activities against external and internal worms and parasites. In tropical Africa, the plant is used as an astringent, insecticide and piscicide agent and to treat coughs, pain and skin diseases. In India, the fruit and flower are employed as remedies against catarrh, while the root-

bark and leaves are believed to have antiphlogistic and anthelmintic activities (Adewole and Ojewole, 2009; Watt and Breye 1962).

In Malaysia, the crushed leaf mixture of Annona muricata together with Annona squamosa and Hibiscus rosa-sinensis is used as a juice on the head to protect against fainting. In South America and tropical Africa, including Nigeria, leaves of Annona muricata are deployed as an ethnomedicine against tumors and cancer (Adewole and Ojewole, 2009). In addition, the anti-inflammatory, hypoglycemic, sedative, smooth muscle relaxant, hypotensive and antispasmodic effects are also attributed to the leaves, barks and roots of A. muricata (Mishra *et al*., 2013; Adewole and Caxton, 2006).

Ethnobotanical studies have indicated that Annona muricata fruit juice and infusions of leaves or branches have been used to treat fever (Betancur-Galvis *et al*., 1999; Dagar and Dagar, 1991), sedative, respiratory illness (Beyra et al., 2004; Kossouoh*et al*., 2007; Vandebroek et al., 2010; Waizel and Waizel, 2009), malaria (Boyom *et al*., 2011; Nguyen-Pouplin*et al*., 2007), gastrointestinal problems (Atawodi, 2011; Samuel *et al*., 2010), liver, heart and kidney diseases (Badrie and Schauss, 2010). In recent years it has become widely used for hypoglycemic (De Souza *et al*., 2011), hypotensive (De Souza *et al*., 2011; Hajdu and Hohmann, 2012; Samuel *et al*., 2010) and cancer treatments (Monigatti*et al*., 2013; Tisott*et al*., 2013).

II. METHOD

2.1 MATERIALS

Female wistar mice
Laboratory coat and gloves
Saw dusts
Centrifuge (search tech instruments, British standard) model 80-2
Thermostat oven DHG-90 23A, PEC medical, USA
Rotary evaporator (digital) TT-52 Techmel and Techmel USA
Thermostatic water bath
Beakers
Measuring cylinders
Syringes
Electronic weighing balance, M-methlar model M311l, china
Watt-man Number1 filter paper
Normal saline
Refrigerator (Nexus)
Cages
Canula
Mice feeds (non pilletized grower)
Snake venom
Methanol

2.2 METHODOLOGY

2.2.1 Location and duration of study

This study was carried out in the animal house, department of human physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Nnewi campus, Nnamdi Azikiwe University. The administration of extract to the experimental animals lasted for 21 days, acclimatization lasted for 14 days (two weeks).

2.2.2 Snake venom extraction process

The venom and a desiccant (calcium chloride) were placed into the vacuum dryer, they were covered with a layer of gauze, with the vacuum dryer sealed and exhausted. While the process was ongoing, a large number of bubbles appeared, so the extraction was suspended to prevent the bubble from spilling and was continued after a moment, the process was repeated several times until the venom was completely drained. The Snake venom was retrieved in a crystallized form of block sizes.

2.2.3 Induction of snake venom

The Indian cobra snake venom was procured in a sample bottle from the University of Maiduguri, Nigeria. The Indian cobra snake venom native preparations were given intraperitoneally (I.P) to the mice at a dose which was proportional to the weight of the animals. The volumes of preparation were identical and the same amounts were injected. The venom dose used in this study was estimated according to our preliminary experiments.

2.3 ETHICAL APPROVAL

Ethical approval was obtained from the faculty of basic medical science, college of health sciences Nnewi campus Nnamdi Azikiwe University. Mice handling and treatment conform to the guidelines of the National institute of health (NIH publication 85-23, (1985) for laboratory animal care and use.

2.4 PLANT SAMPLE COLLECTION

The leaves of soursop were harvested from a local farm in okofia, Nnewi LGA of Anambra state. The identity was confirmed by a botanist. Shade dried leaves were pulverized into fine powder using an electric blender and stored in an air-tight container for further use.

2.5 PREPARATION OF METHANOLIC EXTRACT OF *ANONNA MURICATA*

The *Annona muricata* leaves were harvested from a local farm in okofia, Nnewi LGA of Anambra state.The *Annona muricata* leaves were dried in an ambient temperature. The dried *Annona muricata* leaves were milled into a coarse form using a local grinder. Two hundred and fifty (250) gram of the *Annona muricata* leaf was macerated in 1000mls of 95% absolute ethanol (JHD Chemicals, Guangdong, China) for 48-hours. It was then filtered using a clean porcelain cloth and further filtered using Filter paper (Whatman Qualitative Filter paper, No. 1, Sigma Aldrich; WHA1001042, USA). The filtrate obtained was concentrated using a rotatory evaporator (Digital TT-52; Techmel and Techmel, USA), which was further dried using a laboratory oven (DGH-9023A, PEC MEDICAL, USA) at 45ºC into a gel-like form. The extract was preserved in a refrigerator (Nexus) for further usage. The extract procedure was done according to the method described by Attar and Abu-Zeid (2013).

2.6 Experimental animals

Experimental mice were purchased from the animal house of the Faculty of Basic medical sciences, Nnamdi Azikiwe University, Nnewi campus. The animals were housed in steel cages and kept at room temperature. The mice had no history of drug consumption, that is; they had not been used for any investigation. The mice were put on standard mice non pelletized (feed) and pure drinking water and allowed to get acclimatized for 14 days before the start of the experiment. The study was done in accordance with the guidelines for animal use of the Faculty of Basic Medical Sciences, Nnamdi Azikiwe University.

2.7 NEUTRALIZATION OF VENOM HEMORRHAGIC ACTIVITY

Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of *Annona muricata* extract with different amounts of venom. The plant-venom mixture was centrifuged at 1000rpm x 10 minutes, and 0.1ml of supernatant injected intradermally. The haemorrhagic lesion was estimated after 24 hours.

2.8 EXPERIMENTAL DESIGN

47 female mice were divided into 3 groups of different research subgroups; haemorrhagic effect subgroup, liver function test sub group, histology of the liver sub group. To check for haemorrhagic activity, 10 female mice were used, 12 female mice were used for liver function test and 12 female mice were used for the histology of the liver. 13 mice were used for acute toxicity study.

- Group A: Served as the positive control
- Group B: Served as the negative control and received 600mg/kg of *Naja nigricollis* venom only
- Group C: Received 600mg/kg of *Naja nigricollis* venom + 100mg/kg of *Annona muricata* extract
- Group D: Received 600mg/kg of *Naja nigricollis* venom + 200mg/kg of *Annona muricata* extract
- Group E: Received 600mg/kg of *Naja nigricollis* venom + 600mg/kg of *Annona muricata* extract
- Group F: Received 600mg/kg of *Naja nigricollis* venom + 800mg/kg of *Annona muricata* extract

2.9 ACUTE TOXICITY OF *NAJA NIGRICOLLIS VENOM*

The median lethal dose (LD50) of Naja-Naja snake venom was carried out in the department of physiology, faculty of basic medical sciences, Nnamdi Azikiwe University, okofia campus. This was determined using Dietrich lorke (1983). In this study, 13 rats were used. They received the extract via oral route and it was carried out in two phases.

- ***PHASE 1***
- Group 1 received 10mcg/kg per mice
- Group 2 received 100mcg/kg per mice
- Group 3 received 1000mcg/kg per mice
- The animals were monitored for 24hours for morbidity and mortality. The rats remained normal after 24hours of observation in phase 1. Then, the study proceeded to the second phase where four rats were employed for the study comprising one rat per group.
- ***PHASE 2***
- Group 1 received 1200mcg/kg per mice
- Group 2 received 1600mcg/kg per mice
- Group 3 received 2900mcg/kg per mice

- Group 4 received 5000mcg/kg per mice
- The animals were monitored for another 24 hours for morbidity and motility

PHASE	DOSE	DEATH	OBSERVATION
1	10mc/kg	0/3	The animal remained normal
	100mcg/kg	0/3	The animals were weak but survived
	1000mcg/kg	0/3	The animals were weak but survived
2	1200mcg/kg	0/1	The animal was weak but survived
	1600mcg/kg	1/1	The animal was weak and died within 24 hours
	2900mcg/kg	1/1	The animal was weak and died within 24 hours
	5000mcg/kg	1/1	The animal was weak and died within 12 hours.

Table1 showing the Acute Toxicity Study of Snake venom (Naja nigricollis)

- LD50= √a x b
- A= Maximum dose with 0% mortality
- B= minimum dose with 100% mortality
- LD50=√1200 x 1600= 1385.6mcg/kg
- LD50 of najanaja snake venom is 1385.6mcg/kg

2.10 HISTOPATHOLOGICAL PROCEDURE

Tissues (liver) was fixed in 10% formal saline and were dehydrated in four (4) concentrations of Isopropyl alcohol, i.e., 70%, 80%, 90%, 100% for 1hr each and then cleared in xylene before embedded in molten paraffin wax to remove the isopropyl alcohol. Micro sections of 5micrometer using Leica RM 212 Rt. Rotary Microtome, tissues were stained using Haematoxylin and Eosin (H&E) to demonstrate general tissue structure. Tissues sectioned were examined and interpreted using Leica DM 750 binocular microscope with photomicrographic facilities and then photomicrographed by a histopathologist (Ahmed, 2016).

2.11 STATISTICAL ANALYSIS

Data were analyzed using Statistical Package for Social Sciences (SPSS version 25). An inferential statistics (ANOVA) were used, and values were presented in Mean and Standard error in mean (Mean and SEM). Data for anti-inflammatory, Lethargic and hematological parameterswere analyzed using one-way ANOVA, followed by Post hoc LSD. While bodyweight was analyzed using Student Dependent T-test. Values were considered significant at $P<0.05$.

III. RESULTS

Table 3.1 Effect of methanolic extract of *Anonna muricata* leaf on AST, ALT, and ALP on *Naja nigricollis*induced hepatotoxicity

Groups	Aspartate aminotransferase (UI/L) Mean±SEM	Alanine aminotransferase (UI/L) Mean±SEM	Alkaline phosphatase (UI/L) Mean±SEM
Group A (Positive control)	25.00±5.03 ^{a}	27.67±1.45 ^{b}	620.37±81.69 ^{b}
Group B (600mg/kg of *Naja nigricollis*)	40.67±4.25	35.33±6.88	682.37±14.29
Group C (600mg/kg of *Naja nigricollis* + 100mg/kg of MAM)	24.67±1.20 ^{a}	23.33±4.63 ^{b}	619.87±37.01 ^{b}
Group D (600mg/kg of *Naja nigricollis* + 200mg/kg of MAM)	25.67±1.85 ^{a}	25.33±3.67 ^{b}	830.95±45.04 ^{b}
Group E (600mg/kg of *Naja nigricollis* + 600mg/kg of MAM)	33.00±2.08 ^{b}	30.67±5.48 ^{b}	631.47±112.11 ^{b}
Group F (600mg/kg of *Naja nigricollis* + 800mg/kg of MAM)	30.00±2.64 ^{a}	17.33±3.92 ^{a}	514.74±1.38 ^{b}
F-VALUE	**3.918**	**1.773**	**2.298**

Data was analyzed using ANOVA followed by post Hoc LSD, values were considered significant $p<0.05$. MAM: methanolic extract of *Anonna muricata* leaf, SEM: standard error of mean, [a] (significant: $p<0.05$) [b] (not significant: $p>0.05$).

Table 3.1 result demonstrated a significant ($p=0.004$) increase in the AST level in-group A compared to B, groups C, D, and F had significant decrease ($p=0.004$, $p=0.006$, $p=0.034$) and group E had a non-significant ($p=0.111$) decrease comparedto group B. The ALT results showed a non-significant ($p=0.267$) increase in-group A compared to B, groups C, D, and E had a non-significant ($p=0.093$, $p=0.155$, $p=0.492$) decrease and group F had a significant ($p=0.018$) compared to group B. The ALP findings showed a non-significant ($p=0.513$) increase in-group A compared to B, groups C, E, and F had a non-significant decrease ($p=0.507$, $p=0.131$, *0.128*) and group D had a non-significant increase ($p=0.588$) compared to group B.

Table 3.2 Effect of methanolic extract of *Anonna muricata* leaf on relative liver weight andbodyweight on *Naja nigricollis*induced hepatotoxicity

Groups	Relative liver weight (g/%)	Body weight (g)
	Mean±SEM	**Mean±SEM**
Group A (Positive control)	0.99±0.10[a]	25.90±1.09[b]
Group B (600mg/kg of *N. nigricollis*)	1.64±0.30	25.60±0.64
Group C (600mg/kg of *N. nigricollis*+ 100mg/kg of MAM)	1.18±0.11[a]	24.50±2.32[b]
Group D (600mg/kg of *N. nigricollis* + 200mg/kg of MAM)	1.09±0.06[a]	24.03±0.53[b]
Group E (600mg/kg of *N. nigricollis s*+ 600mg/kg of MAM)	1.16±0.03[a]	24.92±0.54[b]
Group F (600mg/kg of *N. nigricollis* + 800mg/kg of MAM)	1.08±0.02[a]	24.36±0.79[b]
F-Value	**0.076**	**0.507**

Data was analyzed using ANOVA followed by post Hoc LSD, values were considered significant $p<0.05$. MAM: methanolic extract of *Anonna muricata* leaf, SEM: standard error of mean, [a] (significant) [b] (not significant).

Table 3.2 result revealed a significant ($p=0.007$) increase in the relative liver weight in-group A compared to B, groups C, D, E, and F had significant ($p=0.040$, $p=0.017$, $p=0.032$, $p=0.016$) decrease compared to group B. The bodyweight result had a non-significant ($p=0.836$) decrease in-group A compared to B, groups C, D, E, and F had a non-significant ($p=0.450$, $p=0.286$, $p=0.588$, $p=0.328$) compared to group B.

Table 3.3 Effect of methanolic extract of *Anonna muricata* leaf on hemorrhagic diameter on *Naja nigricollis*induced hemotoxicity

Groups	Hemorrhagic diameter (cm)
	Mean±SEM
Group A (Positive control)	0.99±0.01[a]
Group B (600mg/kg of *N. nigricollis*)	3.00±0.11
Group C (600mg/kg of *N. nigricollis* + 100mg/kg of MAM)	1.20±0.11[a]
Group D (600mg/kg of *N. nigricollis* + 200mg/kg of MAM)	1.60±0.11[a]
Group E (600mg/kg of *N. nigricollis* + 600mg/kg of MAM)	1.83±0.08[a]
Group F (600mg/kg of *N. nigricollis* + 800mg/kg of MAM)	0.85±0.07[a]
F-Value	**68.602**

Data was analyzed using ANOVA followed by post Hoc LSD, values were considered significant $p<0.05$. MAM: methanolic extract of *Anonna muricata* leaf, SEM: standard error of mean, [a] (significant) [b] (not significant).

Table 3.3 result revealed a significant ($p=0.000$) increase in the hemorrhagic diameter in-group A compared to B, groups C, D, E, and F had significant decrease ($p=0.000$, $p=0.000$, $p=0.000$, $p=0.000$) compared to group B.

HISTOPATHOLOGICAL REPORT

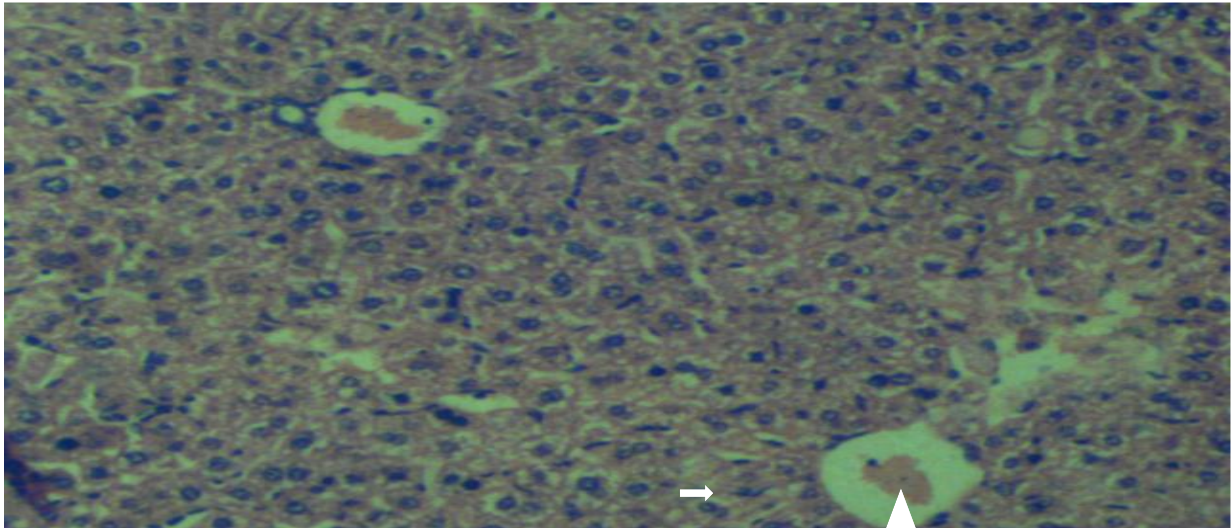
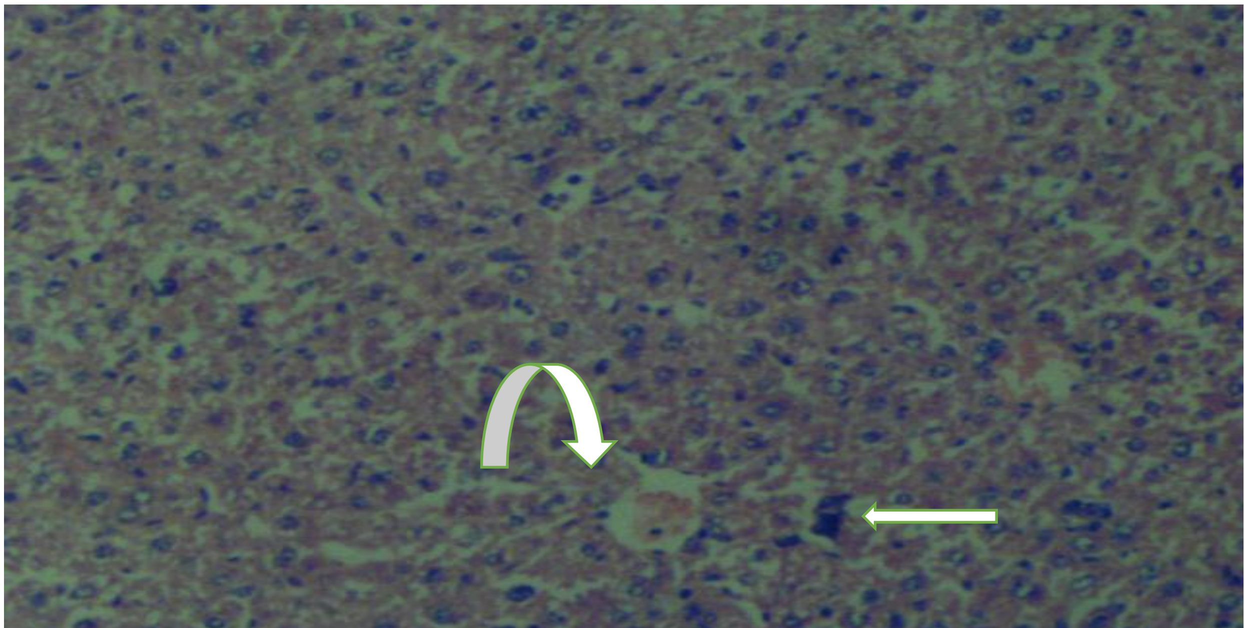


Plate 3.1 Group A (Positive control): Photomicrograph of liver tissue shows morphology consistent with liver histology. The hepatocytes (arrow) and central vein (arrowhead) are normal (H&E).

Plate 3.2. Group B: Photomicrograph of liver tissue shows morphology consistent with liver histology. The



hepatocytes (arrow) and central vein (arrowhead) are normal with mild congestion of blood vessels and inflammatory background (curved arrow) (H&E).

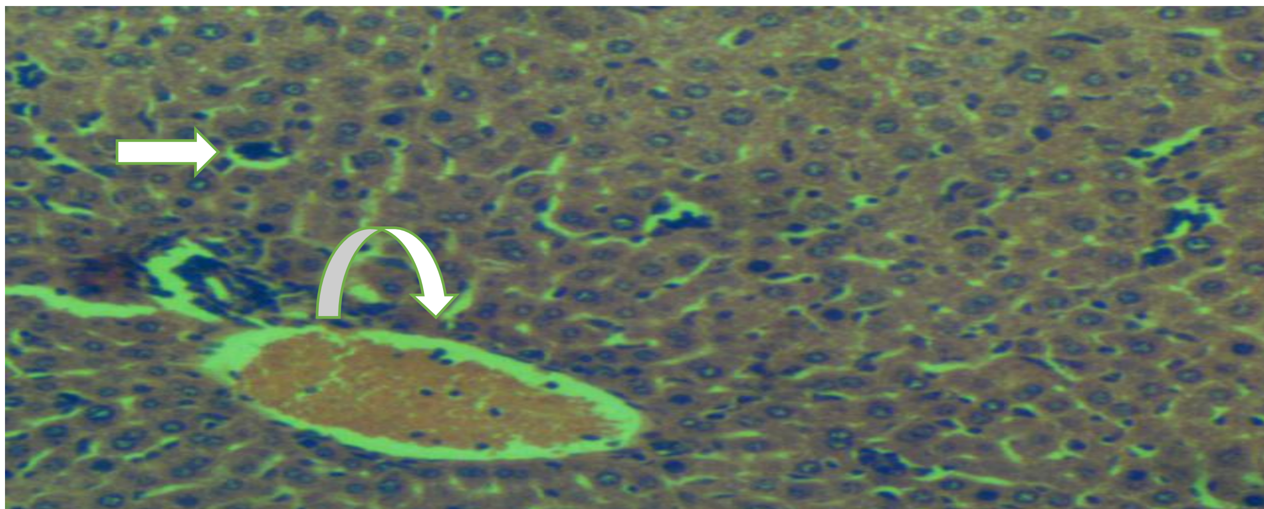


Plate 3.3. Group C: Photomicrograph of liver tissue shows morphology consistent with liver histology. The hepatocytes (arrow) are normal while the central vein show mild congestion (arrowhead) (H&E).

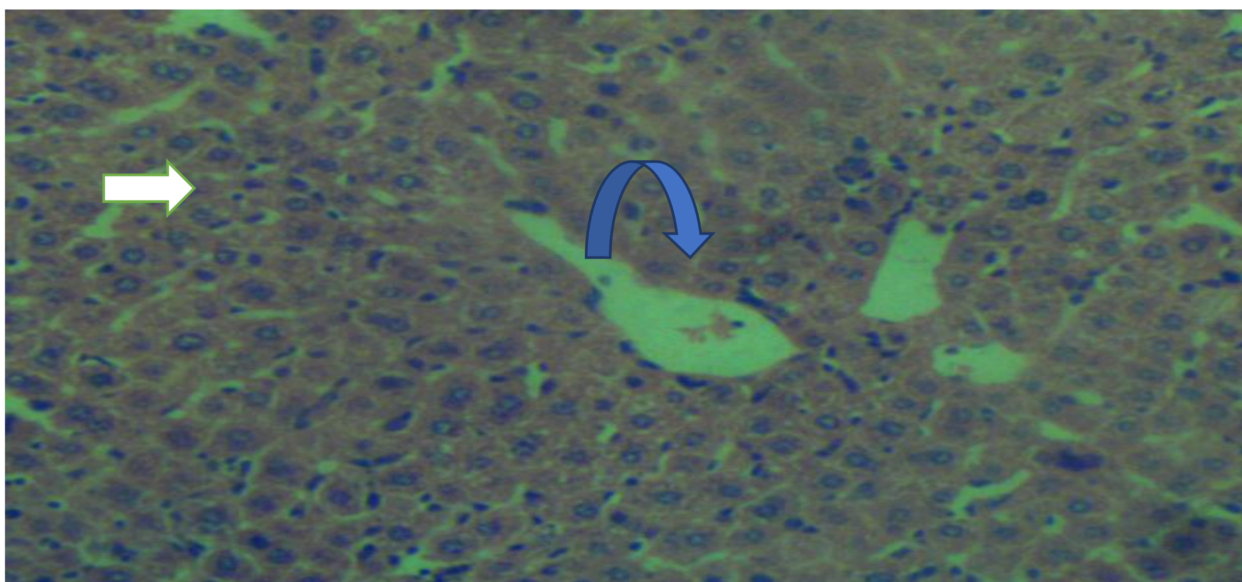


Plate 3.4. Group D: Photomicrograph of liver tissue shows morphology consistent with liver histology. The hepatocytes (arrow) and sinusoid (arrowhead) are normal (H&E).

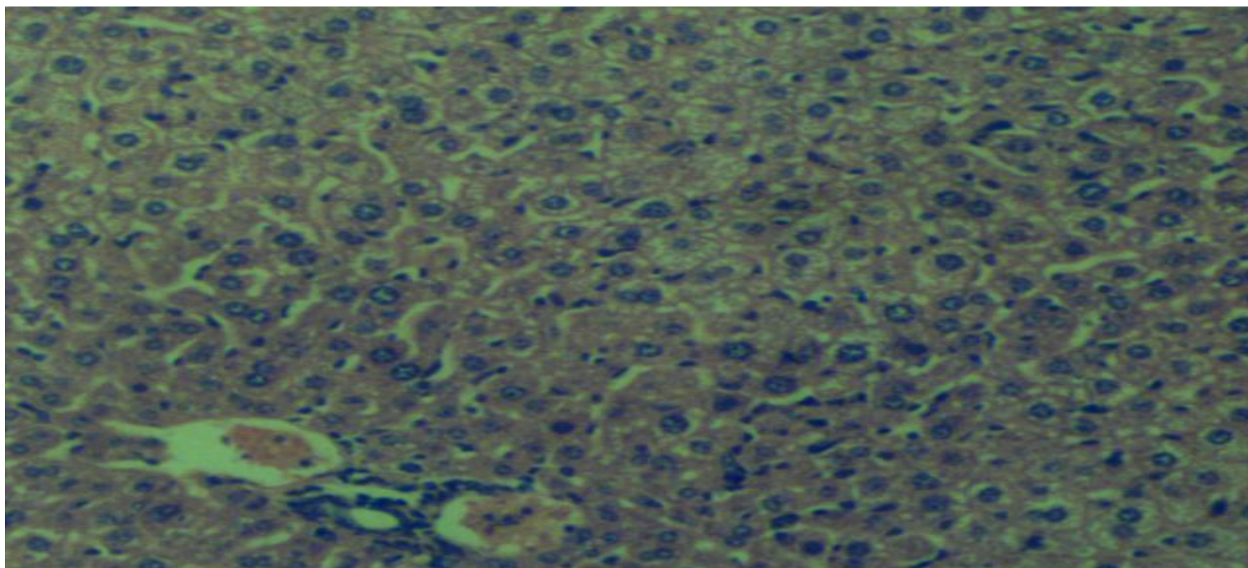


Plate 3.5. Group E: Photomicrograph of liver tissue shows morphology consistent with liver histology. The hepatocytes (arrow) and central vein (arrowhead) are normal (H&E).

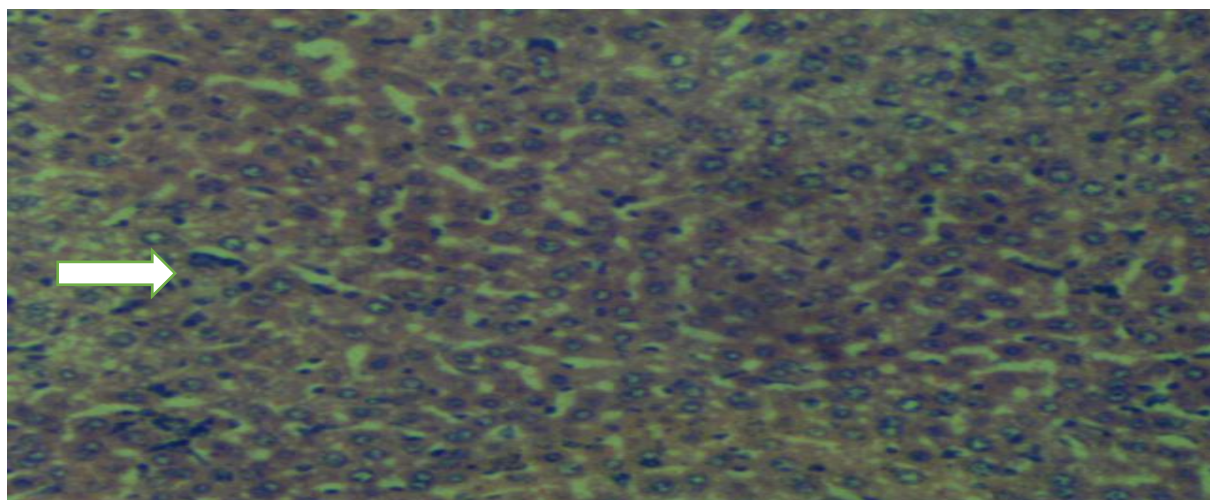


Plate 3.6. Group F: Photomicrograph of liver tissue shows morphology consistent with liver histology. The hepatocytes (arrow) and central vein (arrowhead) are normal (H&E).

IV. Discussion

Snakebite is an important cause of morbidity and mortality and is one of the major health problems in Nigeria (Faruq *et al*., 2002). The black-necked spitting cobra *Naja nigricollis*is considered as one of the most diverse and wide spread species of cobras in the world (Westhoff *et al., 2005).* Liver injury is among the common and most serious symptoms of cobra snake envenoming (Adzu*et al.,* 2005).

Spontaneous bleeding and coagulation disturbances are some of the haematological effects of *Naja nigricollis*in patients (Warrell *et al.,* 1976). AST and ALT enzymes are markers for cellular damage and ALT enzyme is essentially present in hepatocytes (Abdulrahman *et al.,*2015). These enzymes are of importance in assessing and monitoring liver inflammation and necrosis which result in the release of both enzymes in circulation due to increased permeability of the cell membrane or breakdown of the cells (Abdel *et al*., 2013).

Annona muricata possess anti-inflammatory, hepatoprotective, gastro protective, neurotic effect and wound healing, anti-oxidant and anti-tumoral activities (*Coria et al*., 2016).

The result demonstrated a significant increase in the AST level in-group A compared to B, groups C, D, and F had significant decrease and group E had a non-significant decrease compared to group B. The ALT results showed a non-significant increase in-group A compared to B, groups C, D, and E had a non-significant decrease and group F had a significant compared to group B. The ALP findings showed a non-significant increase in-group A compared to B, groups C, E, and F had a non-significant decrease and group D had a non-

significant increase compared to group B. The findings correlate with similar reports in the study outcome of James *et al.,* (2013), which notes that animals inoculated with cobra venom showed an increase in AST and ALT activities for *Naja nigricollis*in mice.

This study also revealed a significant increase in the relative liver weight in-group A compared to B, groups C, D, E, and F had significant decrease compared to group B. The bodyweight result had a non-significant decrease in-group A compared to B, groups C, D, E, and F had a non-significant compared to group B. This is in line with the research carried out by Babafemi *et al.,* (2022), which shows that body weight gain of normal control mice was significantly (P<0.05) higher compared to the envenomed mice.

Furthermore, this study demonstrated a significant increase in the hemorrhagic diameter in-group A compared to B, groups C, D, E, and F had significant decrease compared to group B. Hemotoxic venom disrupts blood clotting, degeneration of organs, tissue damage throughout the body and massive internal bleeding (Neellohit, 2022). The histology of the liver shows a normal histology with no congestion of blood cells and inflammatory background.

V. Conclusion

It can be concluded that the methanolic leaves extract of Annona muricata may have an ameliorative effect and hepatic protective effect on the liver enzymes. In the hemotoxicity hemorrhagic diameter findings, the extract may have an anti-hemorrhagic activity. The mechanism of action of these findings occurs as a result of the presence of flavonoids present in the extract.

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