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Effects of co-administration of *Naja melanoleuca* and *Bitis arietans* venoms on biochemical, hematological and histopothological indices at different concentrations

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Abstract:
Background*: Snakebite is one of the neglected tropical diseases that affects most rural populations with increased mortality. The commercially available antivenoms are expensive and there is less effort to the production of antivenom from indigenous antivenoms that can neutralize various forms of venoms. This study investigated the effects of co-administration of different concentrations of Naja melanoleuca and Bitis arietans venoms on biological parameters.*
Materials and Methods*: The chemicals and reagents used in this study were of analytical grade and established methods were strictly followed.*
Results*: The results revealed significant elevations ($p < 0.05$) in alanine aminotransferase, alkaline phosphatase (ALT, ALP), total and conjugated bilirubin levels. The levels of urea and creatinine in immunized groups showed insignificant increase ($p > 0.05$) compared to the control. On the other hand, the hematological parameters indicated significant reductions ($p < 0.05$) in packed cell volume (PCV) across immunization weeks. Similarly, significant reductions ($p < 0.05$) in absolute platelet counts (APC) were observed at week 4, week 6 and week 8 respectively compared to the control. In addition, significant increase ($p < 0.05$) was observed in white blood cell (WBC) levels at second and sixth weeks. Furthermore, the electrolytes bicarbonate, sodium, potassium and chloride levels fluctuated compared to the control, however, no significant changes observed except for potassium and chloride levels varied significantly across the weeks. Moreover, the histopathology of lung and heart tissues revealed pathologic changes, while liver and kidney tissues showed no effect.*
Conclusion: *It could be concluded that co-administration of N. melanoleuca and B. arietans venoms at different concentrations triggered immune and inflammatory responses.*
Key Word*: Snakebites, Naja melanoleuca, Bitis arietans, antivenom, alanine aminotransferase, alkaline phosphatase*

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I. Introduction

Snakebite is a serious global public health problem considered as a neglected tropical disease affecting almost all the rural populations. It has been estimated that about 5 million people are bitten by a snake annually, with 2 million being envenomed, 81,000 to 138,000 mortality rate and 400,000 with permanent disabilities [1,2]. Interestingly, sub-Saharan Africa have an estimated range of 435,000 to 580 snakebite envenomation annually, while the West African region contributes a substantial proportion of this burden, often characterized by a high frequency of bites from venomous species such as Echis ocellatus and Naja species [3]. In Nigeria, snake bite affects almost all the rural populations of the six geographic regions, however, it mostly affects the rural population of the savannah region [4]. However, the distribution of snakes largely depends on the vegetation and topography, with carpet viper (Echis ocellatus, family Viperidae) dominating in the rocky savanna region of the middle belt and the far north [5]. The puff adder (Bitis arietans, family Viperidae) is also found in these regions [5,6]. On the other hand, the spitting cobra (Naja nigricollis) is the most common and widely distributed African cobra in the savannah terrain. While the forest cobra (Naja melanoleuca) and green mambas (Dendroaspis spp) largely inhabit the southern forests of Nigeria [7].

The *N. melanoleuca's*, (forest cobra) venom is a heterogeneous venom compositions consist of bioactive components including peptides, proteins, enzyme and neurotoxins, phospholipase A (PLA), metalloproteinases, and hyaluronidases [8,9,10]. The venom of *Bitis arietans* contains bioactive molecules that target multiple physiological systems. The major bioactive molecules include snake venom metalloproteinases

(SVMPs), phospholipase A (PLA), serine proteases, L-amino acid oxidases, disintegrins, and various peptides [10,11,12]. Envenomation from these snake species can cause cytotoxic and neurotoxic effects, resulting in clinical manifestations such as bleeding, edema, paralysis, blindness, and other chronic clinical problems such as the cancerous transformation of bite wounds [13]. In addition, SVMPs are responsible for hemorrhage, vascular damage, and local tissue necrosis by degrading extracellular matrix proteins and disrupting endothelial integrity [14]. PLA enzymes also contribute to myonecrosis and inflammation, while serine proteases interfere with blood coagulation pathways, causing either excessive bleeding or thrombosis depending on dose and exposure [15,16]. Polyvalents antivenoms that can neutralize various kinds of venoms could be crucial for effective management of envenomation where the biting species is unidentified [17]. Thus the process of producing polyvalent antivenoms may require controlled immunization of host animals with venoms co-exposure that can elicit hematological and biochemical changes as a result of combined toxicity. Based on foregoing, the intravenous administration of antivenom is the only specific treatment to counteract snake envenoming and yet, less effort is made to domesticate the production of the antivenom. In addition, the importation of the antivenoms is often very expensive. Thus, to minimize death from snakebites, constant and readily available indigenous antivenom production, such as in this study, can serve as the basis for the standard procedure for antivenom production in Nigeria.

II. Material And Methods

Chemicals, Equipment and Reagents:

Paraplast, Plastic tissue cassettes, Forceps, Leica Embedding stage, Paraffin wax, embedding mould, Forceps, sterile surgical blade, EDTA, plain and universal containers, syringe and needle, Cassette scraping knife,10% Neutral buffered formalin, ethanol, Xylene Bursen burner, Microhaematocrit reader, Adjuvants (Freund's complete and incomplete).

Experimental Animals:

Nine (9) New Zealand white rabbits (weighing 2- 2.5 kg, 3-month-old) and Wistar strain albino rats (weighing 175- 180g) were obtained from the Faculty of Veterinary Medicine, University of Maiduguri, Borno State, Nigeria. The rabbits were allowed to acclimatize for 10 days before the immunization protocol. Animal care and handling complied with the National Regulations for Animal Research as well as University of Maiduguri Committee on Animal Use and Care (ethical clearance number FP/03/01/2025). The rabbits were evaluated for common regional rabbit diseases such as *coccidiosis, mastitis*, ear canker, skin mange as well as pneumonia under the strict supervision of a veterinary technician.

Snake Venom Collection and Storage:

Three snakes were captured across Zaria, Kaduna State, Nigeria and identified by a Zoologist at Gombe State University, Gombe State. The snakes were then milked manually by grasping firmly at the back of the head and applying gentle pressure along the temporal region on the head. This make the venom released into a clean glass container which was immediately stored at 4^{o}C for lyophilization. The sample was then lyophilized and kept for further analysis.

Rabbits Immunization using the Venom:

The immunization of the rabbits was conducted at 14 days intervals. For the first immunization, 15 µg of each venom (total = 30 µg) per kilogram was used. For the secondary immunization (day 14th), 60 µg was used while the subsequent immunizations received 120 µg. Freund's Complete Adjuvant (1:1 Adjuvant: Venom ratio) was used for the first immunization. For the subsequent immunizations, 1:4 adjuvant/PBS was used. The primary site for the immunization on the rabbit was the dorsal area of each rabbit. The injection were given at 5 different locations, 2 cm apart [7]. The immunization was carried out five times exactly and lasted for 56 days. Blood samples were collected: Pre-immunization and post-immunization (at two weeks intervals).

Determination of Hematological and Biochemical Parameters:

Biuret method of serum total protein determination was employed in this assay as described by [18]. Albumin was determined using Bromcresol Green (BCG) method as described by [19]. The globulin concentration was obtained by subtracting albumin values from the total protein while the albumin/globulin ratio was obtained by dividing the albumin value by the calculated globulin value. Alkaline Phosphates (ALP), Alanine amino transferase, Aspartate amino transferase activity was determined using spectrophotometric method. The Red Blood Cell (RBC) counts, total White Blood Cell (WBC) counts, hemoglobin (Hb) concentration and Packed Cell Volume (PCV) parameters were determined as describe in [20].

Histopathological Examination:

The Histopathological examination of tissues was performed according to standard procedures of [21]. Briefly, tissues were collected (heart, kidney, liver, and lungs) and immediately fixed in 10% neutral-buffered formalin for 48 hours. The samples were dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax using a Leica TP1020 tissue processor. Sections of 5 µm thickness were cut using a rotary microtome (Mission model) and mounted on glass slides. Slides were stained with hematoxylin and eosin (H and E), dehydrated, cleared, and permanently mounted in DPX. Histological changes were examined under a light microscope, and images were captured for documentation and interpretation.

Statistical analysis:

All data were presented as the mean ± standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons. Statistical analysis was conducted in SPSS software package (SPSS for Windows, version 23, IBM Corporation, NY, USA). Mean values were considered significantly different at $P < 0.05$.

III. Result

Biochemical, and hematological indices as well as the histopathology of some vital organs were examined for the period of eight weeks following the co-administration of the venoms at different concentrations (30µg, 60µg and 120µg). The packed cell volume (PCV) levels were measured for the each sample. A significant difference ($p < 0.05$) was observed in all of the post-immunization samples compared to the control (Pre-treatment) (Figure 1).

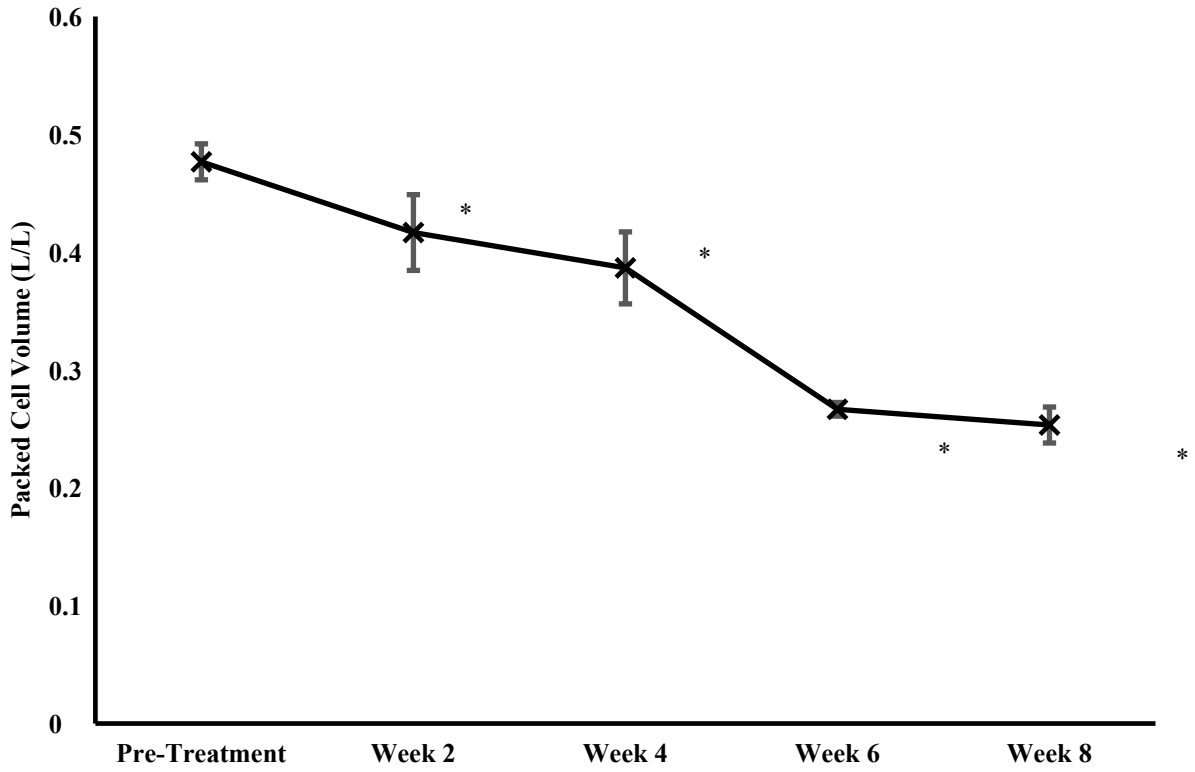

Figure 1: Packed Cell Volume (PCV) levels. Values are means ± standard deviation (n = 3). Means marked with (*) are significantly different compared to the normal control group ($p < 0.05$; ANOVA).

The absolute platelet count showed significant differences ($p < 0.05$) in the fourth, sixth and eighth weeks compared to the control (Pre-immunization) (Figure 2).

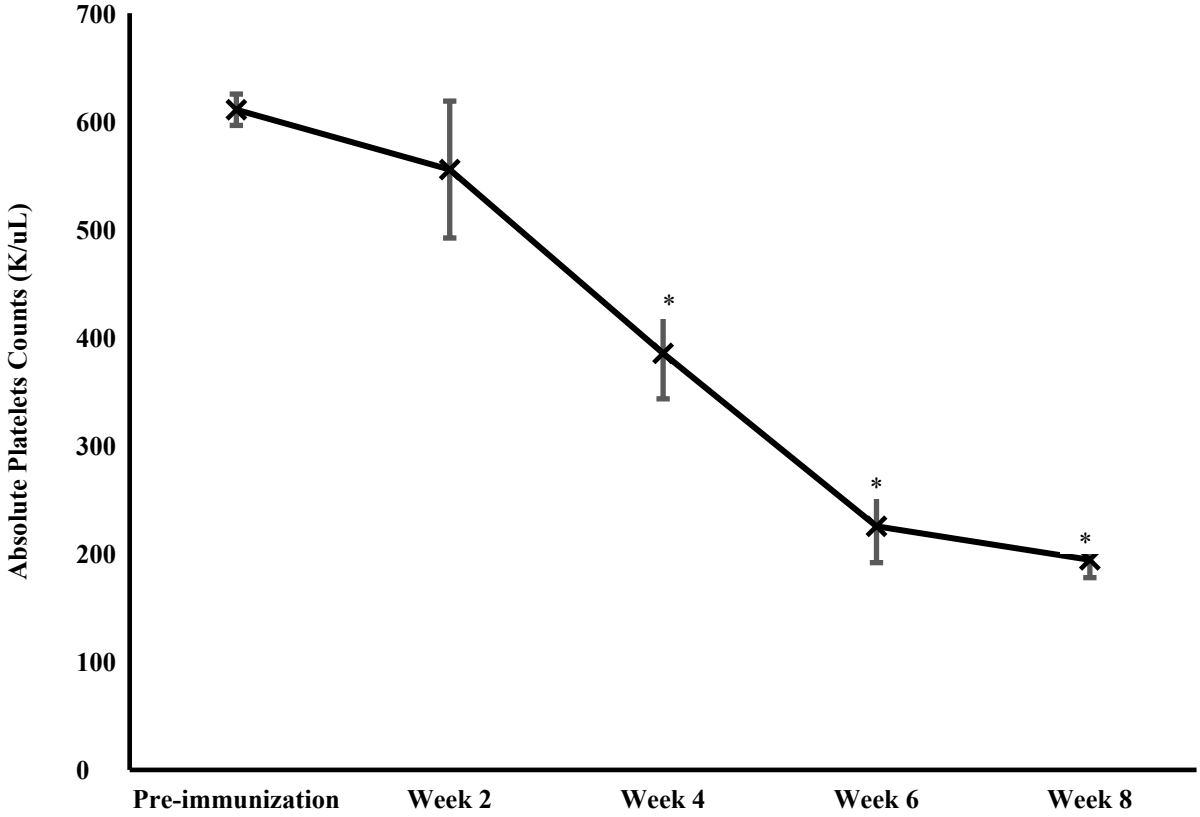

Figure 2: Absolute Platelets Counts (APC) levels. Values are means ± standard deviation (n = 3). Means marked with (*) are significantly different compared to the normal control group ($p < 0.05$; ANOVA)

The white blood cells (WBC) levels at different immunization intervals showed significant differences ($p < 0.05$) at the first and third immunization (2nd and 6th weeks) compared to the control (Figure 3).

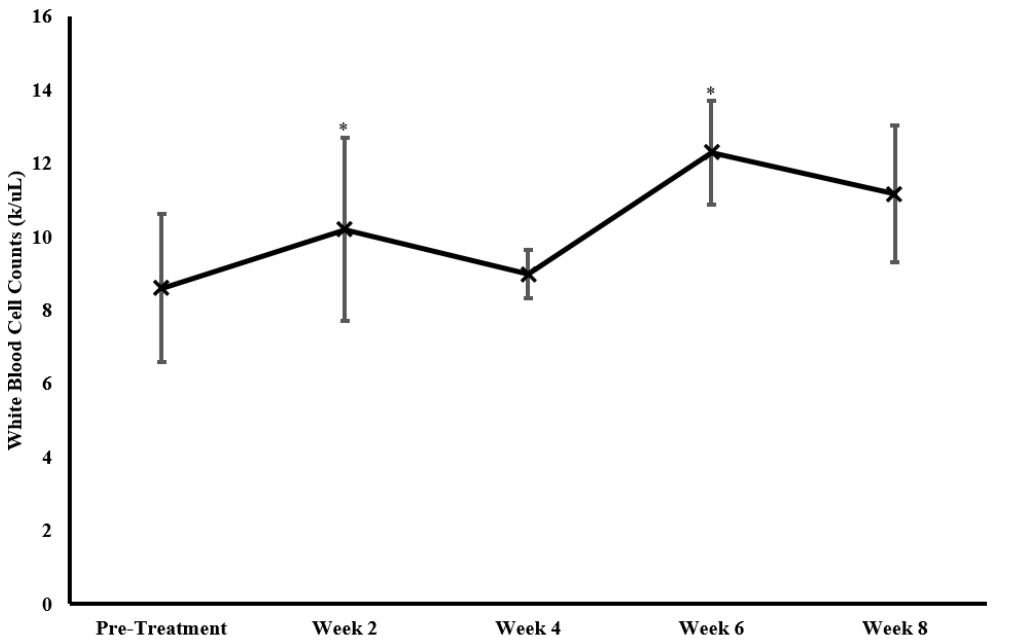

Figure 3: White blood cells (WBC) levels. Values are means ± standard deviation (n = 3). Means marked with (*) are significantly different compared to the normal control group ($p < 0.05$; ANOVA)

Table 1 shows various biochemical parameters in the kidney and liver of the experimental rabbits. The urea levels varied across groups, with means ranging from 5.08 ± 1.23 to 10.56 ± 8.79 mg/dL. The statistical analysis revealed a significant difference across weeks compared to the control (week 6, $p = 0.0205$). Similarly, creatinine concentrations ranged widely between 97.00 ± 22.46 and 183.70 ± 158.10 µmol/L, with all weeks showing insignificant differences compared to the control ($p = 0.1583$). The total bilirubin levels ranged from 13.67 ± 1.23 to 18.22 ± 0.97 µmol/L. Significant differences were observed across the weeks compared to the normal control (p = 0.0003). The conjugated bilirubin on the other hand, varied between 7.00 ± 1.87 and 10.11 ± 1.27 µmol/L, with week 4 and week 6 showing significant differences compared to the control (p = 0.0001). In addition, the liver enzyme activities (ALT, AST, ALP) also showed varying levels. ALT activity ranged from 11.33 ± 1.41 to 14.89 ± 3.18 U/L, with significant intergroup variations noted in week 6 (p = 0.0185), compared to the control. AST activity varied from 17.33 ± 2.83 to 21.00 ± 4.80 U/L, with insignificant differences across all groups compared to the control (p = 0.119). Similarly, The ALP levels showed the widest range, between 30.22 ± 1.48 and 39.67 ± 7.52 U/L, with significance differences across the weeks (p = 0.0002). Furthermore, protein profile markers, such as albumin (ALB), total protein (TP), and hemoglobin (HB) showed that; for albumin, the values ranged between 33.00 ± 1.00 and 34.11 ± 3.33 g/L, with significant differences across the weeks compared to the control (p = 0.0144). The total protein levels on the other hand, fluctuated between 55.33 ± 3.61 and 58.00 ± 3.57 g/L, with significant differences observed across the weeks (p = <0.0001). While the hemoglobin levels varied between 19.00 ± 1.87 and 20.22 ± 1.09 g/dL, with insignificant (p = 0.2605) compared to the control and others showing marginal or not significant changes (p = 0.1432, 0.0286, 0.0513).

Table 1: Biochemical parametrs across the weeks following the co-administtration ***Naja melanoleuca*** **and** ***Bitis arietans*** **venoms**

Parameter	Control	Week 2	Week 4	Week 6	p-value
Urea (mg/dL)	4.14 ± 0.65^{a}	10.56 ± 8.79^{ab}	5.08 ± 1.23^{ab}	6.06 ± 1.39^{b}	0.11
Creatinine (µmol/L)	89.78 ± 2.64^{a}	183.70 ± 158.10^{a}	108.90 ± 30.17^{a}	97.00 ± 22.46^{a}	0.1583
Total Protein (g/L)	63.56 ± 4.28^{a}	58.00 ± 3.57^{b}	56.56 ± 1.67^{c}	55.33 ± 3.61^{d}	<0.0001
Haemoglobin (g/dL)	20.00 ± 1.23^{a}	19.00 ± 1.87^{a}	20.22 ± 1.09^{a}	20.00 ± 1.23^{a}	0.2605
Albumin (g/L)	37.00 ± 3.87^{a}	34.11 ± 3.33^{ab}	33.00 ± 1.00^{b}	33.56 ± 0.88^{b}	0.0144
AST (U/L)	18.22 ± 2.54^{a}	17.33 ± 2.83^{a}	18.22 ± 2.44^{a}	21.00 ± 4.80^{a}	0.119
ALT (U/L)	11.33 ± 1.41^{a}	12.44 ± 1.13^{ab}	13.56 ± 2.88^{ab}	14.89 ± 3.18^{b}	0.0185
ALP (U/L)	28.00 ± 1.73^{a}	30.22 ± 1.48^{ab}	34.44 ± 6.69^{b}	39.67 ± 7.52^{b}	0.0002
Total Bilirubin (µmol/L)	13.67 ± 1.23^{a}	16.67 ± 1.87^{b}	18.22 ± 0.97^{c}	17.00 ± 3.24^{d}	0.0003
Conjugated Bilirubin (µmol/L)	8.11 ± 1.05^{a}	7.00 ± 1.87^{a}	9.89 ± 1.54^{b}	10.11 ± 1.27^{b}	0.0001

Values are means ± standard deviation .Values with different superscripts within the same row differ significantly at $p < 0.05$.

Similarly, there were no significant changes observed in the levels of bicarbonate, sodium, potassium and chloride ($HCO3^{+}$ Na^{+} K^{+} and Cl^{-}) except for K^{+} (P= 0.0002) and Cl^{-} (p = 0.0017) where there levels varied significantly across the weeks (Table 2).

Table 2: Electrolytes levels following the co-administration of ***Naja melanoleuca*** **and** ***Bitis arietans*** **venoms**

Parameter	Control	Week 2	Week 4	Week 6	p-value
$HCO3^{+}$	21 ± 1^{a}	19.33 ± 2.08^{a}	20.67 ± 1.53^{a}	20.33 ± 1.53^{a}	0.6202
Na^{+}	134.1 ± 0.78^{a}	131.6 ± 1.67^{a}	132.2 ± 5.72^{a}	129.7 ± 1.32^{a}	0.0949
K^{+}	5.222 ± 0.58^{a}	4.3 ± 1.11^{b}	6.722 ± 0.51^{c}	5.011 ± 0.40^{ab}	0.0002
CL^{-}	98.22 ± 3.46^{a}	92.44 ± 8.50^{b}	110.7 ± 2.92^{c}	102.4 ± 2.88^{ab}	0.0017

Values are means ± standard deviation. Values with different superscripts within the same row differ significantly at $p < 0.05$.

Similarly, histopathological examination in immunized group revealed myocardial damage and necrosis compared with the control with normal myocardium hiving intact branching muscle fibers, indicating clear cellular architecture without any signs of injury or degeneration (Figure 4).

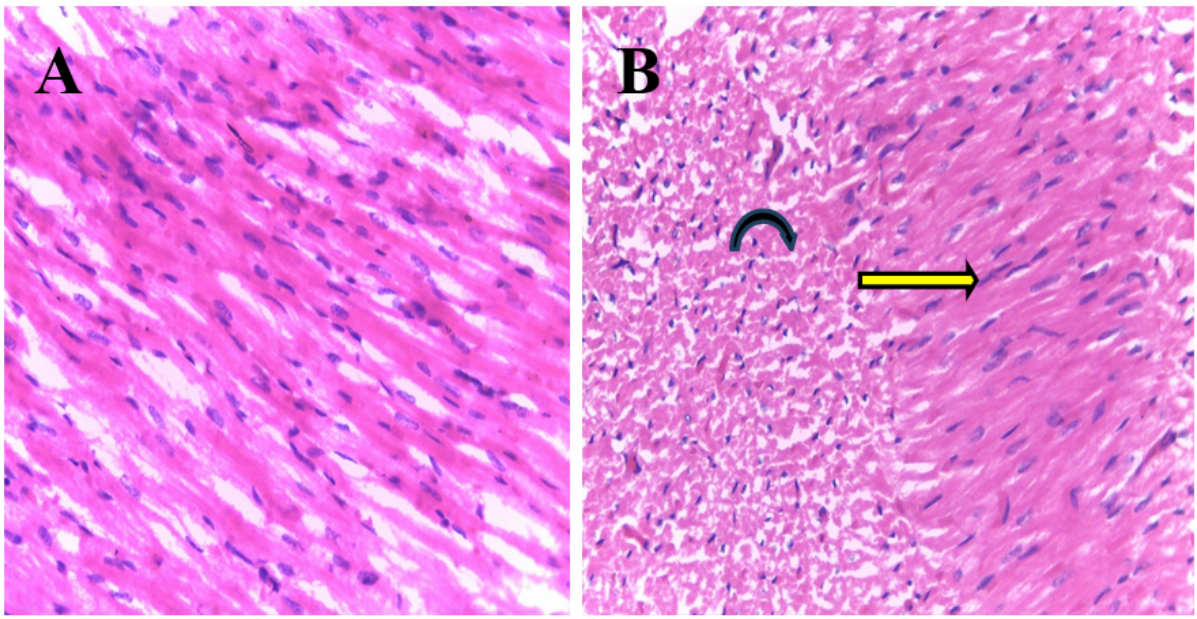

Figure 4: Photomicrograph section of heart muscle tissue composed of branching muscle fiber (A) in control group. The section show heart muscle tissue composed of branching muscle fiber with abrupt area of tissue damage (curved arrow) likely death by apoptosis. The cells show pyknotic nuclei and shruncken cytoplasm. Yellow arrow show remnant of normal tissue (B).

Interestingly, there were no effects observed in the kidney and liver of the immunized group compared to the control (Figure 5).

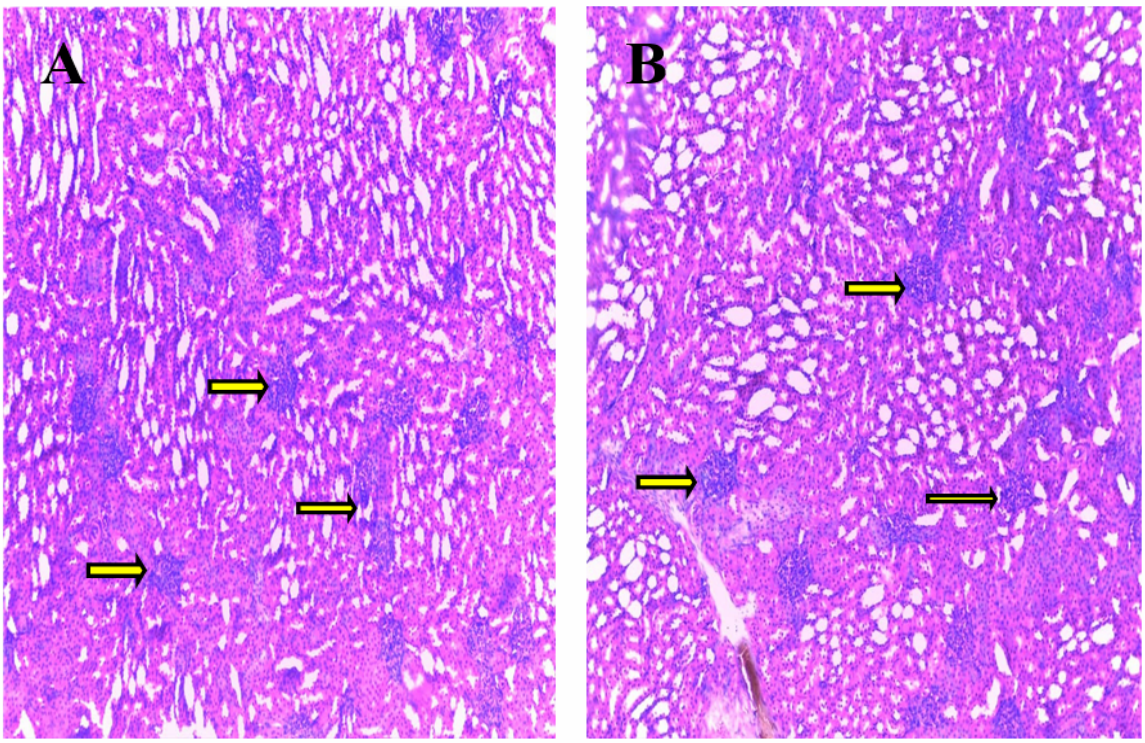

Figure 5 A: Photomicrograph of renal tissue from control rabbit displaying preserved renal parenchyma with no pathological alterations. B Photomicrograph of renal tissue from venom-exposed rabbit showing normal glomerular structure and intact tubular arrangements.

In Figure 6, there were no effects observed in the kidney and liver of the immunized group compared to the control.

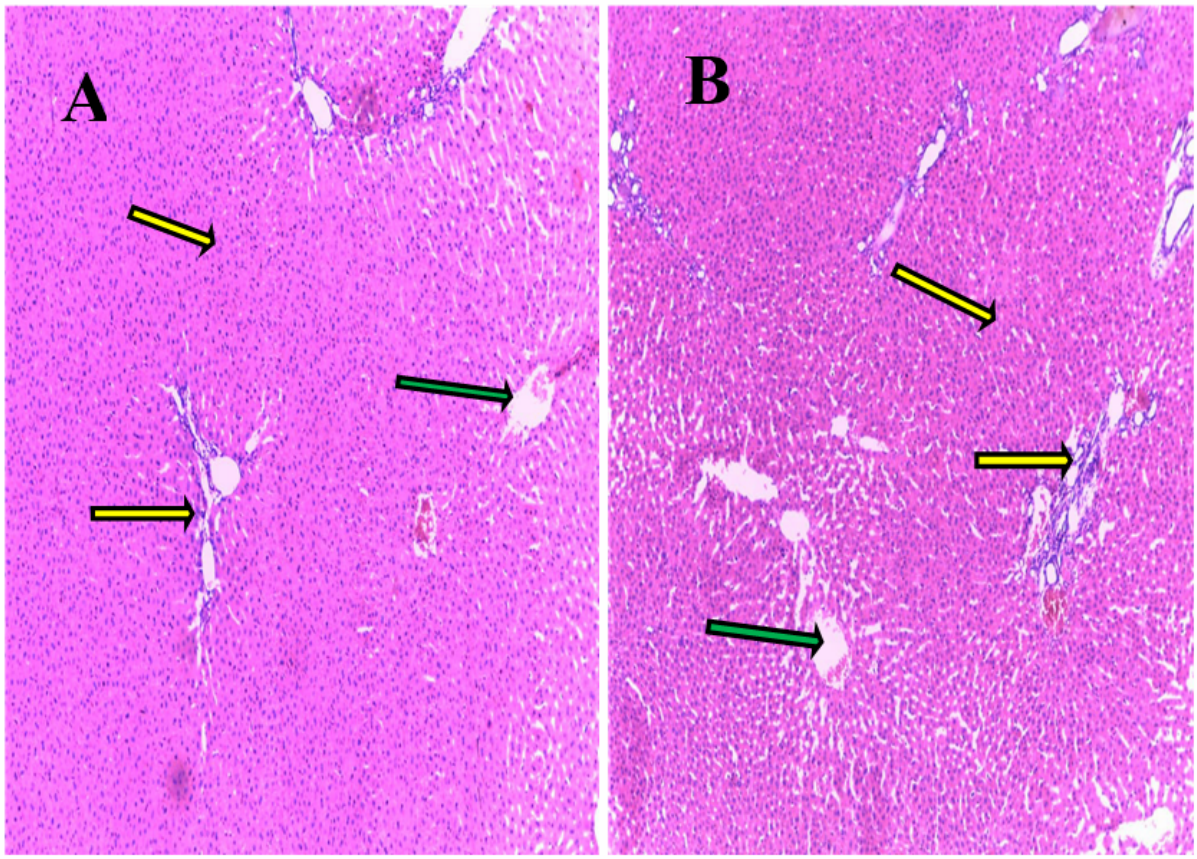

Figure 6 A: Photomicrograph of hepatic tissue from venom exposed rabbit showing showing normal glomerular structure and intact tubular arrangement. B photomicrograph of hepatic tissue from venom exposed rabbit showing well preserved hepatic parenchyma with no pathological alterations.

The lungs on the other hand, showed clear evidence of hemorrhage (yellow arrow) within bronchioles and expansion of the interstitial spaces as a results of infiltration by inflammatory cells and further hemorrhage (green arrows) compared with control showing normal lung parenchyma composed of well-aerated alveolar spaces (Figure 7).

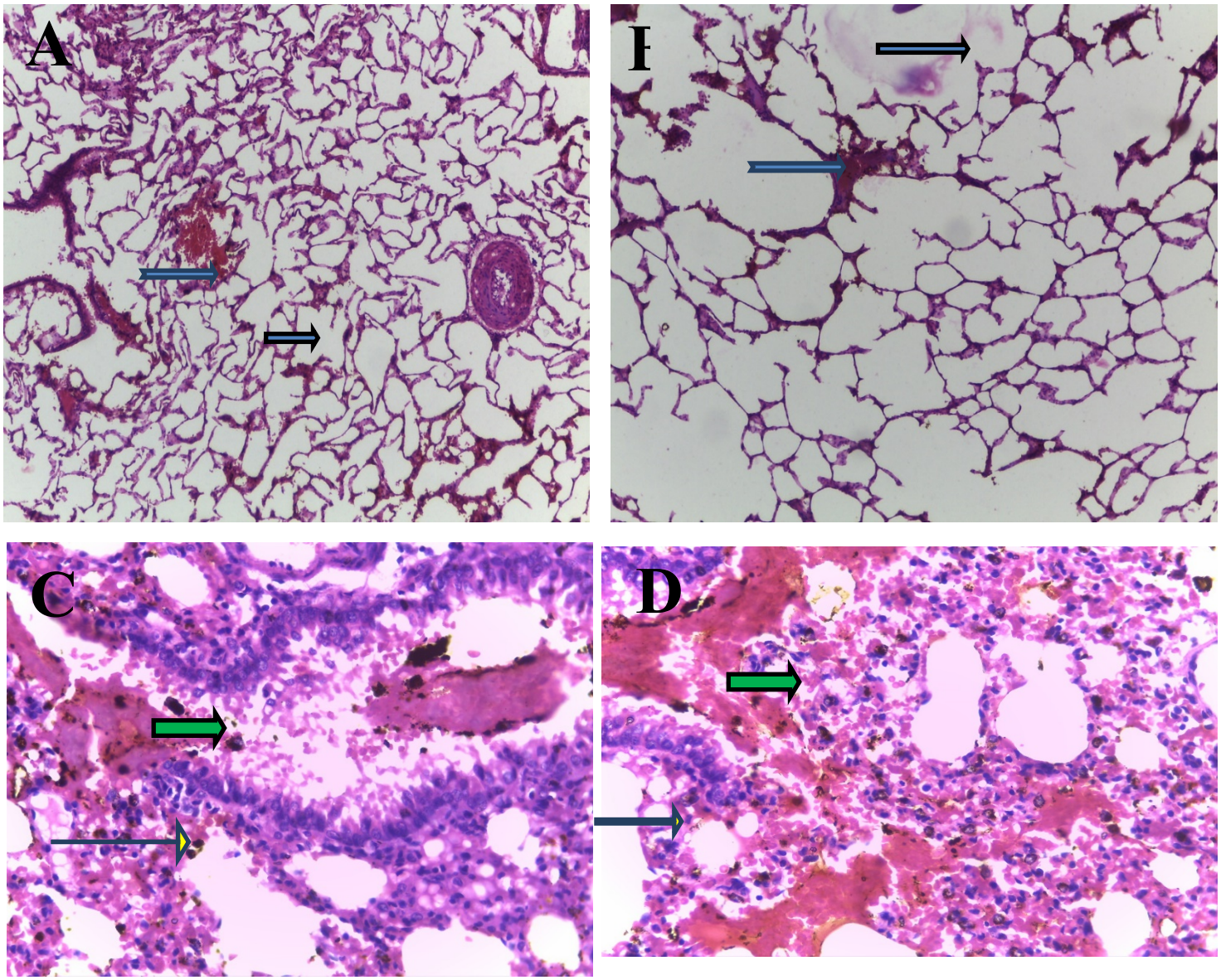

Figure 7: Photomicrograph sections from the lungs show lungs parenchyma composed of alveolar spaces (arrow) and the vasculature (notched arrow) (controls A and B). The section from the lungs show lung parenchyma exhibiting haemorrhage (yellow arrow) within the bronchioles, severe expansion of the interstitium by inflammatory cell and haemorrhage (green arrow), siderophages are also seen as golden brown and narrowing of the alveolar spaces (C and D)

IV. Discussion

Envenomation is accompanied by physiological and immune responses, inflammation, hemolysis and bone marrow activation cytotoxic and neurotoxic effects, resulting in clinical manifestations such as bleeding, edema, paralysis, blindness, and other chronic clinical problems. The evaluation of liver function revealed altered levels of total and conjugated bilirubin, as well as specific liver enzymes (ALT and ALP). These alterations could be associated with co-exposure of venoms which eventually led to hepatic distress and obstructions indicative of venom toxicity [22]. The insignificant increase in urea and creatinine observed herein, might indicate that immunization offers some protection or that the effects of venom varied. These findings could necessitate meticulous evaluation, as renal toxicity continues to be a significant issue in snakebite incidents [23].

The lower levels of albumin and total protein might be indications of liver problems and systemic inflammation. These changes could be due to the effects of various bioactive molecules found in the venom such as venom metalloproteinases (SVMPs) and (PLA_2) which are known to cause hemotoxic, cytotoxic, myonecrosis, and inflammation [12,13]. Also, the changes might be due to multi-system responses to venom immunization which may provide a distinct toxicological and immunological profile that might reveal the complex interactions between the host and venom during antivenom production. The significant decreases in Packed Cell Volume (PCV) across immunization doses showed the recognized detrimental effects of repeated exposure to venom antigens [24]. The immune system while destroying red blood cells and the venom components triggered them directly might probably be the cause of anemia observed herein.

Similarly, a significant reduction in platelet count following each immunization dose could indicate that repeated co-exposure to venom antigens induced progressive hematological stress, even in regulated environments. The loss of platelets might result from mild, persistent disseminated intravascular coagulation (DIC), direct toxicity from the venom, or immune-mediated clearance. These factors are common outcomes of viperid snake venom exposure [25]. Conversely, the notable increases in white blood cell (WBC) counts across the immunization phases might signify effective immune system activation, which is crucial for producing high-quality antivenom. Leukocytosis is associated with increased antibody production; however, it also signifies systemic inflammation, which if not closely monitored could result in significant tissue damage due to inflammatory processes [26,27].

On the other hand, the electrolyte imbalances especially elevations in potassium, and chloride ions during immunizations indicated the impact of venom toxins and immune responses on renal function and fluid-electrolyte homeostasis [28]. These changes could reflect that exposure to the venoms induced ion concentrations variability, while the role of potassium as intracellular ion that is essential in maintaining cellular membrane potential and enzymatic activities cannot be ruled out [29,30]. Also, the histopathological analysis of heart tissue showed branching muscle fiber with abrupt area of tissue damage and subsequently, the cells showed pyknotic nuclei and shruncken cytoplasm. The lung tissue post-immunization revealed significant infiltration of inflammatory cells and an expansion of the interstitium. These phenomena could be attributed to the activity of serine proteases, L-amino acid oxidases, metalloproteinases (SVMPs) and (PLA_2) which are associated with cytotoxicity myonecrosis, inflammation and localized tissue necrosis [11,12]. These results aligned with the previously documented substantial elevations in white blood cell (WBC) counts, indicating strong immune and inflammatory responses to venom protein exposure (Thumtecho et al., 2023). Similarly, the presence of numerous siderophages, which are brown-staining macrophages containing hemosiderin, could indicate ongoing or unresolved haemolysis within the alveolar spaces. These alterations could provide conclusive tissue-level evidence for the systemic reductions in packed cell volume (PCV) and the manifestation of anemia observed in blood tests [25,31].

V. Conclusion

The co-administration of *N. melanoleuca* and *B. arietans* venoms at different concentrations (30µg, 60µg and 120µg) induced significant alterations in biochemical, hematological and histopathological parameters, reflecting systemic toxicity and immune responses activation. Notable elevations in liver enzymes, reductions in packed cell volume and platelet counts, and histological damage to heart and lungs tissues showed the complex pathological effects of these venoms. The findings herein revealed the critical need for developing polyvalent antivenoms capable of neutralizing diverse venom components and emphasize the importance of supporting indigenous antivenom production initiatives to improve snakebite management in endemic regions. It is recommended that further studies can be conducted to establish a lower immunization dose that can tiger immune response.

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