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THE NEUTRALIZING EFFECT OF THE METHANOLIC LEAVES EXTRACT OF ANNONNA MURICATA ON SNAKE VENOM IN FEMALE MICE PUTTING INTO CONSIDERATION ITS ANTI-INFLAMMATORY, LETHARGIC EFFECT, AND HEMATOLOGICAL PARAMETERS

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ABSTRACT

Annona muricata is a plant that has several medicinal properties, however, there is a dearth of scientific information on antivenom activities. This study therefore focused on the neutralizing activities of the methanolic leaf extract of A. muricata against Naja nigricollis venom toxicity in mice. seventy-three mice divided into three research subgroups were used for this study (anti-inflammatory, lethargic, and hematology). For the anti-inflammatory study, fifteen female albino Wistar mice weighing between 22-29g were randomly divided into five groups. Group A was the control group Group B received 600mg/kg of N.nigricollis only while Groups C, D, and E received 600mg/kg of N.nigricollis and 100,200 and 600mg/kg of MAM respectively. The result revealed a significant increase in the paw diameter in Groups B when compared to the control group, while groups C-E had a significant decrease. In the study for the lethargic effect,28 albino female Wistar mice were grouped into 5. Groups A-D were administered the Ld50 of *Naja nigricollis* and 150mg/kg,300mg/kg,600mg/kg, and 1200mg/kg respectively of MAM. The result revealed that there was a significant decrease in mortality rate depending on the doses administered. For hematology, 30 female Wistar mice were allocated into the six groups, Group A was the Negative control, and Group B was envenomed with 600mg/kg of N.nigricollis, with no treatment. Group C-F was envenomed with 600mg/kg of N.nigricollis and given 100mg/kg, 200mg/kg, 600mg/kg, and 800mg/kg respectively. The result showed that A. muricata does not have any impact on hematological parameters.

KEYWORDS: annonna muricata, snake venom, anti-inflammatory, lethargic effect, hematological parameters.

Chapter 1

INTRODUCTION

Venomous snakes bite up to 5·5 million people every year (Kasturiratne et al, 2008). These bites are responsible for causing as many as 1·8 million envenomings and 125 000 deaths annually, with three to five times that number of people thought to suffer from long-term morbidity (Habib et al, 2015). Consequently, snakebite is one of the world's most lethal neglected tropical diseases. Inflammation due to snake venom induces a set of gross inflammatory events, including edema, leukocyte migration, and a complex network of released mediators and the outstanding feature of systemic envenoming is paralysis of the muscles due to rapid action of neurotoxin at the myoneural junction (Chettibi et al.,2000). Venoms contain more than 20 different compounds, mostly proteins and polypeptides (Halliday et al., 2002). A complex mixture of proteins, enzymes, and various other substances with toxic and lethal properties (Bauchot, 2006) serves to immobilize the prey animal (Mattison, 2007), It is injected by unique fangs during a bite, and some species are also able to spit their venom (Bauchot, 2006).

Fig 1.1 shows venom from an Indian cobra.

Venom to the rescue. (https://science.sciencemag.org/content/361/6405/842)

Medicinal plants contain numerous biologically active compounds that have shown considerable pharmacological activities such as antioxidant, antimicrobial, anti-inflammatory, anticancer, antiviral, anti-allergic, and vasodilatory properties (Rustaiyan et al., 2011). Such medicinal plants include Annonna Muricata commonly known as Graviola or Soursop or Gunbanana. The plant-derived herbal compounds have a long history of clinical use, and better patient tolerance and acceptance. Their high ligand binding affinity to the target introduces the prospect of their use in chemo-preventive applications; in addition, they are freely available natural compounds that can be safely used to prevent various ailments. It also has an anticarcinogenic and genotoxic effect. Phytochemical analysis of the plant revealed the presence of tannins, steroids, and cardiac glycosides which are the major phytochemical compounds (Gajalakshmi .S, 2012.).

Therefore, the purpose of this research work is to determine the anti-inflammatory activity of the methanolic extract of Annonna muricata leaves against snake venom and also to confirm its lethal effect against systemic envenomation.

Fig 1.2 Image of *Anonna muricata* (Mishra et al., 2013)

CHAPTER 2

MATERIALS AND METHOD

2.1 LOCATION

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, Anambra State, Nigeria.

2.2 MATERIALS

- Female Wistar mice
- Sawdust
- Laboratory coat and gloves
- Thermostatic water bath
- Beakers
- Syringes
- Measuring cylinders
- Refrigerator (Nexus)
- Watt-man Number1 filter paper
- Normal saline
- Cages
- Canula
- Snake venom
- Methanol
- 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
- Electronic weighing balance, M-methlar model M311l, China
- Mice feeds (non pelletized grower) and water

2.3 PLANT SAMPLE COLLECTION

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

2.4 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.

2.5 EXPERIMENTAL DESIGN

Seventy-three female Wistar mice were purchased from the animal house of the Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi campus and divided into three different research subgroups; lethal effect subgroup (28), the anti-inflammatory subgroup (15) and the hematological parameter subgroup (30). To check for anti-inflammatory properties of Annonna muricata, fifteen female Wistar mice were used, to check for the lethargic effect of Annonna muricta, twenty-eight female Wistar mice were used, while thirty were used to test for the hematological parameters.

2.6 STATISTICAL ANALYSIS

Data were analyzed using Statistical Package for Social Sciences (SPSS version 25). 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 parameters were analyzed using one-way ANOVA, followed by Post hoc LSD. While bodyweight was analyzed using the Student Dependent T-test. Values were considered significant at $P<0.05$.

CHAPTER THREE
RESULTS

Table 3.1 Effect of methanolic extract of *Anonna muricata* leaf on WBC, and platelet count on *Najanigricollis* induced hemotoxicity

Groups	WBC (x10^9/l) Mean±SEM	Platelet count (10^9/L) Mean±SEM
Group A (Positive control)	3.23±0.20[b]	628.33±19.22 [b]
Group B (600mg/kg of *N. nigricollis*)	4.60±0.45	490.33±88.07

Group C (600mg/kg of *N. nigricollis*+ 100mg/kg of MAM)	3.03±0.29[b]	578.33±10.13[b]
Group D (600mg/kg of *N. nigricollis*+ 200mg/kg of MAM)	3.30±0.11[b]	385.66±81.36[b]
Group E (600mg/kg of *N. nigricollis*+ 600mg/kg of MAM)	4.40±0.96[b]	658.00±44.06[b]
Group F (600mg/kg of *N. nigricollis* + 800mg/kg of MAM)	5.63±0.71[b]	855.00±61.54[a]
F Value	3.488	7.442

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 4.1 results revealed a non-significant (*p>0.05*) increase in the WBC in group B compared to group A, groups C and D had a non-significant (*p>0.05*) decrease, and groups E and F had a non-significant (*p>0.05*) increase compared to group B. The platelet count results revealed a non-significant (*p>0.05*) decrease in group B compared to A, groups C and E had a non-significant (*p>0.05*) increase, group D had a non-significant (*p>0.05*) decrease, and group F had a significant (*p<0.05*) increase compared to group B.

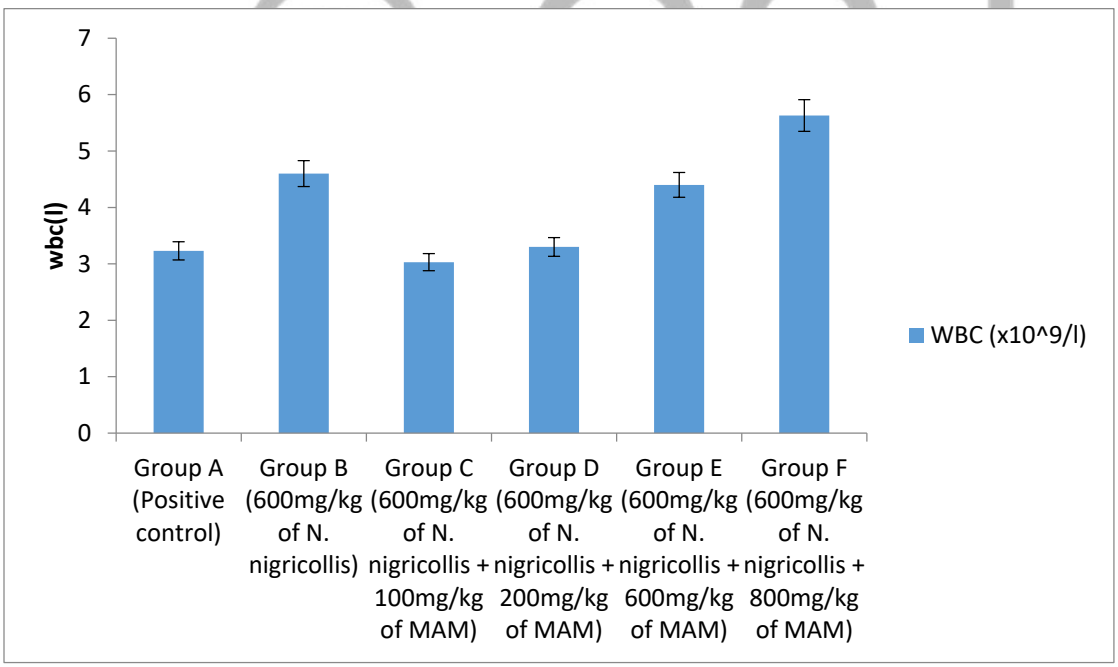

Fig 3.1 Effect Of Methanolic Extract Of *Anonna Muricata* Leaves On WBC On *Najanigricollis* Induced Hemotoxicity

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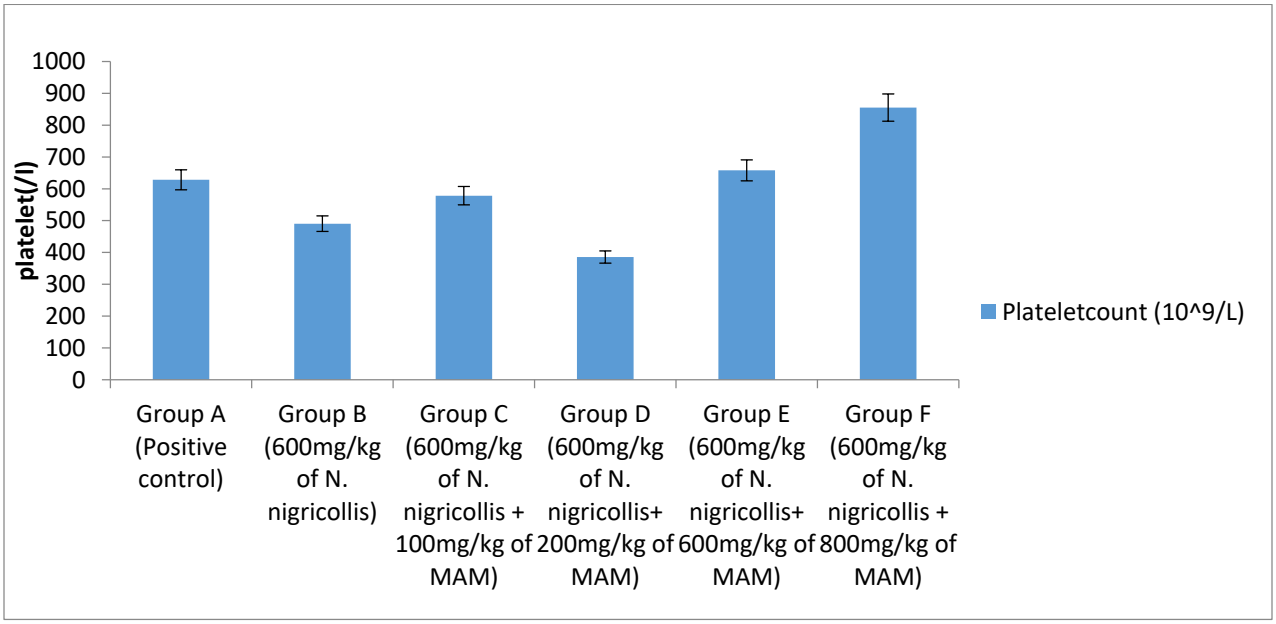

Fig 3.2 Effect Of Methanolic Extract Of *Anonna Muricata* Leaves Platelet Count On *Najanigricollis* Induced Hemotoxicity

Table 3.2 Effect of methanolic extract of *Anonna muricata* leaf on Lymphocytes, monocytes, and granulocytes count on *Najanigricollis*-induced hemotoxicity

Groups	Lymphocytes (%) Mean±SEM	Monocytes (%) Mean±SEM	Granulocytes (%) Mean±SEM
Group A (Positive control)	91.46±0.48 [b]	4.43±0.29 [b]	2.46±0.14 [b]
Group B (600mg/kg of *N. nigricollis*)	92.00±0.57	6.00±0.85	2.47±0.15
Group C (600mg/kg of *N. nigricollis* + 100mg/kg of MAM)	96.17±0.32[a]	4.20±0.35 [b]	0.63±0.24 [a]
Group D (600mg/kg of *N. nigricollis* + 200mg/kg of MAM)	93.57±0.92 [b]	4.07±0.35 [a]	1.86±0.20 [b]
Group E (600mg/kg of *N. nigricollis*+600mg/kg of MAM)	93.43±0.24 [b]	4.30±0.57[b]	2.13±0.40 [b]
Group F (600mg/kg of *N. nigricollis*+ 800mg/kg of MAM)	94.00±0.05 [a]	4.07±0.32 [a]	1.76±0.26 [b]
F Value	10.422	1.538	7.455

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 Shows the lymphocytes result had a non-significant ($p>0.05$) increase in group B compared to A; groups C and F had a significant ($p<0.05$) increase, and groups D and E had a non-significant ($p>0.05$) increase compared to group B.

Monocyte count results showed a non-significant ($p>0.05$) increase in group B compared to A; groups C and E had a non-significant ($p>0.05$) decrease, while groups D and F had a significant ($p<0.05$) decrease compared to group B.

The granulocytes result showed a non-significant ($p>0.05$) increase in group B compared to A; group C had a significant ($p<0.05$) decrease, while groups D, E, and F had a non-significant ($p>0.05$) decrease compared to group B.

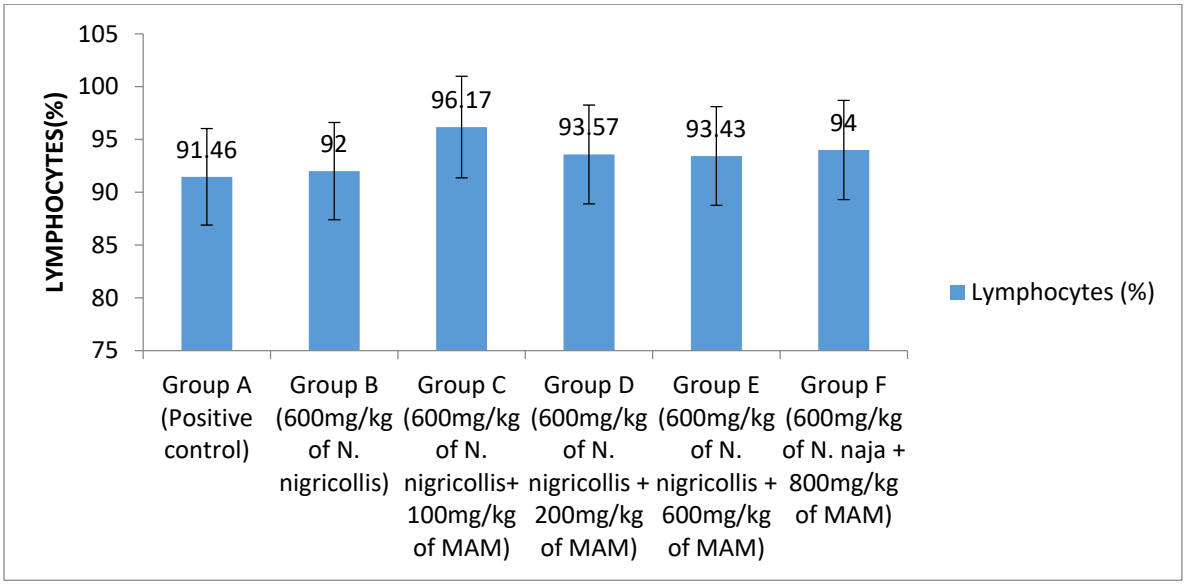

Fig 3.4 Effect Of Methanolic Extract Of *Anonna Muricata* Leaves On Lymphocytes Count On *Najanigricollis* Induced Hemotoxicity

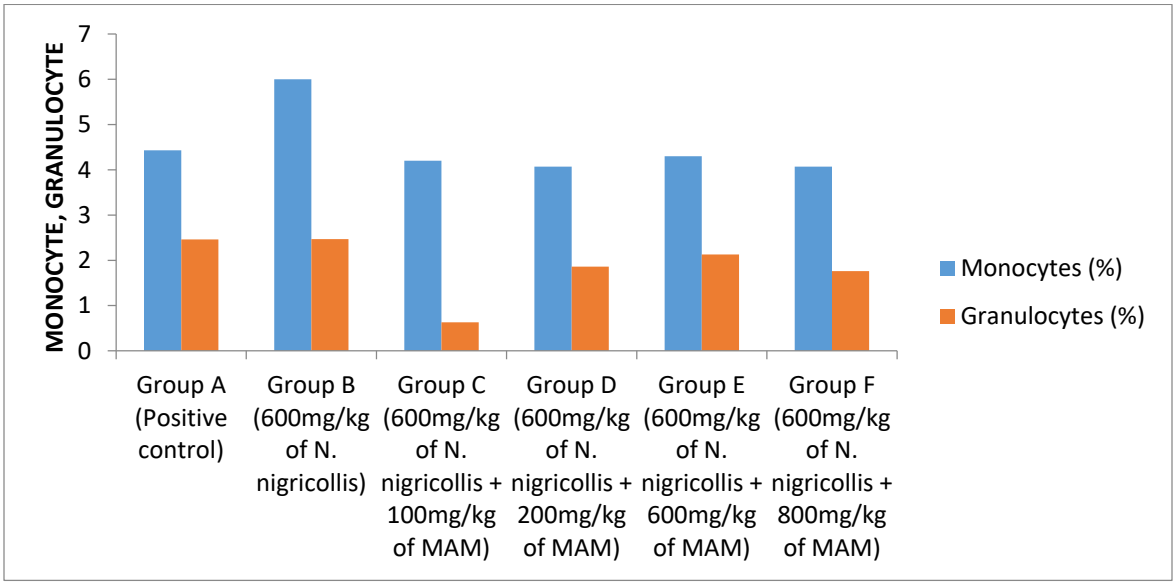

Fig 3.5 Effect Of Methanolic Extract Of *Anonna Muricata* Leaves On Monocytes, And Granulocytes Count On *Najanigricollis* Induced Hemotoxicity**.**

Table 3.3 Effect of methanolic extract of *Anonna muricata* leaf on RBC, hemoglobin level, and pack cell volume on *Najanigricollis*-induced hemotoxicity

Groups	Red blood cell (x10^12/l) Mean±SEM	Hemoglobin (g/dl) Mean±SEM	Pack cell volume (%) Mean±SEM
Group A (Positive control)	9.36±0.24^{b}	10.13±0.20^{a}	45.80±0.91^{b}
Group B (600mg/kg of *N. nigricollis*)	9.65±0.34	13.23±0.24	47.40±0.38
Group C (600mg/kg of *N. nigricollis* + 100mg/kg of MAM)	8.97±0.37^{b}	12.20±0.35^{a}	48.36±0.28^{b}
Group D (600mg/kg of *N. nigricollis* + 200mg/kg of MAM)	8.94±0.25^{b}	12.60±0.63^{b}	47.16±1.99^{b}
Group E (600mg/kg of *N. nigricollis* + 600mg/kg of MAM)	9.91±0.31^{b}	12.46±0.17^{b}	43.06±0.99^{a}
Group F (600mg/kg of *N. nigricollis* + 800mg/kg of MAM)	9.36±0.24^{b}	13.83±0.13^{b}	46.60±1.63^{b}

F VALUE	0.844	14.181	2.351

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 4.3 results revealed a non-significant increase in the RBC level in group B compared to A; groups C, D, E, and F had a non-significant decrease compared to group B. The hemoglobin level showed a significant increase in group B compared to A; group C had a significant decrease, while groups D and E had a non-significant decrease, and group F had a non-significant increase compared to group B. The pack cell volume result showed a non-significant increase in group B compared to A; group C had an insignificant increase, while groups D, E, and F had an insignificant decrease compared to group B.

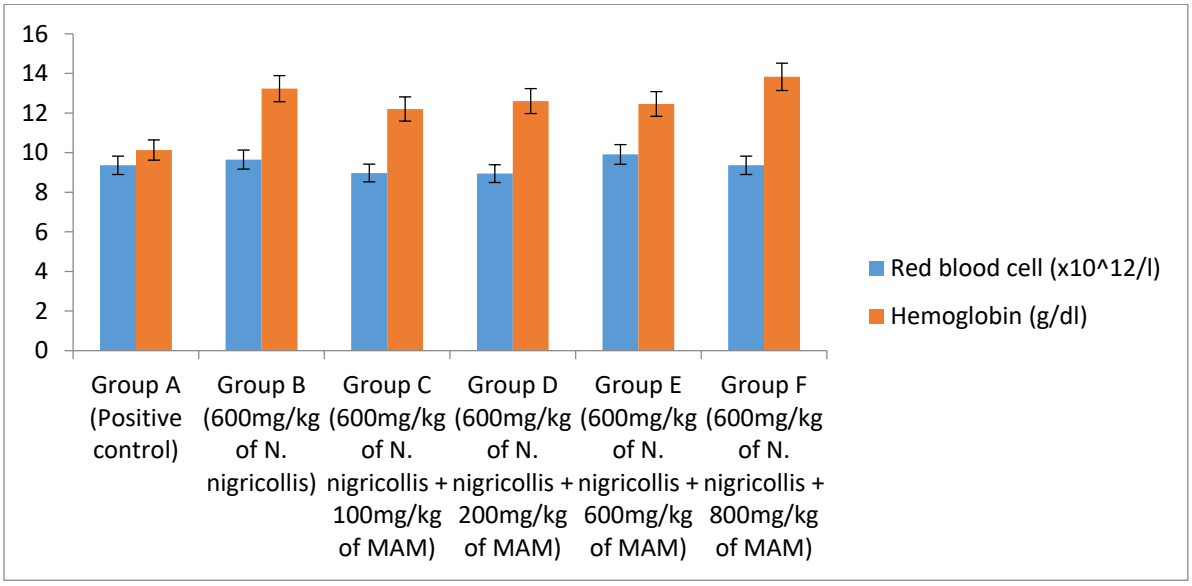

Fig 3.6 Effect Of Methanolic Extract Of *Anonna Muricata* Leaves On RBC, Hemoglobin Level On *Najanigricollis* Induced Hemotoxicity

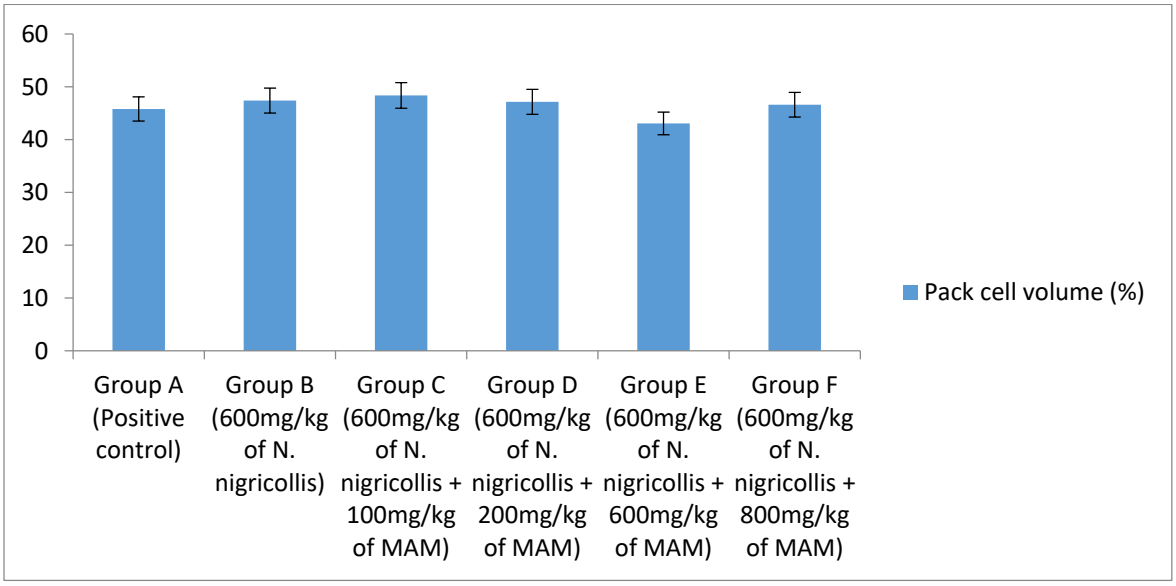

Fig 3.7 Effect Of Methanolic Extract Of *Anonna Muricata* Leaves On RBC, Hemoglobin Level On *Najanigricollis* Induced Hemotoxicity

Table 3.4 Effect of methanolic extract of *Anonna muricata* leaf on MCV, MCH, and MCHC on *Najanigricollis* induced hemotoxicity

Groups	Mean corpuscular volume (fl) Mean±SEM	MCH (pg) Mean±SEM	MCHC (g/dl) Mean±SEM
Group A (Positive control)	47.03±1.01^{b}	10.06±0.46^{a}	22.13±0.20^{a}
Group B (600mg/kg of *N. nigricollis*)	47.10±0.68	14.46±1.02	28.93±0.80
Group C (600mg/kg of *N. nigricollis* + 100mg/kg of MAM)	50.16±0.37^{a}	13.10±0.15^{b}	25.63±0.46^{a}
Group D (600mg/kg of *N. nigricollis* + 200mg/kg of MAM)	52.86±0.68^{a}	13.80±0.35^{b}	26.76±0.46^{a}
Group E (600mg/kg of *N. nigricollis* + 600mg/kg of MAM)	48.46±1.13^{b}	13.96±0.38^{b}	28.90±0.17^{b}
Group F (600mg/kg of *N. nigricollis* + 800mg/kg of MAM)	49.80±0.86^{a}	14.53±0.31^{b}	29.23±0.91^{b}
F VALUE	7.047	10.122	22.724

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 3.4 results showed a non-significant increase in group B compared to A; groups C and D had a significant increase, while groups E and F had a non-significant increase compared to group B in MCV. The MCH level showed a significant increase in group B compared to A; groups C, D, and E had a non-significant decrease, and group F had a non-significant increase compared to group B. The MCHC result showed a significant increase in group B compared to A; groups C and D had a significant decrease, group E had a non-significant decrease and F had a non-significant increase compared to group B.

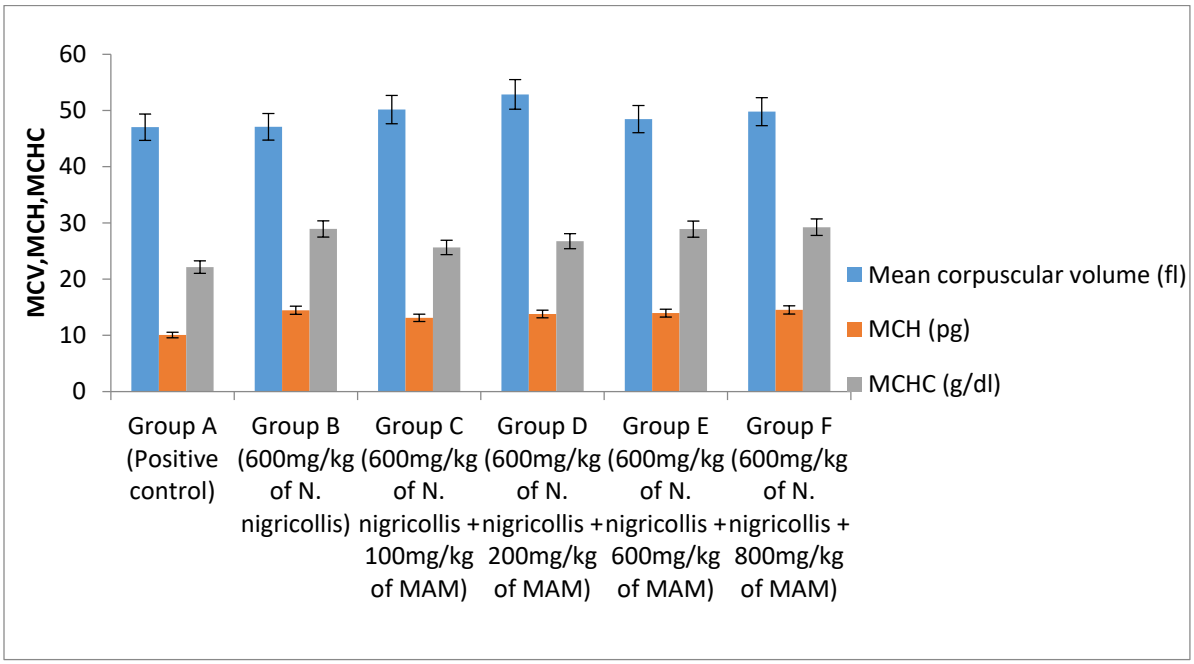

Fig 3.8 Effect Of Methanolic Extract Of *Anonna Muricata* Leaf On MCV, MCH, And MCHC On *Najanigricollis* Induced Hemotoxicity

Table 3.5 Effect of methanolic extract of *Anonna muricata* leaf on edematous activity at zero minutes, 30 minutes, and 60 minutes on *Najanigricollis*-induced hemotoxicity

Groups	Paw size (Zero minutes) Mean±SEM	Paw size (30-minutes) Mean±SEM	Paw size (60-minutes) Mean±SEM
Group A (Positive control)	0.010±0.001	0.010±0.001[a]	0.011±0.001[a]
Group B (600mg/kg of *N. nigricollis*)	0.017±0.001[a]	0.019±0.001	0.017±0.003

Group C (600mg/kg of *N. nigricollis* + 100mg/kg of MAM)	0.015±0.000[a]	0.015±0.001[a]	0.013±0.001[b]
Group D (600mg/kg of *N. nigricollis* + 200mg/kg of MAM)	0.015±0.001[a]	0.015±0.001[a]	0.012±0.002[b]
Group E (600mg/kg of *N. nigricollis* + 600mg/kg of MAM)	0.015±0.001[a]	0.015±0.001[a]	0.014±0.001[b]
F VALUE	6.588	6.776	1.906

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 3.5 results revealed a significant increase in the paw size in groups B, C, D, and E compared to group A at Zero minutes. At 30 minutes, a significant decline in the paw size in groups C, D, and E was noticed, and group A had a significant increase compared to group B. At 60 minutes, a significant increase in the paw size in group B compared to A, and groups C, D, and E had a non-significant decrease compared to group B.

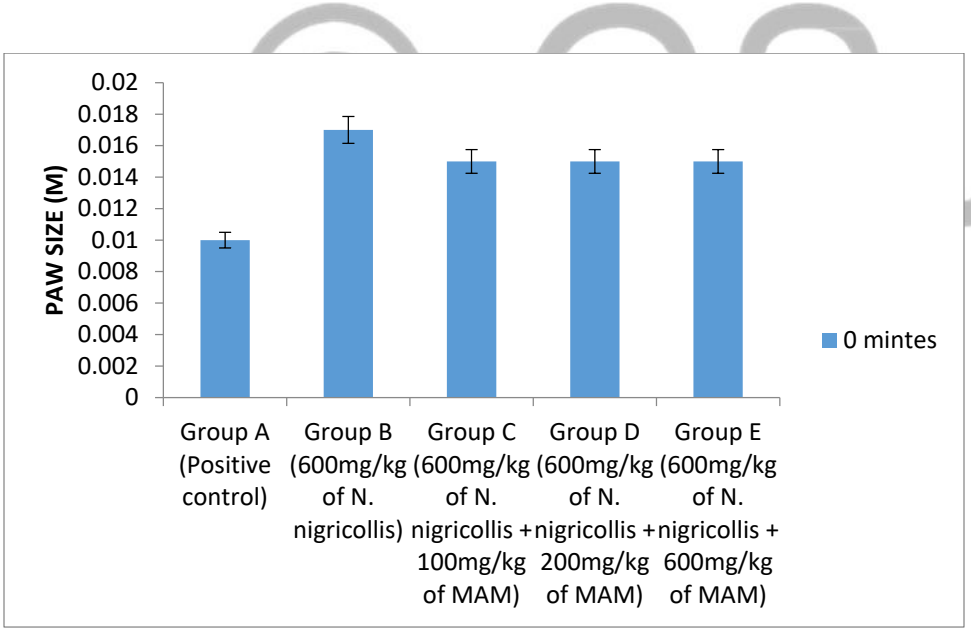

Fig 3.9 Effect of Methanolic Extract of *Annona muricata* at zero minutes following Naja

nigricollis Venom toxicity

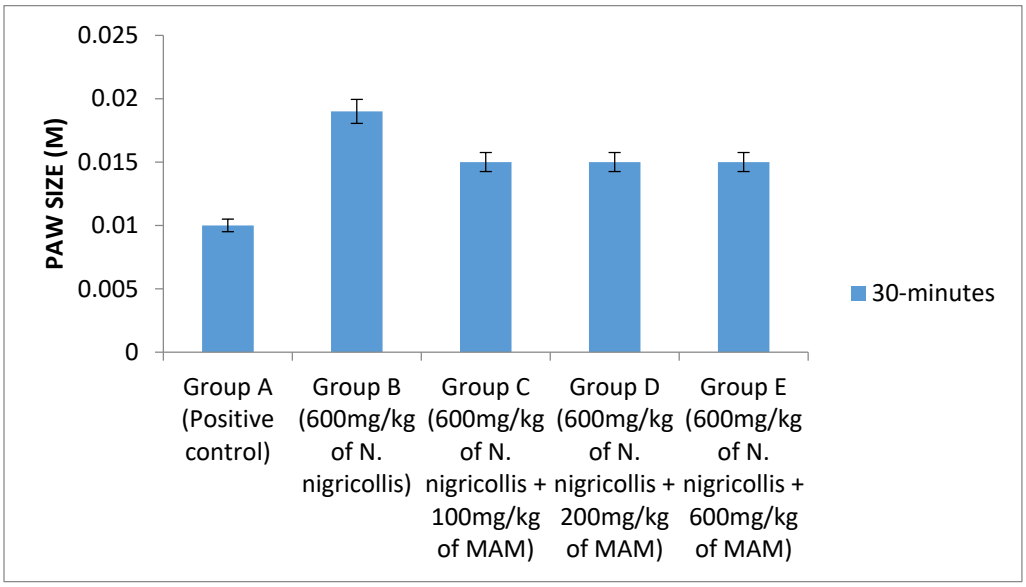

Fig 3.10 Effect of Methanolic Extract of *Annona muricata* at 30 minutes following Naja nigricollis Venom toxicity

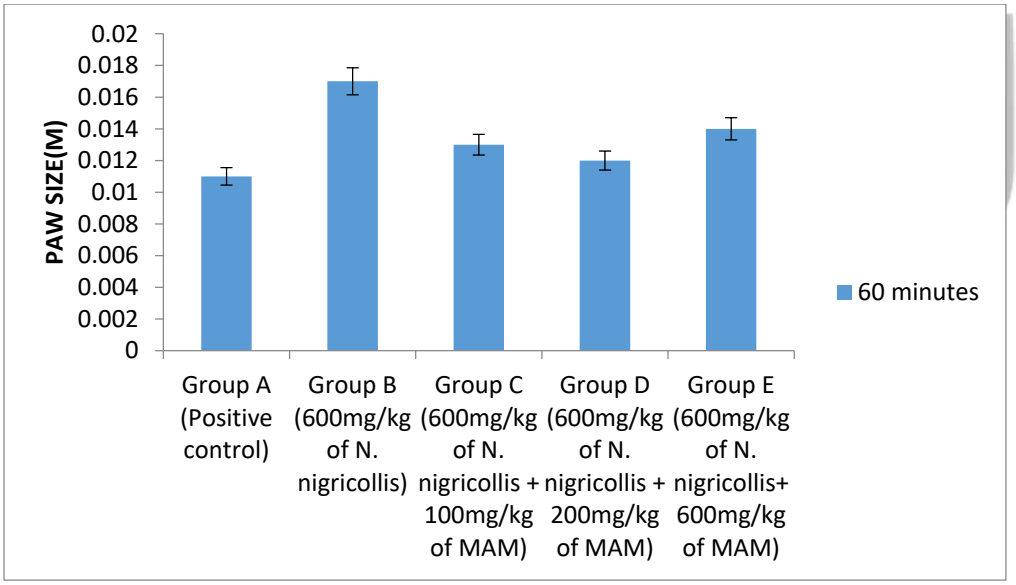

Fig 3.11 Effect of Methanolic Extract of *Annona muricata* at 60 minutes following Naja nigricollis Venom toxicity

Table 4.6 Effect of methanolic extract of *Anonna muricata* leaf on edematous activity at 90 minutes and 120 minutes on *Najanigricollis*-induced hemotoxicity

Groups	Pawsize(90-minutes) Mean±SEM	Paw size (120-minutes) Mean±SEM

Group A (Positive control)	0.010±0.001[a]	0.010±0.001[a]
Group B (600mg/kg of *N. nigricollis*)	0.017±0.002	0.019±0.003
Group C (600mg/kg of *N. nigricollis* + 100mg/kg of MAM)	0.013±0.001[b]	0.012±0.001[b]
Group D (600mg/kg of *N. nigricollis* + 200mg/kg of MAM)	0.012±0.002[b]	0.013±0.003[b]
Group E (600mg/kg of *N. nigricollis* + 600mg/kg of MAM)	0.014±0.001[b]	0.014±0.002[b]
F VALUE	1.842	2.054

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 4.6 results revealed a significant increase in the paw size in group B compared to A, groups C, D, and E had a non-significant decrease compared to group B at 90 and 120 minutes.

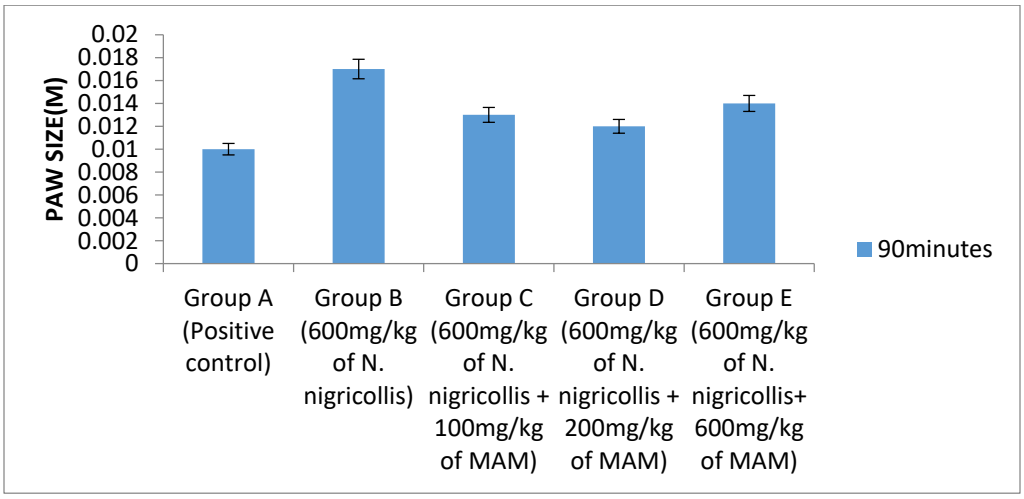

Fig 3.12 Effect of Methanolic Extract of *Annona muricata* at 90 minutes following Naja nigricollis Venom toxicity

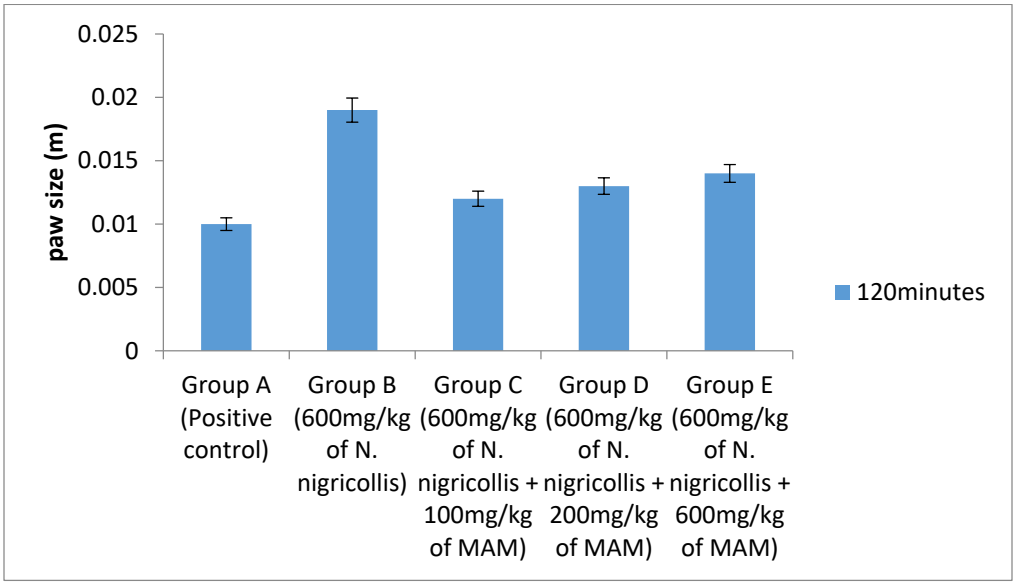

Fig 3.13 Effect of Methanolic Extract of *Annona muricata* at 120 minutes following Naja nigricollis Venom toxicity

Table 3.7 effect of methanolic extract of *Anonna muricata* leaf on the lethargic effect following *Naja nigricollis* venom

Groups	Survival rate	Death %	Correctedformula (%)
Group A (Ld50 of *Naja nigricollis* + 200mcg/kg)	7/7	100	96.4
Group B (Ld50 of *Naja nigricollis* + 400mcg/kg)	6/7	85.7	82.1
Group C (Ld50 of *Naja nigricollis* + 800mg/kg)	4/7	57.1	53.6
Group D (Ld50 of *Naja nigricollis*+ 1200mcg/kg)	1/7	14.3	10.7

Table 3.7 result showed the lethargic effect of *Naja naja* with *Annona muricata* extract, which revealed that group D had the least effect with 10.7% while group A had the highest effect with 96.4%

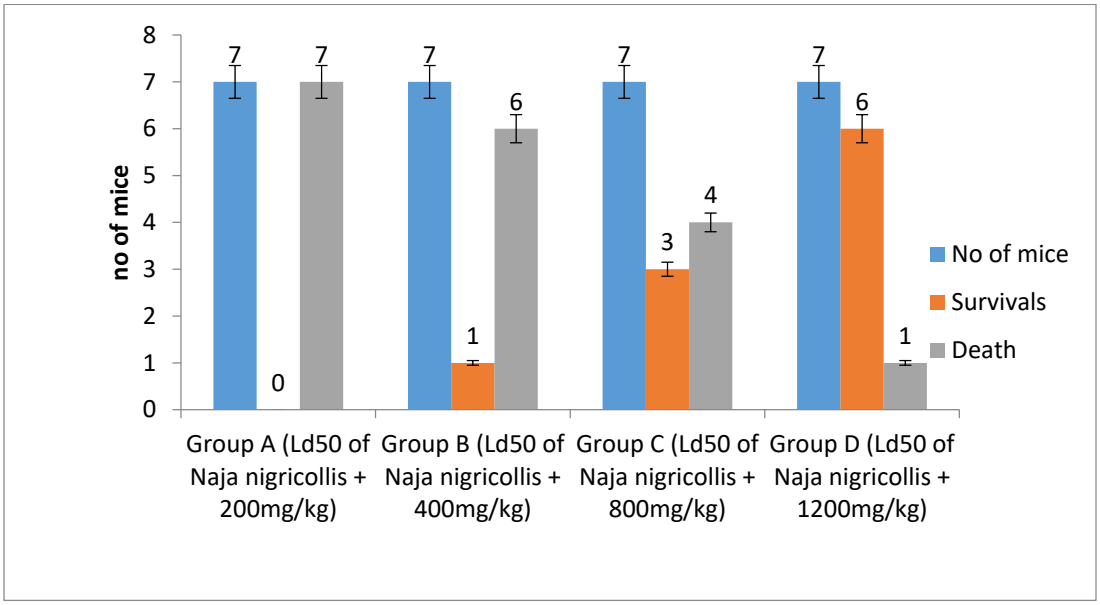

Fig 4.14: chart showing the neutralizing property and prophylactic attributes of Annonna muricata leaf extract against snake venom (Naja nigricollis).

CHAPTER FOUR

DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 DISCUSSION

The current study investigates the effect of methanolic extract of *Annona muricata* on hematological indices, edematous activity, and lethargic effect following Naja*nigricollis* toxicity in female mice. However, the study findings demonstrated a non-significant increase in the WBC in group B compared to group A, groups C and D had a non-significant decrease, and groups E and F had a non-significant increase compared to group B. The mechanism of action linked to the effects may suggest oxidative processes linked to the increase in WBC. (Dissanayake *et al*. 2017) had similar findings with study results revealing a non-significant effect of the venom on WBC levels.

The study findings showed that the platelet had a non-significant decrease in group B compared to A, groups C and E had a non-significant increase, group D had a non-significant decrease, and group F had a significant increase compared to group B. However, the mechanism of action for the insignificant decrease in group B could be due to abnormal coagulation of platelets. (Kini and Evans 1988) had agreement with the study findings revealing a non-significant effect of N. nigricollis venom on platelet count. The study reported that lymphocytes result had a non-

significant increase in group B compared to A; groups C and F had a significant increase, and groups D and E had a non-significant increase compared to group B. Further, *Najanigricollis* venom indicated an insignificant increase in the lymphocyte counts could be linked to one of the inflammatory responses. However, following treatments with the methanolic extract of *A. muricata*, there was a significant increase in groups C and F, which its precise mechanism is not well understood; but, suggests saponin is present.

Monocyte count results showed an insignificant increase in group B compared to A; groups C and E had a non-significant decrease, while groups D and F had a significant decrease compared to group B. However, the significant decline in Monocyte count following treatment with MAM, could result from flavonoids, which combat the toxicity caused by *N. nigricollis.* The granulocytes result showed a non-significant increase in group B compared to A; group C had a significant decrease, while groups D, E, and F had a non-significant decrease compared to group B. The study revealed a non-significant increase in the RBC level in group B compared to A; groups C, D, E, and F had a non-significant decrease compared to group B. The hemoglobin level showed a significant increase in group B compared to A; group C had a significant decrease, while groups D and E had a non-significant decrease, and group F had a non-significant increase compared to group B. The pack cell volume result showed a non-significant increase in group B compared to A; group C had an insignificant increase, while groups D, E, and F had an insignificant decrease compared to group B.

The study findings showed a non-significant increase in group B compared to A; groups C and D had a significant increase, while groups E and F had a non-significant increase compared to group B in MCV. The MCH level showed a significant increase in group B compared to A; groups C, D, and E had a non-significant decrease, and group F had a non-significant increase compared to group B. The MCHC result showed a significant increase in group B compared to A; groups C and D had a significant decrease, group E had a non-significant decrease and F had a non-significant increase compared to group B. The precise mechanism of action of *N. nigricollis* toxicity on MCHC and MCH results from the oxidative stress activity of venom from ROS activation.

The study findings demonstrated a significant increase in the paw size in groups B, C, D, and E compared to group A at Zero minutes. At 30 minutes, a significant decline in the paw size in groups C, D, and E was noticed, and group A had a significant increase compared to group B. At 60 minutes, a significant increase in the paw size in group B compared to A, groups C, D, and E had a non-significant decrease compared to group B. Also, revealed was a significant increase in the paw size in group B compared to A, groups C, D, and E had a non-significant decrease compared to group B at 90 and 120 minutes. The study lethargic effect of *Najanaja*with *Annona muricata* extract revealed that group D had the least effect at 10.7% while group A had the highest effect at 96.4%. The study has a similar report with the findings of Rajesh *et al*. (2017)and (Kazemi-Lomedasht *et al.* 2019).

4.2 Conclusion

The study revealed that the *Annona muricata* (MAM) methanolic extract had no impact on White blood cells. However, it demonstrated a positive impact on platelet count at 800mg/kg of MAM

and positively influenced differential white blood cell count based on dose dependence. In contrast, no impact on Red blood cells, hemoglobin (100mg/kg of MAM) and pack cell volume (600mg/kg of MAM) indicated a positive impact based on dose dependence. Further, MAM indicated no impact on MCH, while MCV had a positive influence at 100, 200, and 800mg/kg of MAM; and MCHC showed positive affluence at 100 and 200mg/kg of MAM following Naja nigricollis toxicity on hematological indices. Further, Naja nigricollis venom showed an inflammatory effect; MAM at 30 minutes positively affected the edematous activities of the venom. MAM possesses an anti-lethargic effect based on dose dependence with the most potency at 1200mg/kg.

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