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Original Article

Hematological and histological effect of fractionated neem leaf extract in healthy Wistar rats

David Chibuike Ikwuka[1,2]*, Ed Nwobodo[2], Bond U. Anyaehie[1], Emmanuel I. Umegbolu[3]

1. Department of Physiology, College of Medicine, University of Nigeria, Enugu, Nigeria
2. Department of Human Physiology, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi, Nigeria
3. Department of General Outpatient, District Hospital Awgu, Enugu State, Nigeria

Abstract

Introduction: In recent years, the growing research towards new drugs has been targeted on plant-based drugs, and Neem (*Azadirachta indica*) is one of the plants that have been extensively researched for its diverse medicinal properties. The study aimed to determine the effects of neem on the hematological parameters (total white blood cells, neutrophil, monocyte and eosinophil counts) and histology of some organs of rats.

Methods: Fifteen healthy male Wister rats divided into control (Nm_0) and experimental groups (Nm_{11} and Nm_{22}). Control group 1 (Nm_0) was given 100mg/200g body weight of normal saline orally twice daily; experimental group 2 (Nm_{11}), 100mg/200g body weight neem extract twice daily for 11 days and experimental group 3 (Nm_{22}), 100mg/200g neem leaf extract twice daily for 22 days. Total number of white blood cells (WBC), lymphocytes, neutrophils, monocytes and eosinophils, packed cell volumes (PCV) and histological changes in the spleen, liver and kidneys were evaluated.

Results: There were no significant differences in mean values of the hematological parameters (total WBC; PCV; neutrophils, lymphocytes, monocytes and eosinophils). We observed the central vacuolation and accumulation of lymphocytes in the spleen, hypertrophy of the central vein in the liver and shrinking of the glomeruli and accumulation of the lymphocytes in the kidney using hematoxylin and eosin staining following prolonged administration of neem extract (Nm_{22}).

Conclusion: Prolonged administration of neem affected the histology of some organs of the rats more than the hematological parameters.

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Keywords:
Hematological;
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*** Corresponding author:**
D.C. Ikwuka
Email:
david.ikwuka.pg80911@unn.edu.ng
Tel: +2348037147603

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Introduction

In recent years, the growing research toward new drug has been targeted on plants based drugs and neem (*Azadirachta indica*) is one of the plants that have been extensively researched for its diverse medicinal properties and potency. Neem tree is

universally accepted worldwide as a wonder tree due to its wide range of medicinal properties and promise. It has been known and documented that the use of neem extract is potent in combating a range of clinical diseases which has led to the increase use of neem as phytotherapy for malaria (Udeinya et al., 2006; Lucantoni et al., 2010; Habluetzel et al., 2019), diabetes (Akinola et al., 2011; Dholi et al., 2011; Patil et al., 2013), cancer (Sharma et al., 2014; Patel et al., 2016; Santos et al., 2018), ulcer (Ofusori et al., 2010; Maity et al., 2014), bacterial (Prashant et al., 2007; Heyman et al., 2017), viral (Mbah et al., 2007; Ahmad et al., 2016; Urade et al., 2019), hypertensive (Peer et al., 2008), inflammatory (Naik et al., 2014; Lee et al., 2017), neurodegenerative diseases (Bamidele et al., 2013; Kandhare et al., 2017) etc. Despite the increased use of neem-based products for therapeutic and other purposes, little information on its toxicity is available as compared to its application level, this has necessitated further studies to ascertain the safety of neem compounds and extract for various applications. The present study aims to investigate the effect of IRACARP® a fractionated neem leaf extract on the hematological parameters and histology of Wister rat tissues.

Materials and methods

IRACARP® (fractionated neem leaf extract)

IRACARP® purchased from Rocitus IJU Intl Limited Enugu, Nigeria; is a 250mg capsule of fractionated neem leaf extract also known as IRAB with registered US Pat No. 5,370,873 issued Dec. 6 1994. It is registered in Nigeria with NAFDAC registration number A7-0319L. Its extraction is done using neem leaves collected in Nigeria in a mixture of acetone and water (1:1 by Vol.) as described by Udeinya (1993). Residue from the bottom layer of the crude extracts is then fractionated by standard high performance liquid chromatography (Udeinya et al., 2006). The end product (IRAB) is a complex molecule (202 Daltons) with functional groups that include sodium salts of carboxylic acid and a non-aromatic dialcohol. Four capsules of IRACARP® (1000mg) were dissolved in 100ml of ethanol and were given to the rats in NM_{11} and NM_{22} according to their different weights.

Experimental animals

The protocol for the animal study was approved by College of Health Science Research Ethics Committee Nnamdi Azikiwe University Nnewi Campus and agrees with the experimental guidelines of the U.S. National Institute of Health (NIH) and Institutional Animal Ethics Committee (IAEC) on the care and use of laboratory animals (070/05/2013). All efforts were used to reduce the number of rats used in the experiments. Experimental animals were 15 healthy male Wistar strain rats (*Rattus norvegicus*), four months of age, with average body weight of 220g. The Wistar rats were reared and maintained under standard conditions at room temperature, 12h light/12h dark cycle and 70% humidity in the animal facility of the animal house of the Department of Human Physiology, Nnamdi Azikiwe University, Nnewi Campus, Nigeria.

Experimental design

The Wistar rats were randomly divided into three groups (n=5/each). Control group 1 (Nm_0), received 100mg/200g body weight normal saline orally, twice a day. Experimental group 2 (Nm_{11}), received 100mg/200g body weight neem leaf extract orally, twice a day for eleven days. Experimental group 3 (Nm_{22}), received 100mg/200g body weight neem leaf extract orally, twice a day for twenty-two days. Following the duration of the experimental period, the rats was anaesthetized with diethyl ether, whole blood was collected by cardiac puncture into EDTA container bottles and pieces of the spleen, liver and kidney were fixed in formaldehyde solution.

Histological processing

The fixed tissues were dehydrated, cleared using xylene, infiltrated and embedded in paraffin, before being sectioned using microtome. The sections slides were deparaffinized xylene to alcohol to water before staining with hematoxylin and eosin stain respectively. The stained section is mounted in D.P.X and dry for micrograph and interpretation.

Hematological analysis

The packed cell volume and differential white blood cell counts and white blood cell counts were determined as outlined by Schalm et al (1975).

Hematocrit determination

The packed cell volume was estimated using the method of Alexander and Griffiths (1993). Hematocrit

Table 1: Total white blood cells (WBCs) counts (10^3/mm^3)

Specimens	Groups		
	Nm_0	**Nm_{11}**	**Nm_{22}**
A	8.7	8.5	8.7
B	9.3	9.0	8.7
C	9.0	8.9	9.5
D	8.5	8.9	9.1
E	9.0	8.8	9.2
Mean	**8.9**	**8.8**	**9.0**
Common Mean	**8.9**		
p-value		**0.69**	
f. ratio value		**0.744**	

Normal saline twice a day (Nm_0), IRACARP® administration for 11days (Nm_{11}), IRACARP® administration for 22days (Nm_{22}).

Table 2: Differential WBC count (%)

Groups	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)
Nm_0	27	72.2	0.2	0.6
Nm_{11}	22.6	76.8	0.0	0.6
Nm_{22}	29.4	67.8	0.8	2.0

Normal saline twice a day (Nm_0), IRACARP® administration for 11days (Nm_{11}), IRACARP® administration for 22days (Nm_{22}).

capillary tubes filled by capillary action to mark with whole blood and the bottom end of the tube were sealed with plasticine. The tubes were centrifuged for 5min using DM1424 hematocrit centrifuge (Scilogex 1275 Cromwell Avenue, C-6 Rocky Hill). The percentage cell volume was read by sliding the tube along the hematocrit reader (Scilogex 1275 Cromwell Avenue, C-6 Rocky Hill) until the meniscus of the plasma intersects the 100% line.

Hemoglobin estimation

Cyamethemoglobin (Drabkin) method (Alexander and Griffiths, 1993) of hemoglobin estimation was used. The 20ul of EDTA anticoagulated whole blood was added to 5ml of Drabkin reagent mixed and incubated for 5min at room temperature for the colour to develop. The absorbance was read against reagent blank at 540nm using optima SP-300 spectrophotometer (PT. Maju Mapan Mandiri Idah Pratama).

Differential leucocyte count

Differential leucocyte count was performed on Leishman's stained thin blood film and read microscopically using immersion oil objective lens (100x magnifications) and a differential manual counter. The different white cells were counted and expressed in cell/l.

Statistical analysis

Data collected were analyzed as descriptive statistics of means and inferential statistics of ANOVA (one-way ANOVA) using MaxStat (version 3.60) statistical software. A *P*-value of ≤0.05 was considered significant.

Results

Effect of IRACARP® administration on total number of white blood cells (WBCs)

Table 1 shows total WBCs (10^3 /mm^3) of the animals. ANOVA analysis revealed that WBC number was not different between groups (F=0.74, *P*=0.5)

Effect of IRACARP® administration on WBC differential counts

As shown in Table 2, administration of IRACARP® over 11days and 22days period caused no significant change in the neutrophil (F=0.81, *P*=0.47), lymphocyte (F=0.96, *P*=0.41), monocyte (F=1.86, *P*=0.20) and eosinophil (F=0.45, *P*=0.65) numbers (%).

Table 3: Packed cell volumes (PCVs) (%)

Specimens	Groups		
	Nm_0	Nm_{11}	Nm_{22}
A	40	33	35
B	42	46	41
C	41	43	44
D	40	41	40
E	41	41	40
Mean	**40.8**	**40.8**	**40**
Common Mean	**40.5**		
p-value	**0.91**		
f-ratio value	**0.09**		

Normal saline twice a day (Nm_0), IRACARP® administration for 11days (Nm_{11}), IRACARP® administration for 22days (Nm_{22}).

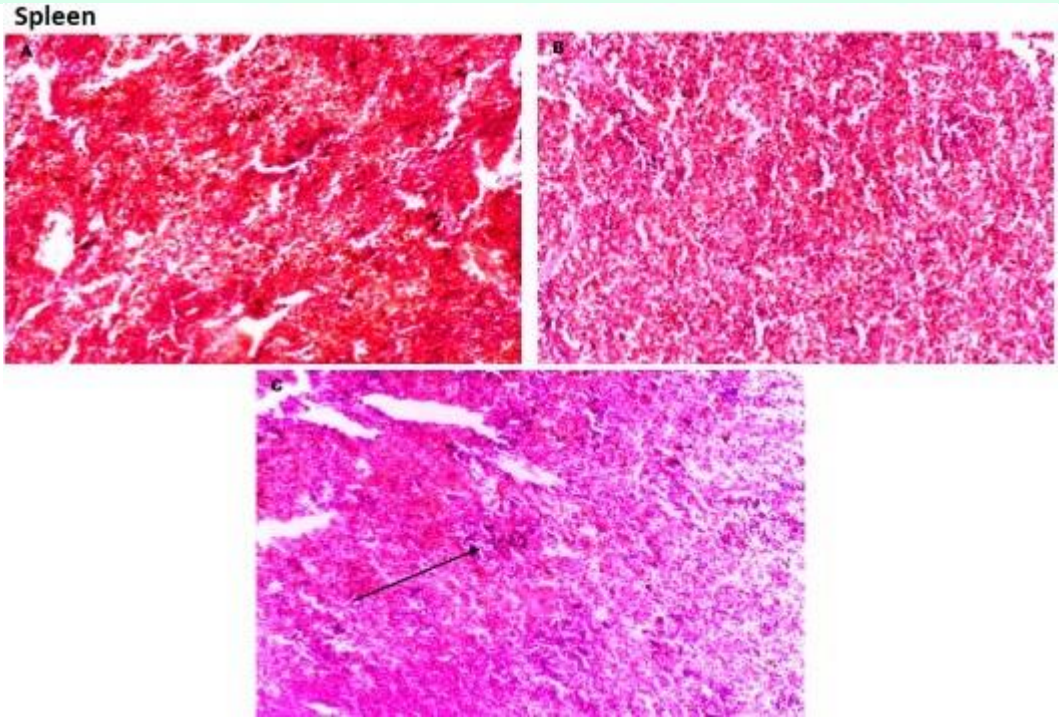

Fig.1. Shows the histopathological changes in rat spleen following long-term administration of IRACARP®. Cross-sectional view of the spleen to represent the cellular outline and architecture. **A.** Nm_0 without IRACARP® administration (Control) **B.** Nm_{11} IRACARP® for 11 days C. Nm_{22} IRACARP® for 22 days.

Effect of IRACARP® administration on packed cell volumes (PCV)

Table 3 shows packed cell volumes (in %) of the animals. There was no significant difference between the three groups (F=0.09, *P*=0.91).

Effect of IRACARP® administration on spleen

The histoarchitecture of the spleen sections stained with hematoxylin and eosin (H&E) are shown in Figure 2. The photomicrograph of Nm_0 (control) and Nm_{11} groups showed normal appearance. In Nm_{22} group cytoplasm vacuolation and mild accumulation of lymphocytes were observed.

Effect of IRACARP® administration on liver tissue

The histoarchitecture of the liver sections stained with H&E are shown in Figure 2. The photomicrograph of Nm_0 and Nm_{11} groups showed normal appearance. In Nm_{22} group slightly hypertrophied central vein were observed.

Effect of IRACARP® administration on kidney

The histoarchitecture of the kidney sections stained with H&E are shown in Figure 3. The photomicrograph of Nm_0 and Nm_{11} groups showed

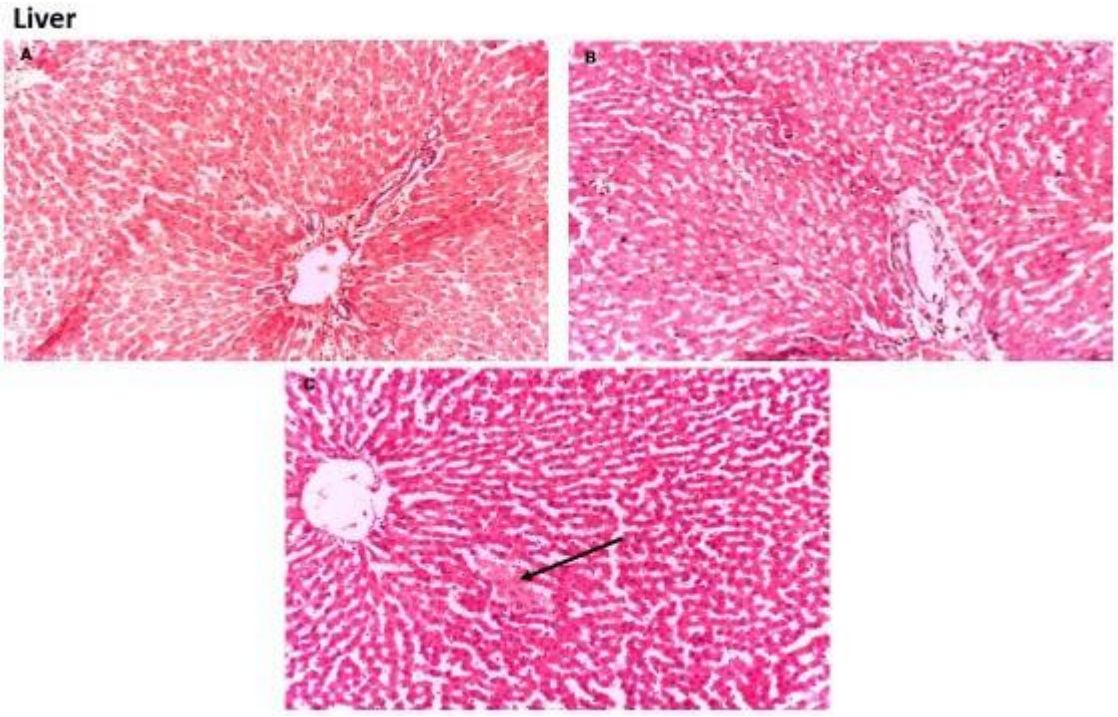

Fig.2. Histopathological changes in rat liver following long-term administration of IRACARP®. Cross-sectional view of the liver to represent the cellular outline and architecture. **A.** Nm_0 without IRACARP® administration (Control) **B.** Nm_{11} IRACARP® for 11 days C. Nm_{22} IRACARP® for 22 days.

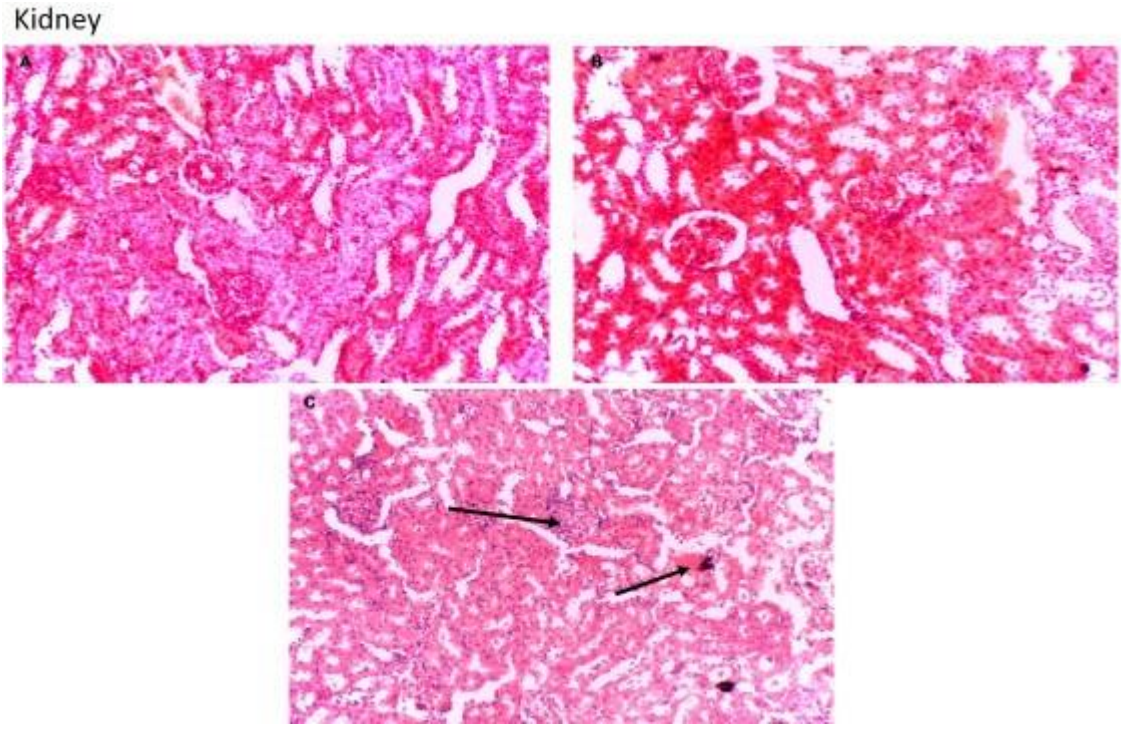

Fig.3. Histopathological changes in rat kidney following long-term administration of IRACARP®. Cross-sectional view of the kidney to represent the cellular outline and architecture. **A.** Nm_0 without IRACARP® administration (Control) **B.** Nm_{11} IRACARP® for 11 days C. Nm_{22} IRACARP® for 22 days.

normal appearance. In Nm_{22} group, shrunken glomeruli with slight aggregation of lymphocytes were observed.

Discussion

Toxicity studies in animals are often used to evaluate potential health risk posed by adverse effects of plant

extract to humans (Ashafa et al., 2012). The present study evaluated the effects of fractionated neem leaf extract derivative IRACARP® on hematological parameters and histology of the spleen, liver and kidney of normal male Wister rats. Hematological parameters are important in diagnosing the structural and functional status of animals exposed to a substance because blood parameters are highly sensitive to environmental and physiological changes and health conditions (Esonu et al., 2006; Kwawukume et al., 2013).

WBCs are involved in the regulation of immunological function and their numbers increase as a protective response to stress (Schalm et al., 1975). The data presented in this report, showed that short term and long term treatment with IRACARP ® caused no significant change in the total white blood cell count. This finding is contrary to the result obtained from previous studies who reported that neem extract increased the total WBC following administration of substances that were toxic to animal hematology (Anokwuru et al., 2011; Kapinga et al., 2018; Kaur et al., 2019). However, Ashafa et al. (2012) observed that neem extract significantly reduced WBC in wistar rats.

Evaluation of WBC differential count are important in identifying the specific effect the administered substance could have on the body immune system, as increase in any of the leucocyte could be an indicator of an immunological reaction to an infection or allergy. In evaluating the effect of neem leaf extract on neutrophil count, neutrophil counts were slightly higher in the control group Nm_0 (27%) than the treatment groups Nm_{11} and Nm_{22} (22.5% and 25.45%); however, the figures were not statistically significant and were within the normal range. Physiologically, neutrophils help heal damaged tissues and resolve infections and its level increase naturally in response to infections, injuries and other types of stress. In rats, they are important during bacterial infections as they act as macrophages and IRACARP ® slightly suppresses neutrophil level. This finding supports the reports that have shown, neem decreases neutrophil count (heterophil in birds) in rabbits (Ogbuewu et al., 2010), birds (Kwawukume et al., 2013) and fish (Kapinga et al., 2018).

Lymphocyte counts in treated groups were higher when compared to the control group but this increase, which may be attributed to neem leaf extract were not statistically significant and still within the normal range of 70-99%. This finding supports the reports that have shown, neem increases lymphocytic proliferative responses in experimental mice, rat and bird (Kwawukume et al., Njiro et al., 1999; Kapinga et al., 2018). There was no significant difference in monocyte count of control and treatment groups following short term and long term administration of neem leaf extract as the values were still within range of normal values of 0-6% for Monocyte. There was also no significant difference between eosinophil counts of control and treatment groups respectively.

In evaluating the effect of neem leaf extract on packed cell volume, there was no significant difference in the mean values of the packed cell volume (40.8%, 40.8% and 40%) following short and long term administration of IRACARP®, the values fall within the normal range of 36% to 46% for PCV. This finding is contrary to the reports from earlier studies done in other animals which stated that neem reduces the packed cell volume (Saravanan et al., 2011; Oloruntola et al., 2019).

The histopathological analysis of the spleen were normal for of Nm_0 and Nm_{11}, however, cytoplasmic vacuolation and mild accumulation of lymphocytes in the lymphoid tissue of Nm_{22} were observed. The cytoplasmic vacuolation in splenic cells is mainly a consequence of considerable disturbances in lipid inclusions and fat metabolism occurring under pathological changes (Banhawy, 1993). The histopathological analysis of the liver were normal for Nm_0 and Nm_{11}. However, the central vein of the liver in Nm_{22} is slightly hypertrophied. This hypertrophic response is indicative of billary obstruction which may be due to chronic exposure of the liver to neem leaf extract (Kim et al., 2011). The histopathological investigation of the kidney were normal for. However, some shrunken glomeruli with slight accumulation of lymphocytes were observed in Nm_{22}. This alteration in the structure of the glomeruli is indicative of decreased renal perfusion which may be alluded to a chronic administration of neem leaf extract (Basile et al., 2011).

Conclusion

Hematological parameters of the Wister rats administered with neem leaf extract for shorter and longer durations did not show any significant

differences with those of the control. However, histopathological changes were observed in the spleen, liver and kidneys following chronic administration of neem leaf extract. Therefore, prolonged administration of neem leaf extract in animal model cause histopathological changes in spleen, liver and kidney. A human model of this study could be designed using full blood count and ultrasound monitoring of the internal organs to determine if the same changes seen in the rats would also be seen in humans.

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Conflict of interest

The authors declare that no conflicts of interest.

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