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EFFECT OF *Azadirachta indica* ON *Plasmodium berghei*-INDUCED NEPHROPATHY OF MALE WISTAR ALBINO RATS

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ABSTRACT

Malaria is a severe febrile illness instigated by *Plasmodium* parasites and spread through bites of infected mosquitoes to humans and animals. This study was carried out to determine the effect of water extract of *Azadirachta indica* on *Plasmodium berghei*-induced nephropathy of male Wistar albino rats. Thirty (30) male albino Wistar rats weighing between 150 and 200 grams were used for the study. Experimental animals were weighed and grouped into six groups (A to F) of five rats per group. Groups A, B, and C were blank, negative, and positive controls, respectively while Groups D, E and F were infected with $1.0x10^7$ *Plasmodium berghei* and treated with 50, 200, and 500mg/kg body weight of ethanol neem leaf extract of*Azadiractha indica, respectively* for twenty-one (21) days post-infection. After treatment, animals were sacrificed and serum collected for biochemical analysis. Results of the experiment showed that *P. berghei* caused a significant hike ($p<0.05$) in mean serum creatinine concentration (4.30±0.96) when compared with the blank control (2.60±0.96).With the administration of *A. indica,* the *P. berghei*-induced creatinine hike reduced significantly ($p<0.05$) (2.80±0.62). The present study also showed that *P. berghei* caused a reduction in urea nitrogen level (11.79±4.03) when compared with the blank control (28.18±5.12). The result of renal histology showed that *P. berghei* caused a focal tubular dilation with brush border depletion and upon the administration of *A. indica,* the kidney cortex still showed some levels of cell-mediated capsular injury and tubular atrophy. We therefore, concluded that *Plasmodium berghei* caused renal dysfunction, as shown by biochemical assay. However, the histological study inferred that the extract of *A. indica* could not entirely reverse the renal damages initiated by the infections.

Keywords: *Azadirachta indica, Plasmodium berghei,* Nephropathy, Electrolytes, Bicarbonate.

INTRODUCTION

Malaria is an acute severe sickness caused by *Plasmodium* parasites transmitted through the bites of infected mosquitoes to humans and animals (WHO, 2014). These parasites are transmitted to humans and other animals through the bites of infected mosquitoes. When a mosquito bites a person, the parasites that live in the mosquito's saliva are transferred into the blood (WHO, 2014). The parasites migrate to the liver, where they mature and multiply. Malaria causes symptoms that commonly include fever, fatigue, vomiting and headaches (Basu et al. 2017).

Malaria is the fourth most prominent cause of mortality among children under five in sub-Saharan Africa, with an estimated 214 million cases and 430,000 deaths globally in 2015 (WHO, 2015). This prominence is associated with the rainfall pattern within the region in addition to man-made environmental conditions that support breeding of mosquitoes (Jamieson *et al.* 2006). In humans, malaria is known to be caused by *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* with *P. falciparum* being responsible for majority of fatalities (Caraballo and King, 2014).

In Africa and Asia, medicinal plants have been in use as alternative medicines in the fight against malaria. According to Ngarivhume et al. (2015), treatment of malaria using African herbs like *Azadirachta indica* (Odugbemi, 2007) has been in existence long before the introduction of Western medications.

Azadirachta indica (Meliaceae), popularly known as Neem tree is traditionally used by Indians as a cure for various illness (Tiwari et al. 2014). It is

rich in bioactive compounds with therapeutic qualities (Biswas et al. 2002). According to Nwafor et al. (2003), it is widely used in the treatment of malaria caused by many strains of the *Plasmodium* parasite because of it is rich in numerous active compounds. The plant's seed are rich sources of plants compounds such as gedunin and azadirachtin (Govindachari et al. 1998) with anti-inflammatory, anti-viral, anti-malarial, anti-pyretic, insecticidal and hypoglycaemic properties Maragathavalli et al. (2012).

In some areas of the world, malaria-carrying mosquitoes have acquired resistance to certain anti-malarial medications (Blasco et al.2017). This has made it challenging to regulate the infection rate and the spread of the disease. It is predicted that the loss of chloroquine to resistance contributed to a more than doubling of malaria-associated mortality in sub-Saharan Africa, which bears more than 90% of the global malaria burden (Trape, 2001).

Due to the growing demand and use of traditional medicines in sub-Sahara Africa nations, the effects of these plant extracts have not been documented. Our study, therefore, focused on investigating the nephropathic effects of neem leaves (*Azadirachta indica*) extracts as alternative in the treatment of *Plasmodium berghei*-infected malaria.

MATERIALS AND METHODS

Collection of Plant Materials

Fresh leaves of *Azadirachta indica* were harvested from a local farm at Ngwo, Enugu South Local Government in Enugu State, Nigeria. The leaves were identified and authenticated by a Professor of Botany, Prof. C.S Eze, in the Department of Applied Biology and Biotechnology, Enugu State University of Science and Technology.

Preparation of Plant Extract

The plant extraction was carried out using the Maceration method described byAbdullahi and Mainul (2020).

Animal Model and Experimental Protocol

Thirty (30) matured male albino Wistar rats weighing between 150 – 200 grams were used for the study. Experimental animals were purchased from the animal house at Faculty of Veterinary Medicine, University of Nigeria Nsukka. Animals were weighed and grouped according to their weight into six (6) groups of five (5) rats each and housed for seven (7) days with food and water provided *ad libitum*. The six groups were:

- Group A: which is the blank control, was not infected with *Plasmodium berghei* and was not treated with *A. indica* extract but was fed with water and feed.
- Group B: the negative control, was infected with 1.0×10^7 *Plasmodium berghei* and was not treated but was fed with water and feed.
- Group C: The positive group was infected with 1.0×10^7 *Plasmodium berghei*, treated with the standard drug, Artemether, at a 100 mg/kg dosage, and fed with water and feed.
- Group D: was infected with 1.0×10^7 *Plasmodium berghei* and treated with 50mg/kg body weight of ethanol neem leaf extract (low dose) while being fed with water and feed.
- Group E: was infected with 1.0×10^7 *Plasmodium berghei* and treated with 200 mg/kg body weight of ethanol neem leaf extract (medium dose) while being fed with water and feed.
- Group F: was infected with 1.0×10^7 *Plasmodium berghei* and treated with 500 mg/kg body weight of ethanol neem leaf extract (high dose) while being fed with water and feed.

All the animals except the blank control group were administered different doses of the neem leaves ethanol extract for a period of twenty-one days (21 days). After which they were sacrificed and blood collected for analysis.

Procurement of *Plasmodium berghei*

Plasmodium berghei was procured from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka in Nigeria. Infected *Plasmodium berghei* mice were moved to animal house in the laboratory, where their blood was used to infect the experimental Wistar albino rats.

Inoculation of *Plasmodium berghei*

A stock of parasitized erythrocytes was obtained from infected mice, with a minimum outlying parasitaemia of 20% by ocular puncture using a capillary tube. The percentage parasitaemia was determined by counting the number of parasitized red blood cells against the total number of red blood cells determined and diluted with normal physiological saline of 9.0 ml such that 0.5ml of the final inoculums contained 1.0 x 10^7parasitized red blood cells, which are the standard inoculums for the infection of a single rat. Twenty-five (25) of the thirty (30) rats were infected with 1.0 ml of *Plasmodium berghei* in a ratio 1:9 in normal saline. A 0.5 ml suspension was induced intraperitoneally in each experimental rat and allowed three (3) days to initiate infection. The determination of parasitaemia was done after five days and the *Plasmodium berghei* infection was confirmed using Giemsa stain.

Determination of Parasitaemia

Blood samples were collected by bleeding through the tail vein of *P. berghei*-infected rats, thick and thin blood smears were made on microscope slides, fixed in ethanol, stained with 10% Giemsa solution, and observed under the binocular microscope. The percentage of parasitaemia was determined by counting the percentage of Red Blood Cells (RBC) for at least ten different fields.

Collection of Serum Samples

Blood samples were collected from the retro bulbar plexus of the rats' medial canthus of the eye. About 5ml of blood sample was extracted from each rat using the ocular puncture method. The blood samples were kept at room temperature for about 30 minutes to clot, after which the test tube containing the clotted blood sample was centrifuged at 4000 revolutions per minute for 20 minutes to separate the serum from the clotted blood. The clear serum was collected carefully using a syringe and needle and stored in a clean sample bottle for the determination of the renal function concentration.

Standard Drug

The standard anti-malaria drug, Artemether, was administered orally by intubation and at 100 mg/kg for twenty-one (21) days.

Biochemical Analysis

Kidney function indices were analysed from the sample. Creatinine was determined using Randox Kit in which the present procedure was based on a modification according to Fabiny and Ertingshausen (1971).

The urea nitrogen was determined using the Randox Kit test method which was based on a modification of the procedure of Talke and Schubert (1965), and the serum electrolytes were analysed using the automated electrolyte analysers.

Histopathological Study

For histopathology, experimental rats were sacrificed and post-mortem examination performed on rats' dissected kidneys. After rinsing the dissected kidneys in normal saline, sections were taken from each organ. The tissue was fixed in a 10% ethanol solution and embedded in paraffin. It was later processed into 4-5Um thick sections stained with haematoxylin-eosin and then observed under a photomicroscope.

Slide Examination and Photomicrography

The prepared slides were examined with an Olympus light microscope using x40 objective lenses. The photomicrographs were taken using an Amscope 5.0 megapixels microscope camera at x200 magnifications.

Statistical Analysis

All Statistical analysis was processed using Statistical Program of Social Science (SPSS) software for Windows, version 18. The values of the measured parameters were expressed as mean ±SEM. A one–way Analysis of Variance (ANOVA) was used to determine the effect of *Azadirachtaindica* at different doses on albino Wistar rats infected with *Plasmodium berghei* and the difference between means were separated using Duncan's multiple range tests. Test for significance was considered at 0.05 probability

level.

RESULTS

Serum creatinine ion

The level of serum creatinine in the blank control group (2.60±0.96) was significantly lower ($p<0.05$) when compared with the negative control (4.30±1.20), low dose (4.00±1.50), and medium dose (3.30±0.66) groups. However, there was no significant difference ($p>0.05$) in the blank control (2.60±0.96) when compared with the positive control (2.62±0.98) and high dose (2.80±0.62) groups. The negative control group (4.30±1.20) was significantly higher ($p<0.05$) when compared with the positive control (2.62±0.98), blank control (2.60±0.96), medium dose (3.30±0.66) and high dose (2.80±0.62) groups. The low dose group (4.00±1.50) was significantly different ($p<0.05$) when compared with the medium dose (3.30±0.66) and high dose (2.80±0.62) groups. The negative control group (4.30±1.20) was significantly different ($p<0.05$) when compared with blank control (2.60±0.96) and positive control (2.62±0.98) (Table 1).

Table 1: Effects of *Azadirachtaindica* extract on serum creatinine of Male Wistar Albino Rats infected with *Plasmodium berghei*

GROUPS	CREATININE LEVEL (mg/dl)
A (Blank control)	2.60 ± 0.96^{a}
B (Negative control)	4.30 ± 0.96^{c}
C (Positive control)	2.62 ± 0.98^{a}
D (Low-dose extract)	4.00 ± 1.50^{c}
E (Medium-dose extract)	3.30 ± 1.50^{c}
F (High-dose extract)	2.80 ± 0.62^{a}

Mean values with different superscripts are statistically significant ($p<0.05$)

Serum urea nitrogen

The blank control group (28.18±5.12) was significantly higher ($p<0.05$) than the negative control (11.79±4.03), positive control (22.91±5.13), low dose (13.49±4.24), medium dose (18.32±4.12) and high dose (21.56±5.16) groups. However, the negative control (11.79±4.03) and the low dose group (13.49±4.24) had no significant difference ($p>0.05$). Likewise, the positive control (22.91±5.13), low dose (13.49±4.24), medium dose (18.32±4.12) and the high dose (21.56±5.16) groups had no significant differences ($p>0.05$) among themselves and all had an ameliorative effect on the urea nitrogen of the rats (Table 2).

Table 2: Effects of *Azadirachtaindica* extract on serum urea nitrogen of Male Wistar Albino Rats infected with *Plasmodium berghei*

GROUPS	UREA NITROGEN LEVEL (mg/dl)
A (Blank control)	28.18 ± 5.12^{c}
B (Negative control)	11.79 ± 4.03^{a}
C (Positive control)	22.91 ± 5.13^{b}
D (Low-dose extract)	13.49 ± 4.24^{a}
E (Medium-dose extract)	18.32 ± 4.12^{b}
F (High-dose extract)	21.56 ± 5.16^{b}

Mean values with different superscripts are statistically significant ($p<0.05$)

Serum sodium ion (Na^+)

The blank control serum level (134.00±21.00) was significantly higher ($p<0.05$) when compared to the negative (105.50±19.50), positive (128.50±20.50), low dose (115.50±18.50), medium (112.50±19.50) and high dose (127.50±20.50) groups. The negative group (105.50±19.50) was significantly low ($p<0.05$) when compared to the blank (134.00±21.00), positive (128.50±20.50) controls as well as the low (115.50±18.50), medium (112.50±19.50) and high dose (127.50±20.50) groups. However, the positive (128.50±20.50) and high dose (127.50±20.50) groups had no significant difference ($p>0.05$) which showed that the plant extract had a curative effect. Likewise, the low (115.50±18.50) and medium dose (112.50±19.50) groups had no significant difference ($p>0.05$) but showed that the plant extract had an ameliorative effect (Table 3).

Table 3: Effects of *Azadirachtaindica* extract on serum sodium of Male Wistar Albino Rats infected with *Plasmodium berghei*

GROUPS	SODIUM LEVEL (mmol/l)
A (Blank control)	134.00 ± 21.00^{d}
B (Negative control)	105.50 ± 19.50^{a}
C (Positive control)	128.50 ± 20.50^{c}
D (Low-dose extract)	115.50 ± 18.50^{b}
E (Medium-dose extract)	112.50 ± 19.50^{b}
F (High-dose extract)	127.50 ± 20.50^{c}

Mean values with different superscripts are statistically significant ($p<0.05$)

Serum potassium ion (K^+)

The blank control group (6.50±2.50) was significantly higher ($p<0.05$) than the negative (2.45±1.15), positive (5.60±2.30), low dose (3.10±0.80), medium dose (3.55±0.85) and high dose (4.40±2.60) groups. The negative group (2.45±1.15) was significantly low ($p<0.05$) compared to the blank (6.50±2.50), positive (5.60±2.30), low dose (3.10±0.80), high dose (4.40±2.60) groups respectively while the positive group (5.60±2.30) had the highest level ($p<0.05$) of serum potassium ion when compared to the other groups.The low (3.10±0.80) and medium (3.55±0.85) dose groups had no significant differences ($p>0.05$) while the high dose (4.40±2.60) had an ameliorative effect and there was a significant difference ($p<0.05$) when compared to the blank (6.50±2.50) and positive (5.60±2.30) controls (Table 4).

Table 4: Effects of *Azadirachtaindica* extract on serum potassium of Male Wistar Albino Rats infected with *Plasmodium berghei*

GROUPS	POTASSIUM LEVEL (mmol/l)
A (Blank control)	6.50 ± 2.50^{c}
B (Negative control)	2.45 ± 1.15^{a}
C (Positive control)	5.60 ± 2.30^{c}
D (Low-dose extract)	3.10 ± 0.80^{a}
E (Medium-dose extract)	3.55 ± 0.85^{a}
F (High-dose extract)	4.40 ± 2.60^{b}

Mean values with different superscripts are statistically significant ($p<0.05$)

Serum chloride ion (Cl^-)

It is observed that the level of serum chloride was significantly low ($p<0.05$) in the negative group (60.50±7.50) compared to the blank control (96.50±8.50), positive control (93.50±7.50), and low (82.50±6.50), medium (90.00±8.00) and high dose (95.00±4.00) groups. However, there was a significant difference ($p<0.05$) in the low dose (82.50±6.50) group when compared to the blank control (96.50±8.50), medium (90.00±8.00) and high dose (95.00±4.00) groups. The positive control (93.50±7.50) shows an ameliorative effect when compared to the blank control (96.50±8.50) group while the high dose (95.00±4.00) group shows that the plant extract has a curative effect (Table 5).

Table 5: Effects of *Azadirachtaindica* extract on serum chloride of Male Wistar Albino Rats infected with *Plasmodium berghei*

GROUPS	CHLORIDE LEVEL (mmol/l)
A (Blank control)	96.50 ± 8.50^c
B (Negative control)	60.50 ± 7.50^a
C (Positive control)	93.50 ± 7.50^c
D (Low-dose extract)	82.50 ± 6.50^b
E (Medium-dose extract)	90.00 ± 8.00^c
F (High-dose extract)	95.00 ± 4.00^c

Mean values with different superscripts are statistically significant ($p<0.05$)

Serum bicarbonate ion (HCO_3^-)

The blank control group (41.50±4.50) had a significant high level ($p<0.05$) when compared to the negative (19.50±3.50), positive (38.50±4.50), low dose (22.50±3.50), medium dose (29.00±4.00) and high dose (36.50±4.50) control groups. The negative group (19.50±3.50) and the low dose groups (22.50±3.50) had no significant difference ($p>0.05$) while the positive drug (38.50±4.50) was significantly different ($p<0.05$) from the low (22.50±3.50) and medium dose (29.00±4.00) groups. However, the positive control group (38.50±4.50) had no significant difference ($p>0.05$) with the high dose (36.50±4.50) and blank control (41.50±4.50) group thereby showing a curative effect of the plant extract (Table 6).

Table 6: Effects of *Azadirachtaindica* extract on bicarbonate of Male Wistar Albino Rats infected with *Plasmodium berghei*

GROUPS	BICARBONATE LEVEL (mg/dl)
A (Blank control)	41.50 ± 4.50^c
B (Negative control)	19.50 ± 3.50^a
C (Positive control)	38.50 ± 4.50^c
D (Low-dose extract)	22.50 ± 3.50^a
E (Medium-dose extract)	29.00 ± 4.00^b
F (High-dose extract)	36.50 ± 4.50^c

Mean values with different superscripts are statistically significant ($p<0.05$)

Histopathology of the Kidney

Blank control (Group A)

Sections of the kidney collected from the animals in this group (blank control) neither infected with *P. berghei* nor treated with *A. indica* showed the glomerulus and the metaplasia convoluted tubules with loss of brush border (arrows) (Plate 1)

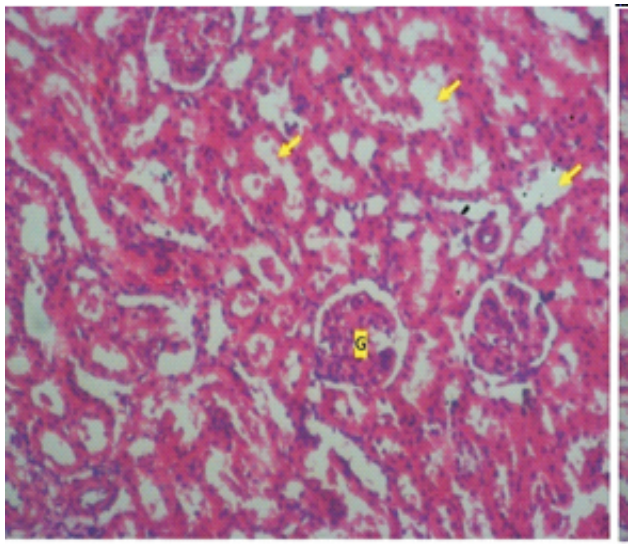

Plate 1: Photomicrograph of the kidney showing Glomerulus (G). There is metaplasia of convoluted tubules with loss of brush border (arrows). H & E. X200.

Negative control (Group B)

Sections of the kidney collected from the animals in this group (negative control) infected with $1.0x10^6$ *P. berghei* and left untreated with *A. indica* extract showed the convoluted tubules (C) and Glomerulus (G) and a focal tubular dilation with brush border depletion (arrows) (Plate 2).

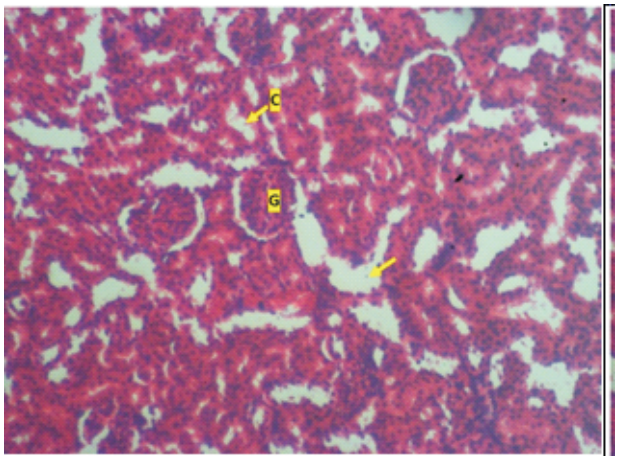

Plate 2: Photomicrograph of the kidney showing convoluted tubules (C) and glomerulus (G). There is focal tubular dilation with brush border depletion (Arrow). H & E. X200.

Positive control (Group C)

Sections of the kidney collected from the animals in this group (positive control) infected with $1.0x10^6$ *P. berghei* and treated with a standard drug; Artemether, showed the kidney cortex showing the Glomerulus (G) and deep pigmented tissues. There was also a brush border loss of the tubules (arrows) and general hyper-cellularity (Plate 3).

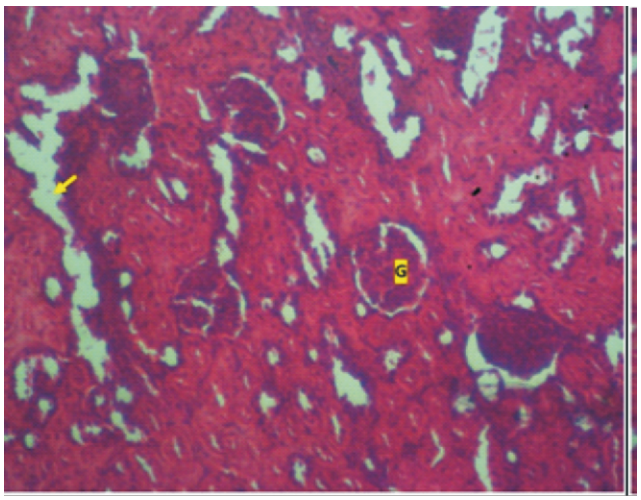

Plate 3: Photomicrograph of the kidney cortex showing glomerulus (G) and deep pigmented tissue. There is also brush border loss of the tubules (arrow) and general hyper-cellularity. H & E. X200.

Low dose control (Group D)

Sections of the kidney collected from the animals in this group (low dose control) infected with $1.0x10^6$ *P. berghei* and treated with *A. indica* plant extract administered in a low dose to the male Wistar albino rats, showed the kidney cortex showing the glomerulus (G) and convoluted tubules (arrows) while the general tissue appeared normal (Plate 4).

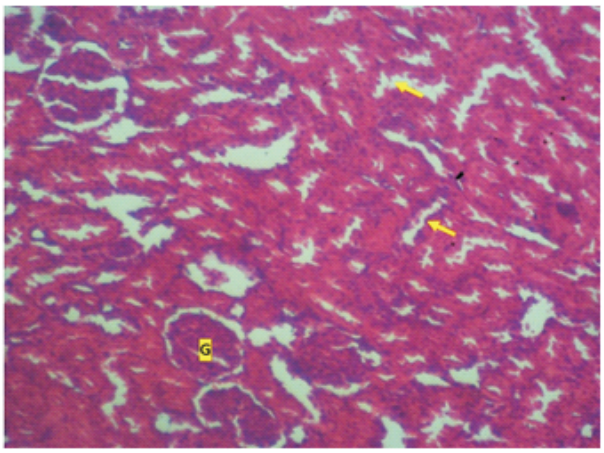

Plate 4: Photomicrograph of the kidney cortex showing Glomerulus (G) and the convoluted tubules (arrow). General tissue appears normal. H & E. X200

Medium dose control (Group E)

Sections of the kidney collected from the animals in this group (medium dose control) infected with $1.0x10^6$ *P. berghei* and treated with *A. indica* plant extract administered in a medium dose to the male Wistar albino rats, showed the kidney cortex showing the Glomerulus (G) and convoluted tubules (arrows) while the tissues showed tubular maladjustments (arrows) (Plate 5).

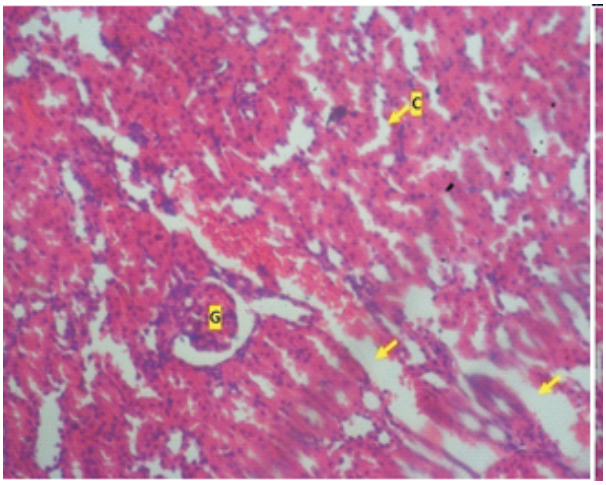

Plate 5: Photomicrograph of the kidney cortex showing the Glomerulus (G) and Convoluted tubules (C). Tissue shows tubular maladjustments (arrow).
H & E. X200

High dose control (Group F)

Sections of the kidney collected from the animals in this group (high dose control) infected with $1.0x10^6$ *P. berghei* and treated with *A. indica* plant extract administered in a high dose to the male Wistar albino rats, showed the kidney cortex showing cell mediated capsular injury (black arrow) and focal area of tubular atrophy (yellow arrow) (Plate 6).

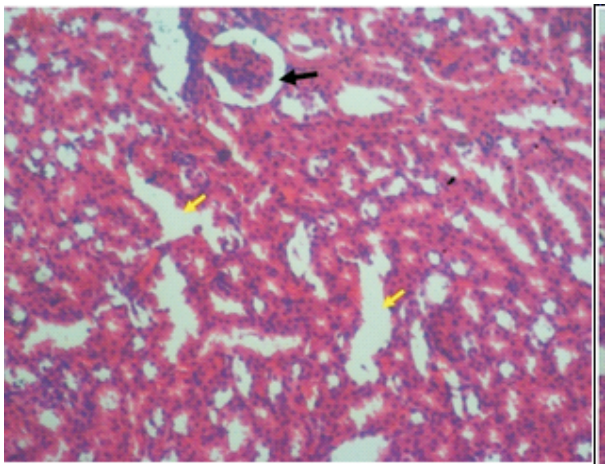

Plate 6: Photomicrograph of the kidney cortex showing cell mediated capsular injury (Black arrow) and focal area of tubular atrophy (Yellow arrow). H & E. X200

DISCUSSION AND CONCLUSION

Discussion

The present study showed that *P. berghei* statistically increased the creatinine level which was reduced upon administration of *A. indica* extract. The present study was consistent with the report of Onyishi et al. (2020) who reported that the extract potentials of *A. boonei* leaves and roots were dependent on both dosage and duration, and have demonstrated satisfactory normalization efficacy to creatinine concentration in malaria treatment. Similarly, the present result agreed with the result of Somsak et al. (2015) who reported that the aqueous crude extract of *A. indica* leaves exerted a dose-dependent protective activity of renal damage induced by *P. berghei.*

The experiment has shown that *P. berghei* significantly decreased the urea nitrogen level which was increased upon administration of *A. indica* extract. The present study was consistent with the report of Irma et al. (2021) who reported that the ethanolic extract of neem leaves did not cause damage to the liver and kidney of the male rat as indicated by the analysis of AST, ALT enzymes, urea, and creatinine concentration and if the kidneys were impaired, urea would accrue in blood.

The present study indicated that *P. berghei* decreased the sodium, potassium, chloride and bicarbonate ion concentrations which were increased upon administration of *A. indica* extract. Results agreed with the findings of Ogbuewu et al. (2009) who reported that there was an increase in the uptake of serum sodium, decrease in potassium, increase in chloride and decrease in uptake of bicarbonate ions with increasing levels of the Neem Leaf Meal (NLM). This suggests that, with up to 15 % inclusion of NLM diets, the ability of the kidney in boosting these cations and anions is not impaired.

After the administration of ethanol extract of neem leaves with doses of 50, 200 and 500 mg/kg, the kidney histology showed that *P. berghei* caused a focal tubular dilation with brush border depletion (Plate 2) and upon the administration of *A. indica,* the kidney cortex still showed some levels of cell-mediated capsular injury and tubular atrophy (Plate 6). The present study supported the work of Katsayal et al. (2008) who observed that lethal agents can cause alterations observed in the kidney

of rats, in the treatment groups, which means that the active constituents in the neem leaves could cause damage to the liver and kidney at high doses, and the extract of *A. indica* has no curative effect on kidney histology of albino rats.

Conclusion

P. berghei caused renal impairment as indicated by the biochemical analysis of creatinine, urea nitrogen, sodium, potassium and chloride and upon the administration of *A. indica*, the renal malfunctions showed dose-dependent amelioration. However, the histological studies showed that *A. indica* could not reverse the renal damages as capsular injury and focal area of tubular atrophy were observed. Further investigation is necessary for a comprehensive safety assessment of the neem leaf extract at various doses. This will highlight the safety of neem leaves for medicinal use as antimalaria chemotherapy.

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