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Toxicological Analysis and Antimalarial Potentials of Secondary Metabolites of *Curvularia lunata*, an Endophyte Obtained from the Leaves of *Azadirachta indica*

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Abstract

Background: Endophytes are symbiotic microorganisms that reside in their hosts. Phytochemicals of endophytes have generated significant interest in drug discovery due to their potentials towards the elucidation of new biologically active molecules. **Objectives:** The study was aimed to evaluate the antimalarial potentials and toxicity profile of endophytic extracts of *A. indica* leaves. **Method:** Endophytic extract was isolated from *Azadirachta indica* leaves using standard extraction protocols. The extract was screened for its potential antimalarial activities using Peter's curative test method, acute (LD50) and chronic toxicity. The chronic toxicity was assessed by evaluating the effect of extracts on the following parameters: AST, ALP, ALT, BUN, creatinine levels, PCV, HB, and RBC. The endophytic extract was subjected to prophylactic antimalarial assay using Peter's prophylactic test method, and ED50 was determined. **Results:** The endophytic extract screened for antimalarial activity showed significant activity ($p \leq 0.05$). At 150 mg/kg/day, the extract displayed a dose-dependent parasitemia clearance of *Plasmodium berghei* by 89% and suppressed parasitemia with ED50 of 333.33 mg/kg. The LD50 was >5000 mg/kg and showed no evidence of hepatotoxicity, nephrotoxicity, and haematotoxicity. **Conclusion:** Endophytic extract of *A. indica* have potent antimalarial activity with no hepatotoxicity, nephrotoxicity, and haematotoxicity.

Keywords: *Phytochemical, Endophytes, Secondary metabolites, Prophylactic, Antimalarial*

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1. Introduction

Endophytes are symbiotic microorganisms (often fungi and bacteria) present in the host plant material, either intracellular or intercellularly (David *et al.*, 2020). Phytochemicals of endophytes have generated significant interest in drug discovery programs due to their immense potential to contribute to the elucidation of new and unique biologically active molecules, especially secondary metabolites (Quang *et al.*, 2020). *A.indica* is used traditionally in the management of several ailments (Debjit *et al.*, 2010). *A. indica* is commonly known as the neem plant. Neem has been reported to show anti-fungal (Debjit *et al.*, 2010), anti-bacterial (Adamu *et al.*, 2019), and antimalarial properties (Ngozi *et al.*, 2018; Carolina *et al.*, 2020). Metabolites of fungal endophytes which were isolated from medicinal plants possess different bioactivities and structures. Hence, they are good sources of novel secondary metabolites that are linked to their therapeutic activities (Sharma *et al.*, 2016; Chutulo *et al.*, 2018; Yu *et al.*, 2010). Endophytic fungi interaction within the host plant, their pharmacological activities, and their biological activities has been positively associated (Gianluca *et al.*, 2020). In other words, host plant isolates (extracts) and their endophytic extract have shown similar bioactivities. This study was aimed to evaluate the antimalarial potentials and toxicity profile of the endophytic extracts isolated from *A. indica* leaves (Figure 1).

Figure 1: Leaves and Seeds of *Azadirachta indica*

2. Methods

2.1. Plant Collection and Authentication

Fresh leaves of *A. indica* were collected from their natural habitat at the Faculty of Pharmacèutical Science. The plant was identified at the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciènces, Nnamdi Azikiwe University, Awka with a herbarium number: PCG474 / A / 054.

2.2. Animals

Healthy Swiss albino mice weighing between 25-30 g, aged 7-8 weeks were used for the analysis. The test animals were gotten from the Pharmacology and Toxicology Department, Faculty of Pharmacèutical Sciences, Nnamdi Azikiwe University, Awka, housed in standard working laboratory conditions of 12 h light, at room temperature, 40-60% relative humidity, and were fed with rodent feed obtained from Guinea Feeds Nigeria Ltd. The animals were allowèd free accèss to foõd and watèr. All animal experimènts were conductèd following the recommendations from the National Institute of Health (NIH) guidelines (Ajaghaku *et al.*, 2016).

2.3. Parasite

The parasite, *Plasmodium berghei* used for the study, was gotten from the Department of Pharmaceutics, University of Nigeria Nsukka, Enugu State; and authenticated by a parasitologist institution.

2.4. Isolation Procedure

The plant leaves were thoroughly rinsed using sterile water to remove debris in the laboratory before being disinfected by immersion using 70% ethanol for 3 min and 2% sodium hypochlorite for 2 min. This was done to achieve surface sterilization and isolation. The isolation procedures reported by Arnold *et al.* were strictly followed (Arnold *et al.*, 2000) with mild modifications. Then, they were rinsed with sterile distilled water and blotted dry on sterile blotting paper in a lamina flow cabinet. A sterile scalpel was used to cut the leaf blade and mid-rib approximately 1 cm in length.

2.5. Endophyte Fermentation and Extraction of Metabolites

The endophyte isolated was subjected to solid-state fermentation in 1 L Erlenmeyer flask containing sterilized rice medium which was prepared by autoclaving a mixture of 100 g of unpolished rice and 200 mL of distilled water. After cooling, blocks of actively growing pure fungal isolates were transferred onto the rice media under aseptic conditions, plugged with cotton wool and foil, and left on the fermentation shelf for 21 days. After fermentation, the fungal secondary metabolites were extracted with ethyl acetate, and flasks were left undisturbed for about 48 h with intermittent agitation. Rotary evaporator was used to concentrate the extract filtrates, at a speed of 7 rpm and at 50 ^{o}C. The filtrates were used for the biological assay.

2.6. Acute Toxicity Test

A total of mice grouped into eight groups 1-8 in two phases (n = 4), were used to evaluate the acute toxicity of the endophyte extracts. Phase 1 was made up of groups 1-4 while phase two was made up of groups 5-8. Group 2, 3, 4, 5, 6, 7 and 8 received 100 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg, 3000 mg/kg, 4000 mg/kg, and 5000 mg/kg of endophyte extract respectively while Group 1 received 10 mL of distilled water as a control. Distilled water was used to dissolve the extracts prior orally administration to the various animal groups after an overnight fast. Test animals were constantly monitored for 2 h, intermittently for the next 6 h, and after 24 h for behavioral changes and mortality. The animals wère left for more 14 days in order to observe any delayed signs or symptoms of acute or chronic toxicity (Lorke, 1983).

2.7. Antimalarial Assay

2.7.1. Prophylactic Assay

Evaluation of the chemoprophylactic potential of *Curvularia lunata* extract was performed accordance with Peters (1975) (with some modifications). Grouped mice (5 mice per group) were administered 100 mg sulfadoxine-pyrimethamine (SP), 250 mg/kg and 500 mg/kg of MR3 endophytic extract daily for four consecutive days (day 0-3) while the negative control (untreated control) mice were given 10 mL/kg of distilled water. Mice (test animals) were injected with *Plasmodium berghei* parasitized erythrocytes in a way that it cont ins $1x10^7$ of parasitized cells. After 72 h of inoculation, serum (blood) samples were obtained from the animals through the vein to make a thin film, fixed and stained using 10% Giemsa stain. The slides were observed under microscope using the X10 objective magnification to determine the level of parasite suppression and calculate percentage suppression, thus:

$$\%\text{Suppression} = \frac{\%\text{Parasitemia Negative Control} - \%\text{Parasitemia Treatement Groups}}{\%\text{Parasitemiain Negative Control}}$$

2.8. Curative Assay

Antimalarial curative study of the endophytic extract was performed in accordance Peters (1975) method with some modifications (Peters, 1975). In this model of study, a total of 55 mice were used. They were grouped into 11 groups of five mice per group. Blood was collected from donor mouse infected with the parasite (*P. berghei*) by oculus puncture and was diluted with normal saline such that 0.2 mL contains approximately $1x10^7$ infected red cells. All the animals in each group were infected with the parasite by intraperitoneal administration of 0.2 mL of the diluted blood and were lèft for 72 h for the infèction to occur. After infection, the animals started receiving treatment according to their respective doses. Artèmisinin Combination Therapy (ACT) at 9.8 mg/kg was used as the positive control. A thin bloõd film was prepared from the tail, blood stained with Gièmsa stain and was examined for parasitemia on day 4 and day 7 post-treatment respectively.

$$\%\text{Parasitemia} = \frac{\text{Number of Parasitized Red Blood Cells}}{\text{Total Number of RBCs Count} \times 100}$$

2.9. Chronic Toxicity Assay

2.9.1. Sub-Acute (30-Days) Toxicity Studies

Albino rats (15) were randomly divided into three groups of five rats per group. The extract was orally administered at doses of 200 mg/kg and 400 mg/kg to groups 1 and 2 respectively once daily for 30days. The control group (3) received 5 mL/kg of 5% tween 80. Collection of blood samples from test animals was through retro-orbital plexus on day 0 before the commencement of treatment and day 31 after treatment. Pre-treatment and post-treatment body weights were recorded. The blood samples were allowed to coagulate for 30 min and serum was separated by centrifuging at 3000 rpm for 10 min and was used for the analysis of biochemical liver markers—Alanine Aminotransferase (ALT), Aspartatè aminotransfèrase (AST), and Alkaline phosphatase (ALP); kidney markers—urea, creatinine, sodium, chloride and bicarbonate ions. Mindray Auto analyzer was used for kidney and liver function tests.

2.10. Kidney Function Test

2.10.1. Blood Urea Nitrogen (BUN) test

BUN Enzyme reagent (1.5 mL containing 10000 µ/l Urease, 6.0 mmol/l Sodium Salicylate, 3.2 mmol/l sodium nitroprusside) was added to 10µl of the test (serum), Standard (20 mg/dL) and Blank (distilled water) followed by incubation for 5 min at 37 ^{0}C. At the timed interval, 1.5 mL of BUN color developer (6 mmol/L of sodium hypochlorite and 130 mmol/l sodium hydroxide) was added to each of the labeled tubes and was incubated for another 5 min at 37 ^{0}C. The absorbance of the tests and Standards were measured spectrophotometrically at 630 nm against a blank (Tietz, 1976).

Urea nitrogen concentration (mg/dL) is calculated as follows:

$$\frac{\text{Absorbance of Test}}{\text{The Absorabance of Standard} \times \text{Concentration of Standard}}$$

2.11. Serum Creatinine

Creatinine working reagent was prepared by combining equal volumes of 10 mM picric acid and creatinine buffer reagent (10 mM sodium borate and 240 nM sodium hydroxide). The creatinine buffer reagent (3 mL) was added to labeled test tubes (test, blank and standard) to which 100 µL of serum (test), 5 mg/dL of Creatinine (Standard), and distilled water (blank) were added and mixed. The tubes were incubated at 37 ^{0}C for 15 min and the absorbance was measured spectrophotometrically at 520 nm against test blank (Morgan *et al.*, 2019).

The concentration of creatinine (mg/dL) was then determined using the formulae:

$$\frac{\text{Absorbance of Test}}{\text{The Absorabance of Standard} \times \text{Concentration of Standard}}$$

2.12. Liver Function Test

Blood samples collected with plain tubes were allowed for 30minutes to clot before they were centrifuged at 3000 rpm for 10 min. The serum (supernatant) was separated into an Eppendorf tube.

2.13. Quantitative Determination of Alanine Aminotransferase (ALT)

Serum alanine aminotransferase concentration was monitored using a RANDOX kit. The enzyme activity was monitored by the concentration of pyruvate hydrazine formed with 2,4-dinitrophenylhydrazine. Mixture of 200 mmol/L L-alanine, α-oxoglutarate (2.0 mmol/L) and phosphate buffer (100 mmol/L) (0.5 mL) was added to 0.1 mL of each sample and blank (distilled water). They were mixed and incubated in a watèr bath at 37 ^{0}C for exactly 30 min (Reitman and Frankel, 1957).

2.14. Quantitative Determination of Aminotransferase (AST)

Serum aspartate aminotransferase concentration was monitored using a RANDOX kit. The enzyme activity was monitored by the concentration of oxaloacetate hydrazine formed with 2,4-dinitrophenylhydrazine. Mixture of L-aspartate (100 mmol/l), α-oxoglutarate (2.0 mmol/L) and phosphate buffer (100 mmol/L) (0.5 mL) was added to 0.1 mL of each sample blank (containing distilled water). They were mixed and incubated at 37 ^{0}C for exactly 30 min in a water bath. Thereafter, 0.5 mL of 2,4-dinitrophenylhydrazine was added into the sample and blank test tubes and incubated again at room temperature for 20 min. 5 mL NaOH was added to all the test tubes and the absorbance of the sample read at 546 nm using a UV spectrophotometer. The AST concentration was extrapolated from a graph of concentration against absorbance of known AST concentrations (Reitman and Frankel, 1957).

2.15. Quantitative Detèrmination of Alkalinè Phosphatase (ALP)

Serum alkaline phosphatase concentration was monitored using TECO DIAGNOSTIC kit. The principle of the test is based on alkaline solution mediated blue chromogen formation upon the reaction of the enzyme with buffered sodium thymolphthalein monophosphate. Alkaline phosphatase substrate (3.6 mM sodium thymolpthalein monophosphate in 0.2 M 2-amino-2-methyl-1-propanol buffer, 0.5 mL) was dispensed into a sample and blank labeled test tubes and equilibrated to 37 ^{0}C for 3 min. Thereafter, 0.05 mL of standard (Thymolphthalein in 0.5 mM/L n-propanol), control (deionized water), and samples were added to their respective test tubes. The mixture was incubated for 10 min at 37 ^{0}C. Alkaline phosphate color developer (0.1 M sodium hydroxide and 0.1 M sodium carbonate, 2.5 mL) was added and the sample absorbance read at 590 nm using a UV spectrophotometer (Reitman and Frankel, 1957).

Calculation of ALP concentration was done using the formulae below:

$$\frac{\text{Absorbance of Unknown}}{\text{The Absorabance of Standard} \times \text{Standard (IU/L)}}$$

2.16. Statistical Analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS Version 23). The results were expressed as mean ± SD. The hematological parameters and antimalarial curative analysis were done using GraphPad Prism (Version 3.10) (GraphPad Prism software, Inc., US. while the kidney function test (creatine and urea), liver function test (ALT, AST, and ALP), and electrolyte balance were done using the one-way ANOVA). The results are presented as the mean ± standard error of the mean (SEM). The differences between means of the

Table 1: Identity of the Endophytes Fungi Isolated from the Leaves of *A. indica*

Plant Source	Plant Parts	Fungi Codes	Identification	Codes	References
A. indica	Leaf	MR3	*Curvularia lunata*	Dothideomycetes	(Frisvad and Samson, 2004; Chemicalbook.com, 2020)

Table 2: Antiplasmodial Prophylactic Effect of the Extract

Group	% Suppression	Mean ± SEM
10mL/Kg Dist H_2O	0	25.0 ± 1.01
100 mg/kg SP	91.32	2.17± 0.22**
250 mg/kg extract	51.60	12.1 ± 1.23**
500 mg/kg extract	46.80	13.3 ± 0.90**

Note: Values are expressed as mean ± Standard *p < 0.01, **extremely significant.

measured parameters were compared using one-way ANOVA. The *p* values < 0.05 at 95% confidence were regarded as statistically significant.

3. Results

The results obtained from the research are presented in tables and figures below:

Table 3: Multiple Comparison of Positive Control and Extracts

Multiple Comparison	*p*-value	Level of Significance
100 mg/kg SP Vs 250 mg/kg extract	**	*p*<0.01>
100 mg/kg SP Vs 500 mg/kg extract	**	*p*<0.01>

Note: Values are expressed as mean ± Standard *$p < 0.01$, **Extremely significant.

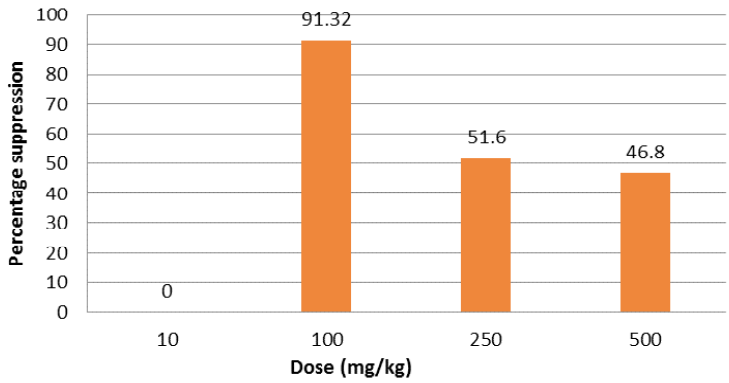

Figure 3a: Antiplasmodial Prophylactic Effect of Extract

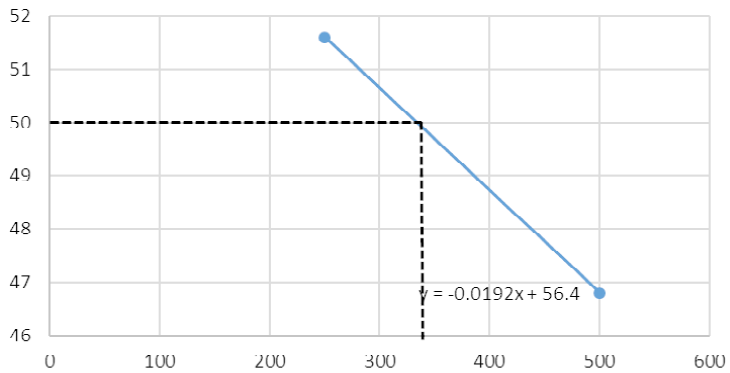

Figure 3b: Effective Dose Concentration (Ed50) of the Extract

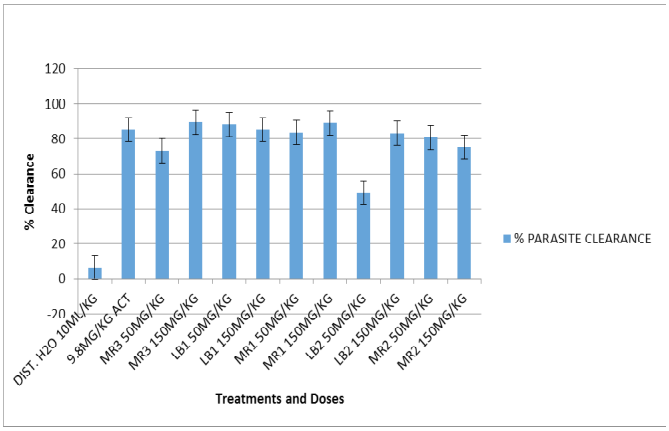

Figure 4: Percentage Parasite Clearance

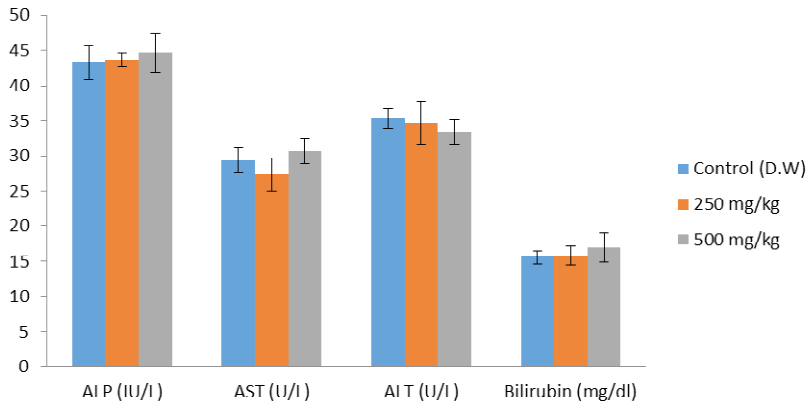

Figure 5a: Effects of Extract on Liver Biomarkers

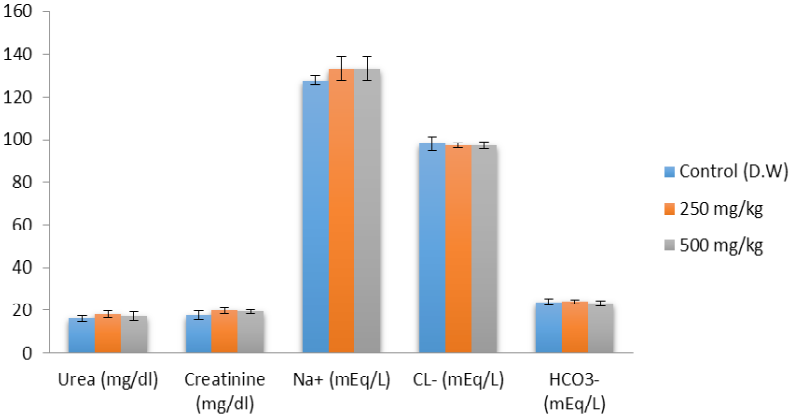

Figure 5b: Effects of Extract on Renal Biomarkers

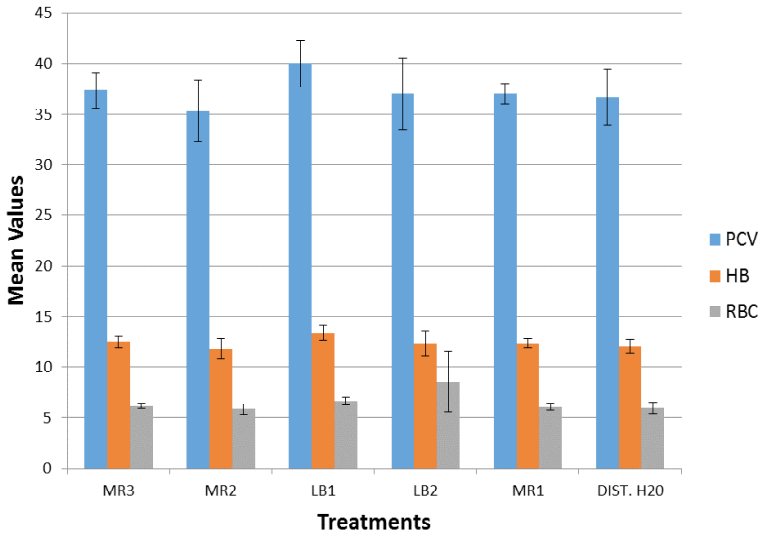

Figure 5c: Heamalogical Effect of the Extract

Plate 1- 3: Kidney histology sections of albino rats treated orally with different concentrations of the extract (H&E-x 400).

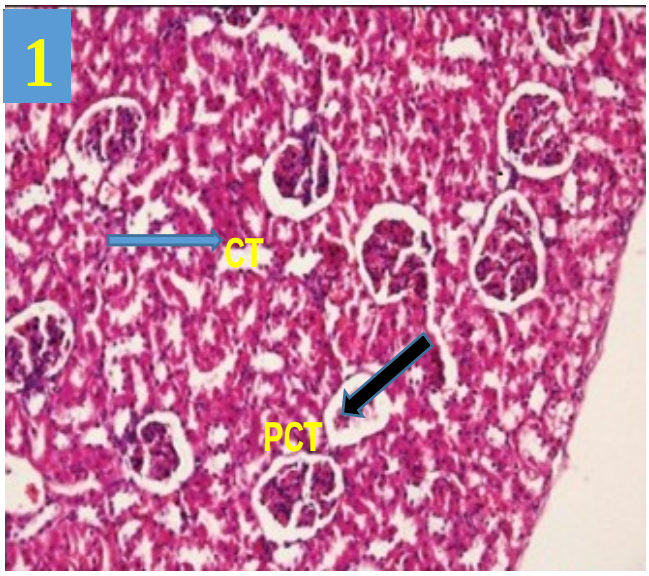

Plate 1: Shows Thick Glomeruli with Normal Vascularity. Collecting Tubules (CT) and Proximal Convoluted Tubule (PCT) are Normal. Histoarchitecture not Affected

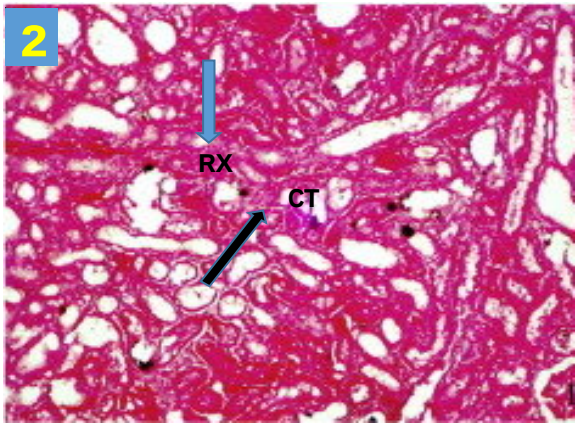

Plate 2: Shows Distal Collecting Tubules with Normal Architecture (CT), and the Interstitial Tissues Show Few Lymphocytes (RX). Histoarchitecture not Affected

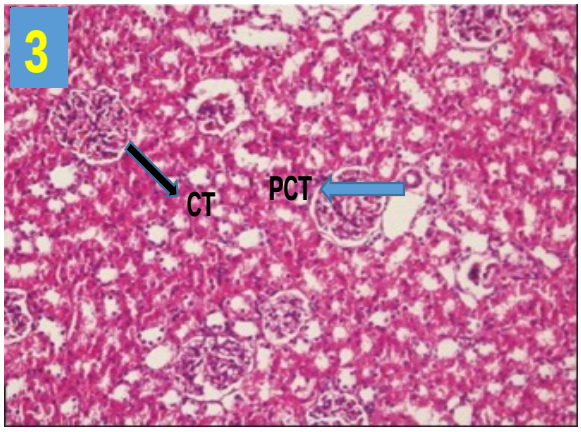

Plate 3: Shows Thick Glomeruli With Normal Vascularity (G), Collecting Tubules (PCT) And Proximal Convoluted Tubule Are Normal (CT). Histoarchitecture Not Affected

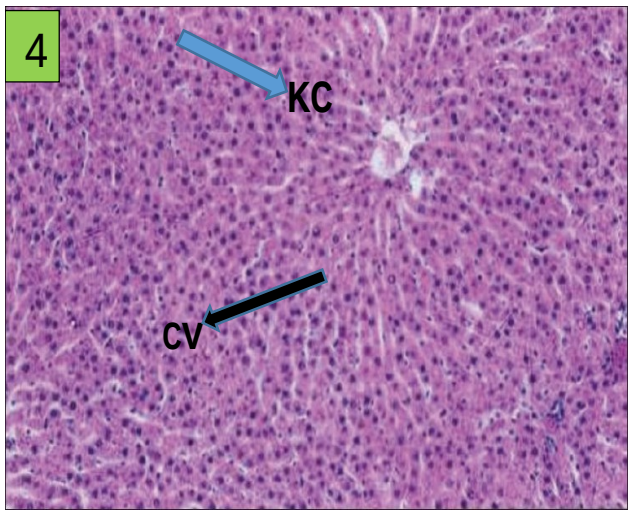

Plate 4: Normal architecture, showing central vein (CV), Kupffer cells (KC), and the hepatocytes are arranged in a regular manner. Histoarchitecture not affected

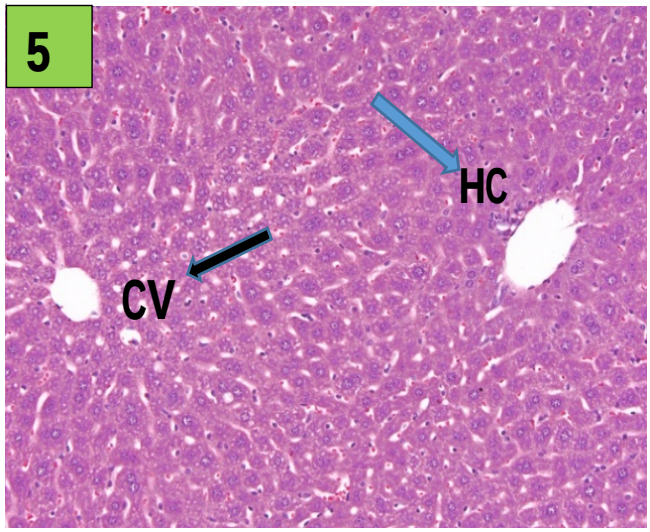

Plate 5: Normal Architecture, Showing Central Vein (CV), and the Hepatocytes (HC) Well Arranged in a Regular Manner. Histoarchitecture Slightly Affected

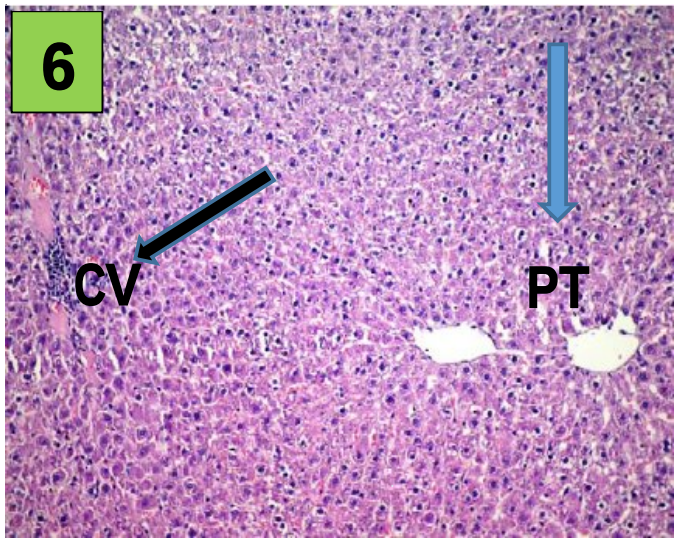

Plate 6: Showing Normal Architecture of Central Vein (CV) and Portal Trait (PT) Appears Normal. Histoarchitecture Not Affected

Plate 4-6: Liver histology sections of albino rats treated orally treated with different concentrations of the extract (H&E-x400).

4. Discussion

Antimalarial potentials and toxicity profile of endophytic extracts isolated from *Azadirachta indica* lèaves were, successfully conducted using Peter's curative test method. The toxicity profile was also evaluated from the most promising extracts was selected for further investigations including acute (LD50) and chronic toxicity testing. The result of acute toxicity study in mice revealed no lethality or toxic reaction at any of the administered doses between 100 mg/kg and 5000 mg/kg. There were no signs of injury, salivation, body weakness or stretching, paw licking, respiratory distress, coma, and mortality in the first three hours and subsequently. This indicates that the extract has a wide therapeutic window, compared to other natural products.

Chemoprophylactic results (Table 2) showed that the extract possessed significant chemoprophylactic activity at the doses of 250 and 500 mg / kg, respectively ($p< 0.05$). Though, a very significant reduction was found in the 100 mg / kg SP treatment of the extract. The mean comparison of the positive control (SP) and the doses of the endophytic extracts used showed very significant suppression of parasitemia (Table 3). The median effective dose (ED50) concentration was calculated at 333.33 mg / kg shown in Figures 3a and 3b. *Plasmodium berghei* was used as the test parasite. Curative antimalarial activity of extracts against *Plasmodium berghei* (Figure 4) showed that endophyte extract treatment resulted in a significant ($p<0.05$) reduction in parasitemia, with parasite clearance of 88.06% and 89.23% at doses of 50 and 150 mg / kg, respectively. The ACT-treated group showed a significant ($p<0.05$) reduction of parasitemia with 85.11% parasite clearance. The chronic toxicity assay of the endophyte extract was seen to be well-tolerated by test animals over the administration of 30 days. Chronic administration of 2000 mg / kg of the extract did not result in any overt signs of toxicity-hepatotoxicity (indicated by AST and ALT), nephrotoxicity (indicated by BUN and creatinine) (Figure 5a, 5b, and 5c and plate 1 - 6).

5. Conclusion

Among the five endophytic extracts screened for antimalarial activities, MR3 showed the highest activity. The extract displayed a dose-dependent parasitemia clearance of Plasmodium berghei at 150 mg / kg / day, by 89%. The endophyte was identified as Curvularia lunata. The LD50 was >5000 mg / kg and showed no evidence of hepatotoxicity, nephrotoxicity, and haematotoxicity in test animals. Therefore, from the results obtained, the endophytic extracts of A. indica have potent antimalarial and antimalarial activities with no organ damage properties.

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