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Study of Hepatoprotective Potential of Fractions and Phytosomes of Ethanol Root Extract of *Millettia aboensis* on Carbon Tetrachloride Induced Hepatoxicity in Rats

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Abstract

Millettia aboensis (Hook. F.) Baker is folklorically used for the treatment of liver diseases, splenomegaly, venereal diseases and constipation. Phytosomes are complexes of plant extracts and phospholipids developed to enhance the bioavailability and therapeutic effectiveness of phytoconstituents. This study aimed to formulate and evaluate phytosomes containing ethanol extract and fractions of *M. aboensis* for enhanced hepatoprotective activity against carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. Pulverized roots of *M. aboensis* were extracted by cold maceration using ethanol and subsequently fractionated with n-hexane, ethyl acetate, n-butanol and water to obtain the respective fractions. Acute oral toxicity was evaluated in mice using a modified Dietrich-Lorke method. Phytosomes were prepared by solvent evaporation using phosphatidylcholine and the respective fractions in 1:1, 1:2 and 2:1 ratios. The 1:1 complexes were formulated into capsules and characterized by

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Fourier-transform infrared (FTIR) spectroscopy. Capsule quality was evaluated according to British Pharmacopoeia specifications, including weight uniformity, assay, stability, disintegration and dissolution. Hepatoprotective activity was assessed in CCl_4-induced hepatotoxicity in rats at 0.75 mL/kg. Data were analyzed using one-way analysis of variance followed by Dunnett's post hoc test, with significance set at $p < 0.05$. Ethanol extraction yielded 39.2%, while fractionation yielded ethyl acetate (14%), n-hexane (12.2%), n-butanol (10%) and aqueous (2.8%) fractions. Acute toxicity studies indicated low toxicity. FTIR analysis confirmed phytosome formation. The formulated phytosomal capsules complied with British Pharmacopoeia specifications. Ethyl acetate fraction and 1:1 ethyl acetate and n-hexane phytosomes demonstrated hepatoprotective activity, with the phytosomes producing significantly greater protection ($p < 0.05$). The findings indicate that *M. aboensis* phytosomes protect against CCl_4-induced hepatic injury and may enhance the hepatoprotective activity of the plant fractions.

Keywords

Millettia aboensis, CCl_4, Phytosomes, Hepatoprotection, Phosphatidylcholine, Rats

1. Introduction

Millettia aboensis is a medicinal plant traditionally used in Nigeria for the management of liver disorders, jaundice, fever, and digestive ailments. Different parts of the plant, including the leaves, roots, and stem bark, are commonly prepared as decoctions or macerations for oral administration [1]-[4]. Its traditional use has generated interest in investigating its phytochemical constituents and potential pharmacological activities, particularly its hepatoprotective properties. Plant-derived compounds with antioxidant and anti-inflammatory activities may offer protective effects against liver injury caused by toxic substances. However, systematic investigations are required to establish the contribution of individual plant fractions and the potential benefits of formulation approaches designed to improve the delivery of bioactive constituents.

Medicinal plants belonging to the Fabaceae family, including several Millettia species, contain diverse secondary metabolites, such as flavonoids, isoflavonoids, phenolic acids, tannins, saponins, alkaloids, and terpenoids. These compounds have been associated with antioxidant and anti-inflammatory activities that may contribute to the protection of hepatic tissue [1]-[3]. In particular, flavonoids and other polyphenolic compounds may scavenge reactive free radicals, inhibit lipid peroxidation, and help preserve hepatocyte membrane integrity. The presence and concentration of these constituents may vary according to the plant part, extraction solvent, and fractionation method, potentially influencing the biological activity of the resulting preparations.

Carbon tetrachloride (CCl_4) is widely employed in experimental studies of hepatotoxicity because its metabolism by cytochrome P450 enzymes generates trichloromethyl and trichloromethyl peroxyl radicals. These reactive intermediates initiate lipid peroxidation, disrupt cellular membranes, promote inflammatory responses, and may ultimately cause hepatocellular necrosis and fibrosis [5] [6]. The findings of the present study are consistent with the work of Boubellouta which demonstrated the hepatoprotective potential of three endemic medicinal plants against CCl_4-induced liver damage in rats [7]. This supports the importance of investigating plant-derived bioactive constituents as potential therapeutic agents for protecting hepatic tissues against chemical-induced oxidative injury. Consequently, CCl_4-induced liver injury provides a useful experimental model for evaluating substances with potential hepatoprotective activity.

The severity of hepatic injury is commonly assessed using serum biochemical markers, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Elevated ALT and AST generally indicate hepatocellular injury, whereas increased ALP may suggest impaired biliary function. Changes in serum bilirubin, albumin, and total protein can provide additional information about hepatic excretory and synthetic functions [8]-[13]. These biochemical measurements are often complemented by histopathological examination to identify structural changes such as hepatocellular necrosis, steatosis, and inflammatory cell infiltration. The combined assessment of biochemical and histological outcomes provides a more comprehensive evaluation of hepatoprotection than enzyme measurements alone.

Phytosome technology represents a formulation approach in which plant-derived bioactive compounds interact with phospholipids to improve their physicochemical properties and potentially enhance absorption and bioavailability. This approach may be particularly beneficial for phytoconstituents with limited aqueous solubility or poor gastrointestinal absorption. Previous studies on phytosomal formulations of medicinal plant extracts, including silymarin, have reported improvements in the delivery and biological activity of selected constituents [14]-[20]. Nevertheless, the extent of improvement depends on the phytochemical composition, phospholipid-to-extract ratio, formulation characteristics, and experimental conditions. Therefore, the superiority of a phytosome formulation should be established experimentally rather than assumed from its formulation alone.

Solvent partitioning provides another means of investigating the hepatoprotective potential of *M. aboensis*. Partitioning an ethanol extract into fractions of different polarities may separate groups of phytochemicals according to their solubility characteristics. The resulting fractions may differ in their concentrations of flavonoids, phenolic compounds, and other metabolites, leading to variations in antioxidant capacity and biological activity [18]-[20]. Comparing the crude extract with its individual fractions can therefore help identify preparations with promising hepatoprotective effects. Further comparison with their corresponding phytosome formulations can establish whether phospholipid complexation provides additional

benefits beyond those attributable to the plant constituents themselves.

Oxidative stress markers are also important in elucidating the mechanisms underlying hepatoprotection. CCl_4-induced free radicals promote lipid peroxidation, which can be assessed through malondialdehyde (MDA) levels. Antioxidant defence may be evaluated through total antioxidant capacity, reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activities [21]-[24]. Improvements in these parameters may indicate attenuation of oxidative damage and preservation of endogenous antioxidant defences. However, biochemical recovery should be interpreted alongside histopathological findings to determine whether improvements in laboratory markers correspond to structural preservation of the liver.

Accordingly, the present study evaluated the hepatoprotective effects of the ethanol extract, solvent-partitioned fractions, and corresponding phytosome formulations of *M. aboensis* against CCl_4-induced liver injury in experimental animals. The assessment integrated serum biochemical parameters, oxidative stress-related endpoints, and histopathological examination to compare the protective effects of the different preparations. This comparative approach was intended to distinguish the intrinsic activity of the crude extract and individual fractions from any additional benefit associated with phytosome formulation. The findings may contribute to a better understanding of the hepatoprotective potential of *M. aboensis* and provide a scientific basis for further investigation of its phytosomal formulations.

2. Materials and Methods

2.1. Experimental Animals

Wistar rats of both sexes were obtained from the department of Veterinary Medicine, University of Nigeria Nsukka. The animals were maintained on standard pellet diet and tap water *ad libitum* and acclimatized for 14 days before use. The use and care of laboratory animals were conducted in accordance with internationally accepted best practices as contained in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and approved by local Ethics Committee of our institution with the ethical approved number: ESUT/AEC/2024/158/AP 132.

2.2. Acute Toxicity Test

This was performed according to modified Dietrich Lorke's method [25]. Here an initial investigation involving administering (10, 100, 1000 mg/kg body weight) of the plant extract fractions to four different groups of three mice each for a particular dose, making a total of thirty six mice as shown in **Figure 2**. After 24 h, the number of deaths was recorded but there was no death. The result was compared to that in a table given in modified Dietrich Lorke's method [25]. Based on the result, from the same table the doses to be chosen for a second acute oral toxicity test was extrapolated. In this second acute toxicity test, four dose levels were used (1000, 1600, 2900 and 5000 mg/kg) using 3 animals per dose-level for each fraction making it a total of forty eight mice for the second acute toxicity rest. Observations were made up to 14 days during which dead animals and toxic manifestations

were noted. The LD_{50} was calculated as the geometric mean of the maximum dose that caused 0% death and the minimum dose that caused 100% death. Fractions of *Milletia aboensis* produced death in the doses of 1600 mg/kg, 2900 mg/kg and 5000 mg/kg body weight. Hence, 1/90th and 1/45th of the lethal dose *i.e.*, 25 mg/kg and 50 mg/kg (Dose ≤ 100 mg/kg) p.o of both fractions and complex capsules of *Millettia aboensis* were used for the next study.

Lorke's method estimates LD_{50} as: $LD_{50} = \sqrt{D_0 \times D_{100}}$

where D_0 is the highest dose at which no mortality occurs and D_{100} is the lowest dose at which all animals die, using the relevant dose groups in the second phase.

D_0 = 1000 mg/kg and D_{100} = 5000 mg/kg.

The estimate would therefore be:

$$LD_{50} = \sqrt{1000\,\text{mg/kg} \times 5000\,\text{mg/kg}} = 2236.07\,\text{mg/kg}$$

2.3. Preparation of Phytosomes

The phytosome of plant extracts or specific active principles with dietary phospholipid is generally prepared by solvent evaporation techniques using alcoholic or organic solvents as reaction medium. In the more frequently used solvent evaporation technique, the drug and the phospholipids are placed in the same flask containing a suitable solvent system such as tetrahydrofuran or ethanol. The reaction is allowed to be carried out at suitable fixed temperature for a fixed duration of time to get maximum possible yield and drug entrapment. The fractions of *Millettia aboensis* root extract and phospholipid were dissolved in dichloromethane by sonication separately. The drug solution was then added drop wise to the phospholipids solution with soniocation for a period of 1 - 2 h after which it was lypholized. 1:1, 1:2 and 2:1of the fractions to the phospholipid were prepared using the method mentioned above to yield 1E:1P ethylacetate phytosome, 1E:2P ethylacetate phytosome, 2E:1P ethylacetate phytosome, 1B:1P n-butanol phytosome, 1B:2P n-butanol phytosome, 2B:1P n-butanol phytosome, 1H:1P n-hexane phytosome, 1H:2P n-hexane phytosome, 2H:1P n-hexane phytosome after 1:1 phytosomes of each fractions was formulated into capsules for further studies. The rationale for selecting the 1:1 phytosome formulation for each fraction was to obtain a manageable and controllable formulation size that would facilitate accurate dose administration to the experimental animals. The 1:1 ratio was considered suitable for balancing the quantities of the plant fraction and phospholipid, thereby supporting the preparation of a formulation that could be administered consistently and conveniently. This approach was intended to improve dose uniformity, minimize handling difficulties, and facilitate reliable evaluation of the biological activities of the phytosomes in the experimental animals.

2.4. Characterization of Phytosome-Loaded Gelatin Capsules

2.4.1. Disintegration Time

Disintegration time was determined using an Erweka disintegration apparatus ac-

cording to European Pharmacopoeia (EP) specifications. Six capsules per batch were tested individually in FeSSIF, FeSSGF, FaSSIF, FaSSGF, simulated intestinal fluid (SIF), and simulated gastric fluid (SGF). The time required for each capsule to disintegrate and pass through the mesh was recorded. Mean, standard deviation, and coefficient of variation were calculated.

2.4.2. Weight Uniformity

Weight uniformity was assessed using ten randomly selected capsules per batch according to USP specifications. Each intact capsule was weighed, emptied, and reweighed. The net content weight was calculated by subtracting the shell weight from the intact capsule weight. Mean net weight and percentage deviation were determined.

2.4.3. Dissolution Study

Dissolution was evaluated using a magnetic stirrer at 100 rpm and 37˚C ± 1˚C in 200 mL of the appropriate medium. One capsule was introduced at zero time, and samples were collected at 30-minute intervals up to 6 hours. Equal volumes of fresh, pre-warmed medium replaced withdrawn samples. Drug release was quantified by UV spectrophotometry at 265, 261, and 264 nm for the ethyl acetate, n-butanol, and n-hexane fractions, respectively.

2.4.4. Drug Content Determination

Drug content was determined by dissolving accurately weighed capsule contents equivalent to 25 and 50 mg of phytosome complex in 10 mL ethanol. After suitable dilution, absorbance was measured at 265, 261, and 264 nm for the ethyl acetate, n-butanol, and n-hexane fractions, respectively. Drug content was calculated using the corresponding Beer's law calibration curves [26].

2.4.5. Stability Studies

Short-term stability was assessed by storing 10 g samples of *Millettia aboensis*-loaded phytosomes in sealed glass vials at 4˚C and 25˚C for 15 days. Residual drug content was determined on days 0, 1, 2, 3, 4, 8, and 15. Long-term stability was evaluated using lyophilized phytosomes stored under the same temperature conditions for six months, with drug content assessed monthly. Corresponding free plant extract preparations served as controls. Physical appearance and consistency were also monitored throughout storage.

2.5. Spectrum Evaluation

Fourier transform infrared (FTIR) (Perkin Elmer, Spectrum XB, USA) spectra date were taken to ascertain the structure and chemical stability of phytosome complex, phospholipids and plant drug (*Millettia aboensis*) root extract and fractions were crushed in KBr to get pellets at 600 kg/cm^2 pressure. Spectral scanning were done in the range between 4000 - 400 cm^{-1} [27]-[29].

2.6. Design for Hepaprotective Assay

The rats were divided into seventeen groups of five rats two male and three female

rats each making a total of 85 rats of both sex thirty four male and fifty one female rats in which each groups were allocated with two male rats and three female rats The hepaprotective activity of the plant extract and solvent fractions was tested using CCl_4 models. Group 1 (normal control) received only food and water. The rats were treated with the fractions as a fine suspension in distilled water and the phytosomes (complexes) for 9 days before injection of CCl_4 on the ninth day [30].

The treatment groups, doses, and administration of the fractions of *Millettia aboensis* and their corresponding phytosome complexes are presented in Table 1.

Table 1. Administration of the drug.

Groups	Administration of drugs
Group I	Served as normal control and received only food and water
Group IIa	Served as induction control and received 0.75 mL/kg b.wt of CCl_4.
Group IIIa	Received 1.0 mg/kg b.wt of LIV 52® +0.75 mL/kg b.wt of CCl_4.
Group IVa	Received 25 mg/kg b.wt of n-hexane fraction +0.75 mL/kg b.wt of CCl_4
Group IVb	Received 50 mg/kg b.wt of n-hexane +0.75 mL/kg b.wt of CCl_4
Group Va	Received 25 mg/kg b.wt of EtOAc fraction +0.75 mL/kg b.wt of CCl_4
Group Vb	Received 50 mg/kg b.wt of EtOAc fraction +0.75 mL/kg b.wt of CCl_4
Group VIa	Received 25 mg/kg b.wt of n-butanol fraction +0.75 mL/kg b.wt of CCl_4
Group VIb:	Received 50 mg/kg b.wt of n-butanol fraction +0.75 mL/kg b.wt of CCl_4
Group VIIa:	Received 25 mg/kg b.wt of aqueous fraction +0.75 mL/kg b.wt of CCl_4
Group VIIb:	Received 50 mg/kg b.wt of aqueous fraction +0/75 mL/kg b.wt of CCl_4
Group VIIIa	Received 25 mg/kg b.wt of 1H:1P complex capsule +0.75 mL/kg b.wt of CCl_4
Group VIIIb	Received 50 mg/kg b.wt of 1H:1P complex capsule +0.75 mL/kg b.wt of CCl_4
Group IXa	Received 25 mg/kg b.wt of 1E:1P complex capsule +0.75 mL/kg b.wt of CCl_4
Group IXb:	Received 50 mg/kg b.wt of 1E:1P complex capsule +0.75 mL/kg b.wt of CCl_4
Group Xa:	Received 25 mg/kg b.wt of 1B:1P complex capsule +0.75 mL/kg b.wt of CCl_4
Group Xb:	Received 50 mg/kg b.wt of 1B:1P complex capsule +0.75 mL/kg b.wt of CCl_4

H; hexhane, E; ethylacetate, EtOAc; ethanol, P; phospholipid, B; butanol.

2.7. Assay for Hepatoprotective Activity of the Fractions and Phytosomal Complex Capsules

The hepatoprotective activity of the extract and fractions were assessed biochemically and histopathologically.

2.8. Biochemical Evaluation of ALT and AST

The rats were treated for nine (9) days and sacrificed under chloroform anesthesia. Blood samples from the rats were withdrawn directly by heart puncture with syringes into properly labelled EDTA bottles and allowed to clot for 1 h at 25˚C. Serum was separated by centrifugation at 3000 rpm and 40˚C for 20 mins. The separated serum was used for estimation of biochemical parameters like alanine

aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin. Serum ALT and AST were determined colorimetrically [31] [32] with slight modification.

Briefly, ALT and AST determination was done by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenyl hydrazine. A 0.5 ml of glutamic-pyruvic transaminase (GPT) substrate phosphate buffer solution was added into test tube labelled blank, sample, control blank and control for ALT and AST, respectively. A 0.1 ml serum sample was added to the test tubes and the tubes incubated at 37˚C for 30 mins. A volume of 0.5 ml each of 2,4-dinitrophenylhydrazine was added into all the test tubes. A 0.1ml of sample and control was added into their respective blank test tube. The contents of each test tube were mixed and allowed to stand for 20 mins at 20˚C - 25˚C. A 5.0 ml of 0.4 N sodium hydroxide was added to each tube, mixed and their absorbencies read at 546 nm using a spectrometer against the respective blank prepared.

2.9. Biochemical Evaluation of Alkaline Phosphatase

Alkaline phosphatase activity was tested by phenolphthalein monophosphate method [33]. Briefly, test tubes were respectively labelled sample, standard and control. A 0.1 ml of distilled Water was put into the test tubes followed by a drop of the substrate (phenolphthalein monophosphate) into each of the test tubes. The contents in the tube were mixed and incubated at 37˚C for 20 mins. A 5.0 ml colour developer was added to each test tubes, mixed, and their absorbance read at 550 nm using water as blank. The activity of sample was calculated using the absorbance of standard multiplied by concentration of standard using the formula [33].

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$$\frac{\text{sample absorbance}}{\text{standard absorbance}} \times \text{Concentration of the standard}$$

2.10. Biochemical Evaluation of Blirubin

Total and direct bilirubin activity was assessed by colorimetric method as outlined in the diagnostic kit [34]. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin by its reaction with diazotized sulphanilic acid.

200 µl of sulphanilic acid (29 mmol/L) and HCl (0.17N) was added into a test tube containing 5 µl of sodium nitrate (38.5 mmol/L) and mixed. Then 1000 µl of 0.26 mol/L caffeine in sodium benzoate was added. 200 µl of serum extracted from the test animal was added to the reaction mixture, mixed and incubated at room temperature for 10 min.

Then, 1000 µl of 0.93 mol/L of tartrate in NaOH was added and incubated further for 10 mins at 25˚C. The sample protocol was used for the sample blank.

After the incubation, the absorbance of the sample against the sample blank was read at the wavelength 580 nm in a colorimeter. The colorimeter read-out multiplied by 185 gives the total bilirubin (mmol/L) in serum.

2.11. Histopathological Study

For histopathological study, liver from each rat was removed, sliced and preserved in 10% neutral formalin solution for 7 days. Thin sections of the liver were washed and dehydrated in ethanol and subsequently immersed in xylene solution for 15 mins. Then representative blocks of the liver tissues were taken and processed for paraffin embedding using the standard micro technique [35]. Thin sections (5 μm) of the livers were stained with hematoxylin and eosin (H&E) and observed microscopically for histopathological conformity.

2.12. Statistical Analysis

The data obtained were analyzed using one way of variance (ANOVA) followed by Dunnett's post hoc test. The values were expressed as means ± SEM an d values of $p \leq 0.05$ were considered significant.

3. Result and Discussion

The crude ethanol extract, along with its ethyl acetate and aqueous fractions, was evaluated for hepatoprotective activity in mice pre-treated orally for seven consecutive days before a single intraperitoneal dose of carbon tetrachloride (CCl_4, 1 mL/kg in olive oil). Serum biochemical markers, including alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin, were quantified 24 hours after toxicant administration, while hepatic malondialdehyde and reduced glutathione levels were determined in liver homogenates to assess oxidative damage and antioxidant defence. Treatment with the crude extract and its fractions produced a dose-dependent reduction in serum transaminase and alkaline phosphatase activities relative to the CCl_4-only group, while hepatic malondialdehyde levels were lowered and reduced glutathione content was restored toward control values. The phytosome-formulated extract groups showed a more pronounced improvement across these indicators than the unformulated extract at equivalent doses, with serum enzyme activities and hepatic oxidative markers approaching those of the normal control group. Total bilirubin and hepatic antioxidant indices followed a similar pattern, indicating that the enhanced activity of the phytosome groups reflects improved delivery and bioavailability of the active constituents rather than a simple additive effect of the parent extract [36].

3.1. Acute Oral Toxicity Study

The acute oral toxicity study was carried out and the results obtained are presented in **Table 2** and **Table 3**.

Acute oral toxicity was performed according to modified Dietrich Lorke Method [25].

An initial investigation was carried out by administering (10, 100, 1000mg/kg body weight) of the plant extract to four different groups of three mice each for a particular dose, making a total of thirty six mice which was observed for 24 h but

here was no death as shown in Figure 2. Based on the results of the preliminary toxicity test, four dose levels (1000, 1600, 2900 and 5000 mg/kg) were selected for the second acute oral toxicity test. Three mice were used at each dose level for each of the four fractions of *Millettia aboensis* root, giving a total of 48 mice. The animals were observed for 14 days for mortality and signs of toxicity, including diarrhoea, reduced food and water intake, weakness, reduced activity, restlessness, abnormal behaviour, difficulty breathing and weight loss.

Table 2. Initial acute toxicity test.

Samples	Dose levels		
	10 mg/kg	100 mg/kg	1000 mg/kg
N-hexane fraction	0/3	0/3	0/3
Ethylacetate fraction	0/3	0/3	0/3
N-butanol fraction	0/3	0/3	0/3
Aqueous fraction	0/3	0/3	0/3

Table 3. Main acute toxicity test.

Samples	Dose levels			
	1000 mg/kg	1600 mg/kg	29,000 mg/kg	5000 mg/kg
N-hexane fraction	3/3	2/3	1/3	0/3
Ethylacetate fraction	3/3	2/3	1/3	0/3
N-butanol fraction	3/3	2/3	1/3	0/3
Aqueous fraction	3/3	2/3	1/3	0/3

At the end of the 14-day observation period, the reported survival pattern for each fraction was three mice at 1000 mg/kg, two mice at 1600 mg/kg, one mouse at 2900 mg/kg and no surviving mice at 5000 mg/kg, as presented in Table 3 [1]. Necropsy and autopsy examinations were performed, and the reported signs of toxicity included diarrhoea and lung congestion with focal areas of necrosis.

Based on the acute toxicity findings, the median lethal dose (LD_{50}) was reported to be 2236.07 mg/kg mg/kg, calculated using Lorke's method [25]. The absence of mortality at 1000 mg/kg suggests that this dose was tolerated by the animals during the observation period. However, the occurrence of mortality at higher doses and the reported pathological findings indicate potential dose-dependent toxicity. Therefore, the safety of the fractions are limited to the observed acute toxicity.

3.2. FTIR Characterization of Phytosomal Complexes

The FTIR spectra of the phytosomal complexes prepared from the n-hexane, ethyl acetate, and n-butanol fractions of *Millettia aboensis* are presented in Figures 1-3, respectively. Variations in the absorption peaks of the fractions, phospholipid, and corresponding complexes suggested possible interactions during phytosome

formation.

N-hexane fraction (Figure 1): The peak at 517 cm^{-1}, assigned to nitro groups, and the peak at 1381 cm^{-1}, attributed to sulfonyl groups, disappeared in the complexes. A new peak at 1134 cm^{-1} was observed in all complexes, while peaks at 3292 and 3288 cm^{-1} appeared in the 1:2 and 2:1 formulations, respectively. Additional peaks at 2833 and 2987 cm^{-1} were attributed to methyl and methylene-related vibrations. The carbonyl-associated peak at 1868 cm^{-1} remained present in the fractions and complexes, suggesting that some structural features were retained after complexation.

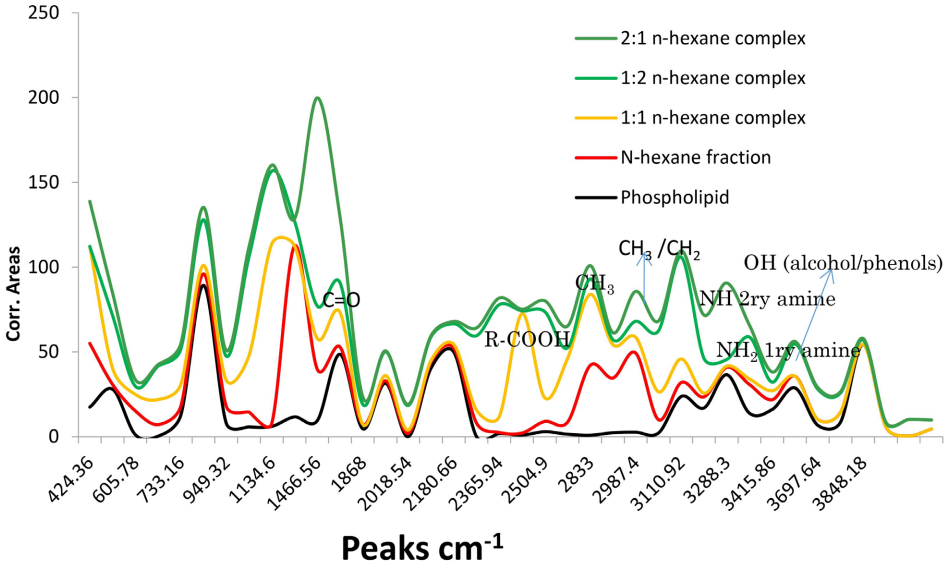


Figure 1. FTIR-derived plot of phospholipid, n-hexane fraction and n-hexane fraction-phospholipid complex.

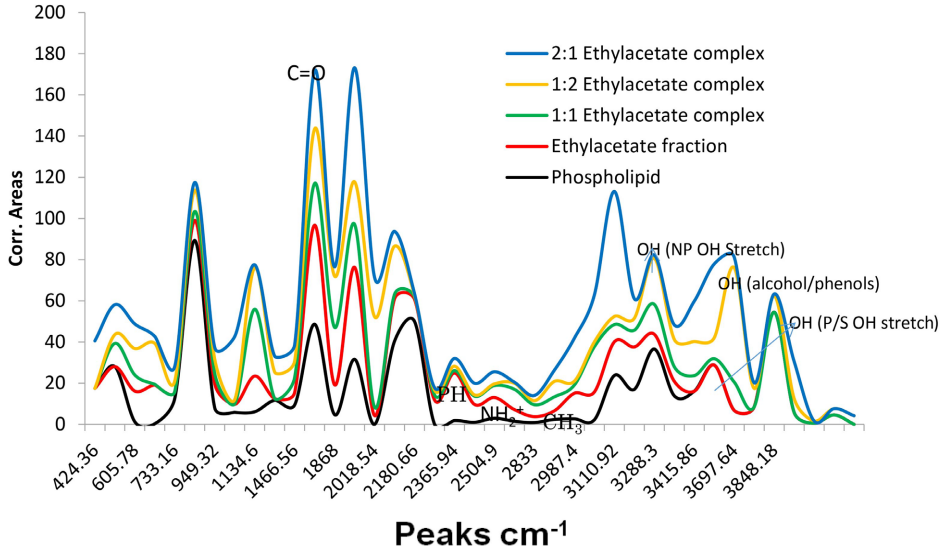


Figure 2. FTIR derived plot of phospholipid, ethylacetate fraction and ethylacetate fraction phospholipid complex.

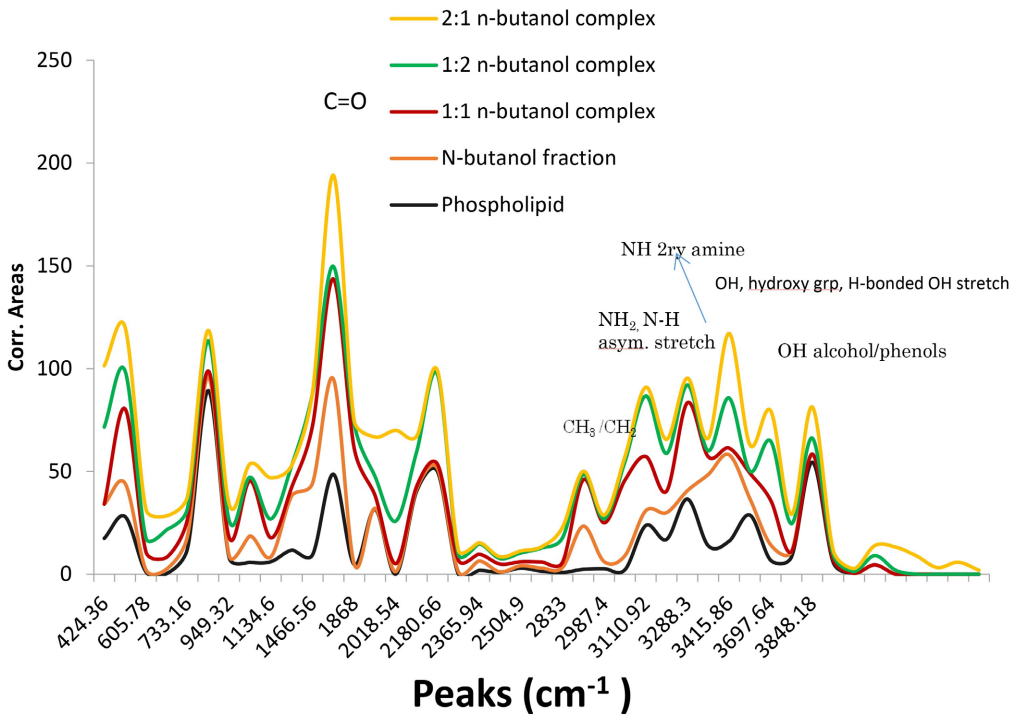


Figure 3. FTIR-derived plot of phospholipid, n-butanol fraction and n-butanol fraction-phospholipid complex.

Ethyl acetate fraction (Figure 2): The peak at 671 cm^{-1}, assigned to an alkyne-related vibration, occurred in the fraction and 1:2 complex. The peak at 733.16 cm^{-1} was associated with long-chain aliphatic structures, while the absorption at 1134 cm^{-1} was observed in the fraction and its complexes but was absent in the phospholipid. A peak at 2365 cm^{-1} was assigned to P-H stretching, and the absorption at 2504 cm^{-1}, attributed to protonated amine groups, occurred in the fraction and 2:1 complex. The peak at 2987 cm^{-1} disappeared in the complexes, whereas the O-H stretching band at 3632 cm^{-1} remained in the fraction and the 1:1 and 1:2 complexes. These changes suggest alterations in the chemical environment of functional groups following complexation.

N-butanol fraction (Figure 3): The peak at 517 cm^{-1}, assigned to nitro groups, and aromatic-ring-associated bands were observed in the fraction, phospholipid, and complexes. The peak at 1381 cm^{-1}, attributed to sulfonyl groups, disappeared in the complexes. Absorption bands at 1072 and 3415 cm^{-1} were assigned to sulfur-containing and hydroxyl-containing groups, respectively. Additional hydroxyl-associated absorption at 3848 cm^{-1} occurred in the 1:2 and 2:1 complexes. Peaks at 3288 and 3110 cm^{-1} were attributed to secondary amine and protonated amine-related vibrations, respectively.

Overall, the FTIR spectra revealed changes in peak positions and intensities, as well as the appearance or disappearance of certain bands, suggesting possible interactions between the plant fractions and phospholipid. These findings support the formation of phytosomal complexes.

3.3. Phytochemical Studies

The phytoconstituents of ethanol extracts and fractions of *Millettia aboensis* root is presented in Table 4.

Table 4. Phytoconsituents of the ethanol extract and fractions of *Millettia aboensis* root.

Phytoconstituents	Relative abundance				
	EE	NH fract.	EA fract.	NB fract.	AQ fract.
Flavonoids	++	++	+++	+++	+++
Saponinns	−	−	−	+++	+
Glycosides	−	+	−	+	+
Tannins	++	++	+++	++	+++
Steroids	+++	+	+	+	++
Alkaloids	+	+	−	++	++
Terpenoids	++	+	−	+	++
Phenolics	++	++	+	++	+++
Resins	++	++	+++	++	−

Key: +++ = high, ++ = moderate, + = low, − = absent, EE: Ethanol extract, NH: N.

From the Table *Millettia aboensis* showed the presence of many phytochemicals. Several phytochemicals might be responsible for several properties that the plant has been identified with. These properties such as anti-inflammatory, antibacterial, antidiarrhea properties, among others could be ascribed to these phytochemicals. The presence of tannin could explain why the leaves are used in preserving grains [37]. Tannins also have allopathic and bactericidal properties. These tannins have free phenolic groups, and have the ability to bind to proteins and form insoluble or soluble tannin-protein complexes [38].

The problem associated with the use of medicinal plant is the inadequate information available on the phytochemistry, pharmacology and toxicity of some of these plants [39] [40].

All these point to the fact that the extract would have antioxidant property which was confirmed in different antioxidant tests. Because of the high tannin content its bactericidal property is an indication that it can be used in food processing to increase shelf-life. Flavonoids, especially quercetin, are the most common group of polyphenolic compounds in the human diet. Flavonoids have relatively low toxicity compared to alkaloids [41]. *In vitro* studies show that flavonoids have anti-allergic, anti-cancer, and anti-diarrhoeal activities [42]. There is evidence that asthma, lung cancer and breast cancer are lower among people consuming higher dietary levels of quercetin-a flavonoid [43]. The chemopreventive mechanisms of anthocyanin a flavonoid include scavenging free-radicals, reducing cell proliferation, up-regulating/inducing apoptosis and modulating mitogen-activated protein kinase (MAPK) activities [44].

On its alkaloid content, it has been observed that alkaloids have multiplicity of host-mediated biological activities, including anti-malarial, antimicrobial, antihyperglycaemic, anti-inflammatory effects [45] [46].

Steroids, on the other hand, have anti-inflammatory effect. Steroids, also, regulate glucose metabolism and salt excretion. They can be used to treat asthma and rheumatoid arthritis [46]. So, steroids content might be a possible reason why *Millettia aboensis* exhibit anti-inflammatory, antirheumatoid arthritic effects, among others. With respect to its antioxidant property, it is observed that natural antioxidants or phytochemical antioxidants are secondary metabolites of plants. These metabolites make the concerned plants to be medically significant [47].

-hexane, EA: ethylacetate, NB: N-butanol, AQ: aqueous.

3.4. Carbon Tetrachloride Induced Acute Toxicity

A significant difference in biochemical markers was observed between normal and CCl_4 treated groups.

Comparative analysis of the effects of the various extracts on serum ALT, AST, and total bilirubin (TB) levels revealed that the fractions and complexes of *Millettia aboensis*, administered at doses of 25 and 50 mg/kg body weight, exhibited hepatoprotective effects against the hepatotoxins. Doses of fractions and complexes of *Millettia aboensis*root extract inhibited the increase of AST when compared with results obtained from rats treated with CCl_4 alone as shown in **Figure 4(a)** and **Figure 4(b)**. The observed effects were dose dependent and are significant at $p < 0.05$ with respect to negative controls (CCl_4).

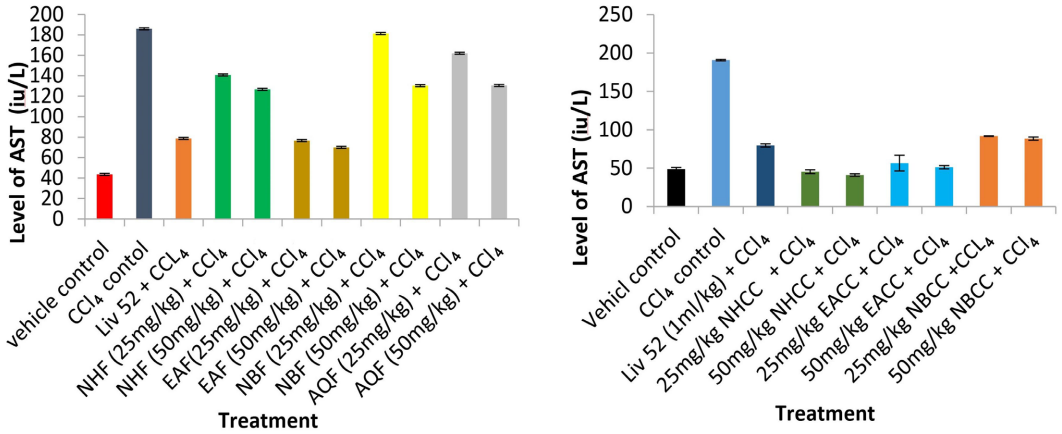


Figure 4. (a) Level of AST in the treated rats; (b) Level of AST in the treated rats.

3.4.1. The Effects of n-Butanol Fraction, n-Hexane Fraction, Ethylacetate Fraction and Aqueous Fraction of *Millettia aboensis* Root on the Level of ALT (U/L) in CCl_4 Treated Rats

The fractions and complexes of *Millettia aboensis* root extract inhibited ithe increase in liver ALT when compared with those obtained from rats treated with CCl_4 alone. The observed effects were dose-dependent and are significantly at $p <$

0.05 with respect to the negative control.

Figures 4-7 show the effect of Liv 52®, n-butanol, n-hexane, ethylacetate and aqueous fraction, n-butanol, n-hexane and ethylacetate complexes of *Millettia aboensis* root on the level of ALT (U/L) in CCl4treated rats. The effects of the n-butanol, n-hexane, ethyl acetate, and aqueous fractions of *Millettia aboensis* root on CCl_4-induced hepatic damage in rats were evaluated by assessing their effects on selected biochemical markers of liver function, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TB) levels.

Figure 5(a) and Figure 5(b), shows that ehtylacetate fraction and 1:1 ethylacetate and 1:1 n-hexane complexes respectively gave the least level of ALT implying that they have more hepatoprotective effect than other fractions and complexes. Doses of 25 and 50 mg/kg of the fraction's complexes of *Millettia aboensis* root inhibited the increase of serum liver function biomarker when compared with those obtained from the rats treated with CCl_4 alone as shown in Figure 6(a) and Figure 6(b), and is significant at $p < 0.05$ with respect to negative controls (CCl_4).

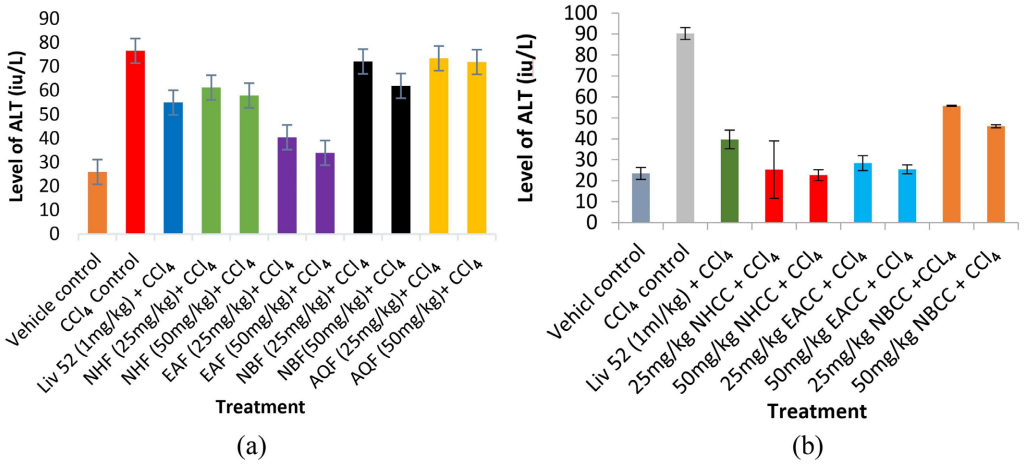


Figure 5. (a) Level of ALT in the treated rat; (b) Level of ALT in the treated rat.

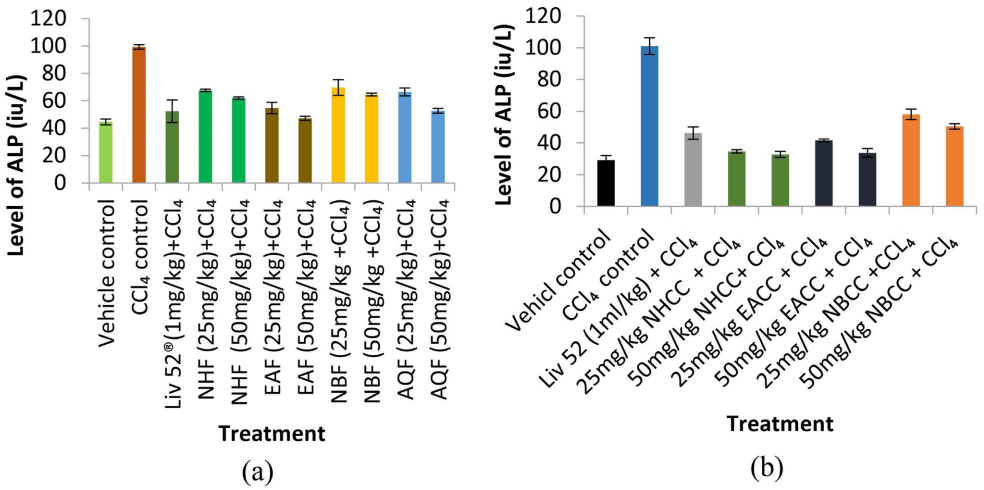


Figure 6. (a) Level of ALT in the treated rats; (b) Level of ALP in treated rats.

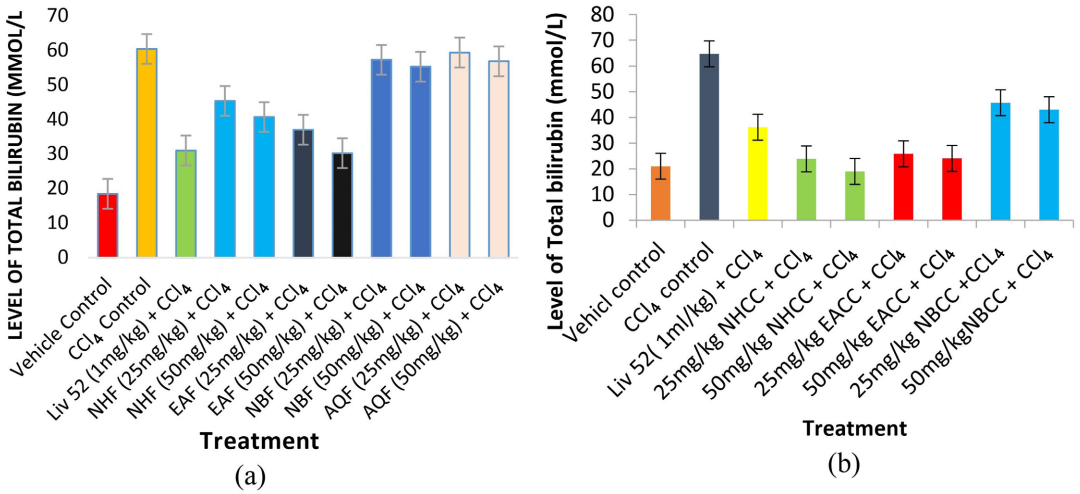


Figure 7. (a) Total Bilirubin Level in the treated rats; (b) Total Bilirubin Level in the treated rats.

3.4.2. The Effects of n-Butanol Fraction, n-Hexane Fraction, Ethylacetate Fraction and Aqueous Fraction of *Millettia aboensis* Root on the Level of ALP (U/L) in CCl_4 Treated Rats

The results of the effect of fractions and complexes of *Millettia aboensis* root on the level of total bilirubin (mg/dl) in CCl_4 treated rats as shown in **Figure 7(a)** and **Figure 7(b)**. The result showed a dose dependent decrease in total bilirubin in all treated rats. At all dose levels the ethylacetate fraction (**Figure 7(a)**), 1:1 ethylacetate and 1:1 n-hexane complexes (**Figure 7(b)**) appear to have greater decrease in total bilirubin when compared to other fractions and complexes. However, the total bilirubin in rats treated with CCl_4 alone was significantly higher than in rats which receives the fractions, the complexes and Liv 52® treated rats.

The liver is very susceptible to damage by xenobiotics and non-xenobiotics induced by oxidative stress, liver damage is manifested in increased serum levels of aspartate transaminase (AST) or serum glutamate oxaloacetate transaminase (GOT), alanine transaminase (ALT) or serum glutamate pyruvate transaminase (GPT), alkaline phosphate (ALP) and serum bilirubin, as well as pronounced necrosis of hepatic cells. Carbon tetrachloride (CCl_4) induced hepatotoxicity model widely used for the study of hepatoprotective effects of drugs and plants extracts [12]. Treatments of animals with the fractions (n-hexane, ethylacetate, n-butanol, aqueous) and the *Millettia aboensis* root extract complexes (n-hexane, ethylacetate and n-butanol capsule) significantly reduce the level of the liver function biomarker (AST, ALT, ALP and serum bilirubin) compared with the CCl_4 treated group. The ehtylacetate fraction, n-hexane complex capsule and ethylacetate complex capsules at 25 mg/kg and 50 mg/kg body weight mediated reduction in levels of AST, ALT, ALP, serum bilirubin as well as repair of hepatic tissue damage caused by CCl_4.

3.4.3. The Effects of n-Butanol Fraction, n-Hexane Fraction, Ethylacetate Fraction and Aqueous Fraction of *Millettia aboensis* Root on the Level of ALP (U/L) in CCl_4 Treated Rats

The effects are in agreements with the commonly accepted view that serum levels

of transaminase return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes [13]. Liver damage is manifested in increased serum levels of AST, ALT, ALP and serum bilirubin, as well as pronounced necrosis of hepatic cells. The rats that received the ethylacetate fraction, n-hexane complex capsule and ethylacetate complex capsule had significant decrease in serum AST, ALT, ALP and bilirubin when compared with those of the normal control rats (**Figures 4(a)-7(a)**, and **Figures 4(b)-7(b)**) These indicate that the *Millettia aboensis* root fractions and complex capsules (ethylacetate fraction, n-hexane and ethylacetate complex capsules at 25 mg/kg and 50 mg/kg body weight) have a hepatoprotective effect that was found to be more pronounced at higher doses.

3.4.4. The Effects of n-Butanol Fraction, n-Hexane Fraction, Ehtylacetate Fraction and Aqueous Fraction of *Millettia aboensis* Root on the Level of Total Bilirubin (mg/dl)

The ethylacetate fraction, n-hexane and ethylacetate complex capsules at 25 mg/kg and 50 mg/kg body weight showed higher hepatoprotective activities than the n-hexane fraction, n-butanol fraction, aqueous fraction and n-butanol complex capsules. Studies have shown that phenolic compounds and flavonoids present in plant extracts enhance their hepatoprotective activities [14]. Thus, the observed increased hepatoprotective activity of the complexes of the *Millettia aboensis* root extract could be attributed to presence of flavonoids and phenolics content which was delivered into the hepatocytes by the phospholipids hence preventing the effects of toxins on the hepatocytes.

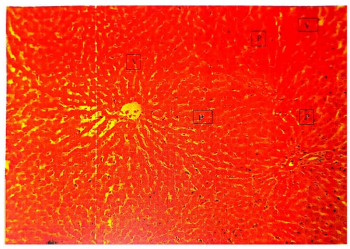
Lipid peroxidation induced by CCl_4 is a commonly used experimental animal model for studying oxidative injury in biological systems [15]. Cytochrome P-450 enzymes are believed to metabolize CCl_4 allows it to cross the cell membrane and to be distributed to the organs. However, the liver is the major target organ of CCl_4 induced toxicity owing to its high content of cytochrome P-450 enzymes. Lipid peroxidation is an oxidative alteration of poly unsaturated fatty acids in the cell membrane that generates a number of degeneration products. Flavonoids have been reported to have antioxidant activity [16]. The antioxidant activity reported in our study could be attributed to these flavonoids present in the roots.

3.5. Histopathological Findngs of the Liver

The histopathological examination of the liver from the experimental animals showed diverse histopathological changes varying from degeneration to necrosis with inflammatory cellular infiltration.

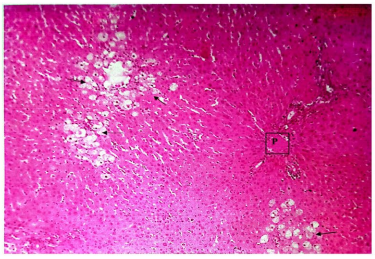
3.5.1. Photographs of Liver Section Taken from Rats of Group-1 (a), Group-2 (b) and Group-3 (c)

The section of the liver showed normal hepatic lobules with central veins (**Figure 8**) surrounded by radiating hepatocytes arranged in chords. The hepatic chords radiate towards the portal area which is composed of the hepatic vein, hepatic artery and the bile duct (**Figure 8(a)**).



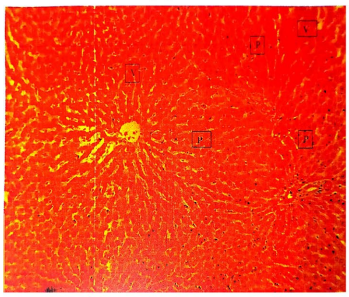
Bile Duct (B), Hepatic chords (arrows). H&Ex400

(a)



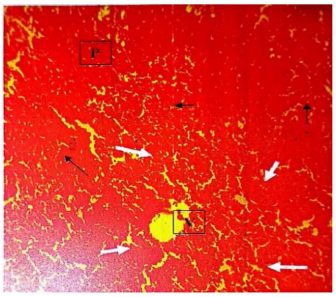
Central vein (V): Portal area (P), H&E×10

(b)



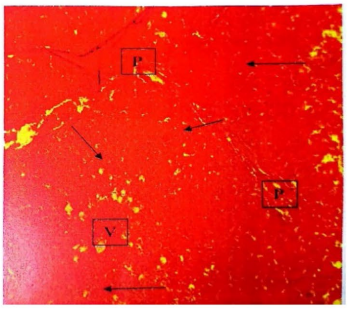
Central vein (V). Portal area (P). H & E x100

(c)



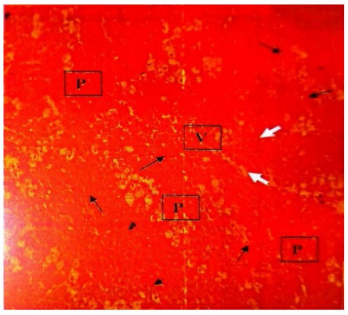
Central vein (V). Portal area (P). H & E x100

(d)



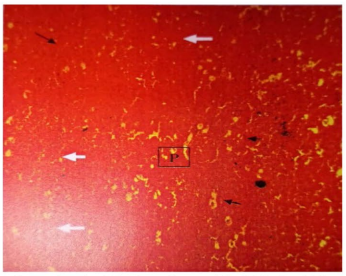
Central vein (V). Portal area (P). H & E x100

(e)



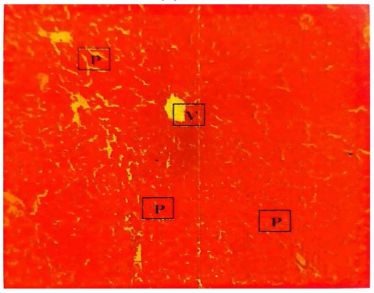
Central vein (V). Portal area (P). H & E x100

(f)



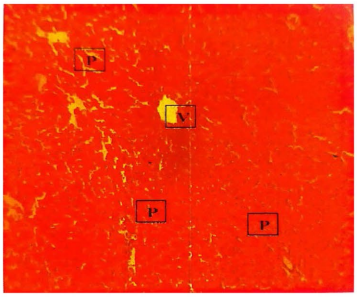
Central vein (V). Portal area (P). H & E x100

(g)



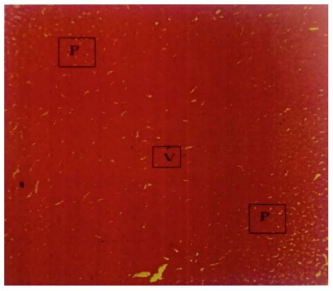
Central vein (V). Portal area (P). H & E x100

(h)



Central vein (V). Portal area (P). H & E x100

(i)



Central vein (V). Portal area (P). H & E x100

(j)

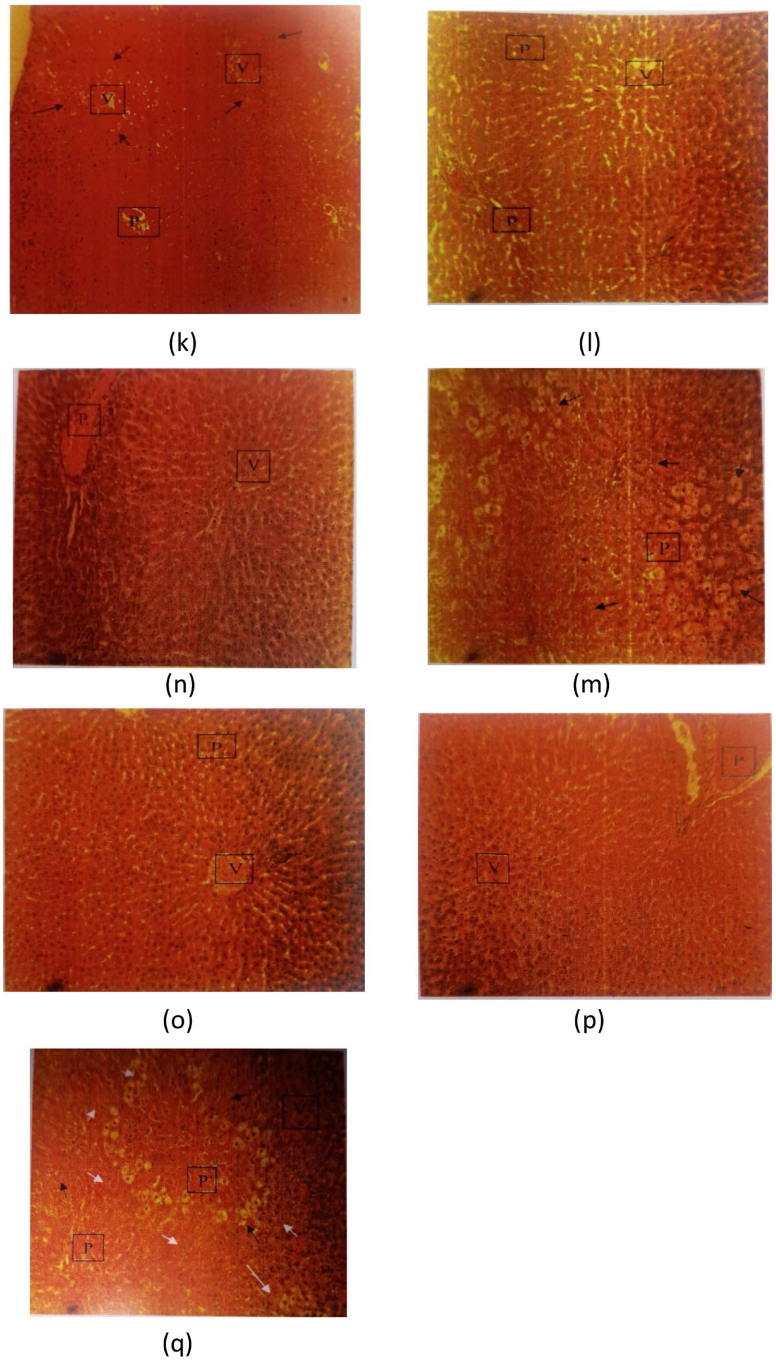


Figure 8. Central veins.

Sections of the liver from the animals in the group showed severe vacuolar degeneration of the hepatocytes around the central vein (arrow). The hepatocytes in the midzonal and periportal areas were relatively normal (**Figure 8(b)**).

Sections of the liver from this group showed the normal hepatic architecture for laboratory rodents.

It showed normal hepatocytes arranged in chords around the central veins. It also showed normal hepatic triad with normal hepatic artery, veins and bile ducts

(Figure 8(c)).

3.5.2. Photographs of Liver Section Taken from Rats of Group-4 (d), Group-5 (e), Group-6 (f) and Group-7 (g)

Sections of the liver from animals in this group showed a coagulative necrosis of the hepatocytes in the centrilobular and midzonal areas of the hepatic lobules (white arrow). The periportal hepatocytes remain unaffected (black arrow) (Figure 8(d)).

Sections of the liver from the animals in this grouped showed coagulative necrosis of the centrilobular and midzonal hepatocytes (arrow). The hepatocytes in the periportal areas are relatively normal (Figure 8(e)).

Sections of the liver from the animals in this group showed coagulative necrosis of the centrilobular hepatocytes (arrow). The hepatocytes in the midzonal and periportal areas are relatively normal (Figure 8(f)).

Sections of the liver from the animal in this group showed a mild vacuolar degeneration of the centrilobular hepatocytes (arrows) (Figure 8(g)).

Section of the liver collected from the animals in this group did not show any deviation from the normal hepatic hist-architecture. It showed the normal structure of the liver.

3.5.3. Photographs of the Liver Section Taken from Rats of Group-8 (i), Group-9 (j), Group-10 (k) and Group-11 (l)

Photomicrograph of the liver collected from these groups, showing normal hepatocytes arranged in interconnecting chords around the central vein (V) (Figure 8(i)). Sections of the liver from this group showed the normal hepatic architecture for laboratory rodents. It showed normal hepatocytes arranged in chords around the central veins. It showed normal hepatic triad with normal hepatic artery, veins and bile ducts (Figure 8(j)).

Sections of the liver collected from the animals in this groups did not show any deviation from the normal hepatic histo-architecture. It showed the normal structure of the liver.

Photomicrograph of the liver collected from these groups, showing normal hepatocytes arranged in interconnecting chords around vein (V) (Figure 8(h) and Figure 8(i)).

Sections of the liver from this group showed the normal hepatic architecture for laboratory rodents. It showed normal hepatocytes arranged in chords around the central veins. It also showed normal hepatic triad with normal hepatic artery. Veins and ducts (Figure 8(j) and Figure 8(k)).

3.5.4. Photographs of Liver Section from Rats of Group-12 (l), Group-13 (m) Group-14 (n) and Group-15 (o)

Samples of the liver collected from the animals in groups 12, 13, 14 and 15 did not show any deviation from the normal hepatic histo-architecture. The hepatocytes in all the atomic zones of the hepatic lobule appeared normal.

Section of the liver showing hepatocytes arranged in chords around the central vein (V) (Figure 8(n) and Figure 8(o)).

3.5.5. Micrographs of the Liver Section Taken from Rats of Group-16 (p), Group-17 (q)

Sections of the liver collected from the animals in this group showed a moderate to severe widespread hepatocellular vacuolar degeneration. Hepatocytes in the centrilobular and midzonal areas showed the most severe changes.

Section of the liver showing a moderate to severe hepatocellular vacuolar degeneration. Random hepatocytes in the central and midzonal areas show the most severe lesions (arrow) (**Figure 8(p)**).

Section of the liver showing mild vacuolar degeneration of the hepatocytes in the periportal area and severe vacuolar degeneration of those in the midzonal area (white arrow) while those immediately surrounding the central vein (V) appear normal (arrow) (**Figure 8(q)**).

4. Conclusions

Phytosomes containing the various fractions of *Millettia aboensis* root were successfully formulated and evaluated for their potential hepatoprotective and antioxidative activities. The acute toxicity studies indicated that the ethanol extract and its derived fractions were relatively safe at the doses investigated, with the median lethal dose (LD_{50}) providing an indication of their safety profile.

Phytochemical screening of the crude ethanol extract and fractions of *Millettia aboensis* root revealed the presence of several bioactive secondary metabolites, including flavonoids, saponins, glycosides, tannins, steroids, alkaloids, terpenoids, phenolic compounds, and resins. The presence of these phytoconstituents may contribute to the biological activities observed in the study.

Administration of the fractions to CCl_4-challenged rats produced significant protective effects on selected biochemical parameters compared with the untreated toxic control group ($p < 0.05$), suggesting that the fractions possess protective activity against CCl_4-induced tissue injury. In particular, the n-hexane and ethyl acetate fractions demonstrated appreciable hepatoprotective and antioxidative effects. Similarly, the corresponding phytosomal formulations, namely the n-hexane complex capsules and ethyl acetate complex capsules, exhibited protective effects, indicating that incorporation of the fractions into phytosomal systems may enhance or improve their biological activity.

The observed hepatoprotective and antioxidative effects may be associated with the combined actions of the secondary metabolites present in the fractions, particularly flavonoids and phenolic compounds, which are known to possess antioxidant properties. These constituents may contribute to the reduction of oxidative stress and attenuation of CCl_4-induced hepatic injury. The findings therefore provide experimental evidence supporting the traditional and potential pharmacological relevance of *Millettia aboensis* root fractions.

However, further investigations are necessary to establish the standardization, quality control, pharmacokinetic properties, mechanism of action, long-term safety, and reproducibility of the *Millettia aboensis* root extracts and their phytosomal

formulations. Future studies should also identify and quantify the specific bioactive constituents responsible for the observed hepatoprotective and antioxidative activities and determine appropriate standardized doses for potential therapeutic application.

Author Contributions

Mercy E. Ugwueze contributed to conceptualization, investigation, data curation, formal analysis, and manuscript preparation. Romanus C. Omeh, Caroline E. Onugwu, and Cyril C. Adonu contributed to the study methodology, investigation, and interpretation of results. Nwodo E. Sunday and Euphemia N. Eke contributed to data analysis, validation, and critical review of the manuscript. Chukwuma E. Ogbuanya contributed to the study design, methodology, and manuscript revision. Anthony A. Attama and Michael U. Adikwu contributed to supervision, scientific review, and critical revision of the manuscript. All authors reviewed and approved the final version of the manuscript and agree to be accountable for the integrity of the work.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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