ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Hepato-Protective Effect Of *Emilia praetermissa* Against Carbon Tetrachloride-Induced Hepatotoxicity In Albino Rats.

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ABSTRACT

Hepatic damage has been reported to disrupt the normal metabolism in the body and incidence of Chronic liver diseases has become an increasing burden to the society. *Emilia praetermissa* aqueous extracts were evaluated for their potential hepatoprotective effects against carbon tetrachloride induced hepatic damage model. Experimental rats in different groups were subjected to different concentrations of *Emilia praetermissa* leaves extract and Silymarin a standard drug for the treatment of liver disorders after carbon tetrachloride induction. The hepatoprotective potentials of *Emilia praetermissa* extract were accessed in the plasma and liver by the estimation of biochemical markers such as; Malondialdehde, Alkaline phosphatase, Aminotransferase, Total Bilirubin, Total Protein and Albumin. Liver histopathological examination was also studied. The aqueous extract of *Emilia praetermissa* brought about significant changes in the level of most biochemical parameters in a dose dependent manner. Generally, the most significant($P<0.05$) protective effect was observed at the highest dose (200mg/kg) of the extract which is more effective than the standard drug. The histopathological study also confirmed the hepatoprotective ability of the aqueous extract of *Emilia praetermissa* which shows that the leaves could play a vital role in the management liver disorders.

Keywords: *Liver disorders, Emilia praetermissa, Histopathology, Aminotransferase*

https://doi.org/10.33887/rjpbcs/2021.12.3.18

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ISSN: 0975-8585

INTRODUCTION

Liver is an abdominal organ which plays a vital role in detoxification and excretion of many endogenous and exogenous substances. The liver is a natural 'chemical factory' which aids in anabolism of complex molecules from simple substances absorbed from the Gastro-Intestinal Tract (GIT). It neutralizes toxic substances and involved in the synthesis of bile which aids fat digestion and eliminates toxins through the bowels[1]. Subsequent exposure and intoxication of the liver to different types of exogenous substances on a daily basis may lead to hepatic dysfunction[2].

Liver diseases remain one of the most serious health problems and modern medicines have little to offer for the reduction of hepatic disorders. Therefore, medicinal plants and herbs are evaluated for their possible hepatoprotective effects due to the limited number of pharmacological agents. Chemicals that cause liver injury are called hepatotoxins. More than 900 drugs have been implicated in causing liver injury[3], and it is the most common reason for a drug to be withdrawn from the market. Hepatotoxicity and drug-induced liver injury also account for a substantial number of compound failures, highlighting the need for toxicity prediction models and drug screening assays, such as stem cell-derived hepatocyte-like cells, that are capable of detecting toxicity early in the drug development process. Chemicals often cause subclinical injury to the liver, which manifests only as abnormal liver enzyme tests[4].

Carbon tetrachloride (CCl_4) is referred to as toxicant that generate free radicals and its capable of inducing liver damage by reacting with the liver cells. Carbon tetrachloride is a haloalkane used in a variety of industrial and chemical applications. It was formerly widely used in fire extinguishers, as a precursor to refrigerants and as a cleaning agent, but has since been phased out because of toxicity and safety concerns. Exposure to high concentrations of carbon tetrachloride (including vapor) can affect the central nervous system, degenerate the liver and kidneys. Prolonged exposure can be fatal[5]. Carbon tetrachloride is a well-known hepatotoxin that is widely used to induce toxic liver injury in a large range of laboratory animals, which is indicated by rises in aminotransferase activities in plasma reflecting release of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) from injured liver cells[6,7]. Many investigations have indicated that reductive dehalogenation of CCl_4 by the P_{450} enzyme system to the highly reactive trichloromethyl radical initiates the process of lipid peroxidation, which ultimately results in the overproduction of reactive oxygen species (ROS) and hepatocyte injuries[6,8].

The popularity of plant-based drug in the modern system of medicine is growing day by day as it is claimed to be safer, economical and efficacious[9]. However, there is need for the development and improvement of oral drugs from medicinal plants for therapeutic purposes. *Emilia praetermissa* which belongs to the family of Asteraceae is a useful plant of west tropical Africa used generally as food and medicine for general healing[10]. The leaves are occasionally eaten as a vegetable, either fresh in salads or cooked. As a vegetable, *Emilia praetermissa* is likely to remain only locally important.

This study is therefore, aimed at verifying the medicinal values of the aqueous extracts of *Emilia praetermissa* using modern scientific methodologies to evaluate the hepatoprotective effects on Carbon tetrachloride-Induced albino rats.

MATERIALS AND METHODS

Sample Collection

The *Emilia praetermissa* (*Milne Redh*) leaves was harvested beside College of Health Technology in Ijero-Ekiti and it was identified at the Herbarium section of Plant Science and Biotechnology Department, Ekiti State University, Ado-Ekiti, Nigeria. A voucher specimen was deposited in the Departmental Herbarium with this voucher Number: UHAE-2017/105.

Apparatus/Equipment and Reagents

Standard apparatus and equipment were used for the study. Standard chemicals and reagent kits was purchased from Randox Laboratories and Fortress kit which was of analytical grade.

Aqueous extract Preparation

The leaves of *Emilia praetermissa* was air dried at room temperature, pulverized with electric blender and then stored in a plastic container in the laboratory prior to analysis. The powdered leaves were extracted with 100g in 1000ml of distilled water for 72hours as described by the method of Farooq [11].

Experimental Rats

Adult female albino Wistar rats, weighing 150–200g each were purchased from the breeding colony of Department of Biochemistry, Ekiti State University College of Medicine. They were acclimatized at 25°C, on a 12h light/12h dark cycle for 2weeks before the experiment. The handling was guided by NIH Guide for the care and use of laboratory animals, and the ethical Committee for Animal Experimentation of the Ekiti State University.

Carbon tetrachloride Preparation and Induction

Carbon tetrachloride (CCl_4) was diluted with olive oil in ratio 1:1 dilution and 1ml/kg body weight was administered intraperitonially, to induce hepatotoxicity.The CCl_4 dose followed was according to the dose that can induce oxidative stress and hepatotoxicity as described by[11] .

Experimental Design

Thirty (30) albino rats were divided into six (6) groups of five (5) rats each and subjected to different doses of aqueous extract of *Emilia praetermissa* leaves: Group I (normal control) received an equivalent volume of water, group II served as Test control, Group III served as the Standard Control and received 100mg/kg Silymarin, Group IV, V and VI were given a single acute dose of carbon tetrachloride(1ml/kg) and treated with 50, 100 and 200mg/Kg body weight of extract respectively for 21days. Animals were sacrificed after 21days by cervical dislocation, the blood sample was collected into a heparinised tube and organ (Liver) was excised and homogenized and centrifuged at 6,000rpm for 10min. The dose was decided after preliminary acute and sub-acute toxicity studies. The authors decided to use the above doses to see the effectiveness at not too low or too high doses. The liver tissue specimen was collected and fixed in formalin for histological studies.

Determination of Malondialdehyde (MDA)

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of [13] with slight modification by [14]. An aliquot of 0.4mL of the plasma or other organ homogenates was mixed with 1.6mL of Tris-KCl buffer to which 0.5mL of 30% trichloroacetic acid (TCA) was added. Then 0.5mL of 0.75% thiobarbituric acid (TBA) was added and placed in a water bath for 45 minutes at 80°C. This was then cooled on ice and centrifuged at 3000*g*. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 10^5 $M^{-1}Cm^{-1}$.

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{\text{E532nm x volume of sample x mg protein}}$$

Determination of Total Protein

The determination was done by the method of[15]. Copper ions react in alkaline solution with protein peptide bonds to give a purple coloured biuret complex. The amount of complex formed is directly proportional to the amount of protein in the specimen. 20µl of sample was thoroughly mixed with 1ml of biuret reagent. The reaction was run at room temperature, thoroughly mixed and incubated for 10 minutes at 20-25°C. The absorbance of the sample against the blank was read at 546nm.

$$\text{Total Protein conc.} = \frac{Absorbace\ of\ sample}{Absorbace\ of\ standard} \text{ X conc. of Standard}$$

ISSN: 0975-8585

Determination of Total Bilirubin

The determination was done by the method of [16], sample (5µl) was gently mixed (to avoid foaming) with 0.5ml of reagent A and reagent B. The reaction was run at room temperature and incubated for 10 minutes at 20-25°C. The absorbance of the sample against the blank was read at 550nm.

$$\text{Total Bilirubin Conc.} = \frac{Absorbace\ of\ sample}{Absorbace\ of\ standard} \text{ X conc. of Standard}$$

Determination of Albumin

The determination was done by the method of [15] briefly, sample (5µl) was thoroughly mixed with 1ml of working reagent. The reaction was run at room temperature and incubated for 10 minutes at 20-25°C. The absorbance of the sample against the blank was read at 630nm.

$$\text{Albumin conc.} = \frac{Absorbace\ of\ sample}{Absorbace\ of\ standard} \text{ X conc. of Standard}$$

Determination of Alkaline Phosphatase

The determination was done by the method of [17]. The alkaline phosphatase acts on the AMP-buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured at 590nm.

Substrate (500µl) was equilibrated at 37°C for 3 minutes. 50µl of sample was added to the equilibrated substrate and incubated for 10 minutes at 37°C. 2.5ml of colour reagent (mixture of NaOH, 0.09M and Na_2CO_3, 0.1m) was added. This mixture was thoroughly mixed. Absorbance of the sample against the blank was read at 590nm.

$$\text{ALP conc.} = \frac{Absorbace\ of\ sample}{Absorbace\ of\ standard} \text{ X conc. of Standard}$$

Determination of Aspartate Transaminase

The determination was done by the method of [17]. AST is measured by the monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. Sample (100µl) was added to 500µl of AST buffer. Mixed well and incubated for 30 minutes at 37°C. 0.5ml of dye reagent (2,4 Dinitrophenyl Hydrazine, 2.0mmol/l) was added. This mixture was thoroughly mixed and allowed to stand for 20 minutes at 20°C. 5.0 ml of diluted NaOH was then added and thoroughly mixed. Absorbance of the sample against the blank was read at 546nm.

$$\text{AST conc.} = \frac{Absorbace\ of\ sample}{Absorbace\ of\ standard} \text{ X conc. of Standard}$$

Determination of Alanine Transferase

The determination was done by the method of [17]. Briefly, 100µl of sample was added to500µl of ALT buffer. Mixed well and incubated for 30 minutes at 37°C in water bath. 0.5 ml of dye reagent (2,4 Dinitrophenyl Hydrazine, 2.0 mmol/l) was added. This mixture was thoroughly mixed and allowed to stand for 20 minutes at 20°C. 5.0 ml of diluted NaOH was then added and thoroughly mixed. Absorbance of the sample against the blank was read at 546 nm.

$$\text{ALT conc.} = \frac{Absorbace\ of\ sample}{Absorbace\ of\ standard} \text{ X conc. of Standard}$$

Histopathology Evaluation

The liver specimen from the control and test groups were collected immediately after sacrifice and fixed in 10% buffered formalin for 48 h. The formalin fixed samples were stained with haematoxylin–eosin. The sections were examined microscopically for changes in the liver's structural architecture according to the method of [18].

Statistical Analysis

The results was analysed using Software Package for Social Sciences (SPSS) version 20 and the results was expressed as mean ±standard deviation (SD). Using Analysis of Variance (ANOVA) and Duncan Multiple Range Test, Means with different superscript are considered significantly different at the value of $P< 0.05$.

RESULTS AND DISCUSSION

Hepatic damage has been reported to disrupt the normal metabolism in the body[19]. The fact that liver is one the vital organs of the body that transform and clear chemicals from the system, it is susceptible to the toxicity from these chemical agents which in turns deplete the hepatocytes. The ability of a hepato-protective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms which have been disturbed by a hepatotoxin is the index of its protective value[20]. The hepatotoxic effects induced by CCl_4 arise from its metabolite $^{\bullet}CCl_3$, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage[21].

Malondialdehyde (MDA) is a degradative product of peroxidation of polyunsaturated fatty acids (PUFA) in cell membrane. Presence of MDA in plasma is often an indicator of oxidative stress. In this study, (Table 1) revealed that, CCl_4 untreated group has a significant($P<0.05$) elevation of MDA in plasma and liver of the experimental rats. Drastic significant reduction ($P<0.05$)was also observed in the group treated with 100mg/kg silymarin and different doses of the extract with the highest reduction at 200mg/kg extract treated group.

Detection of higher levels of MDA in the plasma of CCl_4 control group than in normal control group confirms the report that CCl_4 induces lipid peroxidation and liver damage[22]. This result confirmed the report that administration of 0.5 ml/kg body weight of CCl_4 is able to elevate malondialdehyde (MDA), a product of lipid peroxidation in liver of rats treated with CCl_4 only. [23]attributed the increase in MDA levels to enhanced lipid peroxidation, leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals. The results in this study suggest that treatment and of rats for 21 days with *Emilia praetermissa* aqueous extract at varying doses to CCl_4 significantly ($P<0.05$) reversed these changes. The present results also run in parallel with the findings of [24]who reported that MDA appears at significant higher concentrations in the liver and alters antioxidant levels. The aqueous extract from *Emilia praetermissa* leaves exhibited a protective effect against oxidative stress induced damage by carbon tetrachloride in the liver and plasma of albino rats by inhibiting lipid peroxidation.

The hepatocyte membrane distortion is associated with membrane leakage of the hepatocyte cytosolic contents which is manifested by significant elevation of the plasma marker enzymes of acute hepatocellular damage namely ALT and AST, and ALP as a marker for hepatobiliary damage[15]. In this study, the results as shown in (Table 2-4) indicated that, the induction of hepatic damage with CCl_4 was marked by a significant ($P<0.05$) increase in plasma levels of marker enzymes; Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP).

The untreated CCl_4 induced group shows an elevated level of these enzymes in the plasma and low activities of the enzymes were observed in the liver. The administration of aqueous extract of *Emilia praetermissa* significantly ($P<0.05$) decrease the plasma levels of marker enzymes (ALT, AST and ALP) in a dose dependent manner. The significant reduction of these enzymes in the plasma was more noticeable in the group treated with 200mg/kg extract, and its increase were also noticed in the liver at the same dose, this can be compared with the groups administered with silymarin (100mg/kg) and the normal control group. The treatment of the animals with *Emilia praetermissa* aqueous leaf extract protected the liver from the

deleterious effect of the toxin by decreasing the circulatory levels of AST, ALT and ALP which consequently restore the depleted enzymes activities in the liver. The result obtained in this research are similar to the findings of [25] who observed significant hepatic damage in rats treated with single dose of CCl_4. The results of the total protein, albumin and total bilirubin in the plasma and liver of albino rats are shown on (Tables).

The results show that Total Protein (TP) and Albumin (ALB) were significantly ($p<0.05$) reduced in CCl_4 untreated group. The groups treated with the aqueous extract of *Emilia praetermissa* showed a significant increase in a dose dependent manner but found to be much significant at the highest dose of 200mg/kg as it increased towards the Normal control group and the group treated with standard drug 100mg/kg of silymarin. An increase in the plasma levels of total protein and albumin suggests the stabilisation of endoplasmic reticulum, leading to protein synthesis. The result was in agreement with previous similar work by [26].

Bilirubin is one of the key biochemical parameters used in the investigation of liver function rather than the investigation of hepatic injury. The increase in the level of TB in circulation is an indicator to the diminished function of the liver (Aluko *et al*., 2013). In this study, the elevated level of Total Bilirubin in the CCL_4 untreated group were reduced by the administration of extracts in a dose dependent manner. There were significant reduction in the level of Total Bilirubin in the groups treated with *Emilia praetermissa* extract most especially at the highest dose of 200mg/kg as it reduces almost to the normal control and the standard drug (100mg/kg of silymarin). Reduction in the levels of Total Bilirubin suggests the stabilization of the biliary function.

The magnitude of hepatic injury caused by CCl_4 was further buttressed by the studies on the structural architecture of the experimental rats' liver. Histological examination of the liver sections (Figure 1) showed that the normal liver architecture was disturbed by hepatotoxin intoxication [27]. There were signs of necrosis and irrationality of hepatic cells in the liver section of rats in the CCl_4 untreated group. Whereas in sections obtained from rats treated with *Emilia praetermissa* leave extract and groups administered with standard drug(100mg/kg silymarin), the hepatic structural arrangement were restored towards the normal control. This study reveals the hepatoprotective ability of *Emilia praetermissa* leave extract on the hepatic cell. The possible mechanism of hepatoprotection may be attributed to presence of phytoconstituents in the leaves of this plant. This was similar to the findings of [26].

The results of this study showed that aqueous extract of *Emilia praetermissa* leaves has antioxidant as it ameliorated the effects produced by CCl_4 as shown in its ability to inhibit lipid peroxidation. Treatment with *Emilia praetermissa* exhibited hepatoprotective potentials, hence, the use as food supplement or use in the formulation of drugs for the management of liver complicated disease may be of benefit to mankind as an hepatoprotective agent. The consumption of the plant as vegetable could be encouraged due to its antioxidant potentials.

Table 1: Effect of *Emilia preatemissa* aqueous extract on Malonyladehyde (MDA) in albino rats (Unit/mg protein)

GROUPS	TREATMENT	PLASMA	LIVER
1	NORMAL CONTROL	21.92±2.29a	18.14±1.56a
2	POSITIVE CONTROL	76.14±7.98 b	41.33±3.55 b
3	STANDARD CONTROL	43.23±4.53 c	31.57±2.71 a
4	EXTRACT OF 50mg/kg	57.93±6.07 d	36.39±3.13 b
5	EXTRACT OF 100mg/kg	40.16±4.20 c	31.79±2.73 b
6	EXTRACT OF 200mg/kg	26.52±2.78 ac	24.14±2.07 a

Results are mean of 5 determinations ± Standard Deviation (SD). Using Analysis of Variance (ANOVA) and Duncan Multiple Range Test, Means with different superscript are significantly different at ($P<0.05$).

ISSN: 0975-8585

Table 2: Effect of *Emilia preatemissa* aqueous extract on Alanine Transferase (ALT) in albino rats ((u/l)

GROUPS	TREATMENT	PLASMA	LIVER
1	NORMAL CONTROL	29.64±5.42a	40.49±3.48a
2	POSITIVE CONTROL	44.16±3.80 c	17.16±1.47 b
3	STANDARD CONTROL	26.12±3.96 a	37.26±3.20 a
4	EXTRACT OF 50mg/kg	31.99±5.85 a	22.70±1.95 b
5	EXTRACT OF 100mg/kg	29.91±5.47 a	29.57±2.54 ac
6	EXTRACT OF 200mg/kg	22.51±4.12 a	36.02±3.10 a

Results are mean of 5 determinations ± Standard Deviation (SD). Using Analysis of Variance (ANOVA) and Duncan Multiple Range Test, Means with different superscript are significantly different at (P<0.05).

Table 3: Effect of *Emilia preatemissa* aqueous extract on Aspartate amino Transferase(AST) in albino rats (u/l)

GROUPS	TREATMENT	PLASMA	LIVER
1	NORMAL CONTROL	31.03±9.95a	45.48±6.58a
2	POSITIVE CONTROL	56.81±12.13b	23.07±3.34 b
3	STANDARD CONTROL	35.22±10.24 a	39.42±5.70 a
4	EXTRACT OF 50mg/kg	50.57±11.53 b	26.58±3.84 b
5	EXTRACT OF 100mg/kg	43.75±10.92 a	33.59±4.86 ab
6	EXTRACT OF 200mg/kg	40.87±10.68 a	38.58±5.58 a

Results are mean of 5 determinations ± Standard Deviation (SD). Using Analysis of Variance (ANOVA) and Duncan Multiple Range Test, Means with different superscript are significantly different at (P<0.05).

Table 4: Effect of *Emilia preatemissa* aqueous extract on Alkaline Phosphatase (ALP) in albino rats (u/l)

GROUPS	TREATMENT	PLASMA	LIVER
1	NORMAL CONTROL	21.23±2.22a	36.92±12.47a
2	POSITIVE CONTROL	44.66±12.92 b	25.69±11.85 b
3	STANDARD DRUG	19.18±2.01 a	39.11±12.60 a
4	EXTRACT OF 50mg/kg	32.19±3.37 c	31.22±12.15 ab
5	EXTRACT OF 100mg/kg	29.03±3.04 ac	40.24±12.66 a
6	EXTRACT OF 200mg/kg	22.04±2.31 a	44.66±12.92 a

Results are mean of 5 determinations ± Standard Deviation (SD). Using Analysis of Variance (ANOVA) and Duncan Multiple Range Test, Means with different superscript are significantly different at (P<0.05)

Table 5: Effect of *Emilia preatemissa* aqueous extract on Total Protein (TP) in albino rats (g/l)

GROUPS	TREATMENT	PLASMA	LIVER
1	NORMAL CONTROL	72.66±7.61a	42.48±3.65a
2	POSITIVE CONTROL	40.38±4.23 b	24.75±2.13 b
3	STANDARD CONTROL	76.15±7.98 a	35.25±3.03 a
4	EXTRACT OF 50mg/kg	50.39±5.28 bc	25.31±2.17b
5	EXTRACT OF 100mg/kg	59.42±6.22 c	30.55±2.62ab
6	EXTRACT OF 200mg/kg	70.65±7.40 a	36.45±3.13 a

Results are mean of 5 determinations ± Standard Deviation (SD). Using Analysis of Variance (ANOVA) and Duncan Multiple Range Test, Means with different superscript are significantly different at (P<0.05).

ISSN: 0975-8585

Table 6: Effect of *Emilia preatemissa* (EP) on Albumin in albino rats (mmol/dl)

GROUPS	TREATMENT	PLASMA	LIVER
1	NORMAL CONTROL	35.34±3.70a	29.38±2.52a
2	POSITIVE CONTROL	19.07±1.99b	20.41±1.75 b
3	STANDARD CONTROL	33.20±3.48 c	26.29±2.26 a
4	EXTRACT OF 50mg/kg	25.31±2.65 bc	27.41±2.35 a
5	EXTRACT OF 100mg/kg	32.06±3.36 a	32.74±2.81 ac
6	EXTRACT OF 200mg/kg	36.50±3.82 a	38.63±3.32 c

Results are mean of 5 determinations ± Standard Deviation (SD). Using Analysis of Variance (ANOVA) and Duncan Multiple Range Test, Means with different superscript are significantly different at (P<0.05).

Table 7: Effect of *Emilia preatemissa* aqueous extract on Total Bilirubin in albino rats (mg/dl)

GROUPS	TREATMENT	PLASMA	LIVER
1	NORMAL CONTROL	16.15±1.69a	21.59±1.85a
2	POSITIVE CONTROL	28.65±3.00b	11.64±1.00b
3	STD DRUG (Silymarin)	23.0±2.41 b	12.20±1.05b
4	EXTRACT OF 50mg/kg	27.56±2.88 a	13.86±1.19b
5	EXTRACT OF 100mg/kg	24.02±2.51 b	17.08±1.47ab
6	EXTRACT OF 200mg/kg	19.63±2.05 a	19.25±1.65a

Results are mean of 5 determinations ± Standard Deviation (SD). Using Analysis of Variance (ANOVA) and Duncan Multiple Range Test, Means with different superscript are significantly different at (P<0.05)

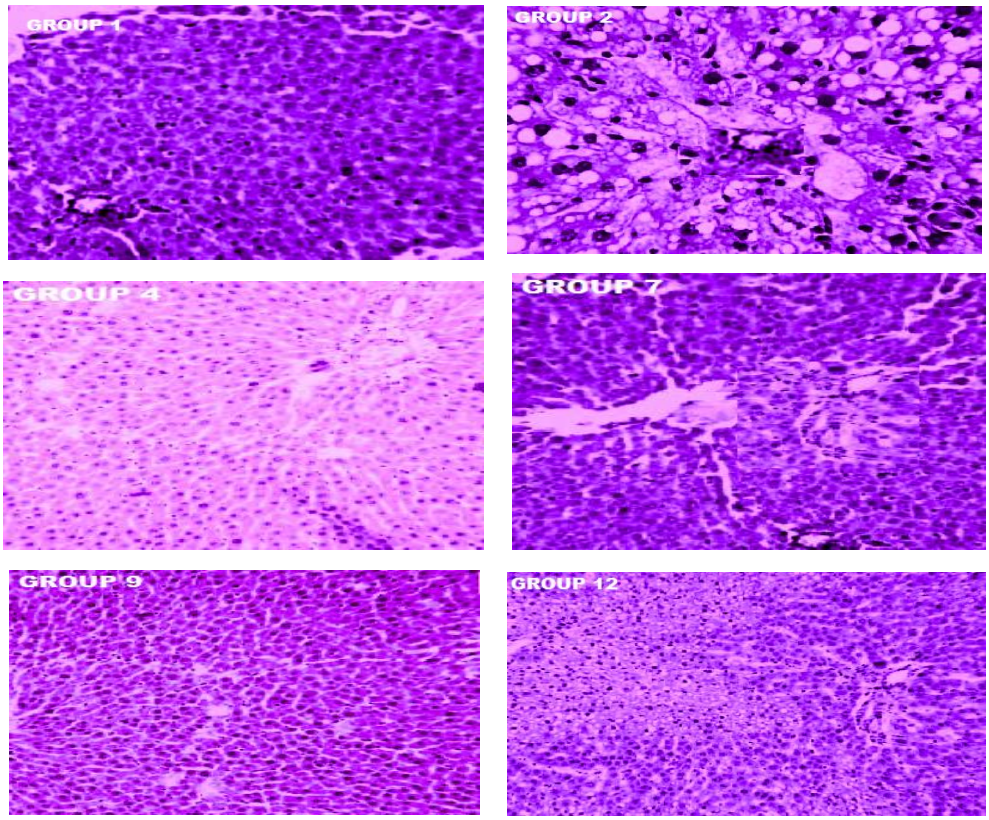

Figure 1: Histopathological Evaluation of Experimental Rats; group 1(Normal control), Group 2(Positive Control), Group 4 (Pre-treatment with Silymarin), Group 7 (Pre-treatment with *Emilia preatemissa* extract of 200mg/kg), Group 9 (Post-treatment with Silymarin) and Group 12 (Post-treatment with Emilia praetermissa extract of 200mg/kg)

Ethical Approval

The handling was guided by NIH Guide for the care and use of laboratory animals, and the ethical Committee for Animal Experimentation of the Ekiti State University.

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