

Assessment of the Hepatoprotective Activity of Ethanol Seed Extract of *Garcinia kola* on Carbon Tetrachloride (CCl₄)-Induced Liver Toxicity in Albino Rat Models

R. B. Ayuba¹, M. O. Enemali², J. P. Mairiga¹, G. S. Haruna^{1*} and O. N. Ani³

¹Department of Biochemistry and Molecular Biology, Faculty of Natural and Applied Sciences, Nasarawa State University, Keffi, Nigeria.

²Department of Applied Biochemistry, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Nigeria.

³Department of Applied Biochemistry, Faculty of Applied Natural and Sciences, Enugu State University of Science and Technology, Enugu State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Authors RBA, MOE, JPM and ONA conceived the research. Authors RBA and MOE designed the experiment. Author GSH analyzed the data and prepared the initial manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To assess the effect of *G. kola* ethanol seed extract on CCl₄-induced liver toxicity in albino rats. The qualitative and quantitative phytochemical analysis of the extract was carried out.

Study Design: Randomized block design.

Place and Duration of Study: Department of Biochemistry and Molecular Biology Laboratory of Nasarawa State University, Keffi, Nasarawa State, Nigeria between January and August, 2019.

Methodology: Thirty male albino rats were randomly distributed into six groups of five rats each. Group 1, normal control, Group 2, standard control, groups 3–6, test groups all administered for seven days. Blood samples were collected for biochemical analysis and liver harvested for histology.

*Corresponding author: E-mail: sundayharuna@nsuk.edu.ng;

Results: Results of phytochemical analysis showed the presence of alkaloids; 1.260±0.00 mg/dl, tannins; 920±0.00 mg/dl, flavonoids; 2.045±0.00 mg/dl, Carbohydrates; 2.00±0.00 mg/dl, Steroids; 0.012±0.00 mg/dl and Cardiac glycosides; 1.25±0.00 mg/dl, saponins, terpenes and anthroquinones were absent. AST in groups 3, 4 and 5 were significantly ($p < 0.05$) higher when compared to control. ALT was significantly ($p < 0.05$) higher in all the treatment groups (4, 5, 6) compared to the control groups (1 and 2). ALP activity increased significantly ($p < 0.05$) in all the test groups compared to the normal control. Total bilirubin increased significantly ($p < 0.05$) in all the treatment groups compared to the controls. Direct Bilirubin was significantly ($p < 0.05$) higher in the treatment groups (5, 6) compared to the normal control. GSH decreased significantly ($p < 0.05$) in all treatment groups compared to the control. CAT and SOD showed no significant ($p > 0.05$) difference in the treatment groups when compared to the control groups. Photomicrographs of the liver showed ballooning degeneration with complete loss of nuclear material.

Conclusion: The administered doses in this study did not protect against CCl₄ induced liver toxicity in albino rats.

Keywords: Evaluation; modulatory effect; Garcinia kola; phytochemical; histology.

1. INTRODUCTION

Plants/plant products have over the years been shown to be an important source of medicine for thousands of years. The World Health Organization (WHO) estimated that 80% of people still rely on traditional remedies such as herb for their medicines [1,2]. Plants are also rich sources of many modern medicines. It is estimated that approximately a quarter of processed drugs contain plant extracts or active ingredient obtained from or modeled on plant substances [2].

The plant, *G. kola* belongs to the family Guittiferae, and it is commonly referred to as 'Bitter Kola'. The plant has the popular title "wonder plant" amongst the South-Western Nigerian people because every part of it have been found to be of medicinal importance and have been found to cure a lot of diseases [3]. In Nigeria, it is referred to as Namijin-goro in Hausa, Orogbo in Yoruba, Akiilu in Igbo and Oro in Ebera languages. *G. kola* is used in folklore remedies for the treatment of ailments such as liver disorders, hepatitis, diarrhoea, laryngitis, and bronchitis [4,5].

The seed can be easily chewed, it has a bitter taste and is used to prevent chest colds, and cough and can be used as well to treat headache [6]. [4] reported the use of this plant for the treatment of jaundice and high fever. The plant has also been found useful in the treatment of stomach ache and gastritis [7]. Liver diseases are some of the deadly diseases in the world today, posing a serious challenge to the international public health [8]. Increase in the incidence of alcoholism, substance abuse (toxic

chemicals) and other unhealthy life style such as eating fatty foods have contributed to the morbidity and mortality due to liver diseases [9]. Some of the commonly known disorders of the liver include viral hepatitis, alcoholic liver diseases, non-alcoholic liver diseases, auto-immune liver diseases, metabolic liver disease, drug-induced liver injury, gall stones, etc. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages [10]. Carbon tetrachloride (CCL₄) has been used extensively to study liver injury induced by free radicals in an animal model system. CCL₄-treated rats are widely used to study liver damage where it was reported that CCL₄ induced not only liver damage but also apoptosis in rats liver [11,12]. Although the mechanism by which CCL₄ causes liver damage is unclear, several lines of evidence suggest that the liver damage could be caused by free radical metabolites [13]. CCL₄ is converted to the trichloromethyl radical by cytochrome *P*-450 through a 1-electron reduction. A fatty acid radical is generated by the reaction between trichloromethyl radical and unsaturated fatty acids, and lipid peroxidation follows [11,12]. In recent times, hepatotoxicity and liver related diseases are becoming prevalent especially in developing countries, such as, Nigeria. Organisms generally possess specific natural pathways to counter the adverse effects posed on the body. However, these mechanisms sometimes the natural repair mechanism fail to keep pace with such harmful effect. There is therefore, the need to seek alternatives from the naturally existing Natural product within the environment to augment the effort of the internal protection and repair mechanisms, hence the rationale behind this study.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

The plant material used for the research was *G. kola* seeds. They were obtained from the Jos central Market in Plateau State, Nigeria and authenticated at the Department of Plant Science and Biotechnology, Faculty of Natural and Applied Sciences, Nasarawa State University, Keffi.

2.1.2 Experimental animals

Male wistar rats weighing between 145 g-200 g were used for the study. A total of 30 male Albino rats were obtained from the animal house of National Veterinary Research Institute Vom. The animals were allowed to acclimatized for 2 weeks before commencement of the study. They were given food (rat chow) and water *ad libitum*.

2.2 Methods

2.2.1 Sample collections and preparation

The *G. kola* seeds were peeled, grated and dried under shade. The dried samples were blended to powder using a mechanical blender to increase the surface area for extraction.

2.2.1.1 Extraction of *G. Kola* seeds

The powdered seed (1200 g) was measured using an Electronic weighing balance and transferred into a stainless bucket. Seventy (70%) ethanol was added and the container was covered and left to macerate for 48 hours. The extract was filtered and collected in a flask and concentrated using a rotary evaporator at 50-70°C.

2.2.1.2 Qualitative phytochemical analysis of *G. kola* extract

The preliminary phytochemical screening of the ethanol extract of *G. kola* seeds was carried out according to the methods of [14,15,16] as described below;

Test for alkaloids: A quantity (0.2 g) of extract was mixed with 10 ml 2% HCl, heated for 5 minutes then filtered. To 1 ml filtrate was added 1 ml of Wagner's reagent. A creamy white precipitate indicates the presence of alkaloids.

Test for steroids: To 0.2 g of methanol extract was added 2 ml of acetic anhydride. The solution was subsequently added to 2 ml of concentrated H₂SO₄ carefully. A colour change from violet to green or bluish green in sample indicated the presence of steroids.

Test for carbohydrate (Molisch's Test): To 0.2 g of extract was added 10 ml of distilled water and then boiled for 5 minutes before filtering. To 1ml filtrate, 100 µl of Molisch solution was added followed by the addition of 1 ml concentrated H₂SO₄. A brown ring formation at interface indicates the presence of carbohydrate.

Test for flavonoids: A quantity of the sample (0.2 g) was heated with 10 ml ethyl acetate in boiling water for 3 minutes. The mixture was filtered, and the filtrate was used for the following test.

(i) Ammonium test: Four millilitres (4 ml) of the filtrate was shaken with 1 ml of dilute ammonium solution to obtain two layers. The layers were allowed to separate. A yellow precipitate observed in the ammonium layer indicated the presence of flavonoids.

Test for tannins (Ferric chloride test): To 0.2 g of extract was added 10 ml of 45% ethanol, boiled for 5 minutes and then filtered. To 1 ml filtrate, 200 µl of ferric chloride was added. An observation of brownish green precipitate indicated the presence of tannins.

Test for saponin: A quantity (0.2 g) of extract was dissolved with 10 ml distilled water, warmed for a minute and then filtered. To 1 ml filtrate was added 4 ml of distilled water, shaken thoroughly for 5 minutes before allowing to stand for 1 minute. Persistence of foam indicates the presence of saponins.

Test for terpenoids: A quantity (0.2 g) of the extract was dissolved in ethanol and 1 ml of acetic anhydride was added to the solution. A few drops of concentrated H₂SO₄ was then added to the solution. A change in colour from pink to violet showed the presence of terpenoids.

Test for phenolics: To 0.2 g of the extract was added 2 ml of distilled water. Then 0.5 ml Na₂CO₃ and 0.5 ml Folin Ciocalteu reagent was subsequently added. Formation of a blue-green colour indicated the presence of phenols.

Acid Test: To 0.2 g of extract was added 10 ml of distilled water, heated for 5 minutes and then

filtered. A blue litmus paper was dipped into the filtrate. A change to red indicated acidity.

Test for cyanogenic glycosides: To 1 g of the extract in a conical flask was added 10 ml water and 1 ml dilute HCl. Picrate paper was suspended above the mixture. The contents of the flask were heated at 45°C for 1 hour. A control without the extract was set up. A colour change from yellow to reddish purple of the picrate paper was a positive test.

2.2.1.3 Quantitative determination of phytochemical content of *G. kola* seeds

This was done according to the methods outlined by [14,15,16].

The concentration of the various phytochemical constituents was calculated using the formula below;

$$\text{Concentration} \left(\frac{\text{mg}}{100\text{g}} \right) = \frac{\text{absorbance of the sample}}{\text{absorbance of the standard}} \times \text{dilution factor}$$

Dilution factor = (total volume/weight) of extract

Determination of Alkaloid Content

To a 10 ml of *G. kola* seed extract in 250 ml separatory funnel followed by 5 ml dilute H₂SO₄ and 5 ml of distilled water were added. The extract was shaken twice with 10 ml CHCl₃ and the combined CHCl₃ extract was transferred to a second separating funnel containing 5 ml dilute H₂SO₄ and 10 ml of distilled water. The CHCl₃ layer was discarded and the aqueous acidic layer was transferred to the contents of the first separating funnel. The extract was made alkaline with ammonia and shaken for about half a minute.

The extract alkaloid was extracted completely by successive portion of CHCl₃ each of 20 ml. (complete extraction was tested using Mayer's reagent). The combined CHCl₃ extract was shaken with 5 ml distilled water. The extract was run through a plug of cotton wool previously mustered with CHCl₃ and covered with a little anhydrous sodium sulphate, the sodium sulphate was washed with 5 ml of CHCl₃. The combined CHCl₃ extract was received into a 250 ml dry conical flask. The CHCl₃ was completely distilled and 5 ml neutral alcohol was added and

evaporated on a boiling water bath. The residue was further heated on the boiling water bath for about 10 – 15 minute (to remove volatile bases). The residue was dissolved in 2 ml of CHCl₃, and 20 ml of N/50 H₂SO₄ was added and warmed on a water bath to remove the CHCl₃ completely and cooled. The excess and was titrated with N/50 NaOH using methyl red as indicator till the first drop of N/50 NaOH caused color change from pink to yellow.

Calculation

1 ml of N/50 NaOH = 0.005787 g alkaloids

%Alkaloids content = [20 ml taken of N/50H₂SO₄] x 0.005787 x 100/10

Determination of Flavonoids

A 5 ml extract was transferred to a small flask and hydrolysed by heating on a water bath with 10 ml of 10% H₂SO₄ for 30 minutes. The original volume was reduced to half and the mixture was cooled on ice for 15 minutes where the flavonoids are pre-evaporated.

The cooled solution was filtered; the residue was dissolved by pouring 50 ml of warm 95% ethanol and further made to 100 ml with 95% ethanol. A 5 ml aliquot was pipetted into a 25 ml volumetric flask and diluted to volume with 50% ethanol. The absorbance of the resulting solution was measured at 370 nm against 50% ethanol blank. Flavonoid concentration was calculated using a reference curve of pure quercetin.

Determination of Tannins

A known weight (0.5 g) of each sample was extracted with 3 ml methanol. The extract was mixed with 5.0 ml water 3 ml of 1.0 ml (FeCl₂ in 0.1 N and 0.8 ml 2 Fe (w₂) was added to 0.1 ml of the solution. The extract was read at 720 nm on a spectrophotometer.

Determination of Saponins

A known weight (0.1 g) of the sample was boiled and filtered with Whatman No.1. A known quantity (5) ml of the filtrate was pipetted into a test tube and 2 ml of olive oil was added. The solution was shaken vigorously for 30 seconds and read at a wavelength of 620 nm against a blank (standard saponin).

2.2.2 Experimental design

A total number of 30 male wistar rats were distributed randomly into six groups of five rats each.

Group 1 (Standard control group) received distilled water and feed

Group 2 (positive control group) received 100 mg/kg of silymarin followed by a single dose of CCL₄ (0.4 ml/kg b.w) on day 7

Group 3 (negative control group) received a single dose of CCL₄ (0.4 ml/kg) on 'day 7

Group 4 (prophylactic) received 400 mg/kg b.w of (*G. kola* ethanol extract) followed by a single dose of CCL₄

Group 5 (prophylactic treatment group) received 800 mg/kg of (*G. kola* ethanol extract) daily followed by a single dose of CCL₄ (0.4 ml/kg b,w) on day 7

Group 6 (prophylactic treatment group) received 1200 mg/kg of (*G. kola* ethanol extract) daily for 1 week followed by a single dose of CCL₄ (0.4 ml/kg b.w) on 'day 7

The animals were sacrificed on day 8 after 8 hours fast under anesthesia and blood collected. The liver were excised and transferred into a buffered solution and other parts kept for histopathology.

Blood samples were collected from ocular vein using capillary tubes in to plain sample bottles and allowed to clot before spinning in a centrifuge to obtain the serum.

2.2.3 Determination of liver function parameters

Determination of Alkaline phosphatase (ALP): The ALP was analyzed according to the methods outlined in Randox kit for ALP assay as described below;

Principle: The principle of this method is based on the reaction of alkaline phosphate and a colourless substrate of phenolphthalein monophosphate, giving rise to phosphoric acid and phenolphthalein which at alkaline pH values, turn pink that can be determined spectrophotometrically.

P-nitrophenylphosphate + H₂O ----- → ALP -----
 - → PO₄²⁻ + P-nitrophenol (pink at pH=9.8)

Method: The blank and sample test tubes were set up in duplicates and 0.05 ml of sample was pipette into the sample test tubes. 0.05 ml of distilled water was pipetted into the blank tube. Three millilitres (3.0 ml) of substrate was pipetted into each tube respectively, which was then mixed and the initial absorbance taken at 405 nm. The stop watch was started and the absorbance of the sample and the blank read again three more times at one minute intervals.

Calculation: alkaline phosphatase activity was calculated as follows:

$$\text{Activity of ALP} \left(\text{in } \frac{U}{L} \right) = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 3300$$

Determination of Alanine Aminotransferase (ALT): The ALT was analyzed according to the methods outlined in Randox kit for ALT assay as described below;

Principle: ALT is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 540 nm.

Method: The blank and sample test tubes were set up in duplicates. 0.1 ml of serum was pipetted into the sample tubes. To these were added 0.5 ml buffer solution containing phosphate buffer, L-alanine and α-oxoglutarate. The mixtures were thoroughly mixed and incubated for exactly 30 minutes at 37°C ml and pH 7.4. A volume, 0.5 ml of reagent containing 2, 4-dinitrophenylhydrazine was later added to both tubes while 0.1 ml of sample was added to sample blank tube. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25°C. Five millilitres of sodium hydroxide solution was then added to each tube and mixed. The absorbance was read against the blank after 5 minutes at 540 nm.

Determination of Aspartate Aminotransferase (AST): The AST was analyzed according to the methods outlined in Randox kit for AST assay as described below;

Principle: AST is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 546 nm.

Method: The blank and sample test tubes were set up in duplicates. A volume, 0.1 ml of serum was pipetted into the sample tubes and 0.5 ml of reagent 1 was pipette into both sample and blank tubes.

The solutions were thoroughly mixed and incubated for exactly 30 minutes at 37°C ml and pH 7.4. 0.5 ml of Reagent 2 containing 2, 4-dinitrophenylhydrazine was added into all the test tubes followed by 0.1 ml of sample into the blank tubes. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25°C and 5.0 ml of sodium hydroxide solution was then added to each tube and mixed. The absorbance was read against the blank after 5 minutes at 546 nm.

Determination of Direct Bilirubin Concentration: The concentration of conjugated and unconjugated bilirubin were determined using themethod of [17] as outlined in Randox Kits.

Colorimetric method: Direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid.

Reagent (R)

R1: Sulphanilic acid (29 mmol/L) and Hydrochloric acid (0.17 N)

R2: Sodium nitrite (25 mmol/L)

R3: Caffeine (0.26 mol/L) and sodium benzoate (0.52 mol/L)

R4 Tatarate (0.93 mol/L) and sodium hydroxide (1.9 N)

Determination of Total Bilirubin (TB) Concentration: The TB was analyzed according to the methods outlined in Randox kit for TB analysis as described below;

Procedure

The samples and reagents were Pipetted into the appropriate test tubes and allowed to stand for 10 minutes at 25°C. This was followed by the addition of 1.0 ml of reagent 4 (tatarate (0.93 mol/L) and sodium hydroxide (1.9 N)). Each was mixed and allowed to stand for 30 minutes at 25°C after which the absorbance was read at 578 nm.

The calculation was done thus:

$$\text{Total Bilirubin } (\mu\text{mol/L}) = 185 \times \text{ATB (578 nm)}$$

ATB = absorbance of total bilirubin at 578 nm

185 = a constant

2.2.4 Estimation of oxidative stress biomarkers

Determination of lipid peroxidation (Malondialdehyde) Concentration: Lipid peroxidation was determined by measuring spectrophotometrically the level of the lipid peroxidation product, malondialdehyde (MDA) as described by [18].

Principle: Malondialdehyde (MDA) reacts with thiobarbituric acid to form red or pink coloured complex, which is phytometrically measured.

Procedure: A volume of the serum (0.1 ml) was mixed with 0.9 ml of H₂O in a beaker. A volume, 0.5 ml of 25% trichloroacetic acid (TCA) and 0.5 ml of 1% thiobarbituric acid (TBA) in 0.3% NaOH were also added to the mixture. The mixture was boiled for 40 minutes in water-bath and then cooled in cold water. Then 0.1 ml of 20% sodium dodecyl sulphate (SDS) was added to the cooled solution and mixed properly. The absorbance was taken at wavelength 532 nm and 600 nm against a blank.

Calculation

$$\%T\text{BARS} = \frac{A_{532} - (A_{600} \times 100)}{(0.5271 \times 0.1)} \text{ mg/dl}$$

Assay of Superoxide Dismutase (SOD) Activity: Determination of the activity of Superoxide Dismutase (SOD) was carried out according to the method described by [19].

Procedure

The ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenaline at pH 10.2, form the basses of this assay, 0.05 M phosphate buffer: 6.97 g of diphosphate K₂HPO₄ and 1.36 g of KH₂PO₄ was dissolved in distilled water and made up to 1000 ml mark in a volumetric flask. The buffer was adjusted to pH 7.8. 0.05 Carbonate buffer: 14.3 g of Na₂CO₃) and 4.2 g Of NaHCO₃) was dissolved in distilled water and

made up to 1000 ml mark in a volumetric flask. The buffer was adjusted to pH 10.2. 0.3 mM Adrenaline: 0.01 g of adrenaline was dissolved in 17 ml of distilled water. The solution was prepared fresh. 0.1 ml of microsome will be diluted in 0.9 ml of distilled water to make 1:10 dilution of microsome. An aliquant mixture of 0.20 ml of the diluted microsome was added to 2.5 ml of 0.05 M Carbonate buffer. The reaction started with the addition of 0.3 ml of 0.3 mM adrenaline. The reference mixture contains 2.5 ml of 0.05 M Carbonate buffer. 0.3 ml of 0.3 mM adrenaline and 0.20 ml of distilled water. Absorbance was measured ever 30 up to 150 s at 480 nm. 1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute.

5.0 ml of R1 was put in test tube 1 i.e. (sample blank) while to the sample test tube 2 (serum) was added 0.5 ml R1 and 0.1 ml of serum. This was incubated at 37°C for 30 mins. After this, 0.5 ml of R2 was then added to all test tubes, in addition to 0.1 serum to the sample blank test tube only. This was allowed to stand for 20 mins at 25°C. Finally, 5.0 ml of sodium hydroxide was added to both test tubes, content mixed and the absorbance of the sample (A_{sample}) was read (at 546 nm) against the sample blank after 5 mins. This was done for all samples obtained from the experimental animals.

Assay of Catalase (CAT) Activity

This was carried out according to the method of [20].

Principle

The ultraviolet absorption of hydrogen peroxide can be easily measured at 240 nm. On the decomposition of hydrogen peroxide with catalase, the absorption decreases with time and from this decrease, catalase activity can be measured.

Procedure

A known volume (2.5 ml) of phosphate buffer + 2 ml H_2O_2 + 0.5 ml of sample were pipetted into a test tube. To 1 ml portion of the reaction, 2 ml of Potassium dichromate and acetic acid were added. The absorbance was determined at 240 nm into 4 places at a minute interval. Catalase concentration was then calculated using the equation:

Catalase concentration (Unit/L) =

$$\frac{0.23 \times (\log\text{Abs1}/\log\text{Abs2})}{0.00693}$$

Determination of Reduced glutathione (GSH) concentration

This was done based on the method of [21].

Principle

Reduced glutathione (GSH) forms the bulk of non-protein sulfhydryl groups. This method is based on the formation of relatively yellow coloration when Ellman's reagent is added to a sulfhydryl compound. The chromophoric product, 2-nitro-5-thiobenzoic acid reagent with reduced glutathione absorbs at 412 nm. The absorbance at 412 nm is therefore proportional to the glutathione content.

Preparation of GSH Calibration Curve

Serial dilutions of the stock GSH were made using the phosphate buffer for dilution (to a volume of 0.5 ml). Ellman's reagent (4.5 ml) was added to each solution. The absorbance of the colored solution developed was read at 412 nm within 5 minutes of the color generation. Optical density was plotted against glutathione concentration.

Procedure

Each sample (1.0 ml) was deproteinezed by adding an equal volume of 4% sulfo-salicylic acid, the mixture was added to 4.5 ml of Ellman's reagent. A blank was prepared by adding 0.5 ml of 4% sulfo-salicylic acid to 4.5 ml Ellman's reagent. The absorbance was read at 412 nm and the equivalent GSH concentration determined from the calibration curve.

Assay of Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase (GPx) activity was assayed spectrophotometrically according to the method described by [22].

Principle

The Cayman chemical glutathione peroxidase assay kit measures glutathione reductase activity indirectly by a coupled reaction with GPx. Oxidized glutathione GSSG, produced upon

reaction with an organic hydroperoxide by GPx is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm. The rate or decrease in A₃₄₀ is directly proportional to the GPx activity in the sample.

Procedure

The instrument was turned on and allowed to stabilize for 20 minutes; the wavelength was set at 340 nm. The instrument was zeroed at 340 nm with distilled water. A known volume of the enzyme, solution A (870 µL) and 60 µL of start solution (enzyme activator) were pipetted into the cuvette. The sample, (30 µL) was added to start the reaction and distilled water was used as blank. The mixture was shaken gently and absorbance measured A₃₄₀ for 1 minute following 30 seconds time interval.

$$\text{GPx activity} = \Delta A_{340} \text{ test} - \Delta A_{340} \text{ blank}$$

2.2.5 Histological examination

This was carried out as described by [23].

Procedure

At the end of the experiment the liver, kidney and testicles from group 1 and 5 were collected for histopathology.

The organs were fixed in 10% formal saline and dehydrated in ascending grades of (70%, 90% and 100%) ethanol respectively. Thereafter, the tissues were cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed and sectioned at 5 – 6 microns. The sections were de-paraffinized in xylene, taken to water and subsequently stained with Haematoxylin and Eosin (H and E) for light microscopy.

2.3 Statistical Analysis

The data obtained from the test parameters (quantitative phytochemical analysis, liver function biomarkers, pro- and anti-oxidant indices) were analyzed using one-way analysis of variance (ANOVA) with the help of IBM statistical product and service solution (SPSS) package, version 20.0 and the results were expressed as mean ± standard deviation. Test for levels of significance was done using Least significant difference (LSD) and Duncan test. The acceptance value of significance was $p < 0.05$ for all the results.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Qualitative and quantitative phytochemical composition of *G. kola* ethanol seeds extract

The results of preliminary qualitative phytochemical analysis ethanol seed extract of *G. kola* showed the presence of alkaloids, tannins, flavonoids, carbohydrates, steroids and cardiac glycosides while saponins, terpenes and anthraquinones were absent. Quantitative phytochemical analysis showed alkaloids to be 1.260±0.00 mg/dl, tannins; 920±0.00, flavonoids; 2.045±0.00 mg/dl, Carbohydrates; 2.00±0.00 mg/dl, Steroids; 0.012±0.00 mg/dl and Cardiac glycosides; 1.25±0.00 mg/dl) as shown in (Table 1).

Table 1. Qualitative and quantitative phytochemical analysis of *G. kola* ethanol seeds extract

Phytochemical	Qualitative comp.	Quantitative comp.
Alkaloids	++	1.260±0.00
Saponins	-	
Tannins	+	0.920±0.00
Flavonoids	+++	2.045±0.00
Carbohydrates	+++	2.00±0.00
Steroids	++	0.012±0.00
Terpenes	-	
Anthraquinones	-	
Cardiac glycosides	++	1.25±0.00

Key; +++ = Present in high amount, ++ = present in moderate amount, + = present in little amount, - = Absent

3.1.2 Effect of *G. kola* ethanol extract on liver function parameters of rats

As shown in Table 2, AST activity in there was no significant ($p > 0.05$) increase in AST activity in groups 2 and 6 when compared to the AST activity of the normal control group, however, the increase in AST activity in groups 3, 4 and 5 were significantly ($p < 0.05$) higher when compared to that of the normal control group. For ALT, the activity was found to be significantly ($p = 0.05$) higher in all the treatment groups (4, 5 and 6) when compared to the control groups (1 and 2) except when compared to the negative control (group 3) group. ALP activity increased significantly ($p < 0.05$) in all the test groups

compared to the normal control group but the increase was not significant ($p > 0.05$) in group 5 when compared to the negative control and was also not significant ($p > 0.05$) in group 6 when compared to the positive control group. Total bilirubin (TB) concentration was observed to increase significantly ($p < 0.05$) in all the treatment groups when compared to all the control groups. DB concentration was significantly ($p < 0.05$) higher in the treatment groups (5 and 6) compared to the normal control group but group 4 showed no significant ($p > 0.05$) increase when compared to the normal control, also the increase in the direct bilirubin (DB) concentration in group 5 was non-significant ($p = 0.05$) when compared to that of the positive control group. No significant ($p > 0.05$) difference was observed in the TP and albumin (ALB) concentrations of all the test groups when compared to the control groups.

3.1.3 Effect of *G. kola* ethanol extract on pro- and anti-oxidant parameters of rats

Table 3 shows MDA concentration non-significantly ($p > 0.05$) higher in group 4 when

compared to groups 1 and 2 but significantly ($p < 0.05$) lower when compared to group 3, the value was found to be significantly ($p < 0.05$) higher in group 5 when compared to groups 1 and 2 and non-significantly ($p > 0.05$) higher when compared to group 3, in group 6, the value showed significant ($p < 0.05$) increase when compared to all the control groups. GSH concentration decreased significantly ($p < 0.05$) in all the treatment groups when compared to the control groups except when compared to the positive control group where the decrease was not significantly ($p > 0.05$). CAT and SOD activities showed no significant ($p > 0.05$) difference in all the treatment groups when compared to the control groups.

3.1.4 Photomicrographs of ethanol extract of *G. kola* Seeds on the liver of rats

Plate 1 (Grp 1) liver of Albino rats showed normal tissue architecture. The hepatocytes, with intact nuclei (white arrows), surrounded by intact cytoplasm (white stars), present with polygonal shapes and interspersed by hepatic sinusoids (black arrows) which contain few red blood cells. H&E **A:** X100 **B:** X400.

Table 2. Effect of *G. kola* ethanol extract on liver biomarkers of rats

Treatment Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	TB (mg/dl)	DB (mg/dl)
Group 1	68.83±9.89 ^a	21.00±4.24 ^c	77.05 ±17.19 [†]	0.54±.07 [†]	0.28± .08 [†]
Group 2	93.10±18.71 ^a	83.50±10.66 ^d	126.10±11.02 ^g	0.83±.19 ⁱ	0.69±.18 ^m
Group 3	191.45±33.90 ^b	109.25±8.34 ^e	203.10±39.60 ^h	1.47±.27 ^j	0.99±.12
Group 4	184.14±31.52 ^b	100.25±12.34 ^e	144.30±38.04 ^g	2.39±.55 ^k	0.46±.15 ^l
Group 5	173.66±38.24 ^b	108.22±10.86 ^e	188.95±34.74 ^h	2.42±.05 ^k	0.74±.08 ^m
Group 6	105.51±6.82 ^a	99.50±10.54 ^e	154.85±20.71 ^g	2.03±.44 ^k	1.52±.18 ⁿ

Results are expressed in Means ± SD (n = 4), TB = Total Bilirubin, DB =Direct Bilirubin

Mean values with different letters as superscripts down the column are considered significant at $p < 0.05$

Group 1 = Normal Control; Group 2 = Positive control; Group 3 = Negative control; Group 4 = 400 mg/kg b.w. of *G. kola* ethanol extract; Group 5 = 800 mg/kg b.w. of *G. kola* ethanol extract; Group 6 = 1200 mg/kg b.w. of *G. kola* ethanol extract

Table 3. Effect of *G. kola* ethanol extract on liver biomarkers of rats

Treatment Group	MDA (mg/dl)	GSH (mg/dl)	CAT (IU/L)	SOD (IU/L)
Group 1	10.88±4.51 ^a	6.56±1.20 ^d	17.53±4.80 ^g	4.74±.84 ^h
Group 2	14.93±1.52 ^a	3.75±.74 ^e	19.12±3.47 ^g	4.30±1.36 ^h
Group 3	23.32±5.99 ^b	4.93±.75 [†]	37.97±35.07 ^g	3.87±.61 ^h
Group 4	17.31±5.76 ^a	3.25±.46 ^e	17.41±2.27 ^g	4.66±1.05 ^h
Group 5	27.58±7.39 ^{bc}	3.00±.50 ^e	24.53±8.60 ^g	4.01±.60 ^h
Group 6	34.50±8.48 ^c	3.18±.24 ^e	30.05±7.75 ^g	4.20±.46 ^h

Results are expressed in Means ± SD (n = 4)

Mean values with different letters as superscripts down the column are considered significant at $p < 0.05$

Group 1 = Normal Control; Group 2 = Positive control; Group 3 = Negative control; Group 4 = 400 mg/kg b.w. of *G. kola* ethanol extract; Group 5 = 800 mg/kg b.w. of *G. kola* ethanol extract; Group 6 = 1200 mg/kg b.w. of *G. kola* ethanol extract

Plate 2 (Grp 2) liver of Albino rat showed ballooning degeneration. The hepatocytes present with swelling and severe shrinkage of nuclei (white arrowheads) while some present with complete loss of nuclei (Black stars). Some of the degenerated cells have completely lost their cellular materials and present only strands (white arrows) of cellular material. Interspersing the degenerated cells are normal cells, presenting with intact nuclei (black arrows). H&E **A:** X100 **B:** X400.

As shown in Plate 3, Group 3 liver of albino rats showed Ballooning degeneration with pyknotic nuclei (white arrows) while some show complete loss of nuclear material (white stars). Black arrows show the nuclei of normal hepatocytes. Few red blood cells (white arrowheads) are seen within the hepatic sinusoids H&E **A:** X100 **B:** X400.

Plate 4 (Grp 4) liver of Albino rats showed severe Ballooning degeneration with complete loss of

cellular materials thereby presenting with remnants of cellular material (white arrows). While the cells increase in size, the nuclei (black arrows) become pyknotic and are completely lost in some of the cells (white stars). H&E **A:** X100 **B:** X400.

In Plate 5, (Grp 5) liver of Albino rat showed Ballooning degeneration. White stars cells showing complete loss of nuclear material, white arrowheads pyknotic nuclei of degenerated cells, black arrows= strands of cellular remnants, white arrows= nuclei of normal hepatocytes. H&E **A:** X100 **B:** X400.

Plate 6 (Grp 6) liver of Albino rats showed Ballooning degeneration (necrosis) showing complete loss of nuclear material (white stars), pyknosis (black arrows) and complete loss of cellular material, leaving hepatocyte strands (white arrows). Intercellular inflammation is seen by the presence of inflammatory cells (white arrowheads). H&E **A:** X100 **B:** X400.

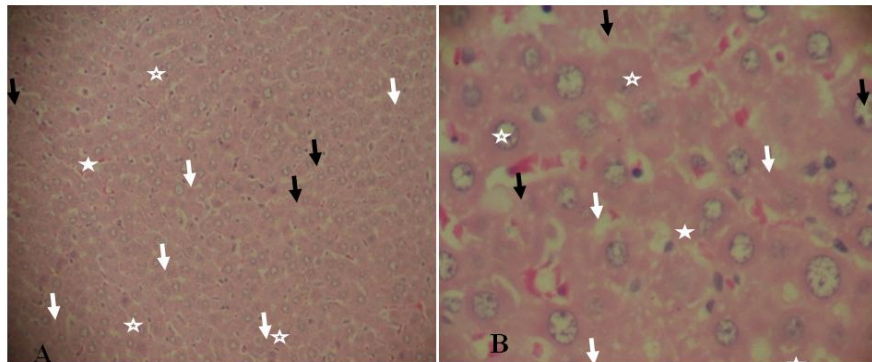


Plate 1 (Grp 1). Liver of Albino rat showing normal tissue architecture

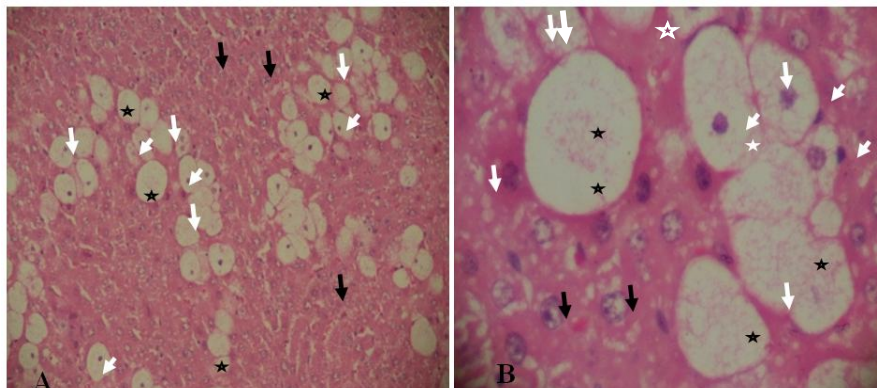


Plate 2 (Grp 2). Liver of Albino rat showing ballooning degeneration

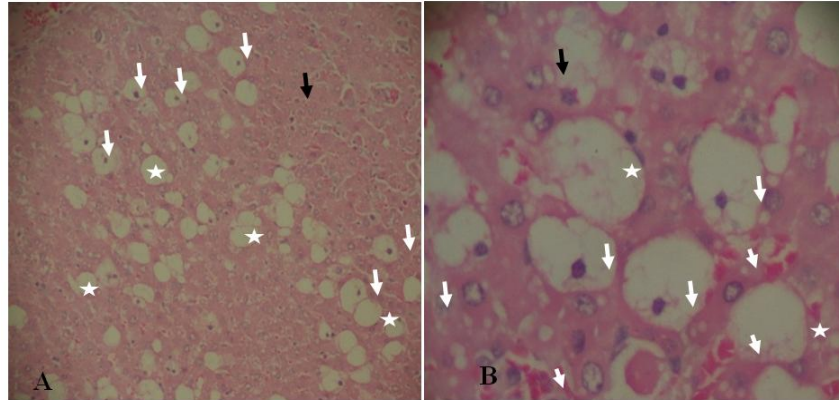


Plate 3 (Group 3). Liver of Albino rat showing ballooning degeneration with pyknotic nuclei

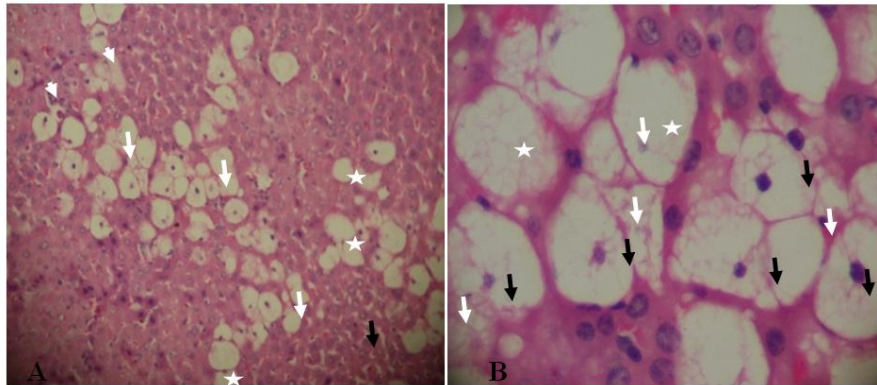


Plate 4 (Grp 4). Liver of Albino rats showing severe ballooning degeneration with complete loss of cellular materials

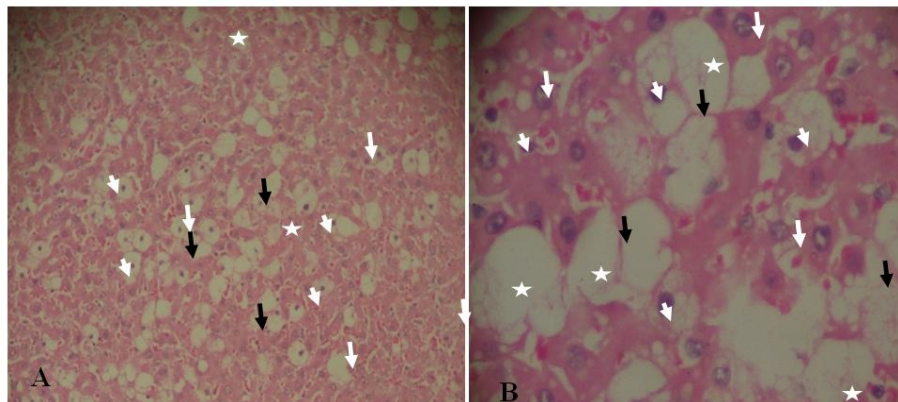


Plate 5 (Grp 5). Liver of Albino rat showing ballooning degeneration

3.2 Discussion

The phytochemical analysis conducted on *G. kola* ethanol seed extract revealed the presence of glycosides, saponins, tannins, alkaloid, flavonoids, steroids, carbohydrates, phenolic

acids, Steroids and terpenoid. These phytochemical compounds are known to support bioactive activities in medicinal plants and may be responsible for the antioxidant activities of *G. kola* ethanol seed extracts used in this study (Table 1).

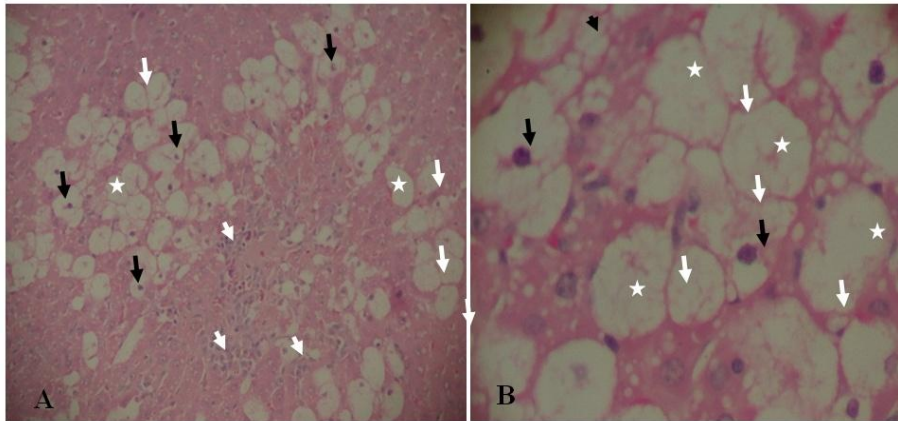


Plate 6 (Grp 6). Liver of Albino rat showing ballooning degeneration (necrosis) showing complete loss of nuclear material

The result obtained for qualitative phytochemical analysis of *G. kola* showed the presence of flavonoids and carbohydrates in high amounts, alkaloids, steroids and cyanogenic glycosides in moderate amounts while tannins were found in small amount. These phytochemical compounds are known to play important roles in bioactivity of medicinal plants. The medicinal values of medicinal plants lie in these phytochemical compounds and as such produce a definite physiological actions on the body. Alkaloids have been shown to have many pharmacological activities including antihypertensive effects (indole alkaloid), antiarrhythmic effect, antimalarial activity (quinine) and anticancer actions. Flavonoids which are part of the phytochemical constituents of *G. kola* exhibit a wide range of biological activities one of which is their ability to scavenge for hydroxyl radicals, and superoxide anion radicals, and thus health promoting in action [24]. Flavonoids have been reported to exert multiple biological property including antimicrobial, anti-inflammatory as well as antitumor activities but the best described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species [25]. Flavonoids also exhibit anti-inflammatory, antiangiogenic, anti-allergic effects, analgesic and antioxidant properties [26]. Tannins exert antimicrobial activities by iron deprivation, hydrogen bonding or specific interactions with vital proteins such as enzymes in microbial cells [27]. Another phytochemical compound observed to be present in the seeds of *G. kola* ethanol extract is tannins. Herbs that have tannins as their component are astringent in nature and are

used for the treatment of intestinal disorders such as diarrhoea and dysentery [28]. Tannin containing plant extracts are used as astringents, against diarrhea as diuretics, against stomach and duodenal tumors among other uses [25]. Tannins have been observed to have remarkable activity in cancer prevention and anticancer [29]. In addition to this, [30] showed tannins to be useful in treatment of inflamed or ulcerated tissues. Cardiac glycosides were also observed to be present in *G. kola* ethanol seed extract. They are important class of naturally occurring drugs whose actions helps in the treatment of congestive heart failure [30]. This compound has been reported to be a novel cancer therapeutic agent [31]. Cardiac glycoside was present in *G. kola* extract, and this plant is used for the treatment of cardiac infections along with other ailments such as cough and chest pain among the Yoruba people of southwestern Nigeria.

The elevation in the levels of liver marker enzymes; alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) maybe due to administration of CCl_4 which probably resulted in a significant hepatic damage leading to the leakage of the enzymes in to the blood. Obviously, the elevated levels of these biochemical parameters are indicators and a direct reflection of alterations of hepatic structural and functional integrity of the hepatocytes [32,33]. Elevation of liver enzymes levels obtained in this study corroborates with reports of previous studies in relation to CCl_4 toxicity; toxicity in liver due to CCl_4 and other chemicals is attributed to the toxic metabolites formed [34,35,36]. ALT is known to be more specific to the liver function test and thus a better

parameter for detecting liver injury [13,35,36]. Remarkable elevation ($p < 0.01$) of ALT enzyme level observed with CCl_4 administration in group II animals is indicative of liver damage. These enzymes are located in the cell cytoplasm and are emptied directly into the blood and undergo circulation once the cellular membrane of the liver is damaged [37].

Total bilirubin (TB) concentration was observed to increase significantly ($p < 0.05$) in all the treatment groups when compared to all the control groups. Direct bilirubin (DB) concentration was significantly ($p < 0.05$) higher in the treatment groups (5 and 6) compared to the normal control group but group 4 showed no-significant ($p > 0.05$) increase when compared to the normal control, also the increase in the DB concentration in group 5 was non-significant ($p > 0.05$) when compared to that of the positive control group.

The levels all the liver function parameters tested (except ALB) were low in the group of rats that was administered silymarin. Silymarin (rated as one of the most powerful antioxidants) would positively influence the position on its antioxidant status. The findings in this study show that pretreatment of rats 14 days prior to CCl_4 administration caused a marked decrease in the levels of liver function markers. This suggests that seeds of *G. kola* may be protective against CCl_4 - induced liver damage in rats. This was ascertained by a comparative analysis of the results obtained in rats pretreated with ethanol extract of *G. kola* seeds.

Furthermore, the AST/ALT ratio has been reported to provide information on the extent and differentiate between causes of liver damage (hepatotoxicity) [38]. It has been reported that the normal ratio is less than 1, and when it is more than 1 but less than 2, it is said to be due to toxins, cirrhosis. When the ratio is greater than 2, it is more likely to be associated with alcoholic hepatitis [39] or hepatocellular carcinoma.

Antioxidant enzymes and lipid peroxidation levels (indicated by the level of MDA) can be used to predict the severity of drug induced liver damage [40]. Antioxidant enzymes such as Superoxide Dismutase (SOD), Catalase (CAT) and Reduced glutathione (GSH) dependently act in the metabolic pathways that involve free radicals. Therefore changes in antioxidant enzymes activity, oxidative stress markers (CAT, SOD, and GSH) are indicators of liver damage [32,41]. Extensive lipid peroxidation leads to

disorganization of membrane by peroxidation of unsaturated fatty acids which also alters the ratio of poly-unsaturated to other fatty acids. This would lead to a decrease in the membrane fluidity and the death of cell. The decrease in CAT, SOD, and GSH is due to the toxic effect of CCl_4 .

Decreased antioxidant enzymes activity (CAT, SOD, and GSH) observed in CCl_4 treated group (group II) shows a treatment related toxicity. This is in agreement with reports from CCl_4 related toxicity studies; CAT, SOD, and GSH decrease levels in liver suggests toxic effect of CCl_4 on the liver [42].

Results show that silymarin is an effective hepato-protective agent. From the results, it can be concluded that the extract at low dose shows a better hepato-protection when compared to the high dose of the extract as demonstrated in liver enzyme levels and oxidative stress indicators.

Histo-pathological examination can be used to show the severity of toxicity of CCl_4 induced liver damage [34]. Histo-architectural distortion manifested as severe alteration in the arrangement of the hepatocytes in the CCl_4 induced group (II), which include areas of inflammatory cells, swelling and shrinkage of nuclei as well as mild necrosis and in some cases complete loss of nuclei (Plate 2). All these are signs of toxicity induced by CCl_4 . Several researchers had reported on histopathological changes that result from CCl_4 administration, such as necrosis [42,41].

Result of photomicrograph image in this study as shown on Plate 1 (Grp 1) liver of albino rats showed normal tissue architecture. The hepatocytes, with intact nuclei (white arrows), surrounded by intact cytoplasm (white stars), present with polygonal shapes and interspersed by hepatic sinusoids (black arrows) which contain few red blood cells while that of Plate 2 (group 2 rats) which were administered silymarin followed by a dose of CCl_4 showed swelling and severe shrinkage of nuclei while some present with complete loss of nuclei. This confirmed the hepatotoxic effect of CCl_4 as stated earlier. The administration of standard drug, 100 mg/kg Silymarin protected the liver cells against the adverse effects of the toxic agent (CCl_4). The histoarchitecture of this group was close to normal with mild necrosis visible in some areas, but the distortion was not as severe as in control. This is possibly due to the hepatoprotective activity of the drug.

Group 3 liver of albino rats was characterized by pronounced degenerative properties as its architecture showed ballooning degeneration with pyknotic nuclei and necrosis, while some show complete loss of nuclear material. This may also be due to the toxic effect of CCl₄ that was administered to the group as observed by many researchers.

No observable improvement in the microscopic appearance of the liver after the administration of ethanol extract of *G. kola* seeds (EEGK) showing no restoration in the hepatocytes, (groups IV, V and VI), (Plates 4, 5 and 6). However, the distortion, was not as severe as manifested in the CCl₄ group when compared. The non-restoration of the hepatocytes in these groups was possibly due to the overwhelming degenerative effect exerted on the hepatocytes by CCl₄.

The structural integrity of the liver is maintained by a delicate meshwork of extracellular matrix in the form of a fine meshwork of reticulin fibres (collage type III). The reticulin meshwork supports both the hepatocytes and the sinusoidal lining cells (endothelial cells) [43].

4. CONCLUSION

The administered doses of ethanol seed extract of *G. kola* in this study did not show to be protective on CCl₄ induced liver toxicity in albino rats as manifested in the results of the biochemical, pro-oxidant (MDA), anti-oxidant and histological parameters analyzed, Therefore, the ethanol extract of the seeds of *G. kola* did not protect the liver against oxidative damages and could not be used as an effective protector against CCl₄ induced hepatic damage. The possible reason for the un protective effect of the ethanol extract of *G. kola* seeds may be due to the inducing intoxicant used or the doses used in this study, further study may be necessary by adjusting the dose and/or trying other intoxicants before a final conclusion may be drawn on the status of the extract visa-viz hepatoprotection.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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