**Hepatoprotective effect of *Anogeissus leiocarpus* against Carbon tetrachloride-induced toxicity in rat liver damage**

**ABSTRACT**

Liver diseases are a critical global health challenge often resulting from the reactive free radicals that can bind covalently to cellular macromolecules forming nucleic acid, protein and lipids adducts. Carbon tetrachloride (CCl4) is leading causes of hepatotoxicity, attributed to oxidative stress, inflammation, and apoptosis through the induction of hypomethylated ribosomal RNA, resulting in inhibition of protein synthesis and CCl4 can affect hepatocellular structure of the liver. The hepatoprotective potential of aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) was evaluated in male Wistar rats using CCl4-induced hepatotoxicity model.

This study evaluated the hepatoprotective, anti-inflammatory, and antiapoptotic effects of aqueous leaf extracts of *Anogeissus leiocarpus* (ALEAL) in mitigating carbon tetrachloride-induced liver damage in male Wistar rats. Statistical analyses were performed using Graph pad Prism 9 with significance different at *P*<0.05.

Elevated liver function enzymes (ALT, AST, and ALP) observed in CCl4 groups was significantly (P<0.05) reversed by ALEAL treatments (115 and 230 mg/kg). The Pro-inflammatory markers (IL-6 and TNF-α) were significantly decreased (P<0.05) in ALEAL-treated groups (115 and 230 mg/kg), but significantly increased (P<0.05) in untreated group. Histological analysis revealed preserved liver architecture and reduced steatosis, necrosis, and fibrosis in ALEAL-treated groups.

The study demonstrated that *Anogeissus leiocarpus* possesses potent hepatoprotective properties mediated through its antioxidant, and its anti-inflammatory activities. The findings validate the traditional use of *Anogeissus leiocarpus* for liver ailments and highlight its potential as a natural alternative for managing hepatotoxicity.

**Keywords**: **Carbon tetrachloride-induced hepatotoxicity**, ***Anogeissus leiocarpus*,** **liver functions assays**, **inflammation**, and **hepatoprotective agents**.

**Introduction**

The liver is a primary structural and functional organ in mammals, performing numerous critical tasks, including bile secretion, bilirubin metabolism, nutrient processing, production of immune agents for infection control, and the metabolism of xenobiotics (Nagy *et al*., 2020). As a metabolically active organ, the liver is responsible for the biotransformation and clearance of foreign substances (xenobiotics and hepatotoxins), making it a key target for drugs and pathogens that could potentially damage liver cells and impair its function (Hong *et al*., 2009). In 2020, Globocan reported more than 75,000 new liver diseases cases in Nigeria. The mortality rate from liver diseases is increasing, and the number of new cases is projected to rise by over 50% in the next 20 years (GLOBOCAN, 2020). Self-medication, drug overdose, contaminated food or water consumption and exposure to pollutants (such as inhaled chemicals) have contributed to a rise in liver damage (Elbery *et al*., 2010).

Carbon tetrachloride (CCl4)-induced hepatic damage results in hepatotoxicity through the generation of free radicals (trichloromethyl peroxyl) and reactive oxygen species (ROS), ultimately causing liver harm. CCl4 is a colorless organic liquid with a sweet odor and minimal flammability at low temperatures (Frank *et al*., 2020). Although CCl4 is not a drug, but when administered in high doses (≥1 mL/kg), it can induce reversible acute liver injury. It is commonly used as a model for xenobiotic-induced liver damage. CCl4 accumulates in the endoplasmic reticulum, where cytochrome P450 enzymes metabolize it into the trichloromethyl radical (CCl3•). These free radicals initiate lipid peroxidation by extracting hydrogen from polyunsaturated fatty acids (Li *et al*., 2015; Wu *et al*., 2018). This process generates an oxidative stress environment, contributing to liver damage and the progression of various liver diseases due to the release of inflammatory cytokines, such as TNF-α and IL-1β, which accelerate disease onset and development.

The relationship between oxidative stress and inflammation plays a significant role in injury progression. Inflammatory feedback, cell death (apoptosis), and immune responses activate the NF-κB signaling pathway (Li *et al*., 2017). In this context, the exploration of natural compounds with potential protective effects against CCl4-induced hepatotoxicity becomes crucial.

Several phytochemicals, including Silymarin, have been identified for their hepatoprotective properties, aiding in the treatment of liver poisoning, cirrhosis and chronic hepatitis (Abenavoli *et* al., 2018). Silymarin, a flavonoid extract from *Silybum marianum*, has been used to prevent a range of liver diseases (Polyak *et al*., 2010). It has been found to act as an antioxidant by reducing free radical production and lipid peroxidation, as well as functioning as a toxin blocker by preventing toxins from binding to the hepatocyte cell membrane receptors (Abenavoli et al., 2018). Additionally, it reduces superoxide levels through its scavenger activity (Papackova *et al*., 2018). Recent studies have indicated that Silymarin can mitigate acute toxic liver injury caused by CCl4 by promoting hepatocyte proliferation and reducing the production of toxic metabolites (Elsayed *et al*., 2020; Yang *et al.,* 2022). However, Silymarin is expensive and not easily accessible. As a result, recent research has focused on the use of natural products derived from plants as an effective therapeutic strategy for liver diseases (Navarro *et al*., 2014; Salomone *et al*., 2016).

Liver-protecting natural products typically exhibit a range of activities including antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective and antiviral effects (Kim *et al*., 2015; Ilyas *et al*., 2016; Abdullah *et al*., 2017; Sharifi-Rigi *et al*., 2019).

*Anogeissus leiocarpus* belongs to the Combretaceae family, a deciduous and tall tree native to the tropical savannas of Africa. Common names for this tree in various regions include Ayin (Yoruba) and Atara (Igbo). *Anogeissus leiocarpus*, an important medicinal tree used in treating in ailments such as; hepatitis, jaundice, hemorrhoids, headaches, toothaches, diarrhoea, jaundice, respiratory diseases, and malaria (Victor, 2013; Ikram Mohammed *et al*., 2015; Muktar *et al*., 2017). Therefore this work aimed to determine the effect of *Anogeissus leiocarpus* leave extract on CCl4-induced liver injury.

**Materials and Method**

**Chemicals and Reagents**

Distilled water, Carbon tetrachloride (CCl4), Tris buffer, homogenizing buffer, phosphate buffer formalin, and phosphate buffered saline (pH 7.4), merchant buffer, sodium hydroxide, ethanol, ethidium bromide, hydrochloric acid, Laboratory kits for quantitative in vivo determination of alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase. Silymarin was a branded drug (Silybon-70) from Micro Laboratory Ltd, India. Carbon tetrachloride was a product of Sigma Chemical Company, USA. All other reagents and chemicals were of analytical grades.

The materials used range from laboratory equipment/apparatus to various preparatory as well as analytical chemicals and reagents.

**Collection and Identification of plant materials**

Fresh plant of *Anogeissus leiocarpus* was collected from the Agricultural farm of Ladoke University of Technology Ogbomoso. The plant was identified and authenticated by Prof. Ogunkunle J.A. of the Department of Pure and Applied Biology LAUTECH with voucher numbers LHO 897 and specimen of the plant was deposited at the Department Herbarium.

**Preparation of aqueous leaf extract of *A. leiocarpus.***

Leaves of *A. leiocarpus* were carefully separated from the stem and other debris, thoroughly washed under running water, air-dried to a constant weight and pulverized into a fine powder with the use of an electric blender. Three hundred grams (300 g) of the leaf powder was soaked in 2 L of distilled water for 72 hours with intermittent shakings. After 72 hours, it was filtered through Whatman No 1 filter paper and the filtrate was concentrated by freeze-drying. The mixture was stored in air-tight containers at 4oC (Christian *et al*., 2021).

**Experimental animals**

A total of twenty-five male Wistar rats with an average weight of 160 g- 180 g were used in this study. The model consisted of five (5) groups of five (5) rats each. Animals in group A received 1 ml distilled water and served as normal control group; group B also received 1 ml distilled water; group C received Silymarin (70 mg/kg) and served as drug control group; groups D and E were treated with aqueous leaf extract of the plant *Anogeissus leiocarpus* 115 and 230 mg/kg body weight respectively. Animals in groups B-E were exposed to CCl4 (3 ml/kg body weight in olive oil 1:1) via the intraperitoneally on days 7 and 14 only. Weight of the animals were recorded on day 0 (24 hours before the first administration) and on the day 15 (24 hours after the last administration) (Supp Plate 1). The rats were housed in plastic cages under the standard laboratory conditions (26 ± 2oC, 30-70 % humidity**,** 12 hours light and 12 hours dark cycle) and were acclimatized for two week to the lab environment. They were fed a standard pellet diet and drinking water *ad libitum* throughout the experiment study.

**Table 1: Animals grouping for CCl4 Model**

|  |  |  |
| --- | --- | --- |
| Group | Treatment | Carbon Tetrachloride |
| A  (Normal Control) | Distilled water for fourteen days | Not administered |
| B  (Negative Control) | No treatment | CCl4 (3 ml/kg body weight in 1:1 olive oil on day 7 and 14) administered (i.p) |
| C  (Positive Control) | Silymarin (70 mg/kg body weight) administered for fourteen days | CCl4 (3 ml/kg body weight in 1:1 olive oil on day 7 and 14) administered (i.p) |
| D | Aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) (115 mg/kg) administered for fourteen days | CCl4 (3 ml/kg body weight in 1:1 olive oil on day 7 and 14) administered (i.p) |
| E | Aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) (230 mg/kg) administered for fourteen days | CCl4 (3 ml/kg body weight in 1:1 olive oil on day 7 and 14) administered (i.p) |

* Given that the LD50 of aqueous leaf extract of *Anogeissus leiocarpus* is 2300 mg/kg of body weight (Somé *et al*., 1996).

**Biochemical Analyses**

The following biochemical parameters were evaluated in the serum using standard methods:

**Alanine (ALT) and Aspartate Aminotransferases (AST)**

Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) was evaluated following the method described on the sigmal manufacturer as kit. 0.5 mL of each of the substrate (DL-Aspartate (R1a) with α-ketoglutarate for ALT and DL- Alanine (R1b) with α-ketoglutarate for AST) were mixed and incubated for 5 minutes at 37oC. 100 µL of serum were added and the reaction mixtures were returned into the water bath for 30 minutes for both ALT and AST. 125 µL of R2 (2, 4-Dinitrophenenylhydrazine (developer)) were added to the reaction mixture and left for 20 minutes at room temperature. Finally the colours were developed by addition of 1.25 mL of NaOH (0.4N) and the products formed were read at 505 nm. Data were expressed as IU/L read from the corresponding calibration curves. The curve was obtained using the same procedure described above, but using supplied calibrator (pyruvic) instead of sample (serum) at a concentration of 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5mL.

**Determination of Alkaline Phosphatase (ALP)**

Determination biochemical parameters such as alkaline phosphatase (ALP) were determined using commercial test kits with strict adherence to procedures prescribed by the producer, Randox Laboratories, UK. They were all determined in serum according to the protocols adopted by Sun, et al. (sun et al., 1988) and Kanu, et al. (kanu et al., 2017).

Inflammatory Cytokines Analyses

**Determination of Rat IL-6**

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat IL-6. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat IL-6 and Avidin-Horseradish Peroxidase (HRP) was conjugated and were added successively to each micro plate well and incubated. Free components are washed away. The substrate solution was added to each well. Only those wells that contain Rat IL-6, biotinylated detection antibody and Avidin-HRP conjugate were appeared blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turns yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value was proportional to the concentration of Rat IL-6. The concentration of Rat IL-6 was calculated in the samples by comparing the OD of the samples to the standard curve.

**Determination of Rat Tumor Necrosis Factor alpha (TNF-α)**

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat TNF-α. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat TNF-α and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Rat TNF-α, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value was proportional to the concentration of Rat TNF-α. The concentration of Rat TNF-α was calculated in the samples by comparing the OD of the samples to the standard curve.

**Histopathological Analysis of Liver Sample**

For the histopathological studies, the male Wistar rats of all the ten groups were sacrificed by cervical dislocation. Liver tissues of the rats were removed and washed properly in 0.9 % saline solution. Tissues were fixed in 10 % buffered formosaline for 48 hours to know the level of damage done by the administered drug using hematoxylin and eosin staining rutting techniques, (Bancroft and Gamble 2002).

**Tissue Processing**

**Grossing:** The tissues were observed and cut into small pieces of not more than 4mm thick into pre-labeled cassettes. These were further immersed in 10 % formal saline for 24 hours to fix.

**Tissue Processing**: This is done automatically using automatic tissue processor (Leica TP 1020). The tissue were allowed to pass through various reagents including; stations 1 & 2 containing 10% formal saline, station 3 to station 7; alcohol (70 %, 80 %, 90 %, 95 %, absolute 1 & absolute 11) for the purpose of dehydration. The tissues passed through station 8 and station 9 containing two changes of xylene for the purpose of clearing and finally transferred into three wax baths for infiltration/impregnation. The machine was programmed to run for 12 hours, the tissues stayed in each station for 1 hour.

**Embedding:** Eachprocessed tissue was given a solid support medium (paraffin wax) and this was done using a semi-automatic tissue embedding center. The molten paraffin wax was dispensed into a metal mold and the tissue was buried and oriented in it, a pre labeled cassette was placed on this and was transferred to a cold plate to solidify. The tissue block formed was separated from the mold.

**Microtomy:** The blocks were trimmed to expose the tissue surface using a rotary microtome at 6micrometer. The surfaces were allowed to cool on ice before sectioning. The tissues were sectioned at 4 micrometer (ribbon section)

**Floating:** The sections were floated on the water bath (Raymond lamb) set at 55 0C and these were picked using clean slides. The slides were labeled appropriately.

**Drying:** The slides were dried on a hotplate (Raymond lamb) set at 60 0C for 1 hour.

**Staining:** The staining technique used was Haematoxylin and Eosin technique

**Procedure for Haematoxylin and Eosin (H and E) Technique**

The slides were dewaxed in xylene for 15 minutes. They were then taken through absolute alcohol, 95 % and 70 % alcohol, after which they were rinsed to section in water.

They were then stained in Harris haematoxylin for 5 minutes. The slides were rinsed in water and differentiated in 1% acid alcohol briefly. This step was followed by rinsing in water and bluing under running tap water for 10 minutes. The slides were counterstained with 1% aqueous eosin for 2 minutes and rinsed in water after which they were dehydrated in ascending grades of alcohol and cleared in xylene. The slides were mounted in DPX. The nuclei and the cytoplasm appeared blue and pink in colour respectively Immunostain formalin-fixed, paraffin-embedded tissue sections

**Immunohistochemical procedure**

The slides were deparaffinized in xylene for 2 times, 5 min each. The slides were transferred to 100% alcohol, for 2 times, 3 min each, and then transferred through 95% twice, 70% once alcohols respectively for 3 min each. They were rinse with Wash Buffer for 2 times, 5 min each. Antigen retrieval was performed to unmask the antigenic epitope. The most commonly used antigen retrieval buffer is a citrate p 6.0 and EDTA pH 9.0. For Preheating, 250-300 ml of 10 mM citrate pH 6.0 /EDTA pH 9.0 buffer was poured into the staining container (24 slide coupling jar) and incubated it at 95-100°C for 5 min. The slides were arranged in a staining hanger and dipped in the preheated buffer; incubated for 10-20 min in the water bath. The staining container was removed to room temperature and allowed the slides to cool in the retrieval buffer for 20 min. The slides were rinsed with Wash buffer for 2 times, 5 min each. Blocking buffer (e.g. 10% fetal bovine serum in PBS or 3 % H2O2) was added onto the sections of the slides and incubated in a humidified chamber at room temperature for 15 minutes. The blocking buffer was drained off from the slides and washed in wash buffer. 130 μL was applied appropriately diluted primary antibody to the sections on the slides and incubated in a humidified chamber at room temperature for 1 hour. The slides were washed with Wash buffer for 2 times, 5 min each. 130 μL was applied appropriately Antibody amplifier + Polymer- HRP Micro-Polymeric-HRP secondary antibody to the sections on the slides and incubated in a humidified chamber at room temperature for 15min each. The slides were further washed with wash buffer for 2 times, 5 min each. 130 μL DAB substrate solution (freshly made just before use: 0.05 % DAB - 0.015 % H2O2 in PBS) was applied to the sections on the slides to reveal the color of antibody staining. The color was allowed to develop for < 5 min until the desired color intensity is reached. The slides were washed under running water 3 times, 2 min each. Slides were counterstained by immersing sides in Hematoxylin for 10-20seconds. The slides were rinsed in running tap water for 10 min. The tissue slides were dehydrated through 4 times of alcohol (95%, 95%, 100% and 100%), 5 min each. The tissue slides were cleared 3 times in xylene and coverslip using mounting solution. The mounted slides can be stored at room temperature permanently. The colour of the antibody staining was observed in the tissue sections under microscopy.

**Data Analysis**

Results were expressed as mean ±SD from at least three replicate measurements. The difference between the means will be analyzed with ANOVA, α value = 0.05. Post-hoc Analysis will be done using Tukey test, using GraphPad Prism software 9, 2022

**RESULTS**

**Effects of aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) on the hepatic parameters of male Wistar rats exposed to Carbon tetrachloride.**

Selected hepatic parameters of rats pretreated with ALEAL and those administered CCl4 (3 ml/kg) are shown in the table 2 below. Result revealed that rats administered with 3 ml/kg of CCl4 presented with a significant increase (*P*<0.05) in serum activities of liver enzymes (AST, ALT and ALP) compared to control group and the groups pretreated with ALEAL.

**Table 2: Effects of aqueous leaf extract of *A. leiocarpus* on the hepatic parameters of male Wistar rats exposed to Carbon tetrachloride.**

|  |  |  |  |
| --- | --- | --- | --- |
| Group | AST (U/L) | ALT (U/L) | ALP (U/L) |
| A: Normal Control | 28.43±0.21a | 36.38±0.88a | 44.17±0.63a |
| B: CCl4 (3 ml/kg) | 38.85±0.12b | 69.12±1.91 b | 55.22±1.49 b |
| C: CCl4 (3 ml/kg) + Silymarin (70 mg/kg) | 31.26±0.70 a | 51.51±0.10 c | 50.44±1.16 c |
| D: CCl4 (3 ml/kg) + AEAL (115 mg/kg) | 32.23±1.62 a | 44.63±1.53 a | 49.18±1.13 c |
| E: CCl4 (3 ml/kg) + ALEAL (230 mg/kg) | 29.23±0.71 a | 49.28±3.13 c | 46.64±0.43 a |

Values were expressed as mean ±SEM (n=5), Different alphabet superscripts indicate significant difference at *P*<0.05, CCl4 (Carbon tetrachloride), AST (Aspartate aminotransferase, ALT- (Alanine aminotransferase), ALP (Alkaline phosphatase), ALEAL (Aqueous leaves extract of *Anogeissus leiocarpus*).

**Effects of aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) on the level of Proinflammatory on Carbon tetrachloride liver damage in male Wistar rats.**

As shown in figure 1 that administration of Carbon tetrachloride significantly (*P*<0.05) elevated interleukin-6 level in rats given only Carbon tetrachloride when compared to the normal control group. However, the groups pretreated with aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) (115 mg/kg and 230 mg/kg body weight) before CCl4 administration had significantly

(*P*<0.05) decrease these pro-inflammatory markers when compared to the group administrated only CCl4. Administration of Carbon tetrachloride significantly (*P*<0.05) elevated Tumor Necrosis Factor- Alpha in rats when compared to the normal control group. However, the groups pretreated with aqueous leaf extract of *Anogeissus leiocarpus* (115 mg/kg and 230 mg/kg body weight) before CCl4 administration had lower level of TNF-α when compared to untreated group (Fig. 2).



**Figure 1:** **Effects of aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) and Carbon tetrachloride administration on inflammatory marker (Interleukin-6) in rats.**



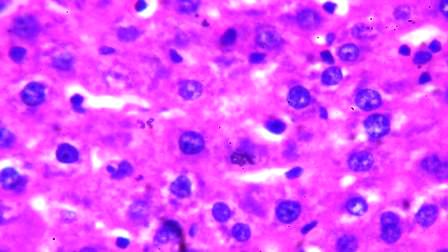
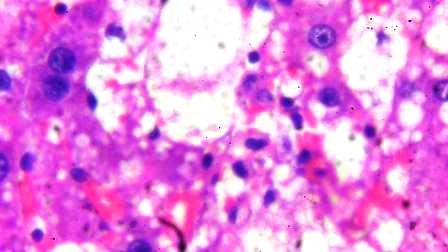
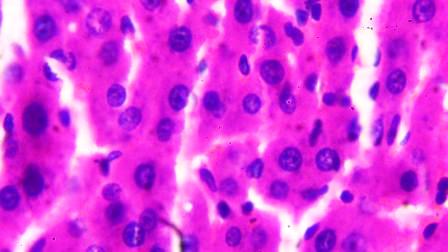
**Figure 2: Effects of aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) and Carbon tetrachloride administration on inflammatory marker (Tumor Necrosis Factor- Alpha) in rats.**

Values were expressed as mean ±SEM (n=5), Different alphabet superscripts indicate significant difference at p<0.05, CCl4 ­(Carbon tetrachloride), ALEAL (Aqueous leaf extract of *Anogeissus leiocarpus*), IL-6 (Interleukin-6), TNF-α (Tumor Necrosis Factor- Alpha).

**Histological overview of liver of rats pretreated with (ALEAL) and exposed to CCl4.**

Histology of the liver shown moderate architecture, the central venules appear normal in all the groups pretreated with aqueous extract of *Anogeissus leiocarpus*, compared to the group induced with Carbon tetrachloride which showed a severe damage to the liver. Indices assessed included hepatocytes, sinusoids, portal tract, inflammation and venules. Both the normal group and the groups pretreated with Silymarin and aqueous leaf extract of *Anogeissus leiocarpus* of the rat’s liver demonstrated normal well-preserved architecture, intact normal histological components of the hepatic lobules, normal portal areas and normal central vein bounded by an intact endothelium. Though the morphology of the hepatocytes of group pretreated with aqueous leaf extracts of *Anogeissus leiocarpus* (230 mg/kg body weight) shows mild cytoplasmic vacuolation probably due to fat infiltration while that of 115 mg/kg body weight appear normal (blue arrow).

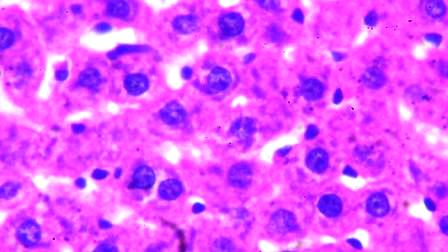
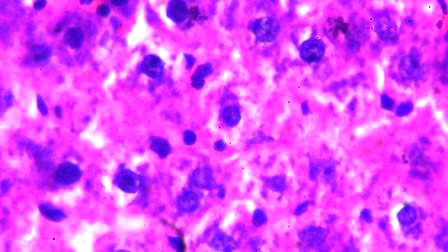
The Carbon tetrachloride induced group revealed indices that shows chronic hepatic steatosis, onset liver cirrhosis with fibrosis and degenerate liver cells (blue arrow).



**C**

**B**

**A**

**E**

**D**

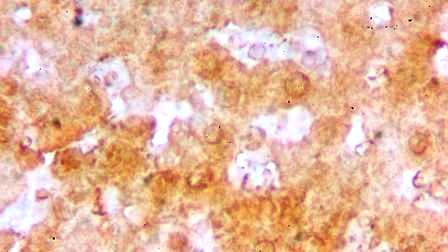
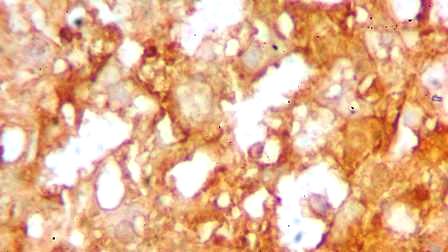
**Plate 1: Photomicrographs representative of male Wistar rats’ liver pretreated with ALEAL and exposed to CCl4 (Photomicrographs of liver section stain by haematoxylin and eosin 400X).**

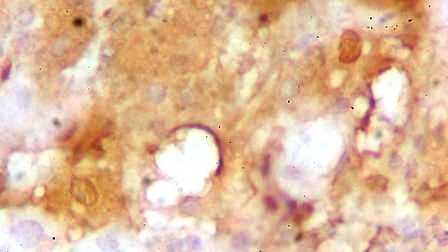
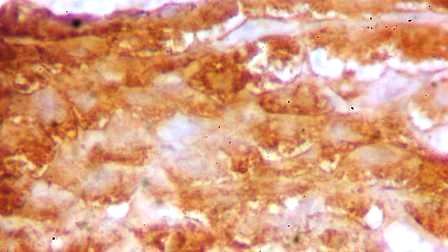
A (Normal Control), B (CCl4 only), C (CCl4 + Silymarin), D (CCl4 + ALEAL 115mg/kg body weight), E (CCl4 + ALEAL 230mg/kg body weight).

CCl4 (Carbon tetrachloride), ALEAL (Aqueous leaf extract of *Anogeissus leiocarpus*)*.*

**Effects of aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) on the immunohistochemical (Bax) staining of livers of rats of Carbon tetrachloride-induced liver damage in male Wistar rats.**

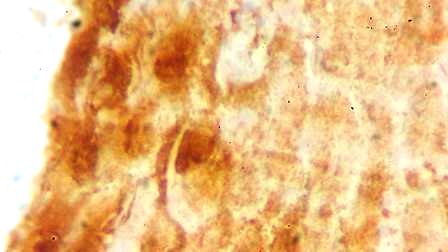
Plate 2 revealed severe expression of Bax in the liver of rats exposed to CCl4. The groups pretreated with Silymarin showed a moderate expression of Bax when compared to the groups pretreated with AEAL which show weak expression of Bax. Analysis of Bax expression when quantified using Image J image processing program also revealed a significant increase in the level of Bax of male Wistar rats induced with CCl4. However, there was a significant difference (*P*<0.05) in the groups pretreated with AEAL and the Silymarin group as revealed in figure 3.

**C**

**D**

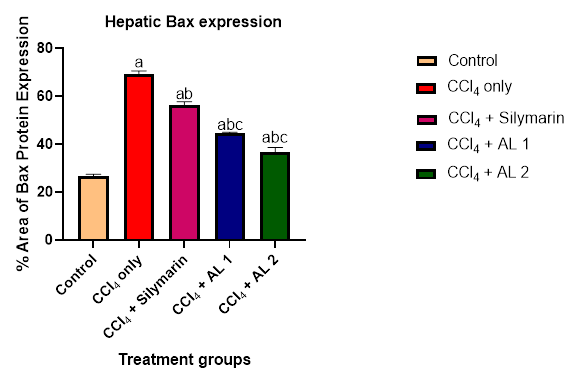


**E**

**Plate 2: Liver images of Wistar rats treated with aqueous leaf extract of *Anogeissus leiocarpus* upon CCl4 toxicity (Bax protein stain 400X).**

A (Normal Control), B (CCl4 only), C (CCl4 + Silymarin), D (CCl4 + AEAL 115 mg/kg body weight), E (CCl4 + AEAL 230 mg/kg body weight).

CCl4 (Carbon tetrachloride), AEAL (Aqueous leaf extract of *Anogeissus leiocarpus*).



**Figure 3: Effects of aqueous leaf extract of *Anogeissus leiocarpus* and Carbon tetrachloride administration on Bax Protein Expression.**

Values were expressed as mean ±SEM (n=3), Different alphabet superscripts indicate significant difference at *P*<0.05, CCl4 ­(Carbon tetrachloride), AL 1 (Aqueous leaf extract of *Anogeissus leiocarpus* 115 mg/kg body weight), AL 2 (Aqueous leaf extract of *Anogeissus leiocarpus* 230 mg/kg body weight).

**Discussion**

Carbon tetrachloride a well-known chemical compound has various uses and exposure to it in a quantified amount can induce severe liver injury which can signify a serious health threat to human beings and animals (Unsal *et al.,* 2022).

The effectiveness of any hepatoprotective drugs depends on its ability to either restore normal hepatic functions impaired by hepatotoxins or minimize its harmful effects (Patrick-Iwuanyanwu et al., 2010). The most common plant portion utilized in traditional medicine is the leaf, which is also the most abundant part of the plant (Priyadarshi and Ram, 2018). The frequency of using leaves rather than other plant components in Saudi Arabian traditional medicine was documented by Ullah *et* al., (2020). This could be as a result of leaves having more phytochemicals than other plant component. Among other plant parts, leaves are simple to evaluate and harvest. Furthermore, rapid leaf regeneration guarantees the maintenance of floristic richness. Plant compound containing saponnin, flavonoid, phenols and tannin are crucial for scavenging reactive oxygen species (ROS) and also reduce systemic inflammation that may arise as a result of hepatotoxins (Ebe *et* al., 2021; Adewole *et al*., 2022). Plants that contain polyphenolic compounds have the potentials of preventing diseases related to oxidative-damage mechanism such as cancer.

Aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL) contains some known phytochemicals which enables it to prevent reactive oxygen species related diseases and thus, it’s examined for in vivo hepatoprotective potentials in rats. In this study, the biochemical assay shows the reduction in systemic inflammation supports protein synthesis in hepatocytes, promoting recovery and maintaining homeostasis. The extract's ability to mitigate weight loss suggests its potential to counteract cachexia and improve overall metabolic function, which has clinical implications for managing liver disease-induced wasting syndromes. The tannin content may also reduce vascular leakage and inflammation, helping maintain systemic energy balance (Nwidu *et al*., 2023).

The serum aminotransferases; Alanine transaminase (ALT) and Aspartate transaminase (AST) serve as widely recognized biomarkers for liver necrosis. ALT and AST are confined to liver cell (Cytoplasm) thus once there is increase in their serum concentration, it’s an indication that the liver membrane is damaged or has experience inflammation or the integrity of the cell membranes has been compromised or is in an infective conditions such as viral hepatitis. When AST is also present in extra-hepatic tissues such as kidney and heart with a simultaneous elevation in serum AST levels in conjunction with ALT level this may indicate hepatic injury (Tala *et al*., 2015). An increased serum AST and ALT concentration signifies inflammation of the hepatocytes due to the buildup of reactive NAPQI radicals from increased carbon tetrachloride metabolism. The elevated levels of ALT, AST, and ALP in untreated groups indicate hepatocellular damage and biliary dysfunction, both hallmarks of liver toxicity. These enzymes were significantly normalized in ALEAL-treated groups, highlighting the extract's hepatoprotective effects. Phytochemicals present in the plant such as Flavonoids stabilize cell membranes and inhibit lipid peroxidation by reducing reactive oxygen species (ROS), while tannins and alkaloids chelate metal ions and prevent ROS-mediated enzyme leakage (Oyedemi *et al*., 2022). The enzymes ALT in particular is increased which is an indication of liver damage. Its increase in the mitochondrial serves as a sensitive indicator of damage to cytoplasmic and /or the mitochondrial membranes suggesting liver injury caused by hepatotoxins. The group administered with CCl4 only experienced and elevated serum liver marker enzymes which is an indication of cellular leakage and loss of liver functional integrity. The groups pretreated with ALEAL (115mg/kg and 230mg/kg body weight) experience a significant reduction (*P*<0.05) in the level of liver enzyme markers concentration when compared with the CCl4 induced group, this correlate with the facts that serum levels of aminotransminase usually return to normal when hepatocytes are been regenerated as well as healing of hepatic parenchyma (Adewale *et al*., 2014). The biochemical principle involves the preservation of hepatocyte integrity by neutralizing oxidative stress, which otherwise disrupts membrane-bound enzymes. Saponins (identified in ALEAL) might also regulate bile acid secretion, alleviating cholestasis observed in the untreated groups (Abdullahi *et al*., 2023). Clinical applications could include managing drug-induced liver injuries and chronic liver diseases, where enzymes elevation serves as a diagnostic marker of damage. Any activity alteration of these enzymes results into cellular impairment, tissue lesion and dysfunction. This healing process could be linked to the livers remarkable capacity for regeneration and rejuvenation following injury or damage. Thus the ALEAL demonstrated the ability to alleviate CCl4 induced hepatotoxicity. These results align with the findings of sa’id *et al.,* (2020).

The elevated levels of IL-6 and TNF-α in untreated group indicate a robust inflammatory response. ALEAL significantly reduced these markers, possibly due to its flavonoid and tannin content, which inhibits NF-κB activation, a key pathway in cytokine production. Saponins further reduce inflammation by modulating macrophage activity and cytokine release (Nwidu *et al*., 2023). Ellagic acid has also been shown to suppress pro-inflammatory mediators through downregulation of COX-2 and inducible nitric oxide synthase (iNOS). It was reported by Eltahire *et al*., (2020) that increase in the expression of several inflammatory cytokine genes such as IL-6, TNF-α, mRNA expression, transforming growth factor- β (TGF-β), xCOX-2, necrosis factor-kappa B (NF-kB) were associated with the administration of CCl4 in rats.

The biochemical principle involves disrupting pro-inflammatory signaling cascades, thereby preventing liver inflammation and fibrosis. This suggests that ALEAL could be a potential therapeutic for chronic liver inflammation and related conditions. Similar findings were reported by Oyedemi *et al*., (2022), where flavonoids suppressed pro-inflammatory cytokines in inflammatory models.

However, the cellular antioxidant response is enhanced by the presence of dietary antioxidants. Antioxidants and anti-inflammatory agents play a crucial role in protecting against CCl­4 toxicity by scavenging reactive oxygen species and free radicals as well as neutralizing lipid peroxides. Histological analysis revealed preserved liver architecture in ALEAL-treated groups, with reduced steatosis, necrosis, and fibrosis. The protective effects are attributed to the combined action of flavonoids (quercetin, kaempferol), tannins, and alkaloids, which prevent lipid accumulation and promote hepatocyte regeneration. Polyphenols in the extract (ALEAL) likely inhibit the activation of hepatic stellate cells, reducing fibrogenesis (Oluwole *et al*., 2023).

Apoptosis activation is linked to mitochondria degeneration (Ahmadian *et al*., 2020). The activation of pro-apoptotic genes like Bax leads to increased mitochondrial permeabilization leading to the released of cytochrome c which further trigger hepatocyte apoptosis and caspase-3 activation (Liuet *et al*., 2018). Analysis of the immunohistochemistry results revealed that the expression levels of Bax protein were quantified in Wistar rats exposed to carbon tetrachloride (CCl4) and treated with aqueous leaf extract of *Anogeissus leiocarpus* (ALEAL). ALEAL treatment showed a dose-dependent reduction in Bax levels compared to the untreated CCl4 group, suggesting its potential protective or anti-apoptotic effects. The results indicate that ALEAL mitigates oxidative stress-induced apoptosis associated with CCl4 toxicity. *Anogeissus leiocarpus*, a medicinal plant with recognized anti-inflammatory and antioxidant properties, present itself as a promising candidate for mitigating the harmful effects of CCl4 on the liver (Motto *et al.*, 2021).Biochemically, this preservation of liver structure supports normal metabolic and detoxification processes, highlighting ALEAL’s potential in preventing progression to cirrhosis. These findings align with studies by Abdullahi *et al*., (2023), demonstrating the antifibrotic effects of tannin-rich extracts.

**Conclusion**

This study highlights the hepatoprotective potential of *Anogeissus leiocarpus* aqueous leaf extract (ALEAL) against carbon tetrachloride (CCl4)-induced hepatotoxicity. The extract demonstrated significant antioxidant, and anti-inflammatory, as evidenced by the normalization of hepatic enzyme levels, along with a reduction in inflammatory cytokines. Histopathological evaluations further confirmed its ability to preserve liver architecture and mitigate tissue damage. These effects are attributed to the phytochemical constituents of *A. leiocarpus*, such as flavonoids, tannins, and saponins, which act through various biochemical mechanisms to protect and restore liver function. These findings underscore the potential of *A. leiocarpus* as a natural, safe, and effective therapeutic agent for the prevention and management of liver diseases.

**List of abbreviations**

* **ALP**: Alkaline Phosphatase
* **ALT**: Alanine Aminotransferase
* **AST**: Aspartate Aminotransferase
* **BAX**: Bcl-2-associated X protein
* **IL-6**: Interleukin-6
* **LD50**: Median Lethal Dose
* **ROS**: Reactive Oxygen Species
* **TNF-α**: Tumor Necrosis Factor-Alpha

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