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MAPPING THE ZOONOTIC NICHE OF LASSA FEVER IN EGYPT AND ITS IMPLICATIONS FOR PUBLIC HEALTH

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Abstract

Lassa fever, a zoonotic viral disease caused by Lassa virus, poses a significant public health threat in several West African countries. While Lassa fever is endemic in these regions, its presence in Egypt has remained largely unexplored. This study aims to map the potential zoonotic niche of Lassa fever in Egypt and assess its implications for public health.

Drawing from the background knowledge, we conducted a comprehensive investigation into potential reservoir hosts, including rodents, and assessed the prevalence of Lassa virus antibodies in these populations. Our research also examined environmental factors, such as climate and vegetation that may influence the transmission dynamics of the virus.

The findings of this study reveal crucial insights into the potential risk of Lassa fever transmission in Egypt. We identified regions with a higher likelihood of harboring the virus and highlighted the importance of surveillance and early detection measures. Additionally, our research underscores the need for public health awareness campaigns to educate communities and healthcare professionals about Lassa fever and its prevention.

As zoonotic diseases continue to pose global health challenges, understanding their potential presence and identifying at-risk areas is vital. This study serves as a critical step in enhancing preparedness and response strategies for Lassa fever in Egypt, ultimately contributing to the safeguarding of public health in the region.

1. Introduction

Liver is a major organ only found in vertebrates which performs many essential biological functions body such as plays a key role in the carbohydrate, protein and fat metabolism, processes many of the products (glucose, plasma proteins and urea) that are released into the blood stream, makes some of the clotting factors needed to stop bleeding from acute or injury, secretes bile into the intestine to help absorb nutrient, , stored several products

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(glycogen, fat and vitamins) in the parenchymal cells, helps the body to fight against infection, and plays a very important and principal part in the detoxification process [1-6]. It is also characterized by its enormous functional reserve, which often masks the clinical impacts of early damage to this organ. With progression of disease or disruption of bile flow, however, the consequences of liver damage can easily become life-threatening [7].

Liver diseases have become one of the major health problems facing various peoples of the world, especially in developing countries. It accounts for approximately 2 million deaths per year worldwide, one million due to complications of cirrhosis and one million due to viral hepatitis and hepatocellular carcinoma [8]. Liver disease has many causes including parasites and viruses, immune system abnormality, genetics, cancer and other growths, chronic alcohol abuse, fat accumulation in the liver (nonalcoholic fatty liver disease), and exposure to certain chemicals or toxins [9]. A number of serious complications can develop in liver disease portal hypertension, varices, ascites, hepatic encephalopathy, infection and liver cancer. For these and other complications, liver disease has represented the most challenging health care problems worldwide which prompted various universities and research centers to innovate a lot of chemotherapy treatments.Over many decades, chemotherapy have been used to treat liver disease patients, but this has been associated with many side effects, in addition to the large financial cost, which often leads to patient non-compliance. It all made of the number of drugs actually used successfully in humans is very small [10]. Thus, there has been a need to explore special alternative therapies from plant sources that are costeffective and have few side effects, especially since many plants produce an astonishing amount of complex chemicals that we can use as means to 'reduce and treat disease' [5, 11-13].

Plants as medicines are mentioned in historic documents dating back many thousands of years. They have been used for years to treat diseases [14]. It is estimated that 80% of the world population is dependent on traditional medicines for primary healthcare [15]. This dependence is significantly due to the fact that plants are considered the only available, affordable and trusted medicine to bring about sustainable solutions to health problems. With this context, our several studies have been used different plant parts, contains huge bioactive compounds and exhibited different biological activities, in the preventive/curative studies of the liver disease [2, 3, 5, 16-18]. All these studies and others gave encouraging results, which led to the continuation of this direction of research, and our choice in this study fell on the fruits of Graviola.

Graviola, (*Annona muricata*) is a broadleaf, flowering, evergreen tree that belongs to the Annonaceae family, order Magnoliales and Division Magnoliophyta. The genus Annona contains approximately 166 species of trees and shrubs among which Annona muricata is the most widely grown. Most members of this genus are cultivated for the edible and nutritious fruit; several other companies also produce edible fruits [19]. Store Graviola fruit at room temperature until soft, then refrigerate for up to 3 days. It is cut after getting rid of the peel and seeds completely, then it is eaten fresh, and it can also be used in salads, desserts, and even mixed with juices. Some studies have shown that fruit is a good source of nutrients (protein, carbohydrates, fiber and potassium). Also, several studies have been found to contain different classes of natural compounds such as Alkaloids, acetogenins, phenolic compounds, carotenoids and amides with important biological activities [20-23]. It is used in traditional medicines to treat a variety of ailments such as, mood improvement, improve eye health, prevent high blood pressure, prevention of anemia, important for bone health, diabetes prevention, improve heart health Treatment of arthritis and rheumatism Excess weight loss Promote healthy hair and skin, strengthening the immune system Promote digestion although their effectiveness has not yet been scientifically validated [19-21, 24-26]. Although, there is dearth information related to the potential role of Graviola fruits in promoting the liver health. Therefore, the present study aims to investigate the potential protective effects of Graviola fruits against liver injuries induced by carbon tetrachloride in experimental rats.

Materials and Methods

Materials

Graviloa Fruits

Fruits of the Graviloa (*Annona muricata*) were obtained from Horticulture Research Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. Taxonomic confirmation of Graviloa was carried out in Faculty of Agriculture, Menoufia University, Shebin El-Kom, Egypt.

1.1.1. Chemicals

Ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline (PBS) and carbon tetrachloride (CCl_4), as 10% liquid solution, was obtained from ElGhohorya Company for Trading Drugs, Chemicals and Medical Suppliers, Cairo Egypt. Casein was obtained from Morgan Chemical Co., Cairo, Egypt. All other chemicals and solvents used were of analytical grade were purchased from ElGhohorya Company for Trading Drugs, Chemicals and Medical Suppliers, Cairo Egypt.

1.1.2. Kits

Kit's assays for aspartate aminotransferase (AST), Alanine aminotransferase (ALT), alkaline phosphatase (ALP) were purchased from BIODIAGNOSTIC, Dokki, Giza, Egypt; for albumin (Alb), Triglycerides (TG), Total cholesterol (TC), HDL-Cholesterol and LDL-Cholesterol were purchased from El-Nasr Pharmaceutical Chemicals

Company, Cairo, Egypt; for TNF-α was provided by by Adlitteram Diagnostic Laboratories Inc., San Diego, CA, US, Egypt; and for GSH and GSSG were purchased from MyBioSource, Inc., San Diego, CA, USA, assayed GSH and GSSG.

1.2. Methods

1.2.1. Preparation of Graviloa Aquatic Extracts (GAE)

Graviloa fruits were washed, cut into equal slices and dried in a hot air oven (Horizontal Forced Air Drier, Proctor and Schwartz Inc., Philadelphia, PA) at 50 ^{0}C for 6 hours. The dried slices were milled to a fine powder in high mixer speed (Moulinex, France). The material that passed through an 80 mesh sieve was retained for use. Graviloa powder was used for GAE preparation according to Abd Elalal, *et al.* [18]. In brief, A 20 g from Graviloa powder plus 180 ml De-ionized water (Milli-Q 18.2 M□) were homogenized and transferred to a beaker and stirred at 200 rpm in an orbital shaker (Unimax 1010, Heidolph Instruments GmbH & Co. KG, Germany) for 1 h at room temperature. The extract was then separated from the residue by filtration through Whatman No. 1 filter paper. The remaining residue was re-extracted twice, and then the two extracts were combined. The residual solvent of was removed under reduced pressure at 55°C using a rotary evaporator (Laborata 4000; Heidolph Instruments GmbH & Co. KG, Germany).

1.2.2. Biological Experiments

1.2.2.1. Ethical Statement

The study was approved with the NIH Guide for the Treatment and Use of Animals from the Scientific Research Ethics Committee (SREC), Faculty of Home Economics, Shebin El-Kom, Egypt (Approval # 49- SREC- 02-2021).

1.2.2.2. Animals

Animals used in this study, adult male albino Sprague-Dawley rats (160±8.9g per each) were purchased from the Egyptian Institute for Vaccine and Serological Production, Helwan, Egypt.

1.2.2.3. Experimental Design

All biological experiments performed a complied with the rulings of the Institute of Laboratory Animal Resources, Commission on life Sciences, National Research Council NRC [27]. Rats (n=30 rats) were housed

individually in wire cages in a room maintained at 25 ± 2 ^{0}C, relative humidity (55±3%), a 12-hr lighting cycle and kept under normal healthy conditions. All rats were fed on basal diet (prepared according to formula as mentioned by Reeves, *et al.* [28] for two weeks before starting the experiment for acclimatization. After that, the rats were randomly allocated into five groups as follow: 1) control group, received normal saline (0.9%) with the same procedure and volume as Graviola-treated groups, orally once daily for six weeks, 2) GAE group, orally administrated Graviola aquatic extract at 300 mg/kg BW daily for six weeks, 3) CCl_4 group, administrated by intraperitoneal (IP) injection of CCl_4 in olive oil, 50% V/V (2 ml/kg bwt), twice a week for two weeks to induce chronic damage of the liver according to Jayasekhar, *et al.* [29], 4) CCl_4+GAE (curative group) CCl4 injection by 2 ml/kg twice a week for two weeks then concomitant with GAE (300 mg/kg BW) daily for next four weeks, and 5) GAE+CCl_4 (prophylactic group), received GAE, 300 mg/kg/day once daily orally four weeks, then concomitant with
CCl_4 injection by 2 ml/kg bwt twice a week for next two weeks. Graviola dry extract was dissolved in normal saline
(0.9%) and administered using gavage needles. The dose of GAE was selected according to the previous study of Shukry, *et al.* [30]. Each of the above groups was kept in a single cage for si weeks. During the experimental period (six weeks), the diet consumed was recorded twice a weekly and body weight was recorded every week. The body weight gain (BWG, %), feed intake (FI) and feed efficiency ratio (FER) were determined according to using the following equations: BWG% = (Final weight – Initial weight)/ Initial weight×100, FER = gain in body weight (g/42 day)/ feed intake (g/42 day).

1.2.2.4. Sampling

At the end of experiment period (six weeks) blood samples were collected after 12 hours fasting using the abdominal aorta and rats were scarified under ether anesthetized. Blood samples were collected part in clean dry centrifuge tubes and left to clot at room temperature, then centrifuged for 10 minutes at 3000 rpm to separate the serum according to Drury and Wallington [31]. The clear, not hemolyzed sera was carefully aspirate, transferred into labeled Eppendorf tubes and stored frozen at -20^{o}C for further biochemical analysis. The other part was collected in sterilized EDTA tubes for hematological analysis. Liver samples from each rat were removed, weighed and used for liver homogenate preparation such as mentioned by Stroev and Makarova [32]. In brief, small pieces of liver were then transferred to a sterile vessel containing cell lysis buffer (FBS 0.025M, pH 7.4) solution and immediately ground to make a tissue homogenate (1g/4ml). The homogenates were centrifuged at 750g for 15 minutes at 4 ^{0}C and the supernatant was collected to a new microcentrifuge tube and the total protein concentration was measure according to the method of Lowry, *et al.* [33]. The samples were diluted to 10 mg protein/mL with 1X FBS and used for biochemical assays. The different samples of the liver tissue were stored in neutral formalin (10%) for histopathological studies.

1.2.2.5. Hematological Analysis

Hematological parameters [hemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs), platelets, neutrophil and lymphocyte] were performed by automatic measurement using an Avantor Performance Materials Inc. Business, Center Valley, USA (H32 VET 3-Part differential analyzer of hematology).

1.2.2.6. Biochemical Analysis

Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) activities were measured in serum using the modified kinetic method of Tietz [34]. Alkaline Phosphatase (ALP) activity was determined using modified kinetic method of Vassault, *et al.* [35]. Albumin (Alb) was determined in serum according to the method of David, *et al.* [36].The total bilirubin (TB) level in the serum was measured by a colorimetric method of Kaplan, *et al.* [37].Triglycerides (TGs), Total cholesterol (TC), HDL-Cholesterol and LDL-cholesterol were determined

in serum according to the methods of Fossati and Prencipe [38], Lopes-Virella, *et al.* [39] and Ahmadi, et al. [40], respectively. TNF-α was determined such as mentioned by Tavakkol, *et al.* [41], by a sandwich enzyme-linked immunosorbent assay (ELISA), utilizing two monoclonal antibodies directed against separate antigenic determinants on rat TNF- α.

1.2.2.7. Analysis of the Antioxidant/Oxidant Status in Hepatic Tissues

Glutathione fractions (GSH and GSSG) were measured colorimetrically in serum samples such as described by Alisik, *et al.* [42]. Glutathione peroxidase (GSH-Px) and catalase (CAT) activities were measured as mentioned by Splittgerber and Tappel [43] and Aebi [44], respectively. Superoxide dismutase (SOD) activity was measured according to the method of Mett and Müller [45]. GSH-Rd activity was determined according to the method recommended by the International Committee for Standardization in Haematology ICSH [46]. Liver nitric oxide (NO) was determined by the method of Montgomery and Dymock [47]. Liver homogenate malonaldehyde (MDA) content was measured using the colorimetric method described by Buege and Aust [48] based on the reaction of thiobarbituric acid (TBA) with MDA, one of the aldehyde products of lipid peroxidation. Reactive oxygen species (ROS) was determined by a colorimetric method described by Erel [49].

1.2.2.8. Analysis of the Liver Cell Apoptosis

Liver samples were prepared and analysis for the B-Cell Lymphoma 2 (Bcl-2), Bcl-2-like Protein 4 (Bax), and caspase-3 by using of flow cytometer instrument (Becton Dickinson, San Jose, CA, USA) according to the method of Gong, *et al.* [50].

1.2.2.9. Histopathological Studies

Liver tissues were prepared for histopathological studies such as described by Drury, *et al.* [51]. The tissues were accurately fixed in a neutral formalin solution (10%). They were dehydrated in an ascending series of ethanol, were cleared in xylene, were embedded in paraffin wax, and were sectioned at 5–7 µm by microtome and were stained with eosin and hematoxylin. The stained sections were examined to detect histopathological changes.

1.3. Statistical Analysis

All data were expressed as means ±SD. Statistical analysis was performed with the Student *t*-test and MINITAB-12 computer program (Minitab Inc., State College, PA). The differences were statistically significant at $p \leq 0.05$.

2. Results and Discussion

2.1. Effect of Graviloa Aquatic Extract (GAE) on BWG, FI and FER of Hepatotoxic Rats Induced by CCl_4

BWG, FI and FER of rats injected by CCl_4 and orally administered GAE were shown in Tables (1). From such data it could be noticed that at the end of the experiment (8 weeks), the CCl_4-treated rats exhibited significantly ($p \leq 0.05$) decreased in BWG, FI and FER compared to the normal group. However, orally administered of the rat diets with GAE (300 mg/kg BW) for daily for four weeks significantly ($p \leq 0.05$) increased the levels BWG, FI and FER with the positive control group. Concerning control one, there were no significant shifts with the GAE treatment. Such data are in agreement with that observed by several authors who found that CCl_4 induced significantly decreasing in all of these factors in rats [2, 5, 16, 52, 53]. Also, Hamzawy, *et al.* [54] and Abd ElRahman [55] reported that hepatic rats reveal significant reduction of the body weight and feed intake. With this context, Morresion and Hark [56] showed that liver disease can lead to malnutrition and the major causes of malnutrition in patients with liver disease are poor dietary/food intake, maldigestion, malabsorption and abnormalities in the metabolism and storage of macro and micro nutrients. Also, Dickerson and Lee [57] reported that many patients with acute or chronic liver disease are ill and commonly lose weight. Oral administration of GAE led to an improvement in body weight close to normal group. Such of these observations are in agreement with that reported by Chokshi [58] and Arthur, *et al.* [59]. In line with these results, several studies have come to

confirm that injected rats by CCl_4 caused decrease in both body weight which improved by consumption of plant parts contains bioactive compounds such as found in GAE [2, 3, 16, 60-63].

2.2. Effect of Graviloa Aquatic Extract (GAE) on the Hematological Parameters of Hepatotoxic Rats Induced by CCl_4

Hematological data of rats injected by CCl_4 and orally administered GAE were shown in Tables (2). Such data indicated that Hb, RBCs, WBCs, platelets, neutrophil and lymphocyte were significantly ($p \leq 0.05$) decreased in the CCl_4-treated group compared to the control and GAE groups. Graviola-treated groups, either prophylactic or curative, showed a significant ($p \leq 0.05$) increase in Hb, RBCs, WBCs, PCV%, platelets, neutrophil and lymphocyte related to the CCl_4-treated group.

The present research data confirmed that CCl_4 has harmful effects on hematological parameters, with characteristic leukopenia. Such data are consentience with Jirova*, et al.* [64] and Mandal*, et al.* [65] who found that depletion in the count of RBC's along with the Hb level was detected in rats treated with CCl_4 which could be attributed to disturbed hematopoiesis, destruction of erythrocytes, and reduction in the rate of their formation and/or enhanced removal from circulation due to CCl_4 toxicity. Also, according to Ballinger [66], depletion in erythrocytes count and Hb level leads to iron deficiency anemia which is characterized by a microcytic hypochromic blood picture, also hyperactivity of bone marrow, which leads to production of red blood cells with impaired integrity that are easily destroyed in the circulation. This could be another reason for decreasing hematological values [67]. The present study showed that the GAE caused an improvement of these parameters to a nearly normalized value in rats treated with CCl_4· this protective effect may be mediated through several mechanisms since the extract itself is a complex mixture of several nutrients (vitamins and minerals) and phytochemicals (phenolics, alkaloids, acetogenins and volatile compounds) [19]. Vitamins C and E are strong antioxidants which play an important role in detoxification process through removing the free radicals, protecting DNA from oxidative damage and reducing micronucleus (MN) frequencies in polychromatic erythrocytes of bone marrow [68, 69]. Phytochemicals detected in GAE exhibited several biological effects including antioxida1nt and scavenging activities and inhibiting the lipid peroxidations [18-20].

2.3. Effect of Graviloa Aquatic Extract (GAE) on Liver Function Parameters of Hepatotoxic Rats Induced by CCl_4

The level of liver enzymes AST, ALT and ALP, and the TB in the GAE- treated group were higher than that of the control and Graviola groups. In the same context, the all GAE-treated groups showed significant ($p \leq 0.05$) decreases in these parameters. On the other side, the opposite direction was observed for the alb. Our data showed that CCl_4 can significantly increase the liver enzymes ALT, AST and ALP, due to its cytotoxic effects, which resulted in damage to the structure and function of the liver cell membrane and intracellular organelles and the release of these enzymes in the circulation [63, 70]. This liver damage is caused by the metabolism of CCl_4 to trichloromethyl radicals (CCl_3^-) by liver cytochrome P_{450} which actively binds to O_2 forming trichloromethyl peroxyl radicals ($CCl3OO^-$), which then causes lipid peroxidation to membrane-bound fatty acids. Serial enzyme measurements, AST, ALT and ALP are often considered sensitive markers for determining the course of liver damage due to their presence of the cytoplasm facilitates blood flow after liver cell damage [71, 72]. Our finding showed that GAE significantly ($p \leq 0.05$) reducing AST, ALT and ALP levels which demonstrating that it can prevent live cells damage. This prophylactic/curative effects could be attributed to GLP content of some important nutrients and bioactive compounds. In similar studies, Graviloa extracts mainly vitamins and phytochemicals (phenolics, alkaloids, acetogenins and volatile compounds) exhibit protective activities against liver injury induced by toxic chemicals including CCl_4 [17, 19]. On the other hand, serum bilirubin levels are often enhanced under GAE intervention. Bilirubin is an endogenous compound that can be toxic under certain conditions but, on

the other hand, mild unconjugated hyperbilirubinaemia might protect against tumour development. Several studies indicated that plant parts contains different classes of bioactive compounds such as found in GAE which have the ability to reduce bilirubin levels through the extract active regulators increase the activity of enzymes, synthesis of transporter, and steps related to bilirubin clearance pathway [19, 63, 73-75]. On the other hand, serum albumin levels are often enhanced under GAE intervention. In similar studies, CCl_4-induced significant decrease in the serum albumin content as the consequence of liver injury [55, 61, 62, 76]. Another study of Koneri, *et al.* [77] indicated that hypoalbuminaemia is most commonly in the case of advanced chronic liver diseases and a useful index of the severity of cellular dysfunction in such diseases. Our findings indicated that GAE significantly ($p \leq 0.05$) increased serum Alb levels which demonstrating that it can prevent or repair the liver cells damage. Such a role of the GAE in manipulating the serum albumin level is of high importance, since human serum albumin is the major protein of blood plasma and constitutes about 50%. It is the transport protein that binds to and carries around various ligands such as water, fatty acids, hormones, bilirubin, thyroxin (T4), pharmaceuticals, and cations (Na+, K+, and Ca2+). Thus, one of the main functions of albumin is to regulate the oncotic pressure of blood [78, 79].

2.4. Effect of Graviloa Aquatic Extract (GAE) on Serum Lipid Profile Parameters of Hepatotoxic Rats Induced by CCl_4

The level of serum lipid profile parameters (TG, TC and LDL-c) in the CCl_4-treated group was higher than that of the control and GAE groups. In the same context, the all GAE-treated groups showed significant ($p \leq 0.05$) decreases in these parameters. On the other side, the opposite direction was observed for the HDL-c. This result was according with that obtained by Abd El-Rahman [55] and ElSamouny [63] who reported that CCl_4 increased cholesterol synthesis and hyperlipidemia, with increased serum TG and TC, shifting the glucose metabolism towards lipogenesis. The prophylactic and curative effects of GAE in enhanced the serum lipid profile parameters could be attributed to the involvement of hypolipidemic effect in the GAE [59, 80]. The different biological effects (hypolipidemic, antioxidant and scavenging activities) of GAE probably due to the presence of bioactive compounds such as alkaloids, acetogenins and polyphenolic compounds cause decreasing cholesterol absorption by deactivating the coenzyme-A (HMG-CoA) reductase hydroxymethylglutaryl [81]. Also, McAnlis, *et al.* [82] suggested that quercetin found in GAE, having a high affinity for protein, was bound to albumin and never incorporated into the LDL particle. In recent years, however, the possible hypocholesrerolemic effects of several dietary components, such as found in Graviloa including, alkaloids, polyphenoles, acetogenins, carotenoids, amides, cyclopeptides and megastigmanes etc have attracted much interest. Such compounds found in GAE exert their beneficial effects on cardiovascular health by antioxidant and anti-inflammatory activities [83-85]. LDL oxidation and endothelial cell damage is believed to be involved in the early development of atherosclerosis [86] Researchers found that presence of phenolics such quercetin significantly reduced LDL oxidation *in vitro* from various oxidases including 15lipoxygenase, copper-ion, UV light, and linoleic acid hydroperoxide [84, 86, 87]

2.5. Effect of Graviloa Aquatic Extract (GAE) on Liver Oxidative Status Parameters of Hepatotoxic Rats Induced by CCl_4

Data presented in Table (5) showed the effect of GAE on liver oxidative status parameters of hepatotoxic rats induced by CCl4. From such data it could be noticed that a significant ($p \leq 0.05$) decreasing in hepatic GSH, GSSG, GSH-Px, SOD and CAT levels in the CCl_4 group compared to the control and GAE groups. The GAE intervention to rat groups resulted in a significant ($p \leq 0.05$) increase in all of those parameters compared to the CCl_4 group. Also,

significant increasing was showed in MDA and NO concentrations in the CCl_4 group compared to control and GAE groups. GAE intervention (300 mg/kg BW) to the rat groups resulted in a significant ($p \leq 0.05$) decreasing in MDA and NO concentration compared to the CCl_4 group. IP injection of CCl_4 led to increase oxidative stress markers, such as MDA and NO, and decrease GSH, GSSG, GSH-Px, SOD and CAT. Such data are supported by several studies [2, 16-18, 88, 89]. CCl_4-induced liver damage came through metabolizes CCl_4 to many harmful free radicals which subsequently cause lipid peroxidation of membrane-bound fatty acids [90]. As a result of lipid peroxidation, harmful degredative products, namely MDA can be formed in cell membranes. With the same context, nitric oxide synthase catalyzes the conversion of L-arginine to citrulline and highly reactive free radical species, nitric oxide (NO) [91]. The elevation levels of MDA and NO return to the difficulty of glutamate transportation across the cell membrane, which initiates lipid peroxidation and alter the cell redox state leading to membrane damage [92]. The membrane damage due to cross linking of MDA with the membrane components leads to changes in its properties including inactivation of the enzymes and receptors, disturbance in membrane fluidity, cell injury and may cause the formation of atherosclerotic plaques [61, 62, 93-95]. Also, MDA exhibited mutagenic effect via reacting with nitrogenic bases in DNA [96]. Furthermore, NO can react with O_2 and H_2O to form nitrite (NO_2) and nitrate (NO_3); with the amino and thiol groups (-SH) of protein to produce nitrosylated species; with hemoglobin to form ironnitrosyl adducts and NO_3 in blood; and with superoxide anion (O_2^-) to make NO_3 [90, 97]. Therefore, highly significant decreasing rate on the formation of MDA and NO in liver as the result of GAE intervention proposed that liver prophylactic/curative effects may also be mediated by the radical-scavenging properties, and lipid peroxidation and nitric oxide synthase inhibition by GAE. With the same context, George, *et al.* [23] found that GAE intervention normalized the oxidant status of the liver cells due its antioxidant activity. Also, Baskar, *et al.* [98] reported that Graviola extract has a protective role against free radicals species, OH and H_2O_2. The protective roles of GAE due to the presence of bioactive compounds including alkaloids, polyphenols and acetogenins which leads to decrease the levels of lipid peroxidation and increase the levels of enzymatic and nonenzymatic antioxidants. Finally, the conversion of the majority of GSH in the liver to glutathione disulfide (GSSG) by the glutathione reductase enzyme to protect the liver cells from toxic material damage decreased the GSH level. GSH role in the CCl_4 detoxifications process represent the main function through as a key conjugate of xenobiotics electrophilic metabolites and as an important antioxidant [1, 53]. The antioxidant functions of GSH include serving as a nonenzymatic scavenger of oxyradicals and its role in the activities of the antioxidant enzymes system (glutathione peroxidase, GSH-Px and glutathione reductase, (GSH-Rd) [3, 18, 99]

2.6. Effect of Graviloa Aquatic Extract (GAE) on Liver ROS and TNF-α Level of Hepatotoxic Rats Induced by CCl_4

Data in Table (6) indicated that ROS level was significant ($p \leq 0.05$) increasing in the CCl_4 group compared to the control and GAE groups. Also, the TNF-α level recorded the opposite direction. In addition, both GAE prophylactic/curative treatment groups showed a significant ($p \leq 0.05$) decrease and increase in ROS and **TNF-α** levels, respectively. Comparable research has examined biomarkers or end-products of free radical-mediated oxidative processes to identify oxidative stress associated with CCl_4 [2, 5, 16-18, 88, 89]. Thus, ROS is often utilized as a biomarker to provide an acceptable indication of oxidative stress state. Our findings revealed that the degree of oxidative stress in CCl_4 injected rats was reduced as a result of GAE-induced ROS decrease. Several previous studies has also shown a substantial correlation between ROS liver concentrations and the pathogenic phases of a number of illnesses, including liver diseases [1, 18, 76]. Maintaining an appropriate level of oxidative stress would be eased by the AEG-induced regulation of ROS levels throughout the body. In accordance with our data, Shukry, *et al.* [30] found that oxidative stress indices including ROS was enhanced in CCl4-injected rats

and that Graviloa had an antioxidant and chemoprotective impact on the liver due to its high bioactive component content. On the other side, data of our findings indicated that GAE significantly ($p \leq 0.05$) decreased liver TNF-α levels which demonstrating that it can prevent tissue damage. With this context, Abd Elalal, *et al.* [18] reported the same behavior of TNF-α levels in liver with other plant parts applied and contain the same bioactive compounds found in GAE. Such role of GAE in suppression the TNF-α could be of a high degree of importance because it is a proinflammatory cytokine which plays an important role in initiating the tissue inflammatory reaction [100-102].

2.7. Effect of Graviloa Aquatic Extract (GAE) on CCl_4-Induced Liver Cell Apoptosis

Data in Table (7) and Figure (1) are shown the effect of GAE on CCl_4-induced liver cell apoptosis. From such data it could be noticed that the apoptotic (Bax) proteins and caspase-3 levels of the CCl_4 group were significantly ($p \leq 0.05$) higher than the control and GAE groups. Besides, the GAE intervention by 300 mg/kg BW in both prophylactic and curative treatment groups resulted in a significant ($p \leq 0.05$) decrease in their expression levels compared to the CCl_4 group. Conversely, the GAE-treated groups showed normalization to antiapoptotic (Bcl-2) proteins level, which was significantly ($p \leq 0.05$) decreased by CCl_2 injection. This study shows that the injection of CCl_4 led to significant ($p \leq 0.05$) increases in caspase-3, and Bax proteins and a substantial reduction in Bcl-2 proteins. This was corresponding with Cai, *et al.* [103] and Lee, *et al.* [104] who explained that oxidative damage is one of the essential mechanisms of CCl_4 hepatotoxicity, which triggers apoptosis via the mitochondria-initiated pathway. This implies that CCl_4-induced Ca^{2+} influx and destruction of the inner mitochondrial membrane potential, leading to disorganized mitochondrial permeability of pores to markers of apoptosis. Also, DNA fragmentation results revealed apoptosis and necrosis following CCl_4 treatment. GAE was found to decrease the higher levels of Bax and caspase, and significantly ($p \leq 0.05$) increase Bcl2 in CCl_4-treated rats. This observation was in convenient with Mansour, *et al.* [105] who found that GAE induced the overexpression of Bcl-2, which prevents DNA fragmentation due to its antioxidant activities, blocks the cytochrome c release and mitochondrial permeability.

2.8. Effect of GAE on the Histopathological Alterations Induced by CCl_4 in Rat's Liver

Photomicrograph of the liver of the control and different treated groups of rats were shown in Figure 2. The control and GAE showed normal hepatocytes arranged in cords around the central vein and normal histological structure of hepatic lobule. CCl_4 treated group showed hepatocellular vacuolar degeneration (steatosis), congestion of hepatic sinusoids, hyperplasia of biliary epithelium and fibroplasia in the portal triad, and focal hepatocellular necrosis and a loss of cellular details. The CCl_4 + GAE group showed slightly of necrosis of sporadic hepatocytes. In GAE + CCl4 group, slight Kupffer cells activation , only change was observed. Such observations were similar to the study of Elhassaneen, *et al.* [17] who reported that CCl_4 induced several histopathological alterations in rats including focal hepatocellular necrosis associated with inflammatory cells infiltration, congestion of hepatic sinusoids, marked fibroplasia in the portal triad, appearance of newly formed bile ductuoles and portal infiltration with inflammatory cells. Also, Badawy [106] liver of rats treated with CCl4 revealed steatosis of hepatocytes, hyperplasia of biliary epithelium and focal hepatocellular necrosis associated with mononuclear inflammatory cells infiltration. Furthermore, Ding, *et al.* [107] mention that liver fibrosis induced by injecting CCl_4 IP in rats for eight weeks and observed that disruption of tissue architecture, large fibrous septa formation, pseudolobe separation and collagen accumulation. These changes were accompanied by increase of the levels of ALT and AST, while albumin was decreased significantly. On the other study, Ahmed, *et al.* [108] mentioned that CCl_4 induced histopathological changes by includes regenerative nodules, deteriorated parenchyma, and the lobules were infiltrated with fat and structurally altered. Such changes were accompanied by increased serum liver enzymes ALT, AST, and ALP, lactate dehydrogenase, level of nitric oxide, tumor necrosis

factor alpha (TNFα) and liver malondialdehyde content, collagen fiber percent and decreased liver reduced glutathione content as endogenous antioxidant. Finally, Elhassaneen, *et al.* [53] reported that rats treated with CCl_4 showed congestion of central vein and cytoplasm vacuolization of hepatocytes. Also, feeding on diet containing Cape Gooseberry fruits and Mulberry leaves powder showed Kupffer cells activation and slight hydropic degeneration of some hepatocytes. Such histopathological changes were confirmed by the biochemical results.

Abbreviations

Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bax, B-cell lymphoma 2 (Bcl-2)-like protein 4, Bcl-2, B-cell lymphoma 2, BWG, body weight gain; CCl_4, carbon tetrachloride; FER, feed efficiency ratio; FI, feed intake; GAE, Graviola aqueous extract, GSH, reduced glutathione; GSSG, oxidized glutathione; GSH-Rd, glutathione reductase; GSH-Px, glutathione peroxidase; Hb, hemoglobin; IP, intraperitoneal (IP), HDL-c, high density lipoprotein cholesterol, LDL-c, low density lipoprotein cholesterol, MDA, malondialdehyde; NO, nitric oxide; RBCs, red blood cells; ROS, reactive oxygen species; OS, oxidative stress, TBA, thiobarbituric acid; TNF-α, tumor necrosis factor-alpha; TB, total bilirubin, TC, total cholesterol (TC), TG, triglycerides; WBCs, white blood cells

Table-1. Effect of Graviloa aquatic extract (GAE) on BWG, FI and FER of hepatotoxic rats induced by CCl_4

Parameters	Control	GAE	CCl_4	CCl_4+GAE	GAE+CCl_4
BWG (%)	0.88 ± 0.06^{a}	0.91 ± 0.07^{a}	0.48 ± 0.03^{d}	0.69 ± 0.02^{c}	0.78 ± 0.03^{b}
FI (g/day/rat)	11.51 ± 0.54^{a}	11.73 ± 0.61^{a}	7.79 ± 0.48^{c}	9.47 ± 0.89^{b}	10.01 ± 0.91^{b}
FER	0.069 ± 0.004^{a}	0.071 ± 0.003^{a}	0.049 ± 0.002^{c}	0.061 ± 0.005^{b}	0.063 ± 0.004^{b}

Each value represents the mean of six rats ±SD. Means with different superscript letters in the same raw indicate significant differences at $p \leq 0.05$. BWG, body weight gain, FI, feed intake; FER, feed efficiency ratio.

Table-2. Effect of Graviloa aquatic extract (GAE) on the hematological parameters of hepatotoxic rats induced by CCl_4

Parameters	Control	GAE	CCl_4	CCl_4+GAE	GAE+CCl_4
Hb (g/L)	156.78 ± 2.56^{a}	157.35 ± 3.77^{a}	79.14 ± 2.96^{d}	99.56 ± 3.56^{c}	111.32 ± 4.56^{b}
RBCs (10^{12}/L)	9.32 ± 0.56^{a}	9.34 ± 0.85^{a}	3.84 ± 0.77^{c}	5.84 ± 0.63^{b}	5.91 ± 0.75^{b}
WBCs (10^{9}/L)	10.01 ± 1.02^{a}	10.12 ± 0.98^{a}	5.13 ± 0.81^{c}	6.92 ± 0.84^{b}	6.97 ± 0.84^{b}
Platelets (10^{9}/L)	762.43 ± 29.56^{b}	771.52 ± 30.25^{a}	261.81 ± 12.43^{e}	391.67 ± 22.75^{d}	401.53 ± 23.89^{c}
Neutrophil (%)	18.32 ± 1.23^{a}	18.31 ± 0.87^{a}	6.82 ± 0.69^{c}	15.73 ± 0.65^{b}	16.01 ± 0.99^{b}
Lymphocyte (%)	74.67 ± 3.98^{a}	74.86 ± 2.31^{a}	59.92 ± 0.88^{c}	66.09 ± 3.07^{b}	65.98 ± 4.06^{b}

Each value represents the mean of six rats ±SD. Means with different superscript letters in the same raw indicate significant differences at $p \leq 0.05$. Hb, hemoglobin; RBCs, red blood cells; WBCs, white blood cells.

Table-3. Effect of Graviloa aquatic extract (GAE) on liver function parameters of hepatotoxic rats induced by CCl_4

Parameters	Control	GAE	CCl_4	CCl_4+GAE	GAE+CCl_4
AST (U/L)	50.32 ± 3.21^{c}	50.02 ± 2.65^{c}	98.27 ± 5.21^{a}	76.32 ± 4.11^{b}	69.15 ± 3.71^{b}
ALT (U/L)	28.94 ± 2.56^{d}	26.45 ± 1.98^{d}	67.83 ± 3.08^{a}	42.52 ± 3.17^{b}	37.45 ± 2.87^{c}
ALP (U/L)	108.77 ± 9.17^{d}	104.65 ± 8.21^{d}	276.56 ± 11.31^{a}	187.64 ± 7.59^{b}	171.88 ± 10.56^{c}
Alb (g/L)	36.14 ± 2.17^{a}	37.04 ± 3.98^{a}	18.37 ± 2.97^{d}	28.52 ± 2.18^{c}	30.54 ± 3.10^{b}
TB (mmol/L)	6.69 ± 0.87^{c}	6.49 ± 0.98^{c}	22.78 ± 2.67^{a}	15.38 ± 0.84^{b}	14.71 ± 0.91^{b}

Each value represents the mean of six rats ±SD. Means with different superscript letters in the same raw indicate significant differences at $p \leq 0.05$. AST, aspartate aminotransferase; ALT, alanine aminotransferase. ALP, alkaline phosphatase; Alb, albumin, TB, serum total bilirubin.

Table-4. Effect of Graviloa aquatic extract (GAE) on serum lipid profile parameters of hepatotoxic rats induced by CCl_4

Parameters	Control	GAE	CCl_4	CCl_4+GAE	GAE+CCl_4
TG (mmol/L)	1.14 ± 0.10 ^{c}	1.14 ± 0.09 ^{c}	1.51 ± 0.13 ^{a}	1.38 ± 0.08 ^{b}	1.33 ± 0.03 ^{b}
TC (mol/L)	2.91 ± 0.09 ^{c}	2.81 ± 0.05 ^{c}	3.44 ± 0.11 ^{a}	3.16 ± 0.04 ^{b}	3.12 ± 0.06 ^{b}
HDL-c (mmol/L)	1.41 ± 0.03 ^{a}	1.45 ± 0.06 ^{a}	0.72 ± 0.04 ^{c}	0.99 ± 0.02 ^{b}	1.08 ± 0.03 ^{b}
LDL-c (mmol/L)	0.91 ± 0.02 ^{c}	0.90 ± 0.03 ^{c}	2.03 ± 0.01 ^{a}	1.64 ± 0.04 ^{b}	1.54 ± 0.04 ^{b}

Each value represents the mean of six rats ±SD. Means with different superscript letters in the same raw indicate significant differences at $p \leq 0.05$. TG, triglycerides, TC; total cholesterol; HDL-C, high-density lipoproteins; LDLC, low-density lipoproteins.

Table-5. Effect of Graviloa aquatic extract (GAE) on liver oxidative status parameters of hepatotoxic rats induced by CCl_4

Parameters	Control	GAE	CCl_4	CCl_4+GAE	GAE+CCl_4
Antioxidants					
GSH (mmol/g)	6.01 ± 0.56 ^{a}	6.09 ± 0.68 ^{a}	2.69 ± 0.19 ^{c}	3.86 ± 0.35 ^{b}	3.90 ± 0.61 ^{b}
GSSG (mmol/g)	0.58 ± 0 02 ^{a}	0.61 ± 0.01 ^{a}	0.32 ± 0.09 ^{b}	0.37 ± 0.08 ^{b}	0.39 ± 0.03 ^{b}
GSH-Px (U/g)	1.33 ± 0.04 ^{a}	1.38 ± 0.09 ^{a}	0.71 ± 0.07 ^{c}	0.81 ± 0.04 ^{b}	0.89 ± 0.05 ^{b}
SOD (U/g)	87.18 ± 2.98 ^{a}	88.17 ± 4.12 ^{a}	48.52 ± 7.18 ^{c}	63.32 ± 5.27 ^{b}	67.28 ± 6.06 ^{b}
CAT (U/g)	179.55 ± 11.56 ^{a}	181.96 ± 9.88 ^{a}	114.54 ± 8.61 ^{c}	159.45 ± 8.05 ^{b}	163.08 ± 10.72 ^{b}
Oxidants					
MDA (nmol/g)	691.05 ± 22.67 ^{c}	689.34 ± 19.03 ^{c}	1384.14 ± 31.45 ^{a}	789.58 ± 18.67 ^{b}	775.45 ± 15.98 ^{b}
NO (mmol/g)	17.46 ± 1.03 ^{c}	17.44 ± 0.97 ^{c}	41.55 ± 2.61 ^{a}	31.91 ± 3.32 ^{b}	29.06 ± 2.05 ^{b}

Each value represents the mean of six rats ±SD. Means with different superscript letters in the same raw indicate significant differences at $p \leq 0.05$. GSH, reduced glutathione; GSSG, oxidized glutathione; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; MDA, Malonaldehyde; NO, nitric oxide.

Table-6. Effect of Graviloa aquatic extract (GAE) on liver ROS and TNF-α level of hepatotoxic rats induced by CCl_4

Parameters	Control	GAE	CCl_4	CCl_4+GAE	GAE+CCl_4
ROS (nmol/g)	0.55 ± 0.08 ^{c}	0.46 ± 0.04 ^{d}	2.01 ± 0.09 ^{a}	1.35 ± 0.04 ^{b}	1.19 ± 0.06 ^{b}
TNF-α (pg/mg)	14.05 ± 1.11 ^{c}	13.91 ± 1.32 ^{c}	30.67 ± 2.01 ^{a}	24.43 ± 1.42 ^{b}	22.12 ± 1.98 ^{b}

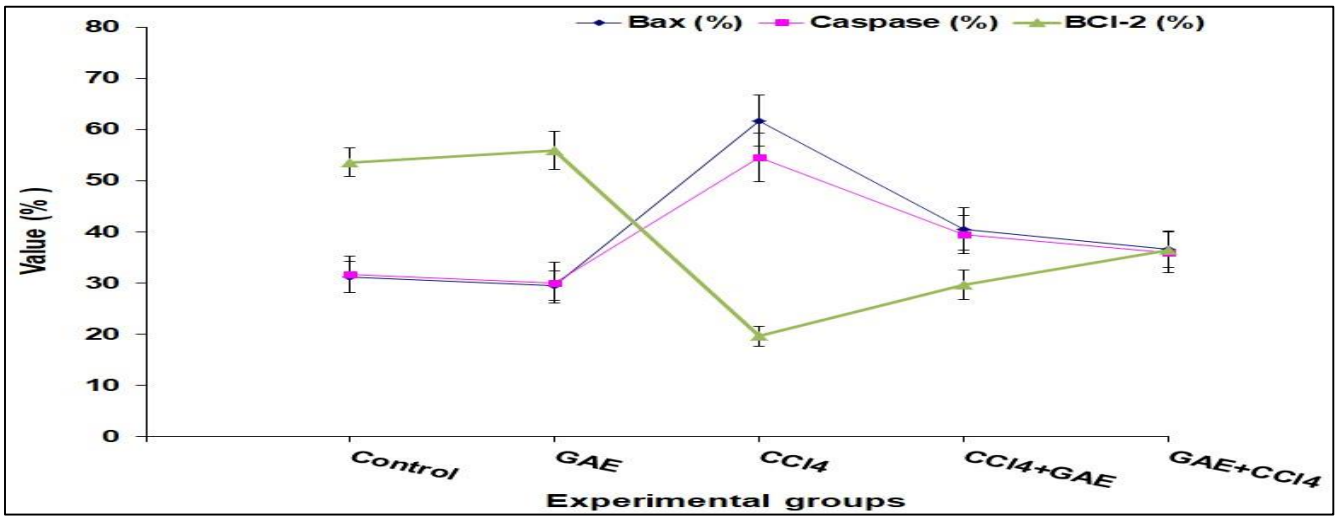
Each value represents the mean of six rats ±SD. Means with different superscript letters in the same raw indicate significant differences at $p \leq 0.05$. ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha.

Table-7. Effect of Graviloa aquatic extract (GAE) on CCl_4-induced liver cell apoptosis

Parameters	Control	GAE	CCl_4	CCl_4+GAE	GAE+CCl_4
Bax (%)	31.21 ± 3.54 [c]	29.52 ± 3.98 [c]	61.71 ± 4.69 [a]	40.56 ± 3.76 [b]	36.54 ± 4.02 [b]
Caspase (%)	31.74 ± 3.07 [c]	30.09 ± 2.88 [c]	54.57 ± 5.01 [a]	39.53 ± 4.17 [b]	36.00 ± 3.56 [b]
Bcl-2 (%)	53.61 ± 4.01 [a]	55.90 ± 2.76 [a]	19.65 ± 3.67 [d]	29.72 ± 1.98 [c]	36.45 ± 2.84 [b]

Each value represents the mean of six rats ±SD and % of change. Means with different superscript letters in the same raw indicate significant differences at $p \leq 0.05$. Bax, apoptosis regulator Bax/Bcl-2-associated X protein, Bcl2, antiapoptotic protein B-cell lymphoma 2.

Figure-1. Effect of Graviloa aquatic extract (GAE) on CCl_4-induced liver cell apoptosis



Each value represents the mean of six rats ±SD and % of change. Means with different superscript letters in the same raw indicate significant differences at $p \leq 0.05$. Bax, apoptosis regulator Bax/Bcl-2-associated X protein, Bcl2, antiapoptotic protein B-cell lymphoma 2.

Figure-2. Effect of GAE on the histopathological alterations induced by CCl_4 in rats liver (H and E X 400)

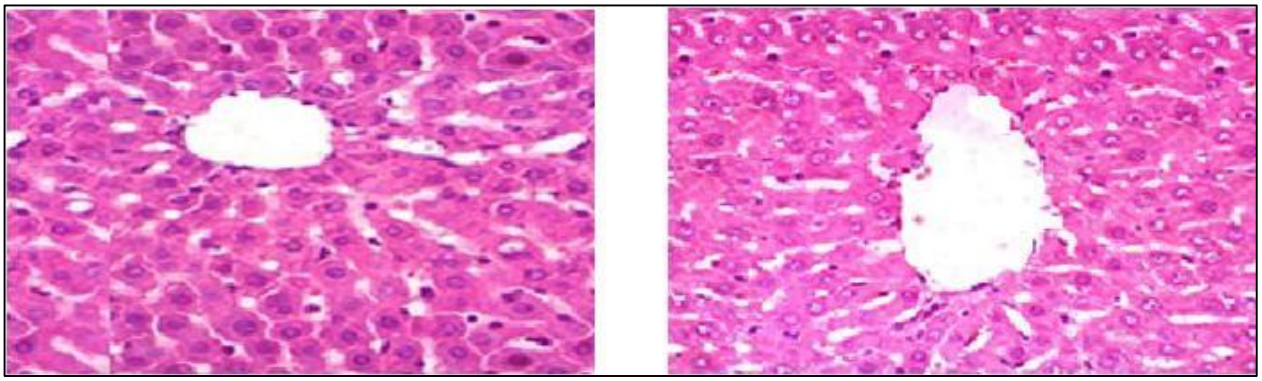


Photo 1. Control group showing normal hepatocytes arranged in cords around the central vein

Photo 2. GAE group showing the normal histological structure of hepatic lobule

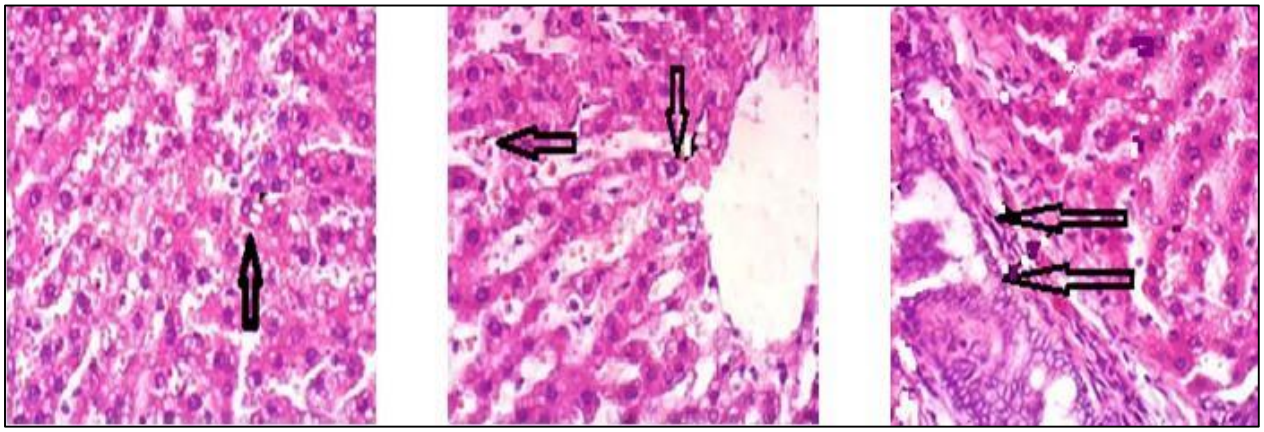


Photo 3. CCl4 group showing hepatocellular steatosis

Photo 4. CCl4 group showing congestion of hepatic sinusoids

Photo 5. CCl4 group showing hyperplasia of biliary epithelium and fibroplasia in the portal triad

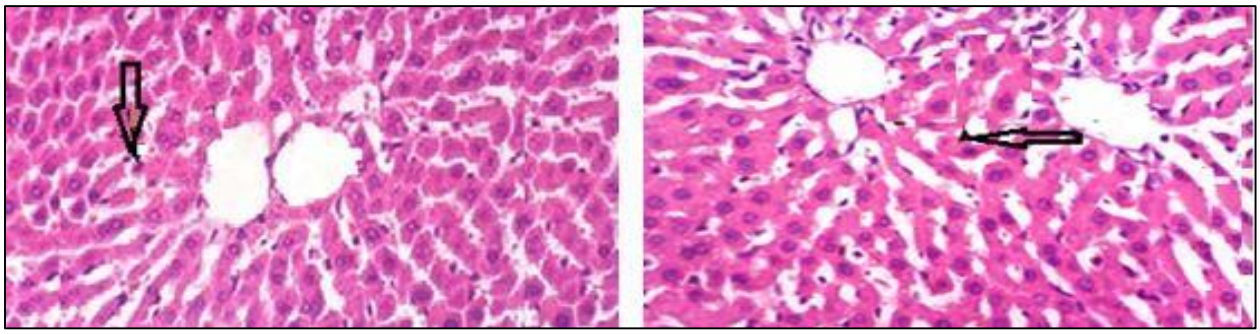


Photo 6. CCl4 + GAE group showing necrosis of sporadic slight hepatocytes

Photo 7. GAE + CCl4 group showing the only change, Kupffer cells activation.

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