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RESEARCH

Prospective Hepatoprotective Actions of Novel Nano-formulations of Sildenafil and Neem Extract in Counteracting Oral Carbon Tetrachloride-induced Liver Injury in Rats

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Abstract

Purpose A multitude of inflammatory cells and chemical mediators initiate a complex cascade that ultimately leads to hepatocyte death and a systemic inflammatory response. This research aimed to investigate the potential effects of sildenafil and neem (Azadirachta indica) extract, in both conventional and nanoparticle (NP) forms, in the treatment of moderate acute liver damage induced by orogastric carbon tetrachloride (CCL_4).

Methods To induce moderate acute hepatic damage a single oral dosage of CCL_4 (2.5 mL/kg body weight) was provided 24 h before euthanasia. In liver damage-induced CCL_4, sildenafil and neem extract were given in conventional and nanoparticle (PLGA or niosome) forms. To find histological anomalies and hepatic changes, behavioral, biochemical, histopathological, and immunohistochemical methods were used.

Results The findings indicated that sildenafil and/or neem extract, especially in NP combination, significantly mitigated CCL_4-induced acute moderate liver damage. Indicators of liver function, including aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), albumin, bilirubin and gamma-glutamyl transferase (GGT), shown improvement, particularly with the nanoparticulation of both therapies. Treatment, particularly in NP forms, improved the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase activity (GPx) in liver tissues. A significant reduction in NF-κB expression in hepatic tissue was shown in treatment groups. Also, medication resulted in lower levels of interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), caspase-3, and transforming growth factor-beta (TGF-β) in the liver tissue homogenates. Liver function was more significantly improved by the drug-NP combination.

Conclusions This study verified the beneficial therapeutic effects of the combination of sildenafil and neem extract, particularly in NP forms, using biochemical, histological, and immunohistochemical analyses in a rat model of liver damage.

Keywords Sildenafil · Neem · CCL_4 · Oxidative stress · TGF-β · NF-κB

Abbreviations

ALT	Alanine aminotransferase
ALP	Alkaline phosphates
AST	Aspartate aminotransferase
CCL_4	Carbon tetrachloride
cGMP	Cyclic guanosine monophosphate
GGT	Gamma-glutamyl transferase
GPx	Glutathione peroxidase activity
IL-1β	Interleukin- 1 beta
MDA	Malondialdehyde
NP	Nanoparticle
NF-κB	Nuclear factor kappa B
PLGA	Polylactic-co-glycolic acid
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor alpha

Introduction

The death of hepatocytes and the development of systemic inflammation are the results of a complicated chain of events that include a wide range of inflammatory cells and chemical mediators [1]. Both the length of time that the ischemia has been present and the underlying liver disease are factors that determine the severity of the inflammatory response and the

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functioning of the organs [2]. To prevent harm to the liver, numerous pharmaceutical treatments have been developed. Antioxidants, ozone, nitric oxide donors, adenosine agonists, sildenafil, and vardenafil are all examples of such agents [3]. CCl_4 is an example of a xenobiotic industrial solvent that has been shown to cause injuries to the liver and chemical hepatitis in animals. The majority of drugs' hepatoprotective effectiveness has been tested in experiments involving CCl_4-induced liver injury [4]. Acute liver necrosis and steatosis are caused by a single dosage of CCl_4, a powerful xenobiotic that is toxic to the liver [3, 5].

The proposed route of action involves converting CCl_4 to highly reactive free radical metabolites via CYP2E1, which can covalently bond to biological macromolecules, particularly phospholipid fatty acids in cell membranes [6]. Lipid peroxidation initiates a domino effect of free radicals. The orogastric route is the most popular for CCl_4 due to its small doses and direct transport to the liver, reducing extrahepatic effects caused by selective accumulation in the liver [3].

Phosphodiesterase type 5 inhibitors are used to treat pulmonary arterial hypertension and erectile dysfunction in men due to their vasodilatory effects [7]. These inhibitors, like sildenafil, vardenafil, and tadalafil, prevent cyclic guanosine monophosphate (cGMP) breakdown, resulting in broader effects [8, 9]. Phosphodiesterase type 5 inhibitors protect kidneys from ischemia–reperfusion injury by improving endothelial function, making them a potential treatment for ischemia–reperfusion injury in various organs [10].

In a study conducted by Ali, Azouz [11], it was revealed that the liver deficiencies caused by cholestatic liver cirrhosis, which is caused by bile duct ligation, could be greatly improved when diosmin and sildenafil were administered together. Additionally, a study conducted by Adeyanju, Molehin [12] indicated that a high dose of sildenafil (20 mg/kg) administered prior to CCl_4 treatment may provide protection against oxidative stress. The pharmacological protective agent sildenafil, when administered at modest levels, may be effective in preventing hepatotoxicity caused by CCl_4 (0.5 ml/kg/i.p.), according to research by Molehin, Adeyanju [13]. When CCL_4 given intraperitoneally at doses of 3 mg/kg twice for four weeks, sildenafil restores antioxidant status and considerably down-regulates mRNA expressions of profibrotic genes, thereby alleviating hepatic dysfunctions produced by CCl_4 [14].

Natural xenobiotic/drug hepatoprotection may be found in natural medicinal products from the neem tree [15–17]. Extracts have potential medical uses like insect repellant, anti-inflammatory, diabetes medication, and cancer prevention. Flavonoids in neem suppress inflammatory enzymes and endoperoxides [18]. Limonoid, a primary bioactive compound, inhibits inflammatory mediators and enhances opioid pathways, making it a well-known pain anesthetizer [18, 19]. In addition, the chemicals included in neem leaf extract also helped restore the antioxidant system and reduced damage to the liver and kidneys, as reported by Shailey and Basir [20]. Researchers found that the active ingredient in neem, nimbolide, protected rats' livers from CCl_4-induced damage [21]. Another study showed that azadirachtin-A, when administered as a pretreatment at higher dose levels, somewhat returns the liver to normal in rats [22].

Nanoparticles are increasingly used in drug delivery formulations, particularly in targeting liver cells for homeostasis and disease treatment. Nano-delivery technologies have shown promise in resolving issues related to hydrophobic medication distribution. The use of NPs for liver illnesses has significantly improved since the advent of tailored delivery, offering a fresh approach to treating fatal illnesses [23]. Niosomes are self-assembly nanovesicles with higher stability, ease of preparation, and low cost compared to liposomes. They are attractive for drug delivery systems due to their ability to encapsulate hydrophilic and lipophilic active drug products due to their hydrophobic bilayer and hydrophilic aqueous core [24].

Our study's goal is to provide potentially helpful information on the therapeutic potential of treating acute liver damage in rats with a combination of sildenafil and neem extracts in conventional and NP forms using an orogastric CCL_4 delivery method.

Materials and Methods

Materials, Neem Seeds Collection and Seeds Extract Preparation

This study's neem seeds were kept in sealed containers. Span 40, Tween 80, were obtained from Adwic, El-Naser Chemical Co., Egypt. Cholesterol was purchased from Sigma Chemical Co, St. Louis, MO, USA. The plant seeds were extracted according to the method reported in previous study [25]. The seeds were collected and washed with distilled water. After the seeds were cleaned, they were dried in the air. 10 g of seeds were weighed and then grounded to obtain fine powder. The amount of neem seed powder (10 g) was placed in a conical flask. The measured quantity of seed was mixed with an organic solvent mixture (100 ml) consisting of 70% ethanol. The mixture was placed for 72 h at room temperature, then the resulting extract was filtered with Whatman filter paper. The organic solvent was evaporated using a rotary evaporator at 40 °C to produce a crude extract. The extract was dried and stored at −20 °C for future studies.

Synthesis of Sildenafil-loaded Polylactic-co-glycolic Acid Nanoparticles

The National Research Centre in Egypt created NPs that loaded sildenafil. A previous study dictated that the solid-in-oil-in-water (s/o/w) emulsion approach is used to create NPs loaded with sildenafil [10].

Preparation of Neem Seed Loaded Nanovesicles (Niosomes)

The thin film hydration method was utilized to fabricate Neem extract loaded-niosomes that contain mixture of cholesterol and non-ionic surfactants at molar ratios of 1:1. A 500 µmol of non-ionic surfactants mixture (Span 40: Tween 80 (1:1)) and cholesterol were dissolved in a chloroform/methanol mixture (2:1 v/v) in a round bottom flask. The dried neem extract was added to the organic phase. Then, organic solvents were evaporated by rotary evaporator (HAHNVAPOR HS- 2005 V, HAHNSHINE Scientific, South Korea) at 55 °C, 900 rpm for 30 min. The rounded bottom flask containing dried film was placed in the desiccator for 24 h and then, hydrated with distilled water. The hydrated dispersion was subjected to slow shaking for 45 min at 55 °C. The prepared niosomal dispersion was stored at 4 °C for complete hydration and further investigation. The above fabrication procedure was performed according to previous research work [24].

Measurement of Particle Size, Polydispersity Index (PDI) and Zetapotential

The mean vesicle size and poly dispersity index of the nano system dispersion were measured using dynamic laser light scattering (DLS) technique at room temperature using the Zetasizer (Nano ZS)- (Malvern Instruments-Worcestershire, UK). Also, zeta potential was detected by Laser Doppler anemometry on a Malvern Nano ZS. All the measurements were performed in triplicates.

Transmission Electron Microscopy (TEM)

The fresh dispersion was diluted (10-folds) by distilled water. A dilute dispersion drop was placed on a carbon-coated 300 mesh copper grid and left for 1 min to allow adherence to the carbon substrate. Excess dispersion was removed by a piece of filter paper and the sample was air-dried. Then, the sample was imaged by the microscope at 10–100 k magnification power using an accelerating voltage of 100 kV using the JEOL TEM (Model 100 CX II; Tokyo, Japan) (electron Microscope Unit -Assiut University).

Animals and Acute Hepatic Damage Induction

This experiment was carried out in accordance with the guidelines that were established for the care and treatment of laboratory animals, after receiving approval from the Ethical Committee (approval number:06/2025/0299). In this investigation, adult male albino rats that were 9 to 11 weeks old were employed. They came from Faculty of veterinary medicine at Egypt's animal house in Assiut, where they were initially collected. They were roughly 170 g in weight. The temperatures ranged from 28 degrees Celsius to 31 degrees Celsius, and they were housed in a room that was kept clean and had lights on from five in the morning until seven in the evening. Both food and water were made available to the animals without any restrictions. For the purpose of inducing moderate acute hepatic damage, a single oral dosage of CCL_4 (2.5 mL/kg body weight) (50 percent saturated in olive oil) was utilized, twelve hours prior to the sacrifice [3, 26].

Behavior Analysis and Evaluation

In order to explore behaviors that are connected with anxiety, a test that is known as the raised plus maze was carried out. The purpose of this test was to determine the potential influence that each treatment could have on a variety of animal patterns. According to previous research, the test was carried out [3].

Experimental Design

Randomly, they were split up into ten groups, each of which had eight animals. For a period of seven days, rats in group 1 (Control) were administered 1 ml/kg of body weight of olive oil and normal saline through oral gavage. This group served as the control rats. Oral administration of CCl_4 (50 percent in olive oil) at a dose of 2.5 ml/kg of body weight was administered to group 2 (CCl_4) twenty-four hours prior to the sacrifice. Both acute liver damage and carrier-based NPs of PLGA were administered orally to the animals in group 3 (PLGA), which was likewise produced with the condition. Niosome-based nanoparticles were orally administered to the animals in group 4 (Niosome), and they were also allowed to experience acute liver damage. Oral administration of sildenafil dissolved in saline at a dosage of 1 mg/kg [3] body weight was administered to group 5 (S) for a period of one week. Group 6 (NE) received an oral dose of neem extract at a dosage of 25 mg/kg body weight [27, 28] for a period of one week. Group 7 (O) administered a treatment consisting of sildenafil and neem extract for a period of one week.

The animals in group 8 (S-NP) were given polylactic-co-glycolic acid (PLGA) nanoparticles that were loaded with sildenafil in addition to being induced with acute liver damage. Nanovesicles NPs loaded with neem extract were given to animals in group 9 (NE-NP), and they were additionally stimulated with acute hepatic damage while they were being treated. In addition to being subjected to acute liver damage, animals in group 10 (O-NP) were given a mixture of NPs loaded with sildenafil and neem extract. CCl_4 was administered to all of the animals, with the exception of the control group Fig. 1.

The Process of Collecting Blood, Preparing Samples, and Storing

Following an overnight fast (which lasted around 12 to 15 h), after the rats had been put under anesthesia through the eye canthus, blood samples were taken from the retro-orbital venous plexus of the rodents. In order to euthanize rats prior to scarification, the following procedure was utilized: animals were completely sedated by breathing in 5% isoflurane. After that, blood samples were extracted from the canthus of the eye. In the event that rats did not react to stimulation of their heads and limbs, they were quickly put to death via cervical dislocation. In the event that rats stopped breathing and did not react to systemic stimulation after ten seconds after cervical dislocation, it was determined that they had committed suicide. In a potassium phosphate buffer with a concentration of 0.1 M and a pH of 7.4, liver tissues were chopped and homogenized at a weight-to-volume ratio of 10%. After centrifuging the homogenate at a speed of 3000 g for ten minutes at a temperature of 4 degrees Celsius, the supernatant that was obtained was utilized for the purpose of determining the levels of oxidative stress, antioxidants, and inflammatory markers. Additionally, histological and immunohistochemical examinations were performed on other sections of the liver tissue [16, 29].

Evaluation of the Indicators of Liver Function

Aspartate Aminotransferase (AST) Activity in the Rat Serum

Formerly glutamate oxaloacetate transaminase, now AST. AST activity was measured using Reitman and Frankel's

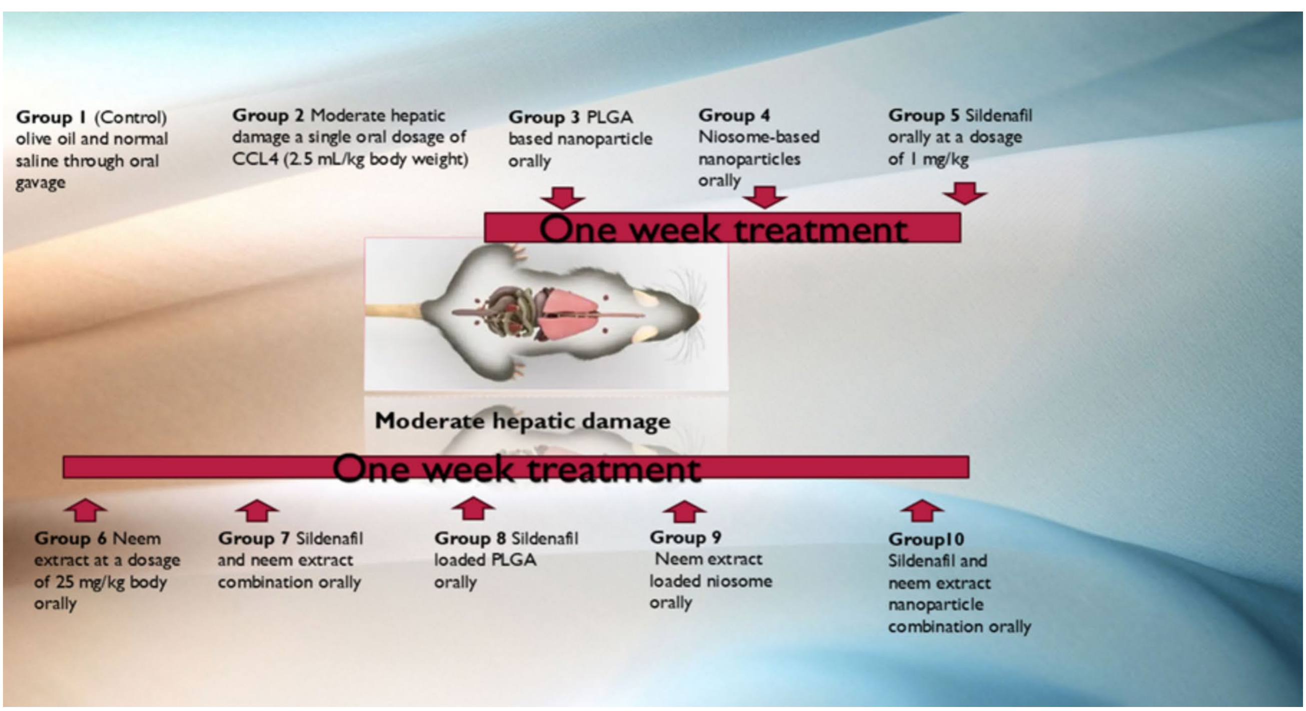


Fig. 1 The experimental groups' design

kinetic technique. The assay was done with AST enzyme activity assay kit. Aspartate and ketoglutaric acid are converted into oxaloacetate and glutamate aspartic acid by the PLP-dependent AST enzyme, which results in the production of a colorimetric active product that is proportionate to the enzymatic activity of the AST enzyme [30, 31].

Alanine Aminotransferase (ALT) Activity in the Rat Serum

The kinetic technique of Reitman and Frankel [30] was utilized to measure ALT activity. ALT Activity Assay kit was used. GPT, a pyridoxal phosphate-dependent enzyme, catalyzes the reversible transfer of an amino group from alanine to ketoglutaric acid, producing pyruvate, glutamate, and a colorimetric active product proportional to the pyruvate generated.

Activity Levels of Total Proteins in Rat Serum

A colorimetric method was developed by **Gornall, Bardawill** [32] for the purpose of identifying serum proteins. The reagent kit states that the protein produces a violet color when it is in the presence of alkaline cupric sulfate, and the intensity of this color is proportional to the concentration of the sulfate.

The Determination of the Activity of Serum Alkaline Phosphates (ALP)

In order to carry out the experiment, reagent kits were utilized. These kits are dependent on the presence of alkaline phosphatase, which is necessary for the transformation of phenyl phosphate into phenol. The released phenol is then measured using calorimetric analysis in the presence of 4- aminophenazone and potassium ferricyanide [33].

The measurement of the gamma-glutamyl transferase (GGT) level in the serum

The kinetic method that Bergmeyer, Herder [34] developed was utilized in order to determine the level of GGT activity in the serum.

Assessment of the Serum Concentration of Bilirubin

The detection of serum bilirubin was accomplished through the utilization of the colorimetric method that was reported by Belfield and Goldberg [33].

Measurement of the Serum Albumin Concentration

The colorimetric method that was described by Doumas, Watson [35] was utilized in order to determine the amount of serum albumin that was present.

Peroxidation of Lipids in Liver Tissue Analysis and Measurement

The technique that Ohkawa, Ohishi [36] described was utilized in order to ascertain the degree of MDA that might be found in the homogenate of liver tissue. Spectrophotometric analysis was used to determine the MDA level after a colorimetric reaction with thiobarbituric acid was initially performed [37, 38].

The Determination of the Antioxidant Enzyme Activity in Liver Tissue

The Determination of the Activity of GPx

The technique that was described by Paglia and Valentine [39] was utilized in order to calculate the amount of GPx. In order to initiate enzyme action, the substrate, hydrogen peroxide, is initially introduced, and then the A340 has been measured. One may observe a direct correlation between the GPx activity of the sample and the rate of decline in the A340.

The Determination of the Activity of SOD

Kuthan, Haussmann [40] method was utilized in order to determine the amount of SOD. For the purpose of this experiment, the enzyme's ability to prevent the reduction of nitroblue tetrazolium dye that is mediated by phenazine methosulfate is utilized.

Determination of the Inflammatory Markers in Liver Tissue

Estimation of Transforming Growth Factor-beta

For the purpose of determining the levels of TGF-β in the liver tissue of rats, an ELISA kit (Cat. no:SL0708Ra, supplied by SunLong Biotech Co., China) that was specifically created for rat TGF-β was utilized, and the directions prescribed by the manufacturer were strictly adhered to [41].

Assessment of the Levels of Tumor Necrosis Factor α

The concentrations of TNF- α in the tissue of the liver of rats were determined by utilizing ELISA kit (Cat. no:

SL0722Ra, supplied by SunLong Biotech Co., China) specifically designed for rat TNF- α and following the instructions provided by the manufacturer [3].

Assessment of the Levels of Interleukin 1 Beta

In accordance with the instructions provided by the manufacturer, rat IL-1β ELISA kit (Cat. no: SL0402Ra, supplied by SunLong Biotech Co., China) and monoclonal anti-rat antibodies for IL-1β were utilized in order to determine the level of IL-1β which can be found in the tissue of rats' livers. There is a correlation between the absorbance and the amount of rat IL-1β that was collected in the plate [3].

Assessment of the Levels of Caspase-3

An ELISA kit (Cat. no: SL0152Ra, supplied by SunLong Biotech Co., China) particularly designed for rat caspase-3 was employed to ascertain the amounts of caspase-3 in rat liver tissue, following the manufacturer's instructions meticulously [42].

Histopathological Examination and Analysis

Formalin with a neutral buffer at a concentration of 10% was used to fix liver tissue samples. In the following steps, the substance is dehydrated using alcohol of increasingly higher grades, clarified using xylene, and then embedded in paraffin. Sections of tissue were cut at a thickness of 5 microns, and they were stained with H&E [3].

Immunohistochemical Examination of Nuclear Factor-κB Detected in Liver Tissue

The immunohistochemical analysis approach was utilized in the investigations that we had previously carried out [3]. After computing the mean number for each slide, the mean ± standard error for each group was calculated. This was done after the computation of the mean number. There were five distinct microscopic regions that were used to count the number of immunopositive cells that were present on each slide.

Statistical Analysis

As a consequence of the fact that the Shapiro-Wilks normality test revealed that each of the variables has a normal data distribution, parametric statistical analysis was carried out. When comparing all of the variables, the Tukey's multiple comparisons test was utilized in conjunction with the one-way analysis of variance (GraphPad Prism, version 8.0.2). When the value of P was less than 0.05, it was determined that statistical significance had finally been attained.

Results

Features of Nanoparticles Loaded With Sildenafil and Neem Extract

The TEM pictures in Supplementary Fig. S1 reveal that the NPs containing sildenafil have a regular spherical shape and a suspension form, and their diameter is 200 ± 50 nm. The charge density of PLGA NPs was found to be − 0.778 mV, as shown in Supplementary Fig. S2A, using a Zeta Potential Analyzer. After loading sildenafil into PLGA, the charge density decreased to − 0.453 mV, as shown in Supplementary Fig. S2B.

The results of Zetasizer instrument demonstrated that the vesicle size of the freshly prepared niosomes dispersion was found to be 210.5 ± 13.65 nm and PDI was 0.345 ± 0.11 (Fig. 2A). Furthermore, the value of zetapotential of neem extract loaded niosomes was found to be −21.60 ± 1.3 mV (Fig. 2B). Niosomes loaded with formulation neem extract are seen in TEM images (Fig. 3A, B). There is no evidence of aggregation, and the niosome vesicles are round, uniformly dispersed, and of uniform size.

Elevated Plus Maze Test

The elevated plus maze test showed that rats with CCL_4-induced liver damage had a significant ($p < 0.0001$) decrease in both the number of times they entered and the amount of time they spent in the open arms. Nevertheless, the frequency of entrances and duration in closed arms escalated relative to the negative control group. Compared to the CCL_4-induced liver damage group, rats that were given PLGA and niosomes did not show any significant changes in the number of times they entered or stayed in open or closed arms over time. Utilization of traditional treatments or NP formulations of sildenafil and neem extract, either individually or in combination, resulted in a significant increase in the frequency of entries and duration spent in open arms, alongside a reduction in the frequency of entries and duration spent in closed arms, when compared to the CCL_4-induced liver damage group. In the elevated plus maze test (Fig. 4), the combination-NP group did better than the rats that were given the same standard treatments.

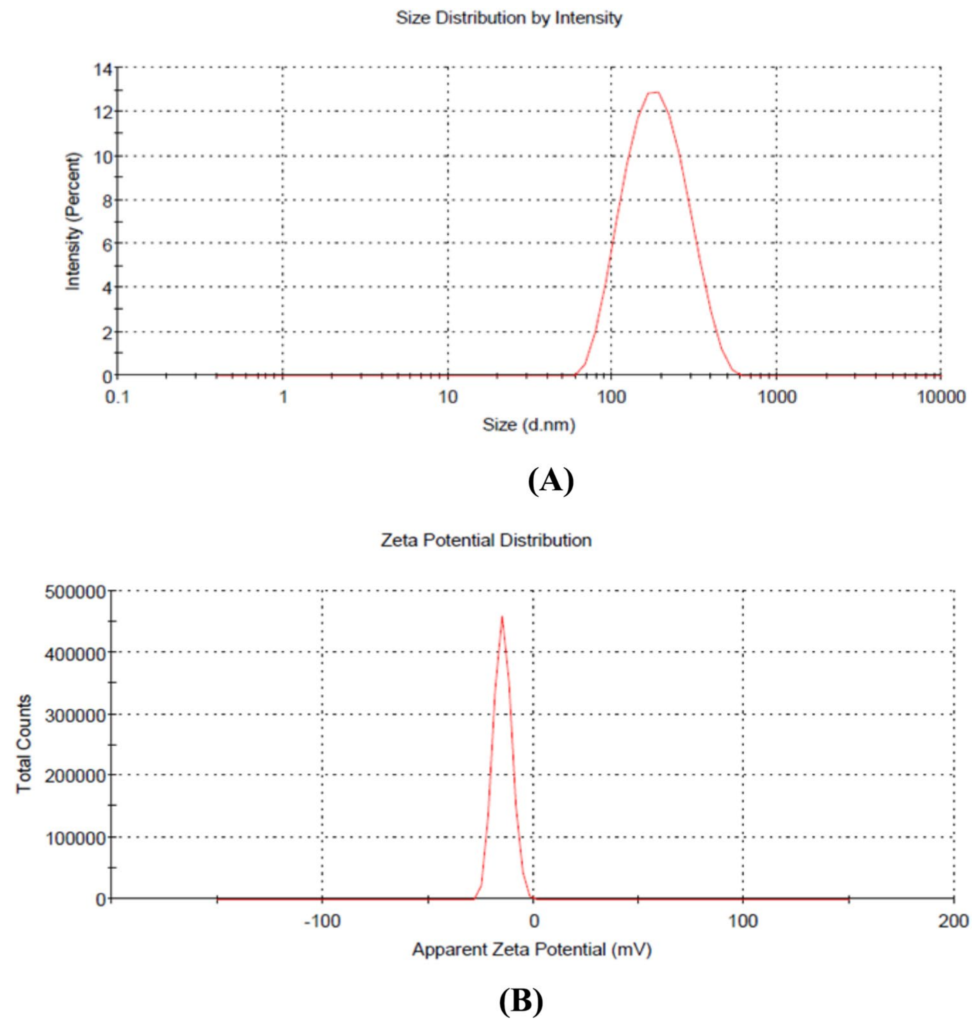


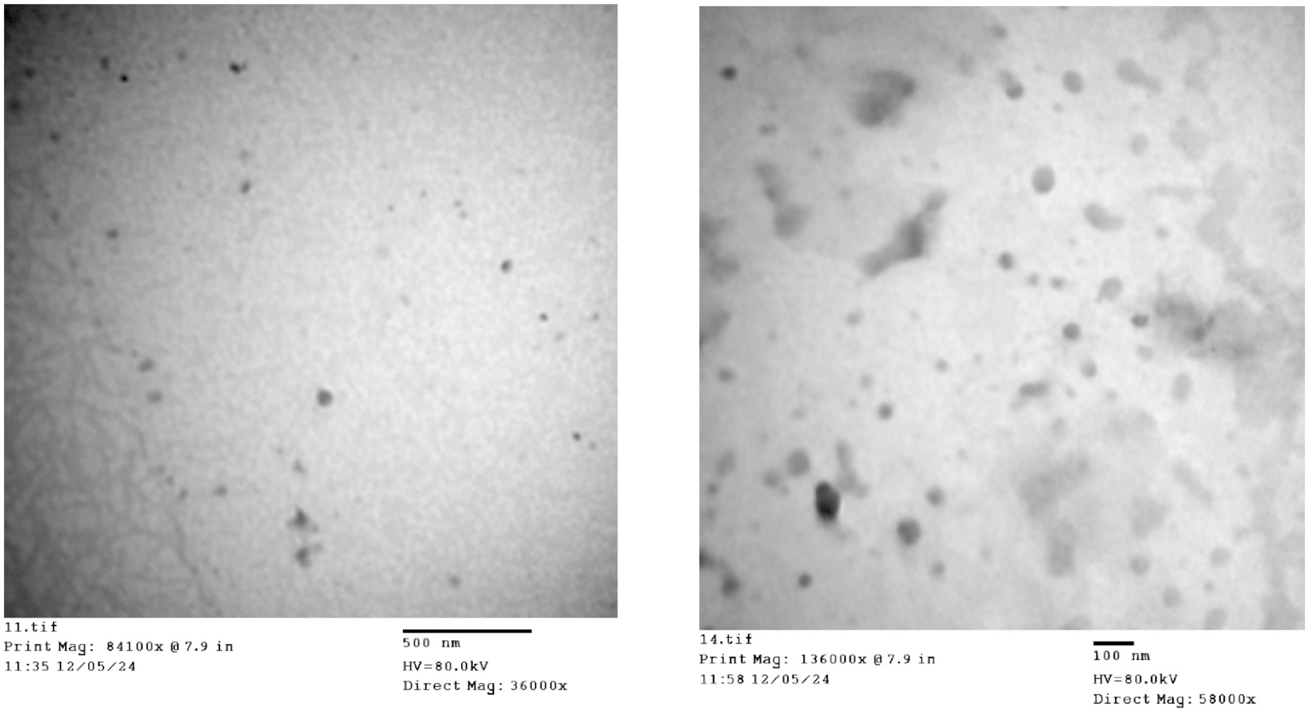
Fig. 2 **A**) Vesicle size distribution and **B**) Zetapotential distribution of the prepared neem extract loaded with niosomes

The Impact of Sildenafil, Neem Extract, and Their Nanoparticle Formulations Alone or in Combination On Blood Albumin, Total Protein and Bilirubin (direct, Total) Levels

Rats treated with CCl_4 had significantly elevated levels of blood albumin ($p < 0.0001$), direct bilirubin ($p < 0.0001$), and serum total bilirubin ($p < 0.0001$), with reduced total protein levels ($p < 0.0001$) compared to the negative control group. In contrast to the CCl_4-induced liver damage group, rats administered PLGA or niosomes exhibited no significant alterations in the levels of the four blood indicators. In comparison to the CCl_4-induced liver damage group, both conventional and NP versions of sildenafil, neem extract, or their combination led to a significant reduction ($p < 0.0001$) in blood albumin, direct bilirubin, and total bilirubin levels, alongside an elevation in total protein levels. It is interesting that, compared to rats treated with their respective conventional medications, animals that were provided with NP versions of sildenafil, or neem extract, or their combination, demonstrated a considerable reduction in blood albumin, direct bilirubin, total bilirubin, and an increase in total protein, as indicated in Fig. 5.

The Impact of Sildenafil, Neem Extract, and Their Nanoparticle Derivatives On the Liver Function Markers

The group that was exposed to CCl_4 showed considerably greater levels of the liver enzymes AST, ALT, GGT, and ALP than the negative control group ($p < 0.0001$). In rats treated with PLGA and niosomes, serum AST, ALT, GGT, and ALP levels were not substantially different from rats with liver damage. In comparison to the CCl_4-induced



(A) **(B)**

Fig. 3 Transmission electron microscopy photomicrographs of the fabricated neem extract loaded with niosomes at various magnifications

liver damage group, both conventional and NP forms of sildenafil, neem extract, or their combination resulted in a substantial reduction in AST, ALT, GGT, and ALP levels ($p < 0.0001$). Compared to their conventional forms, the decline was particularly significant in rats administered NP formulations of sildenafil, neem extract, and their combination (Fig. 6).

The Impact of Sildenafil, Neem Extract, and Their Nanoparticle Formulations On Tissue MDA, SOD and GPX Levels in Rats Induced With Acute Liver Damage

CCl_4-induced liver damage revealed a large rise in tissue MDA levels ($p < 0.0001$) and a major drop in SOD and GPX levels ($p < 0.0001$) compared to the negative control group, as depicted in Fig. 7. Rats treated with PLGA and niosomes did not reveal significant variations in MDA, SOD, and GPX levels compared to the CCl4-induced liver damage group. When traditional treatments or NP forms of sildenafil, neem extract, or their combination were given, there was a major drop in tissue MDA levels ($p < 0.0001$), while SOD and GPX levels dramatically increased ($p < 0.0001$) compared to the acute liver damage-positive control group. In comparison to animals who received the same conventional treatments, the combination-NP group displayed a large rise in tissue SOD and GPX and a considerable drop in tissue MDA levels ($p < 0.0001$) (Fig. 7).

The Impact of Sildenafil, Neem Extract, and Their Nanoparticle Formulations On Pro-inflammatory Cytokines (TNF-α and IL-1β), Profibrotic Factor (TGF-β), and Apoptotic Factor (caspase-3) Levels in Rats Induced With Acute Liver Damage

When compared with the negative control group, the CCl_4-induced liver damage group revealed a substantial rise in the levels of tissue TNF-α ($p < 0.0001$), IL-1β ($p < 0.0001$), TGF-β ($p < 0.0001$), and caspase-3 ($p < 0.0001$). However, when these results were contrasted with the acute liver damage-positive control

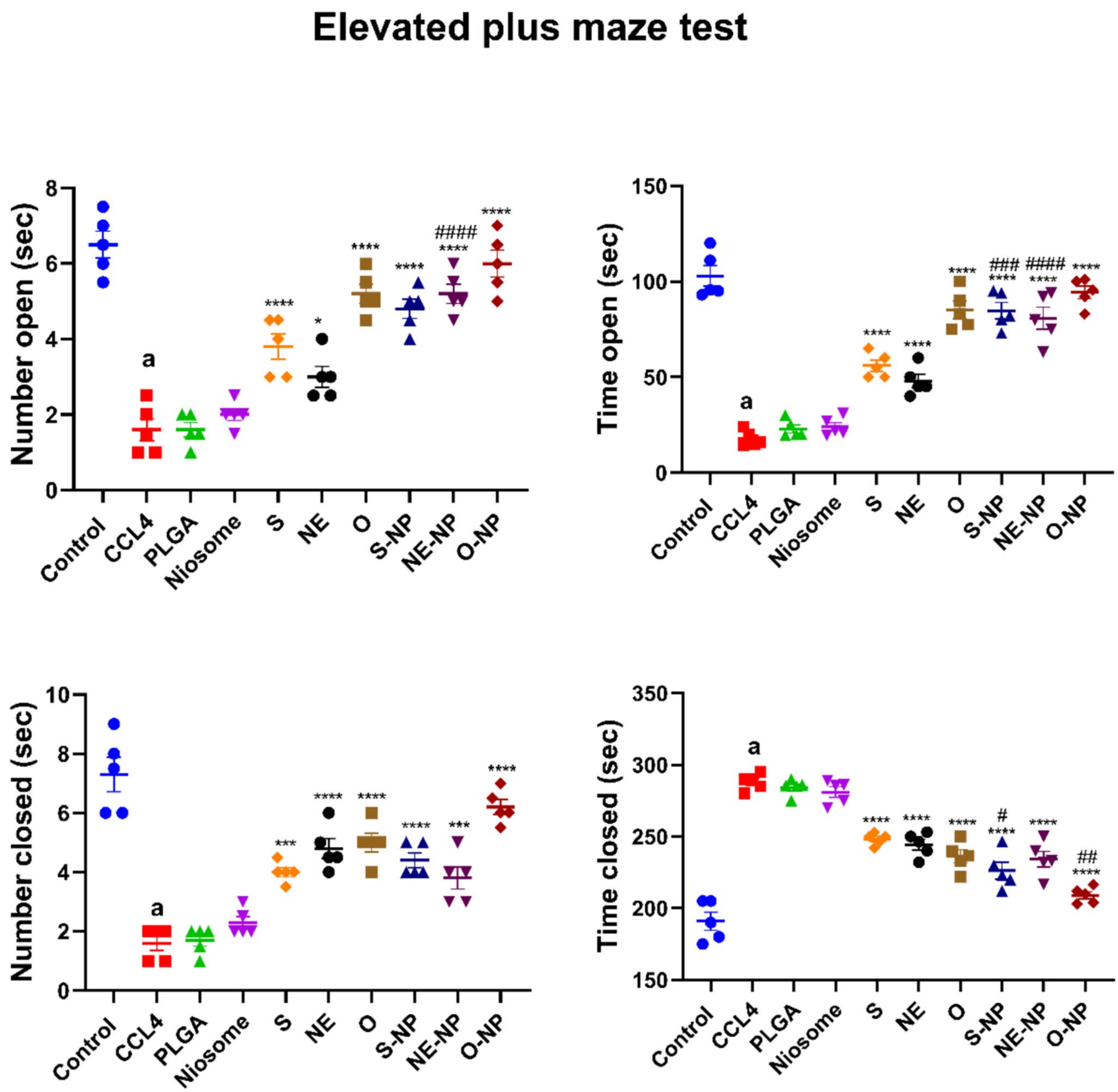


Fig. 4 Acute liver damage in rats caused by carbon tetrachloride (CCL_4) and the effects of various treatments on the elevated plus maze behavioral test. [a]$P < 0.0001$ in comparison to with the control group. $^{*}p < 0.05$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ in comparison to the group with CCL_4. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and. $^{\#\#\#\#}p < 0.0001$ in contrast to the related nanoparticle category. PLGA (polylactic-co-glycolic acid), S (sildenafil), NE (neem), O (sildenafil-neem combination), S-NP (sildenafil nanoparticle), NE-NP (neem nanoparticle) and O-NP (treatment combination nanoparticle)

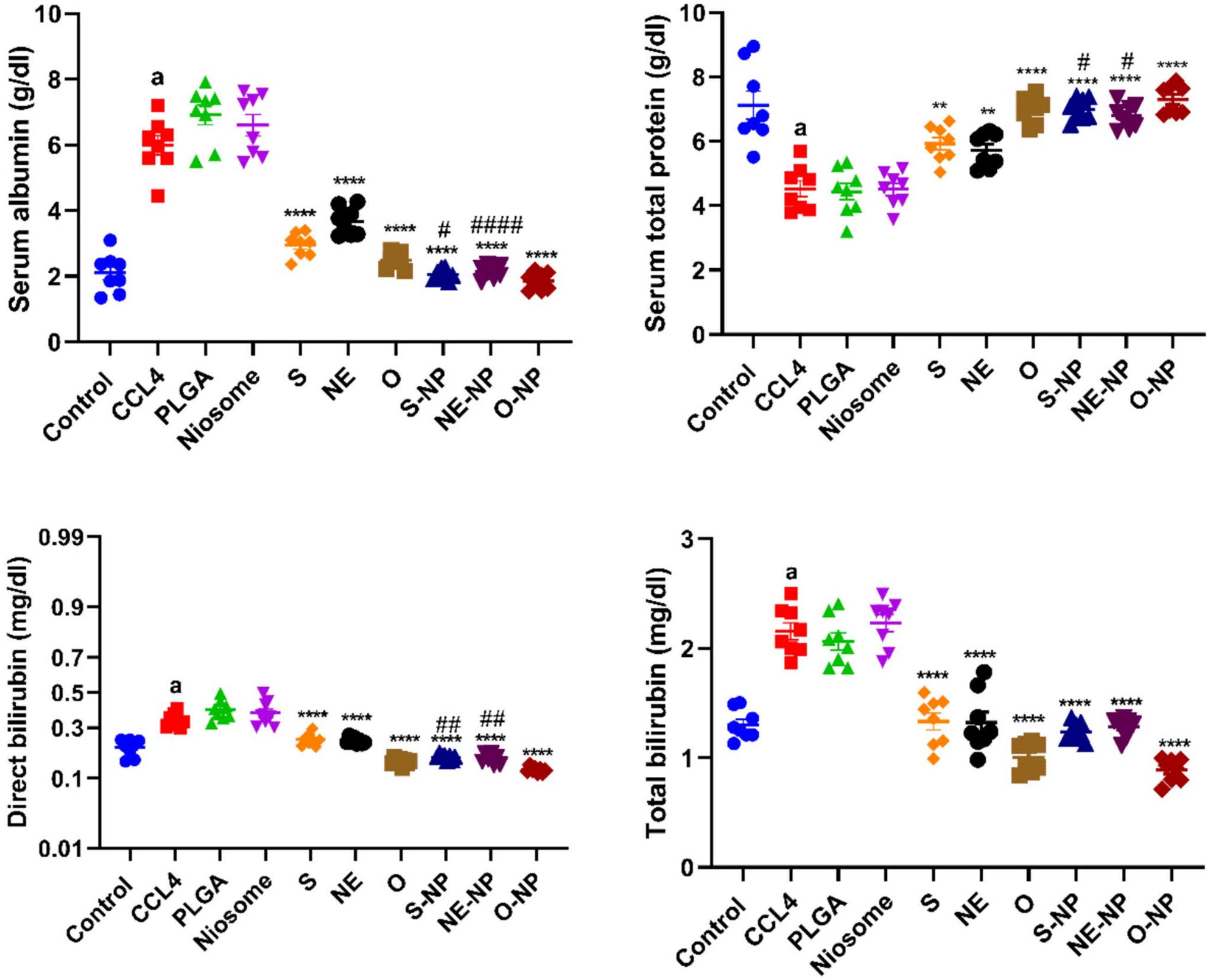


Fig. 5 Acute liver damage in rats caused by carbon tetrachloride (CCL_4) and the effects of various treatments on the serum levels of albumin, total proteins and bilirubin. $^{a}P<0.0001$ in comparison to with the control group. $^{**}p<0.01$ and $^{****}p<0.0001$ in comparison to the group with CCL_4. $^{\#}p<0.05$, $^{\#\#}p<0.01$ and.$^{\#\#\#\#}p<0.0001$ in contrast to the related nanoparticle category. PLGA (polylactic-co-glycolic acid), S (sildenafil), NE (neem), O (sildenafil-neem combination), S-NP (sildenafil nanoparticle), NE-NP (neem nanoparticle) and O-NP (treatment combination nanoparticle)

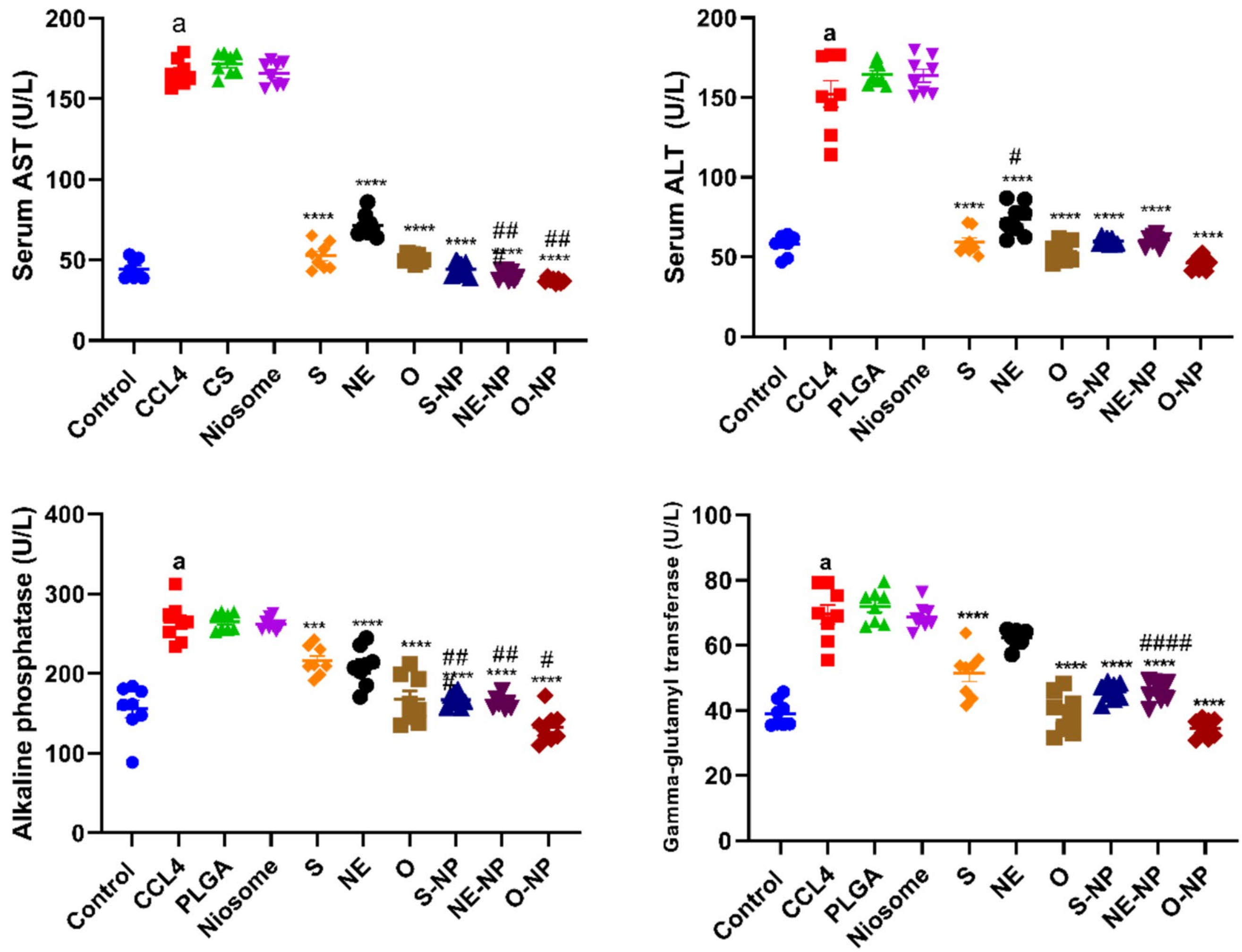


Fig.6 Acute liver damage in rats caused by carbon tetrachloride (CCL_4) and the effects of various treatments on serum levels of alanine aminotransferase (ALT), alkaline phosphates, aspartate aminotransferase (AST) and gamma-glutamyl transferase. $^{a}P<0.0001$ in comparison to with the control group. $^{***}p<0.001$ and $^{****}p<0.0001$ in comparison to the group with CCL_4. $^{\#}p<0.05$, $^{\#\#}p<0.01$ and.$^{\#\#\#\#}p<0.0001$ in contrast to the related nanoparticle category. PLGA (polylactic-co-glycolic acid), S (sildenafil), NE (neem), O (sildenafil-neem combination), S-NP (sildenafil nanoparticle), NE-NP (neem nanoparticle) and O-NP (treatment combination nanoparticle)

group, no substantial changes were found in the levels of tissue of these four markers in the rats treated with PLGA or niosomes. In contrast to the acute liver damage-induced group, a significant reduction (p < 0.0001) in the levels of tissue TNF-α (p < 0.0001), IL-1β (p < 0.0001), TGF-β (p < 0.0001), and caspase-3 (p < 0.0001) were observed when either the conventional or NP versions of sildenafil, neem extract, or their combination were administered. It is of particular interest to note that, when compared to rats that received their respective conventional drugs, the animals treated with nanoparticle versions of sildenafil or neem extract, or their combination, displayed a significant reduction in the levels of the four liver tissue markers, as illustrated in Fig. 8.

Histopathological Analysis

Table 1 lists the lesion score of the histopathological test findings for each of the groups that were investigated. Normal architecture was seen when the hepatic tissue in the control negative group was examined. The group treated with CCL_4 exhibited either parenchymal or vascular alterations. Vascular

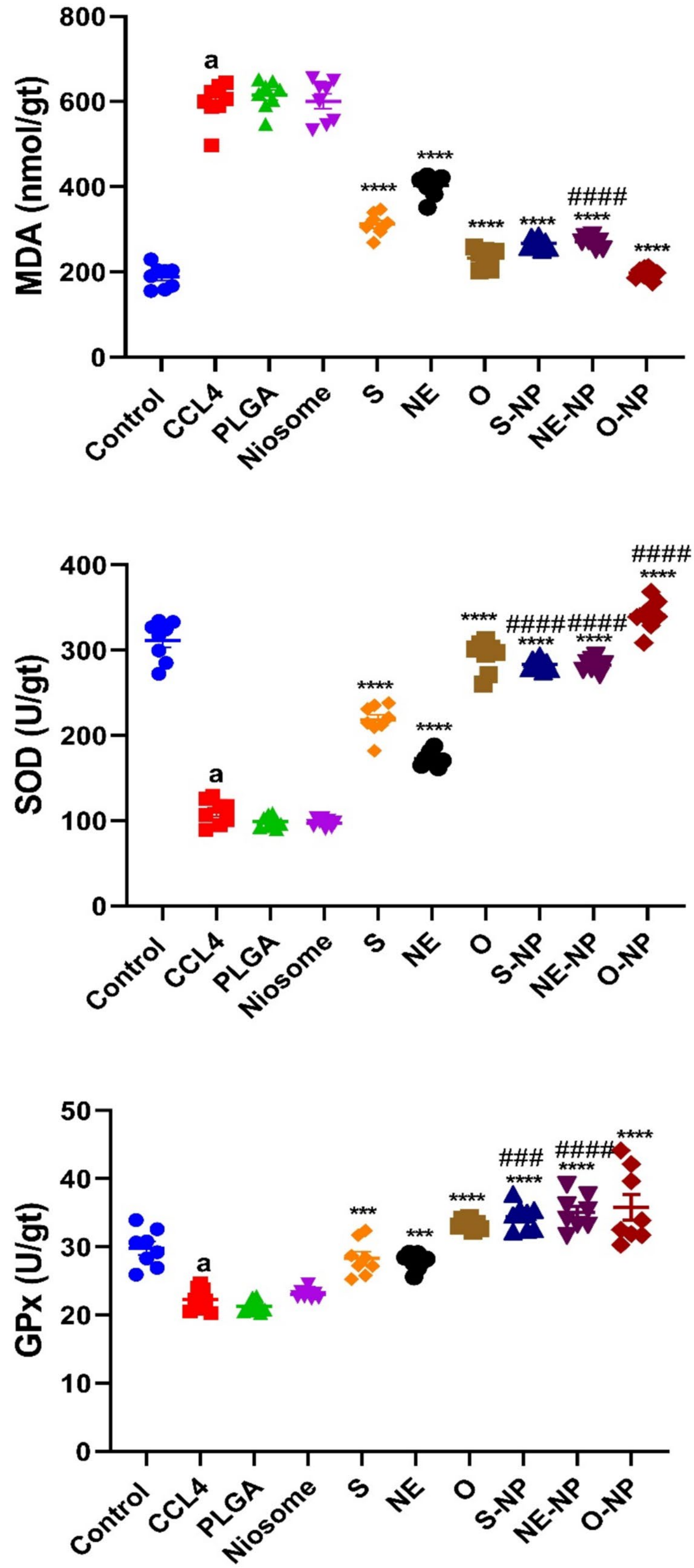


Fig. 7 Acute liver damage in rats caused by carbon tetrachloride (CCL_4) and the effects of various treatments on tissue levels of malondialdehyde (MDA), glutathione peroxidase activity (GPx) and superoxide dismutase (SOD). $^{a}P < 0.0001$ in comparison to with the control group. $^{***}p < 0.001$ and $^{****}p < 0.0001$ in comparison to the group with CCL_4. $^{###}p < 0.001$ and.$^{####}p < 0.0001$ in contrast to the related nanoparticle category. PLGA (polylactic-co-glycolic acid), S (sildenafil), NE (neem), O (sildenafil-neem combination), S-NP (sildenafil nanoparticle), NE-NP (neem nanoparticle) and O-NP (treatment combination nanoparticle)

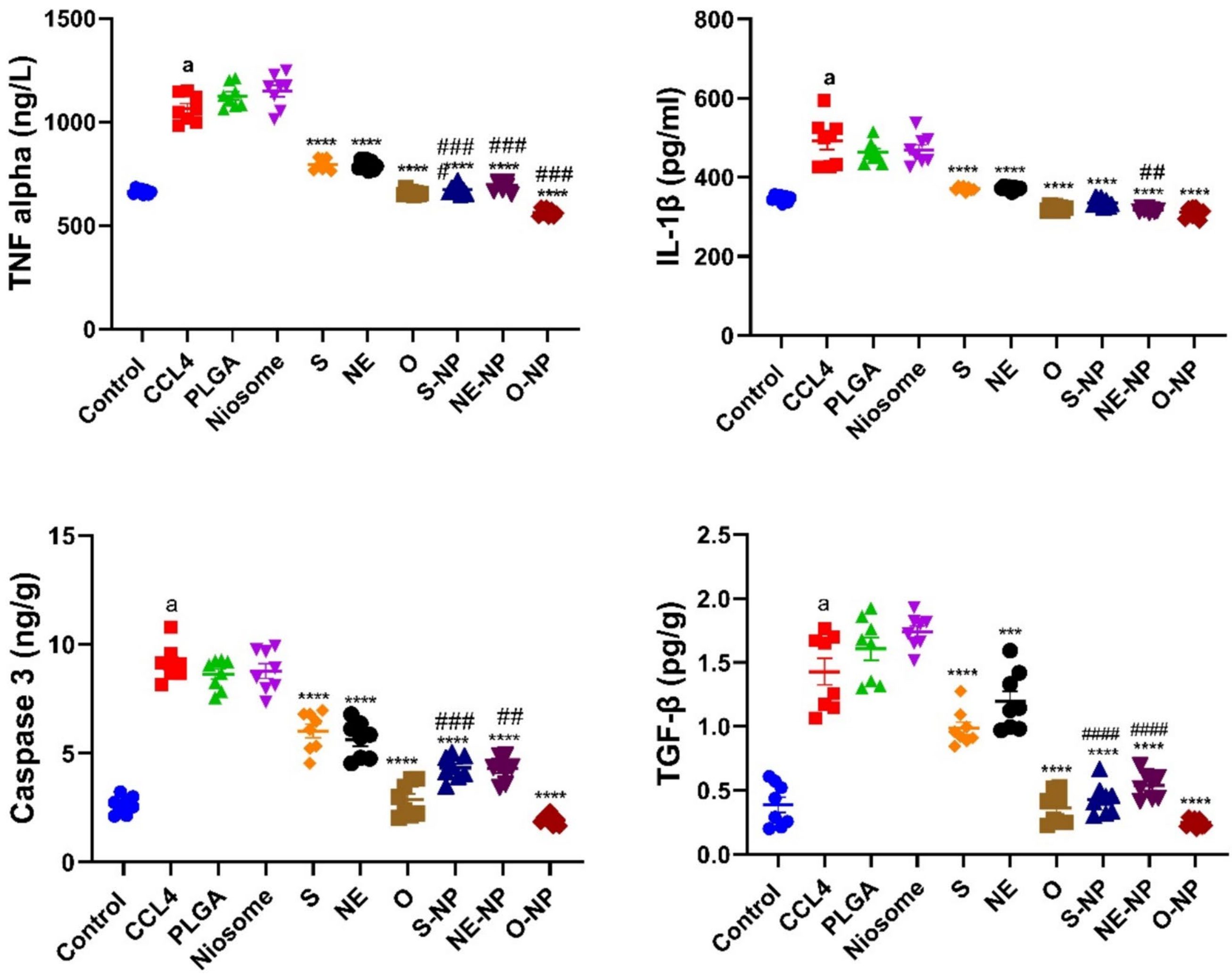


Fig. 8 Acute liver damage in rats caused by carbon tetrachloride (CCL_4) and the effects of various treatments on tissue levels of tumor necrosis factor alpha (TNF-α), interleukin- 1 beta (IL-1β), caspase-3 and transforming growth factor-beta (TGF-β). [a]P < 0.0001 in comparison to with the control group. ****p < 0.0001 in comparison to the group with CCL_4. ##p < 0.01, ###p < 0.001 and.####p < 0.0001 in contrast to the related nanoparticle category. PLGA (polylactic-co-glycolic acid), S (sildenafil), NE (neem), O (sildenafil-neem combination), S-NP (sildenafil nanoparticle), NE-NP (neem nanoparticle) and O-NP (treatment combination nanoparticle)

alterations include perivascular cuffing, thrombosis, and congestion of the blood vessels. Hepatocyte fatty changes and mononuclear cell focal regions were the hepatocellular alterations. The hepatic system improved in the groups treated with neem (normal form and niosomes), while there were some fatty alterations in the hepatocytes. Both the normal and nanoforms of sildenafil showed improvement in the hepatic system, but some hepatocytes showed lipid alterations and some central vein erythrocytes aggregated. The hepatic portal triad with normal architecture improved in the combination-treated group in both traditional and nanoforms (Fig. 9).

Analysis of Immunohistochemistry

Concerning NF-κB in rat liver that has been immunohistochemically displayed. NF-κB immunoreactivity was significantly elevated in CCL_4-treated groups compared to those treated with neem and sildenafil (normal and nanoforms). No expression was seen in the control negative group or the combination treated groups in the normal and NP forms (Fig. 10) (Table 2).

Table 1 Damage ratings for liver tissue histology in the groups under study

Lesions Groups	Congestion of CV	Erythrocyte aggregation	Fatty changes	Mononuclear cell infiltration	Detached fibrosed tissue
Control	-	-	-	-	-
CCL_4	+ + +	+ +	+ + +	+ + +	+ +
PLGA	+ +	+ +	+ + +	+ +	+ +
Niosome	+ +	+ +	+ + +	+ +	+ +
NE	-	-	+	+	+
NE-NP	-	-	+	+	+
S	-	+	+ +	+	+
S-NP	-	+	+ +	+	+
O	-	+	+	+	+
O-NP	-	+	+	+	+

- No lesions, + lesions present in 2–3 sections, + + lesions present in 4–7 sections and + + + lesions present in 8–10 sections

-CCL_4 (carbon tetrachloride), NE (neem), NP (nanoparticle), S (sildenafil), O (combination of NE and S), O-NP (combination nanoparticle group) and PLGA (polylactic-co-glycolic acid)

Discussion

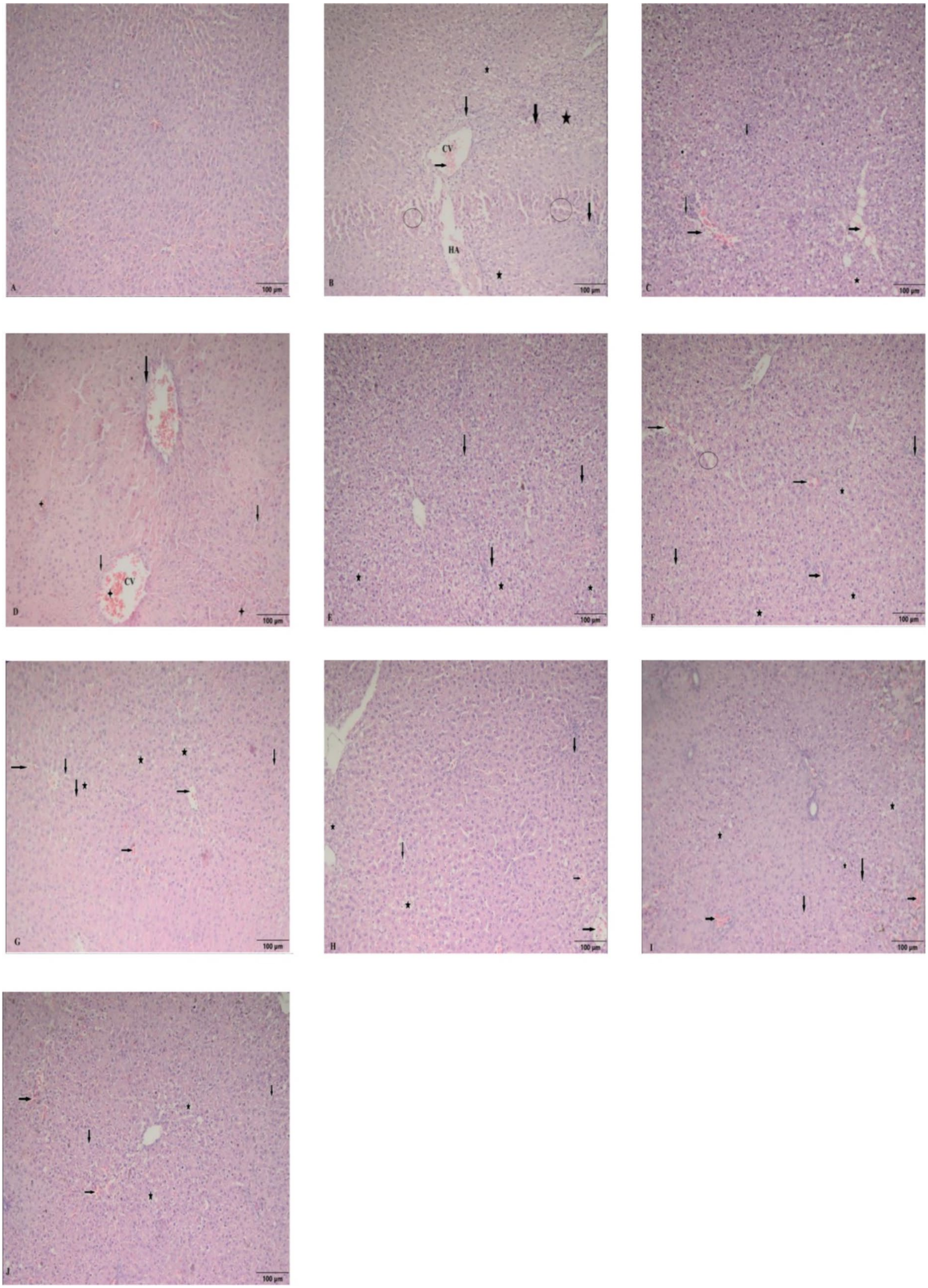
In recent years nano-particulate based systems have been utilized as a promising alternative to conventional drug delivery systems. Nano formulations were developed to deliver drug products into targeted sites that were achieved through the interesting physicochemical properties regarding small particles size and high surface area [16].

The current work presents the development of niosomes formulation loaded with neem seed extract using mixture of cholesterol and nonionic surfactants (Span 40 and Tween 80) with a view to improving the oral delivery of therapeutic active molecules [43]. The prepared vesicles were found in nanosized range which would reveal the improvement of oral delivery and systemic absorption of neem extract. The detected Lower value of PDI for the prepared neem extract loaded vesicles is considered appropriate for drug delivery due to the homogenous distribution of nanoplatforms [44]. Also, the higher negative value of zetapotential suggests the adequate physical stability of nonionic surfactant vesicles. The results obtained are in agreement with previous studies [45, 46]. Furthermore, since there is no force separating the particles, those with a low zeta potential that emerged with the sildenafil-PLGA nanocomposite will flocculate in accordance with previous study [10].

Damage to the liver is produced by a number of mechanisms, including oxidative cellular stress, cellular membrane peroxidation, and inflammation. These are all induced by CCl_4. In terms of both physiological and pathological characteristics, the hepatotoxic experimental rat model of CCl_4 damage is very similar to the hepatotoxic liver injury that occurs in humans [47]. The liver contains an enzyme called CYP2E1, which is responsible for converting CCl_4 into potentially harmful reactive trichloromethyl and trichloromethyl peroxide radicals. After that, these reactive radicals bind themselves to unsaturated fatty acids that are present in the membranes of hepatic cells, mitochondria, and the endoplasmic reticulum. This causes a chain lipid peroxidation process to begin, which ultimately results in structural damage and death of hepatocytes as well as intracellular cells [48].

The occurrence of this event reveals that CCl_4 is a potent hepatotoxin that has the potential to be utilized in the process of defining medications that cure liver disease. One of the biomarkers of hepatotoxicity is a change in the amounts of transaminase and phosphatase contained inside the liver [3]. Stabilization of these parameters represents an improvement. A high level of AST indicates that the functional integrity of the liver has been damaged, which is similar to the effects that viral hepatitis, myocardial infarction, and muscle injury have on the body. ALT enzymes are released from hepatocytes into the bloodstream in the same manner that AST enzymes are [49]. A high level of ALP indicates that there is a blockage of the biliary system either infiltrative, intrahepatic, or extrahepatic, or that the liver is sick [50]. The measurement of the concentration of bilirubin in the serum is an indication of the liver's ability to take bilirubin from the blood and transport it to the hepatocyte [51].

The current research revealed that the combination of sildenafil and neem with both conventional and NP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CV

Fig. 9 Representative micrograph of the liver of the examined groups stained by with H&E. A) Control negative group exhibited ideal normality of hepatic tissue. B) CCL_4 induced group exhibited congestion of the central vein (CV), congestion of the hepatic arteries, detached fibrosed tissue (black circles), focal areas of mononuclear cell infiltration (down-head arrows), fatty changes of hepatocytes (black stars) and erythrocytes aggregations (right-handed black arrows). C, D) nano-particles base groups exhibited more congestion of CV with less fatty change (black stars) and less mononuclear cell infiltration (down-head arrows). E, F) CCL_4 + neem (normal and nano-forms) treated group exhibited improvement of the liver cells with some fatty changes. G, H) CCL_4 + sildenafil (normal and nano-forms) exhibited fewer fatty changes of hepatocytes (black stars), with few areas of mononuclear cell infiltration (down-head arrows) and less aggregation of erythrocytes (right-handed black arrows). I, J) CCL_4 + neem + sildenafil (normal and nano-form) treated groups exhibited improvement of the hepatic cells with normal architecture

forms, either alone or in-combination, was able to normalize elevated levels of AST, ALT, ALP, GGT, albumin and bilirubin, as well as increase total protein activity in a rat model of liver damage that was produced by orogastric CCL_4 administration. Similarly, Nasr, Hegazy [14] 's research found that sildenafil is effective in alleviating the hepatic dysfunctions that are brought on by CCl_4 (i.p.). The restoration of normal levels of ALT, AST, and GGT, in addition to the restoration of the antioxidant status, contributes to the achievement of this goal. In addition, pretreatment with sildenafil at dosages of 5 mg, 10 mg, and 20 mg/kg, orally, resulted in a moderate reduction in the activities of ALP, AST, and ALT, while also causing a minor increase in the total protein level in CCl_4 induced hepatotoxicity when administered intraperitoneally [13]. The findings of previous research also lend weight to these conclusions [3].

Previous research has demonstrated that pretreatment with neem leaves or seed oil at higher dose levels can restore normal function to the liver of rats [22, 52, 53]. In the context of CCl_4-induced hepatotoxicity, there is no research that we are aware of that has explored the nanoparticulation of sildenafil and neem. There is no such research. In this particular investigation, nanoparticle formulations demonstrated a hepatoprotective impact that was superior to that of alternative formulations.

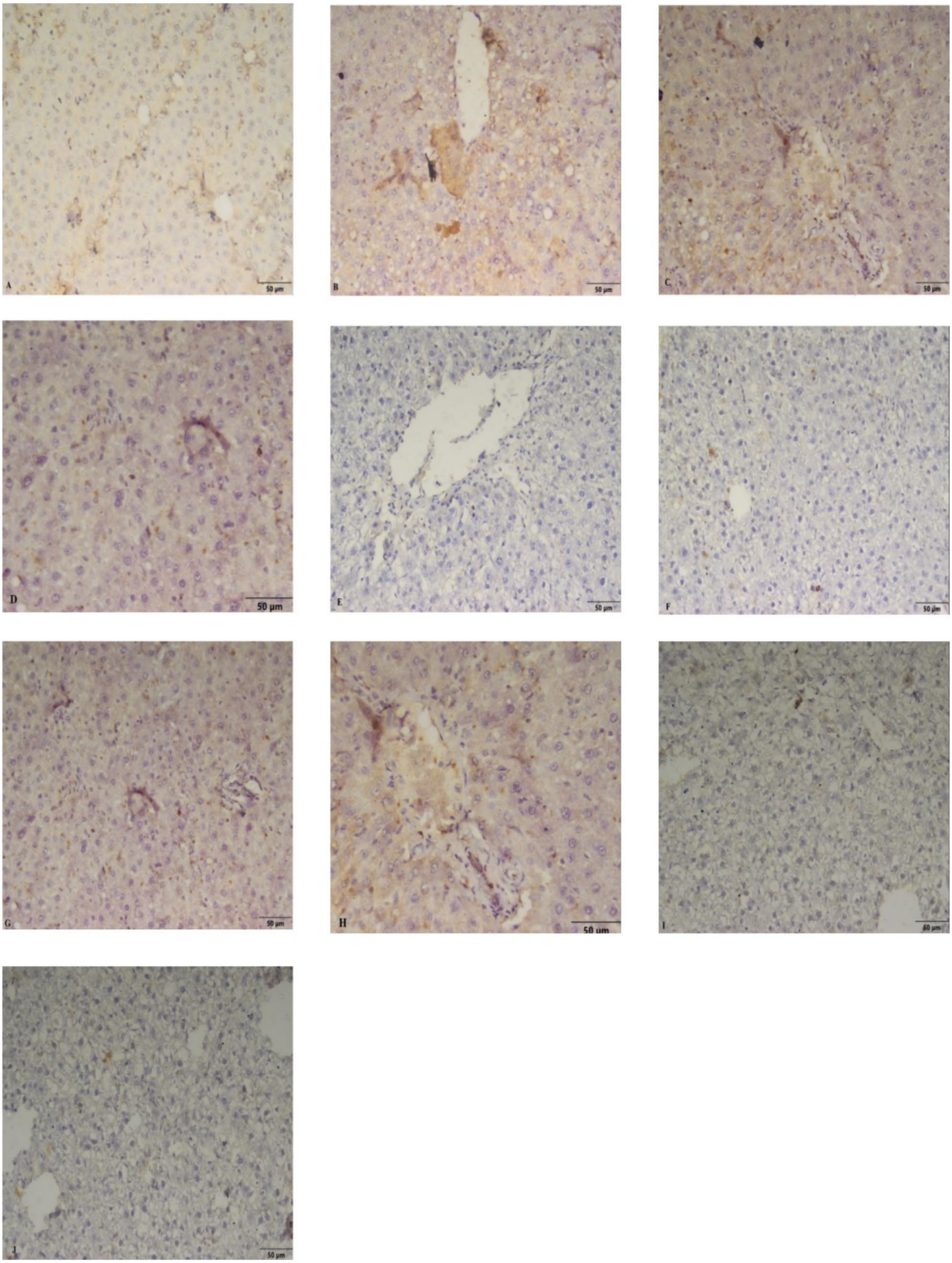
A well-known rat behavioral test for examining the anti-anxiety effects of pharmaceutical drugs is the elevated plus maze. Anxiogenic medications that selectively decrease open arm exploration while increasing closed arm exploration, and anxiolytic medications that preferentially increase open arm exploration while lowering enclosed arm exploration. Additionally, there is growing evidence that nitric oxide may contribute to anxiety in the raised plus maze anxiety test. It is commonly recognized that the cGMP-NO pathway controls rat anxiety. There is conflicting evidence, nevertheless, about whether activating the NO-cGMP pathway causes anxiety-like behavior to grow or decrease [3].

The majority of data suggests that the NO-cGMP pathway is anxiogenic when activated and anxiolytic when inhibited [54]. Rats given sildenafil over an extended period of time experience antidepressant-like effects, but only when muscarinic receptor antagonism is also present [55]. Our findings were supported by a publication that suggested steroidal extract of dried neem could have anxiolytic effects in rats without inducing motor adverse effects [56]. Additionally, neem flower aqueous extract showed anxiolytic and antidepressant-like actions in stressed rats [57].

The antioxidant enzymes in the cells become inactive as a result of the oxidative stress that is generated by CCl_4. The enzymes peroxidase, catalase, and SOD are the primary antioxidative enzymes that are responsible for neutralizing free radicals. Enzyme peroxidase is responsible for the transformation of hydrogen peroxide and lipid peroxides into species that are not reactive. The enzyme known as SOD is responsible for producing molecular oxygen or hydrogen peroxide by catalyzing the transition of superoxide radicals. The reduction of anti-oxidative enzymes results in the accumulation of oxygen dioxide and hydrogen peroxide, which is a free radical cascade process that leads to damage to the liver [47]. Oxidative stress is being imposed on lipid membranes as a result of the presence of large concentrations of polyunsaturated fatty acids and transition magnetic elements. In addition to their ability to cause damage to cellular nuclear proteins and DNA, transition metals, such as iron, have the capacity to inhibit antioxidant enzymes and breakdown lipid membranes through the oxidative process through Haber–Weiss reaction [58].

Carbon tetrachloride poisoning was found to be responsible for a considerable increase in MDA levels as well as a significant drop in the activity of antioxidant enzymes SOD and GPx., according to the findings of the current analysis. After receiving treatment with sildenafil and neem in both conventional and NP forms, either alone or in-combination, these changes that had become more severe recovered to their usual state. The findings presented here are in agreement with those presented in [13, 14, 22, 53].

Pro-inflammatory cytokines, such as TNF-α and IL-1β, play a crucial role in the development of various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◂**Fig. 10** Immunohistochemical staining of nuclear factor kappa B (NF-κB) in rat liver. A) Control negative groups showing seldom expression of NF-κB. B) CCL_4 treated showing a significant increase in NF-κB immunoreactivity in the cytoplasm of hepatocytes. Brown color indicates NF-κB positivity. C, D) nano-particles base groups showing a non-significant increase in NF-κB positivity. E, F) CCL_4 + neem (normal and nan-form) treated groups showing nearly no expression of NF-κB. G, H) CCL_4 + sildenafil (normal and nano-form) treated groups showing non-significant reduction in NF-κB immunostaining. I, J) CCL_4 + neem + sildenafil (normal and nano-form) treated groups showing a significant reduction in NF-κB immunostaining

characteristics associated with non-alcoholic fatty liver disease in human beings [59]. With its ability to regulate adaptive immunity, IL-1β is a pleiotropic cytokine that has a wide range of impacts, including those on inflammation, liver regeneration, and the immune system's ability to fight off infections. The cytokine known as IL-1β is frequently considered to be harmful because of its high prevalence in inflammatory environments. On the other hand, an increasing amount of study is being conducted that lends credence to the idea that IL-1β has a helpful function in a variety of liver diseases. This is because of its roles in liver regeneration and in supporting an anti-inflammatory response in specific situations [60]. Additionally, cytokines and mediators that are pro-inflammatory and inflammatory, such as TNF-α, IL-1β, NF-κβ, and TGFβ, which are produced by Kupffer cells, are believed to play significant roles in the process of hepatic inflammation Additionally, caspase-3 is a sensitive marker that detects damage to the liver and is also linked to fibrosis of the liver [61].

Carbon tetrachloride poisoning was found to result in a considerable increase in TNF-α, TGFβ, caspase 3 and IL-1β, according to our study findings. In addition, the conditions that had become more severe reverted to their usual state following therapy with sildenafil and neem, particularly when they were administered together in NP forms. The findings presented here revealed that the NP combination of sildenafil and neem exhibited powerful anti-inflammatory effect by inhibiting the production of pro-inflammatory mediators that are associated with acute hepatotoxicity.

Similarly, the research conducted by El Awdan, Abdel Rahman [61] lends confirmation to our findings.

Thrombosis, cellular congestion, vacuolar deterioration, and mononuclear cell permeation were some of the vascular and hepatic alterations that occurred as a consequence of hepatotoxicity brought on by CCL_4 in the current experiment. Injury to the liver caused by CCl_4 is frequently utilized in the process of evaluating hepatoprotective medications. By converting CCl_4 into free radicals through the process of biotransformation, hepatotoxicity is produced. According to our findings, the effects of sildenafil and neem in hepatoprotection against CCl_4-induced hepatotoxicity are consistent with those found in prior studies [4, 14, 52]. On the other hand, there is no research that reviews the hepatoprotective effects of their nanoparticle formulations, either on their own or in conjunction with other substances.

Interleukins, TNF-α, lipopolysaccharide, and antigen are the primary proteins that are responsible for activating the canonical NF-κB signaling pathway. In response to a number of bridging proteins, these activators will cause the activation of NF-κB signaling by binding to cell surface receptors and causing the signaling pathway to commence [62]. Additionally, it has been brought to light that TGF-β, via activating the NF-κB signaling pathway, is responsible for the stimulation of cardiac inflammation and fibrosis [63]. Therefore, due to the activation of interleukins, TNF-α, and TGF-β, the NF-κB signaling pathway is influenced. In the present work, we investigated the immunoexpression of NF-κB in liver tissues.

According to the findings of research conducted by Toriumi, Horikoshi [64], the production of diacylglycerol-O-(OH) occurs during the process of liver caused by CCl_4. This process ultimately results in the triggering of the protein kinase C/ NF-κB pathway and the demonstration of TNF- α-induced worsening of liver injury. According to the findings of the present investigation, the immunohistochemical staining for NF-kB was significantly higher in the group that received CCL_4 treatment, whereas it decreased after receiving treatment with sildenafil and neem, particularly in NP forms.

Table 2 Effects on the percentage expression of nuclear factor-κB (NF-κB) in the liver of rats subjected to CCL4 hepatotoxicity of traditional neem (NE) extract, sildenafil (S), and their nanoparticles (NP) both separately and together

Groups	Control	CCL_4	PLGA	Niosome	NE	NE-NP	S	S-NP	O	O-NP
NF-κB	5.412 ± 0.3421^a	81.08 ± 1.146^b	78.36 ± 0.653^b	79.74 ± 1.264^b	26.53 ± 0.423^c	32.265 ± 0.362^d	33.89 ± 0.643^d	25.97 ± 0.642^c	19.965 ± 1.086^e	7.085 ± 0.875^a

[a,b,c,d,e] values are not sharing a common superscript letter differ significantly at $P < 0.05$

-CCL_4 (carbon tetrachloride), O (combination of NE and S), O-NP (combination nanoparticle group) and PLGA (polylactic-co-glycolic acid)

In conclusion, important evidence for the effectiveness of sildenafil and neem extract NP formulations in treating acute liver damage in rats has been presented in this study. These NP formulations show great potential as a new treatment option for acute liver damage since they outperform traditional forms on a wide range of parameters, such as inflammatory cytokines, liver function markers, measures of oxidative stress, and histological characteristics. It appears that there is a potential pharmaceutical strategy that could enhance liver regeneration and protection. New therapeutic strategies for the treatment of acute liver damage may be possible as a result of this novel approach.

Limitation Section

The treatment duration was limited to just seven days, preventing assessment of long-term therapeutic effects relevant to chronic liver conditions. Sample size was notably small at only eight animals per experimental group. Additionally, the exclusive use of male rats' neglects potential sex-based differences in drug metabolism and treatment response.

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Author Contribution Dr. Mahmoud S Sabra and Dr. Essmat A. H. Allam developed the design, methodology and analysis of the study. Dr. Madeha H. A. Darwish and Dr. Dalia Hassan contributed to data collection, behavioral analysis and animal rearing. Dr. Al-Hassan Mohammed Mostafa and Dr. Abeer S. Hassan contributed to data collection, nanoparticulation and histopathology. Dr. Marwa G. Gamea and Dr. Mohamed M. Elbadr contributed to data collection, biochemical analysis, writing and publishing. All authors are aware of the submission and agree to it. All authors reviewed the manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

References

1. Yan M, Man S, Ma L, Guo L, Huang L, Gao W. Immunological mechanisms in steatotic liver diseases: An overview and clinical perspectives. Clinical and Molecular Hepatology. 2024.
2. Clària J, Arroyo V, Moreau R. Roles of systemic inflammatory and metabolic responses in the pathophysiology of acute-on-chronic liver failure. JHEP Reports. 2023;5(9):100807.
3. Sabra MS, Mohammed AA, Hassanein KMA, Ahmed AA, Hassan D, Abdel-Lah ES. Novel drug therapy of acute hepatic failure induced in rats by a combination of tadalafil and Lepidium sativum. BMC Complementary Medicine and Therapies. 2024;24(1):104.
4. Munir F, Khan MKA. Hepatotoxicity Induced by Carbon Tetrachloride in Experimental Model: Hepatotoxicity Induced by Carbon Tetrachloride. Pakistan BioMedical Journal. 2023:10–5.
5. Dua TK, Ashraf GJ, Palai S, Baishya T, Nandi G, Sahu R, et al. The protective role of probiotics in the mitigation of carbon tetrachloride (CCl4) induced hepatotoxicity. Food Chemistry Advances. 2023;2:100205.
6. Kim M, Jee S-C, Sung J-S. Hepatoprotective Effects of Flavonoids against Benzo [a] Pyrene-Induced Oxidative Liver Damage along Its Metabolic Pathways. Antioxidants. 2024;13(2):180.
7. Samidurai A, Xi L, Das A, Kukreja RC. Beyond erectile dysfunction: cGMP-specific phosphodiesterase 5 inhibitors for other clinical disorders. Annu Rev Pharmacol Toxicol. 2023;63(1):585–615.
8. Kaltsas A, Dimitriadis F, Zachariou A, Sofikitis N, Chrisofos M. Phosphodiesterase Type 5 Inhibitors in Male Reproduction: Molecular Mechanisms and Clinical Implications for Fertility Management. Cells [Internet]. 2025; 14(2).
9. Crescioli C, Paronetto MP. The Emerging Role of Phosphodiesterase 5 Inhibition in Neurological Disorders: The State of the Art. Cells [Internet]. 2024; 13(20).
10. Sabra MS, Allam EA, Hassanein KMA. Sildenafil and furosemide nanoparticles as a novel pharmacological treatment for acute renal failure in rats. Naunyn-Schmiedeberg's Archives of Pharmacology. 2024:1–15.
11. Ali FE, Azouz AA, Bakr AG, Abo-Youssef AM, Hemeida RA. Hepatoprotective effects of diosmin and/or sildenafil against cholestatic liver cirrhosis: The role of Keap-1/Nrf-2 and P38-MAPK/NF-κB/iNOS signaling pathway. Food Chem Toxicol. 2018;120:294–304.
12. Adeyanju AA, Molehin OR, Ige ET, Adeleye LO, Omoniyi OV. Sildenafil, a phosphodiesterase-5-inhibitor decreased the oxidative stress induced by carbon tetrachloride in the rat kidney: A preliminary study. Journal of Applied Pharmaceutical Science. 2018;8(2):106–11.
13. Molehin OR, Adeyanju AA, Adefegha SA, Aina OO, Afolabi BA, Olowoyeye AO, et al. Sildenafil, a phosphodiesterase-5 inhibitor, offers protection against carbon tetrachloride-induced hepatotoxicity in rat. J Basic Clin Physiol Pharmacol. 2018;29(1):29–35.
14. Nasr HE, Hegazy AM, El-Shaer NO, El-Shafey RS, Elgendy SA, Elnoury HA, et al. Ameliorative effects of sildenafil against carbon tetrachloride induced hepatic fibrosis in rat model through downregulation of osteopontin gene expression. Sci Rep. 2024;14(1):16902.

15. Enyindah BO. Investigating the relationship between vitamin C, cGMP and Ca2+ with regard to hepatoprotection: The University of Liverpool (United Kingdom); 2020.
16. Allam EAH, Darwish MHA, Abou Khalil NS, El-Baset SHAA, El-Aal MA, Elrawy A, et al. Evaluation of the therapeutic potential of novel nanoparticle formulations of glutathione and virgin coconut oil in an experimental model of carbon tetrachloride-induced liver failure. BMC Pharmacol Toxicol. 2024;25(1):74.
17. Allam EAH, Sabra MS. Plant-based therapies for urolithiasis: a systematic review of clinical and preclinical studies. Int Urol Nephrol. 2024;56(12):3687–718.
18. Islas JF, Acosta E, Zuca GB, Delgado-Gallegos JL, Moreno-Trevino MG, Escalante B, et al. An overview of Neem (Azadirachta indica) and its potential impact on health. Journal of Functional Foods. 2020;74:104171.
19. Soares DG, Godin AM, Menezes RR, Nogueira RD, Brito AMS, Melo IS, et al. Anti-inflammatory and antinociceptive activities of azadirachtin in mice. Planta Med. 2014;80(08/09):630–6.
20. Shailey S, Basir SF. Strengthening of antioxidant defense by Azadirachta indica in alloxan-diabetic rat tissues. Journal of ayurveda and integrative medicine. 2012;3(3):130.
21. Baligar NS, Aladakatti RH, Ahmed M, Hiremath MB. Evaluation of acute toxicity of neem active constituent, nimbolide and its hepatoprotective activity against acute dose of carbon tetrachloride treated albino rats. Int J Pharm Sci Res. 2014;5(8):3455.
22. Baligar N, Aladakatti R, Ahmed M, Hiremath M. Hepatoprotective activity of the neem-based constituent azadirachtin-A in carbon tetrachloride intoxicated Wistar rats. Can J Physiol Pharmacol. 2014;92(4):267–77.
23. Meng X, Zhu G, Yang Y-G, Sun T. Targeted delivery strategies: The interactions and applications of nanoparticles in liver diseases. Biomed Pharmacother. 2024;175:116702.
24. Mohamed AM, Toaleb NI, Allam AM, Hekal SHA, Abdelgayed SS, Hassan AS. Preparation and Characterization of Alginate Nanocarriers as Mucoadhesive Intranasal Delivery Systems for Ameliorating Antibacterial Effect of Rutin Against Pasteurella Multocida Infection in Mice. OpenNano. 2023;13:100176.
25. Gbotolorun S, Oremosu A, Noronha C, Okanlawon O. The effect of alcohol extract of Neem seed on ovulation, estrous cycle, and the fertility of adult cyclic Sprague-Dawley Rats. Niger J Health Biomed Sci. 2004;3(2):116–9.
26. Frank D, Savir S, Gruenbaum BF, Melamed I, Grinshpun J, Kuts R, et al. Inducing Acute Liver Injury in Rats via Carbon Tetrachloride (CCl4) Exposure Through an Orogastric Tube. Journal of visualized experiments : JoVE. 2020(158).
27. Kaur G, Sarwar Alam M, Athar M. Nimbidin suppresses functions of macrophages and neutrophils: relevance to its antiinflammatory mechanisms. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives. 2004;18(5):419–24.
28. Batra N, Kumar VE, Nambiar R, De Souza C, Yuen A, Le U, et al. Exploring the therapeutic potential of Neem (Azadirachta Indica) for the treatment of prostate cancer: a literature review. Annals of translational medicine. 2022;10(13):754.
29. Sabra MS, Allam EAH, El-Aal MA, Hassan NH, Mostafa A-HM, Ahmed AAN. A novel pharmacological strategy using nanoparticles with glutathione and virgin coconut oil to treat gentamicin-induced acute renal failure in rats. Naunyn-Schmiedeberg's Archives of Pharmacology. 2024.
30. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol. 1957;28(1):56–63.
31. Maghraby N, El-Baz MAH, Hassan AMA, Abd- elghaffar SK, Ahmed AS, Sabra MS. Metformin Alleviates Doxorubicin-Induced Cardiotoxicity via Preserving Mitochondrial Dynamics Balance and Calcium Homeostasis. Applied Biochemistry and Biotechnology. 2025.
32. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem. 1949;177(2):751–66.
33. Belfield A, Goldberg D. Revised assay for serum phenyl phosphatase activity using 4-amino-antipyrine. Enzyme. 1971;12(5):561–73.
34. Bergmeyer H, Herder M, Ref R. International federation of clinical chemistry (IFCC). J clin Chem clin Biochem. 1986;24(7):497–510.
35. Doumas BT, Watson WA, Biggs HG. Albumin standards and the measurement of serum albumin with bromcresol green. Clin Chim Acta. 1971;31(1):87–96.
36. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95(2):351–8.
37. Sabra MS, Hemida FK, Allam EAH. Adenine model of chronic renal failure in rats to determine whether MCC950, an NLRP3 inflammasome inhibitor, is a renopreventive. BMC Nephrol. 2023;24(1):377.
38. Elbadr M, Sabra M, Ahmed DH, Hassanein K, Saber E. The role of nuclear factor kappa B signaling in the therapeutic effect of tadalafil against dexamethasone-induced gastric ulcer in rats. Journal of Advanced Veterinary Research. 2024;14(6):996–1003.
39. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med. 1967;70(1):158–69.
40. Kuthan H, Haussmann H-J, Werringloer J. A spectrophotometric assay for superoxide dismutase activities in crude tissue fractions. Biochemical Journal. 1986;237(1):175–80.
41. Jokar A, Sheikhani Shahin H, Moqaddasi M, Jowhari A. The effect of eight weeks of high-intensity intermittent swimming training on tumor necrosis factor alpha and transforming growth factor beta 1 in the liver tissue of rats fed with high-fat diet. Journal of Arak University of Medical Sciences. 2023;26(5):0-.
42. Saka WA, Adeogun AE, Adisa VI, Olayioye A, Igbayilola YD, Akhigbe RE. L-arginine attenuates dichlorvos-induced testicular toxicity in male Wistar rats by suppressing oxidative stress-dependent activation of caspase 3-mediated apoptosis. Biomed Pharmacother. 2024;178:117136.
43. Miatmoko A, Faradisa AA, Jauhari AA, Hariawan BS, Cahyani DM, Plumeriastuti H, et al. The effectiveness of ursolic acid niosomes with chitosan coating for prevention of liver damage in mice induced by n-nitrosodiethylamine. Sci Rep. 2022;12(1):21397.
44. Hashem FM, Elkhateeb D, Ali MM, Abdel-Rashid RS. In-vivo and in-vitro assessment of curcumin loaded bile salt stabilized nanovesicles for oral delivery. DARU Journal of Pharmaceutical Sciences. 2024;33(1):9.
45. Abou Youssef NAH, Labib GS, Kassem AA, El-Mezayen NS. Zolmitriptan niosomal transdermal patches: combating migraine via epigenetic and endocannabinoid pathways and reversal of migraine hypercoagulability. Drug Delivery and Translational Research. 2024:1–21.
46. Sadeghi S, Ehsani P, Cohan RA, Sardari S, Akbarzadeh I, Bakhshandeh H, et al. Design and physicochemical characterization of lysozyme loaded niosomal formulations as a new controlled delivery system. Pharm Chem J. 2020;53:921–30.
47. Ritesh KR, Suganya A, Dileepkumar HV, Rajashekar Y, Shivanandappa T. A single acute hepatotoxic dose of CCl(4) causes oxidative stress in the rat brain. Toxicol Rep. 2015;2:891–5.
48. Khan RA, Khan MR, Sahreen S. CCl4-induced hepatotoxicity: protective effect of rutin on p53, CYP2E1 and the antioxidative status in rat. BMC Complement Altern Med. 2012;12:178.

49. Ndrepepa G. Aspartate aminotransferase and cardiovascular disease—a narrative review. Journal of Laboratory and Precision Medicine. 2021;6.
50. Lopes Vendrami C, Thorson DL, Borhani AA, Mittal PK, Hammond NA, Escobar DJ, et al. Imaging of biliary tree abnormalities. Radiographics. 2024;44(8): e230174.
51. Rahman A, ul Haq I, Ullah I, Manzoor F, Younas M, Khan F, et al. Serum Bilirubin Levels as a Diagnostic Indicator for Various Ailments in Patients with Hyperbilirubinemia. Pak-Euro Journal of Medical and Life Sciences. 2024;7(Special 2):S231-S8.
52. Nikolova G, Ananiev J, Ivanov V, Petkova-Parlapanska K, Georgieva E. The Azadirachta indica (Neem) Seed Oil Reduced Chronic Redox-Homeostasis Imbalance in a Mice Experimental Model on Ochratoxine A-Induced Hepatotoxicity. 2022;11(9).
53. Okojie S, Idu M, Ovuakporie-Uvo O. Protective effects of neem (Azadirachta indica A. Juss) seed oil on carbon tetrachloride-induced hepatotoxicity in Wistar rats. Journal of Medicinal Plants for Economic Development. 2017;1(1):1–5.
54. Liebenberg N, Harvey BH, Brand L, Wegener G, Brink CB. Chronic treatment with the phosphodiesterase type 5 inhibitors sildenafil and tadalafil display anxiolytic effects in Flinders Sensitive Line rats. Metab Brain Dis. 2012;27:337–40.
55. Liebenberg N, Harvey BH, Brand L, Brink CB. Antidepressant-like properties of phosphodiesterase type 5 inhibitors and cholinergic dependency in a genetic rat model of depression. Behav Pharmacol. 2010;21(5–6):540–7.
56. Thaxter K, Young LE, Young R, Parshad O, Addae J. An extract of neem leaves reduces anxiety without causing motor side effects in an experimental model. West Indian medical journal. 2010;59(3).
57. Hawiset T, Sriraksa N, Kamsrijai U, Wanchai K, Inkaew P. Anxiolytic and antidepressant-like activities of aqueous extract of Azadirachta indica A. Juss. flower in the stressed rats. Heliyon. 2022;8(2):e08881.
58. Sousa L, Oliveira MM, Pessôa MTC, Barbosa LA. Iron overload: Effects on cellular biochemistry. Clin Chim Acta. 2020;504:180–9.
59. Rafaqat S, Gluscevic S, Mercantepe F. Interleukins: Pathogenesis in Non-Alcoholic Fatty Liver Disease. Metabolites. 2024;14(3):153.
60. Barbier L, Ferhat M, Salamé E, Robin A, Herbelin A, Gombert JM, et al. Interleukin-1 Family Cytokines: Keystones in Liver Inflammatory Diseases. Front Immunol. 2019;10:2014.
61. El Awdan SA, Abdel Rahman RF, Ibrahim HM, Hegazy RR, El Marasy SA, Badawi M, et al. Regression of fibrosis by cilostazol in a rat model of thioacetamide-induced liver fibrosis: Up regulation of hepatic cAMP, and modulation of inflammatory, oxidative stress and apoptotic biomarkers. PLoS ONE. 2019;14(5):e0216301.
62. Guo Q, Jin Y, Chen X, Ye X, Shen X, Lin M, et al. NF-κB in biology and targeted therapy: new insights and translational implications. Signal Transduct Target Ther. 2024;9(1):53.
63. Szabo P, Inci M, Hilber K, Abraham D, Trojanek S, Costantino S, et al. Tenascin-C provokes cardiac fibrosis and endothelial impairment in Duchenne muscular dystrophy. Cardiovascular Research. 2022;118(Supplement_1):cvac066. 152.
64. Toriumi K, Horikoshi Y, Yoshiyuki Osamura R, Yamamoto Y, Nakamura N, Takekoshi S. Carbon tetrachloride-induced hepatic injury through formation of oxidized diacylglycerol and activation of the PKC/NF-κB pathway. Lab Invest. 2013;93(2):218–29.

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