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The Role of Curcumin – Chitosan Nanoparticles in the Prevention and Treatment of liver Fibrosis in Mice.

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ABSTRACT

Background: Nanotechnology is one of the most rising up branch in modern science. A few years ago, natural product therapies play an important role in health care. The major application of nanotechnology in medicine was the development of nanoparticles as drug delivery system. The main advantages of using nanoparticles are large surface area, controlled particle size, site specific targeting, bioavailability, stability, biodegradable and controlled release of drug. **Aim of the study:** The present study had tested the potential of curcumin – chitosan nanoparticles as a great chance to apply the modern nanotechnology techniques in the prevention and treatment of liver fibrosis induced by carbon tetrachloride (CCl_4) in male albino mice. **Materials and Methods:** 30g mice were divided into four groups; normal control (NC), CCl_4, prevention (Prevent) and treatment (Treat) group. Mice were intraperitoneal (ip) injected by CCl_4 three times a week for 4 weeks with dose of 0.5 ml / kg body weight to induce liver fibrosis injury. While, mice were orally administrated nano curcumin – chitosan mixture (100 mg : 100 mg) / 1 kg body weight 5 times a week for 4 weeks as a technique of prevention and treatment. **Results:** The results showed that nano curcumin – chitosan mixture exerted a great potential as antifibrotic agent and it was showed clearly through regulation of different parameters measured in this study such liver enzymes (ALT, AST & ALP), AFP, Caspase-3, oxidative stress biomarker (MDA), antioxidant activities (GSH & CAT) and histopathological study.

Keywords
Curcumin, Chitosan, Nanoparticles, Liver fibrosis, CCl_4.

1. INTRODUCTION

Nanotechnology is one of the most interesting areas of research in modern science. In biochemistry, nanotechnology-based drug delivery system is an advanced method for treating number of dreadful diseases. In the recent years, plant-derived medicines play an important role in health care. The major application of nanotechnology in medicine was the development of nanoparticles as drug delivery system. The advantages of using nanoparticles are large surface area, controlled particle size, site-specific targeting, bioavailability, stability, biodegradable and controlled release of drug. A long term goal in nano medicine has been to design drug delivery systems that improve the narrow therapeutic window [1-3]. Conceptually, several nano techniques based entity candidates, including biosynthetic nanoparticles (NPs) [4,5].

Curcumin is a polyphenolic compound (1, 7-Bis-(4-hydroxy-3-methoxyphenyl)-hepta-1, 6-diene-3, 5-dione) which is found in turmeric of *Curcuma longa* which is familiar in the indian spice turmeric, a

perennial herb belonging to the Zingiberaceae family, is a traditional medicine in Asia. The typical yellow colour of turmeric is due to the presence of curcuminoids. The curcuminoids are polyphenols which contain three major components: curcumin (77%), demethoxycurcumin (17%) and bisdemethoxycurcumin (3%). Curcumin is bioactive agent and many studies had been performed to discover its therapeutic properties [6,7]. Curcumin exhibits anti-inflammatory, antioxidant, immunomodulatory, pro-apoptotic, and antiangiogenic properties that have been exploited in the treatment of cancer in various models [8,9]. It has poor bioavailability, similar to other natural agents, due to poor oral absorption and fast metabolism in the liver and intestines [10,11].

Chitosan is a polysaccharide generated from chitin. It is a natural polymer that can be obtained from the exoskeletons of shellfish and insects. Several remarkable properties of chitosan offered unique opportunities for the development of biomedical applications that will lead to a better understanding of chitosan medical and pharmaceutical interest, due to the presence of amino groups in the chitosan structure. Because of its positive charges, chitosan can interact with the negative part of the cell membrane, causing reorganisation and opening of the tight junction proteins, explaining the permeation enhancing property of this polysaccharide is biodegradable, biocompatible, nontoxic, abundant, renewable polymer has the unique characteristic of providing higher affinity for negatively charged molecules, explaining the permeation enhancing property of this polysaccharide is biodegradable, biocompatible, nontoxic, abundant, renewable polymer.

Because CCl_4 is a major contributor to stratospheric ozone depletion, its production and consumption are regulated under Montreal Protocol's Article 2D. Further changes to the Protocol resulted in a 100 percent reduction in CCl_4 production and consumption for emissive uses from 2010 onwards, resulting in steadily decreasing global CCl_4 levels [12]. Although CCl_4 is biologically inert, the liver microsomal mixed function oxidase (MFO) system converts it to highly reactive hepatotoxic compounds [13,14].

The liver is a vital organ with numerous functions. It has several synthetic roles, including blood proteins (albumin, globulin, and so on), coagulation proteins (fibrinogen and prothrombin), transport proteins such as transferrin, which can transport iron ions, and immunoproteins synthesis, which is involved in complement system creation. In addition, the liver protects against foreign antigens such as bacteria, viruses, and toxins by metabolising xenobiotic chemicals and converting them from inactive to active form or vice versa, as well as clearing them from the body system. Hepatic fibrosis is defined by a scarring process that is associated with increased and altered extracellular matrix deposition in the liver, where it is connected with cellular activation of hepatic stellate cells and atypical transforming growth factor-1 (TNF-1) activity [15,16]. Injured epithelium and/or endothelial cells release proinflammatory factors after an injury, which promote blood clotting and the creation of a conditional extracellular matrix (ECM). Injured tissues regenerate in a normal state and are characterised by fibre organisation, blood vessel restoration, and scar tissue elimination. However, an aberrant phase, which is marked by chronic inflammation, tissue necrosis, myofibroblast activation, excessive ECM accumulation, and eventually permanent fibrosis formation, is more common. Hepatic fibrosis is triggered by necrotic parenchymal cell breakdown caused by a variety of causative substances and mechanisms that cause inflammation, which causes "resting" hepatic stellate cells to become active (HSC). HSCs change phenotypic from fat-storing cells, vitamin-A-storing quiescent cells, or retinoid-storing cells to myofibroblast-like active cells during the activation process [17,18].

The aim of present study is to assess the potential of curcumin – chitosan nanoparticles in the prevention and treatment of liver fibrosis induced by carbon tetrachloride (CCl4) in male albino mice.

2. MATERIALS AND METHODS

1. Materials

a. Animals

Six weeks old male albino mice 30 ± 10 g obtained from National Cancer Institute (NCI), Cairo University, Egypt. Animals were divided randomly in plastic cages under standard conditions of temperature is between 20 – 26 ^{o}c , humidity is between 30% – 70% and a 12 hours light / dark cycle along experimental period. They were left to adapt for one week before start of experiment. They were maintained and used in accordance with the animal ethics and the guide for the care and use of laboratory animals (National Research Council, 1996). The animal treatment protocol has been approved by the Bioethics Committee of the National Center for Radiation Research and Technology, Cairo, Egypt.

b. Diet

Mice were allowed to have diet as pellets (from Al-Farouk co. – Benha - Egypt), with formula of 21 gm protein per 100 gm pellets.

c. Chemicals

Nano curcumin was purchased from Herbal-Cure co. India, chitosan, sodium tripolyphosohate (TPP), tween 80 and CCl_4 from Sigma-Aldrich. All chemicals and solvent used in this study were of analytical grade.

2. Methods

a. Experiment design

6 weeks old male albino mice 30 ± 10 g were divided randomly into four groups in plastic cages, each group has 15 mice and were left to adapt for one week before start of experiment. Normal control (NC) group had free access of water and feed and never injected or orally administrated any chemicals. Positive control group (CCl_4) that is intra-peritoneal injected with CCl_4 at a dose 0.5 ml / kg three times per week lasts for four weeks [19]. Prevention group (Prevent) extended for four weeks where mice are intra-peritoneal injected with CCl_4 at a dose 0.5 ml / kg three times per week and orally administrated curcumin – chitosan nanoparticles mixture (Cur-Chito NP) at a dose 100 mg / kg five times a week. Treatment group (Treat) extended for seven weeks; where mice are intra-peritoneal injected with CCl_4 at a dose 0.5 ml / kg three times per week for four weeks of the experiment time frame and orally administrated curcumin – chitosan nanoparticles mixture at a dose 100 mg /100 mg / kg five times a week for another four weeks at the beginning of the fourth week of seven weeks overall duration.

b. Curcumin – Chitosan nanoparticles mixture preparation

Preparation of nanoparticles mixture is based on electrostatic interaction between negatively and positively charged molecules. Stock solution of nano chitosan was dissolved in acidified distilled water and homogenized using vortex for 1 minute. Nano curcumin was dissolved in ethanol and tween 80 was added and homogenized using vortex for 1 minutes. TPP solution as a cross linker was added to emulsified nano curcumin-chitosan solution in a drop-wise manner and stirred for 10 min. At last, the mixture solution was transferred into a new flask and kept for subsequent steps.

c. Sample collection

At the end of experiment time frame, waiting for 72 hours after the final dose, the mice were anesthetized by using of diethyl ether, dissected and exsanguinated by cardiac puncture to obtain blood serums, liver tissues were washed with ice-cold phosphate buffered saline (PBS) and dissected into smaller pieces, some were snap frozen at liquid nitrogen and stored at $-80^{\circ C}$ for biochemical parameters, while other parts were fixed in 10% buffered formalin and embedded in paraffin for histopathological study.

d. Preparation of liver samples

Livers were washed with ice-cold phosphate buffered saline (PBS) and dissected into smaller pieces. Aliquots were snap frozen at liquid nitrogen and stored at −80°C, or fixed in 10% buffered formalin and embedded in paraffin.

e. Tissue sampling:

The livers of experimental animals were dissected out and divided into two parts, one part was kept in 10% formalin for histopathological studies and the other part was homogenized in (10% w/v) phosphate-buffered-saline composed of (0.02 M sodium phosphate buffer with 0.15 M sodium chloride, pH 7.4) was prepared with a portion of liver tissue homogenized in a glass tissue homogenizer with a Teflon pestle.

f. Test methods:

The study figured out the effect of curcumin – chitosan nanoparticles on several parameters such:

I. Biochemical parameters:

- liver enzymes:

- Alanine aminotransferase (ALT) from Spectrum co. according to the colorimetric method of [20].
- Aspartate aminotransferase (AST) from Spectrum co. according to the colorimetric method of [20].
- Alkaline phosphatase (ALP) from Spectrum co. according to the colorimetric method of [21].

- Apoptotic markers:

- Alpha feto protein (AFP) according to [22].
- Caspase-3 (Cas-3) was assayed by ELISA kit Komabiotech co. [23].

- Oxidative stress & Antioxidants:

- Malondialdhyde (MDA) was measured in 10% liver homogenate according to the colorimetric method of [24,25].
- Reduced glutathione (GSH) was measured in 10% liver homogenate as described by the colorimetric method of [26].
- Catalase activity (CAT) was measured in 10% liver homogenate according to the colorimetric method of [27].

II. Histopathological study:

- Hematoxylin-eosin (H&E) stain was followed by technique according to [28].
- Masson's trichrome stains was followed by technique according to [29].
- Acridine orange / propidium iodide stain was followed by technique according to [30].

g. Statistical Analysis:

The obtained data is presented as mean ± standard error (SE) for normally distributed continuous data is performed using Statistical Package for Social Science (SPSS) version 21.0 for windows [31]. All data is analysed statistically using one-way analysis of variance (ANOVA). Comparisons between groups are performed using Post-Hoc LDS tests. A P value < 0.05 is considered to indicate a statistically significant difference.

3. RESULTS

liver enzymes:

Experimental date in Table 1, Figure 1 & Figure 2 showed the role of nano curcumin – chitosan mixture in protection of liver tissue against fibrogenesis induced by CCl_4 administration where, ALT, AST and ALP

values increased in CCl_4 group compared to normal control mice. On the other hand, liver enzymes values decreased dramatically in prevention and treatment mice groups compared to CCl_4 group.

As recorded, the experimental data of (NC) group showed that, the mean value of serum ALT was 16.15 ± 0.69 U/L. Meanwhile mice of (CCl_4) group represented a significant increase in serum ALT (555 %) in compared to (NC) group value. On the other hand experimental mice of (Prevent) group recorded a significant increase in serum ALT (371 %) in compared to (NC) group and a significant decrease in serum ALT (-28%) against to (CCl_4) group. Meanwhile, experimental animals of (Treat) revealed a significant increase in serum ALT (320 %) in compared to (NC) group and a significant decrease in serum ALT (-36%) against (CCl_4) group.

According to experimental data of (NC) group showed that, the mean value of serum AST was 14.41 ± 0.67 U/L. Meanwhile in CCl_4 administrated represents a significant increase in serum AST (607 %) in compared to (NC) group. When experimental mice of (Prevent) group injected intra-peritoneal with CCl_4 and orally administrated by Cur-Chito NP mixture recorded a significant increase in serum AST (488 %) in compared to NC group and a significant decrease in serum AST (-17 %) in compared to CCl_4 group. Meanwhile, experimental animals of (Treat) group revealed a significant increase in serum AST (273 %) in compared to normal control (NC) group and a significant decrease in serum AST (-47 %) against to CCl_4 group.

The experimental data of (NC) group showed that, the mean value of serum ALP was 101.25 ± 5.85 U/L. Meanwhile, mice in (CCl_4) group represents a significant increase in serum ALP (146 %) in compared to (NC) group. When experimental mice of (Prevent) group injected intra-peritoneal with CCl_4 and orally administrated by Cur-Chito NP mixture recorded a significant increase in serum ALP (80 %) in compared to (NC) group. Meanwhile a significant decrease in serum ALP (-27 %) in compared to CCl_4 group was detected. On the other hand, experimental animals of (Treat) revealed a significant increase in serum ALP (96 %) in compared to control (NC) group and a significant decrease in serum ALP (-20 %) against to CCl_4 group.

Table 1. Effect of nano curcumin & chitosan mixture on serum ALT, AST and ALP of mice liver fibrosis induced by CCl_4

Parameter / Group	ALT	AST	ALP
NC Mean ± S.E. (U/L)	16.15 ± 0.69	14.41 ± 0.67	101.25 ± 5.85
CCl_4 Mean ± S.E. (U/L)	105.75 ± 0.94	101.85 ± 1.54	248.70 ± 9.86
% change to NC	555% [c]	607% [c]	146% [c]
Prevent Mean ± S.E. (U/L)	76.00 ± 0.74	84.80 ± 0.95	182.10 ± 3.32
% change to NC	371% [c]	488% [c]	80% [c]
% change to CCl_4	-28% [c]	-17% [c]	-27% [c]
Treat Mean ± S.E. (U/L)	67.85 ± 0.12	53.75 ± 0.65	198.55 ± 2.49
% change to NC	320% [c]	273% [c]	96% [c]
% change to CCl_4	-36% [c]	-47% [c]	-20% [c]

Normal control (NC), Carbon Tetrachloride (CCl_4), Prevention (Prevent) and Treatment (Treat) groups.
a = Significant (P<0.05); b = Highly significant (P<0.01); c = Very highly significant (P<0.001).

Apoptotic markers:

Regarding to Table 2, Figure 3 & Figure 4 Alfa fetoprotein and caspase-3 mean values also increased in CCl_4 group in comparison with normal control group. But, the action of nano curcumin – chitosan mixture was observed on the prevention and treatment groups where their mean values downregulated compared to CCl_4 group.

The experimental data of (NC) group showed that, the mean value of serum AFP was 0.256 ± 0.012 IU/mL. Meanwhile mice revealed a significant increase in AFP (203 %) in compared to (NC) group value. On the other hand experimental mice of (Prevent) group recorded a significant increase in AFP (21 %) in compared to (NC) group while a significant decrease in AFP (-60 %) in compared to (CCl_4) group. Meanwhile, experimental animals of (Treat) revealed a significant increase in AFP (137 %) in compared to (NC) group and a significant decrease in AFP (-22 %) in compared to compared to (CCl_4) group.

The experimental data of (NC) group showed that, the mean value of serum Cas-3 was 1.906 ± 0.038 ng/mL. Meanwhile mice IP treated (CCl_4) group revealed a significant increase in Cas-3 (259 %) in compared to (NC) group. On the other hand when experimental mice of (Prevent) group figured out a significant increase in Cas-3 (42 %) in compared to (NC) group while a significant decrease in Cas-3 (-61 %) in compared to (CCl_4) group. Meanwhile, experimental animals of (Treat) group that revealed a significant increase in Cas-3 (76 %) in compared to (NC) group and a significant decrease in Cas-3 (-51 %) in compared to compared to (CCl_4) group.

Table 2. Effect of nano curcumin & chitosan mixture on liver injury related markers (serum AFP and Caspase-3) of mice liver fibrosis induced by CCl_4.

Parameter / Group	AFP	Caspase-3
NC		
Mean ± S.E.	0.256 ± 0.012	1.906 ± 0.038
CCl_4		
Mean ± S.E.	0.776 ± 0.023	6.852 ± 0.230
% change to NC	203% [c]	259% [c]
Prevent		
Mean ± S.E.	0.310 ± 0.007	2.706 ± 0.108
% change to NC	21% [b]	42% [c]
% change to CCl_4	-60% [c]	-61% [c]
Treat		
Mean ± S.E.	0.606 ± 0.019	3.346 ± 0.090
% change to NC	137% [c]	76% [c]
% change to CCl_4	-22% [c]	-51% [c]

Normal control (NC), Carbon Tetrachloride (CCl_4), Prevention (Prevent) and Treatment (Treat) groups.
[a] = Significant (P<0.05); [b] = Highly significant (P<0.01); [c] = Very Highly significant (P<0.001).

Oxidative stress:

According to Table 3, Figure 5 & Figure 6 Malondialdehyde is the final product of lipid peroxidation, which is a highly reactive compound. It was clear that MDA value in CCl_4 group elevated highly compared to normal group. On contrary, oral administration of nano curcumin – chitosan mixture in prevention and treatment groups lowered MDA values compared to CCl_4 group.

The experimental data showed that mean value of (NC) MDA was 430.14 ± 7.98 μM/g tissue. Meanwhile mice in (CCl_4) group revealed a significant increase in MDA (85 %) in compared to (NC) group. On the other hand, when experimental mice of (Prevent) group figured out a significant increase in MDA (37 %) in compared to (NC) group while a significant decrease in MDA (-26 %) in compared to (CCl_4) group. Meanwhile, experimental animals of (Treat) group revealed a significant increase in MDA (26 %) in compared to (NC) group and a significant decrease in MDA (-32 %) in compared to compared to (CCl_4) group.

Antioxidants biomarkers:

According to Table 3, Figure 5 & Figure 6 Reduced glutathione (GSH) and catalase activity (CAT) are cellular enzymes that scavenge reactive oxygen species (ROS). Where, CCl_4 lowered GSH and CAT values compared to normal control group. While, mice in prevention and treatment groups had been administrated with nano curcumin – chitosan mixture had higher values of GSH and CAT compared to CCl_4 group.

The experimental data showed mean value of (NC) group GSH level was 45.32 ± 0.71 mg/g tissue. Meanwhile in CCl_4 group revealed a significant decrease in GSH level (-45 %) in compared to NC group value. On the other hand, when experimental mice of (Prevent) group showed a significant decrease in GSH (-29 %) in compared to (NC) group while a significant increase in GSH (28 %) in compared to (CCl_4) group was detected. Meanwhile, experimental animals of (Treat) group revealed a significant decrease in GSH (-27%) in compared to (NC) group and a significant increase in GSH level (33 %) in compared to compared to (CCl_4) group.

The experimental data showed that, the mean value of (NC) group CAT activity was 0.407 ± 0.004 μM/g tissue. Meanwhile in CCl_4 group revealed a significant decrease in CAT (-61 %) in compared to (NC) group. On the other hand, when experimental mice of (Prevent) group showed a significant decrease in CAT activity (-36 %) in compared to (NC) group while a significant increase in CAT activity (65 %) in compared to CCl_4 group was recorded. Meanwhile, experimental animals of (Treat) group revealed a significant decrease in activity of CAT (-15 %) in compared to NC group and a significant increase in CAT activity (118 %) in compared to compared to CCl_4 group.

Table 3. Effect of nano curcumin & chitosan mixture on oxidative stress biomarkers (MDA, GSH, CAT) of mice liver fibrosis induced by (CCl_4)

Parameters / Group	MDA (µM/g)	GSH (mg/g)	CAT (µM/g)
NC			
Mean ± S.E.	430.14 ± 7.98	45.32 ± 0.71	0.407 ± 0.004
CCl_4			
Mean ± S.E.	795.66 ± 15.16	24.94 ± 0.46	0.159 ± 0.005
% change to NC	85% [c]	-45% [c]	-61% [c]
Prevent			
Mean ± S.E.	589.81 ± 8.86	31.97 ± 0.27	0.262 ± 0.003
% change to NC	37% [c]	-29% [c]	-36% [c]
% change to CCl_4	-26% [c]	28% [c]	65% [c]
Treat			
Mean ± S.E.	542.35 ± 15.46	33.21 ± 0.55	0.346 ± 0.003
% change to NC	26% [c]	-27% [c]	-15% [c]
% change to CCl_4	-32% [c]	33% [c]	118% [c]

Normal control (NC), Carbon Tetrachloride (CCl_4), Prevention (Prevent) and Treatment (Treat) groups.
a = Significant (P<0.05); b = Highly significant (P<0.01); c = Very highly significant (P<0.001).

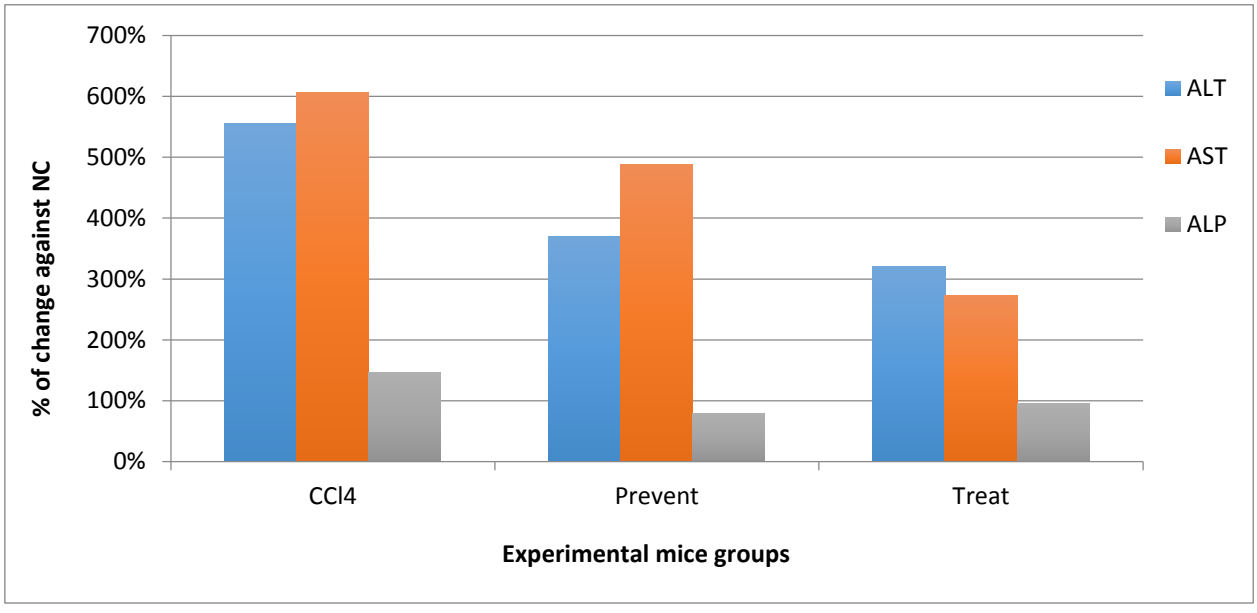

Figure 1 Percent of changes of ALT, AST & ALP in mice bearing CCl_4 treated with nano curcumin - chitosan mixture (Cur+Chito NP) against normal control (NC) group.

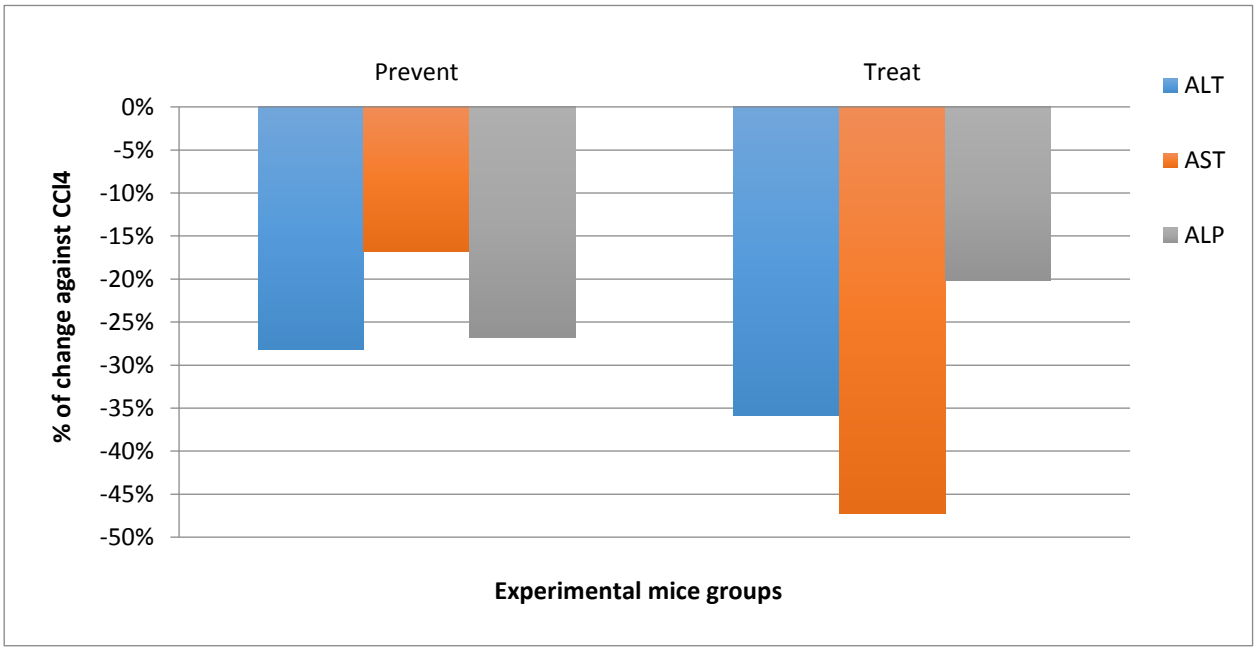

Figure 2 Percent of changes of ALT, AST & ALP in mice bearing CCl_4 treated with nano curcumin - chitosan mixture (Cur+Chito NP) against (CCl_4) group.

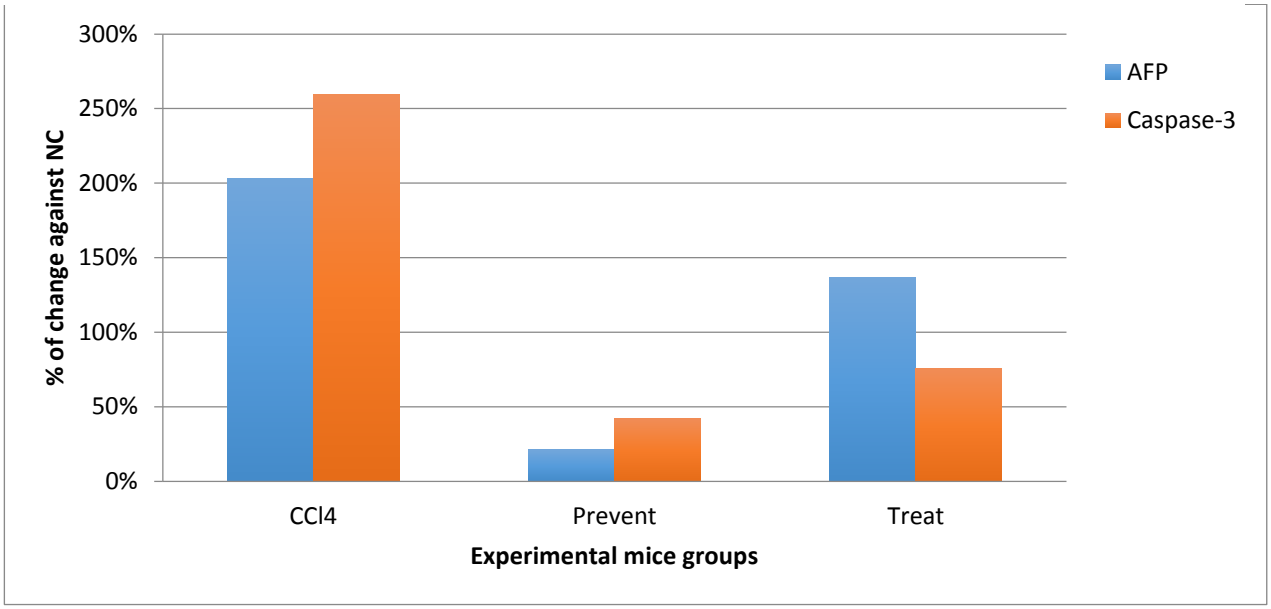

Figure 3 Percent of changes of AFP & CAS-3 in mice bearing CCl_4 treated with nano curcumin - chitosan mixture (Cur+Chito NP) against normal control (NC) group.

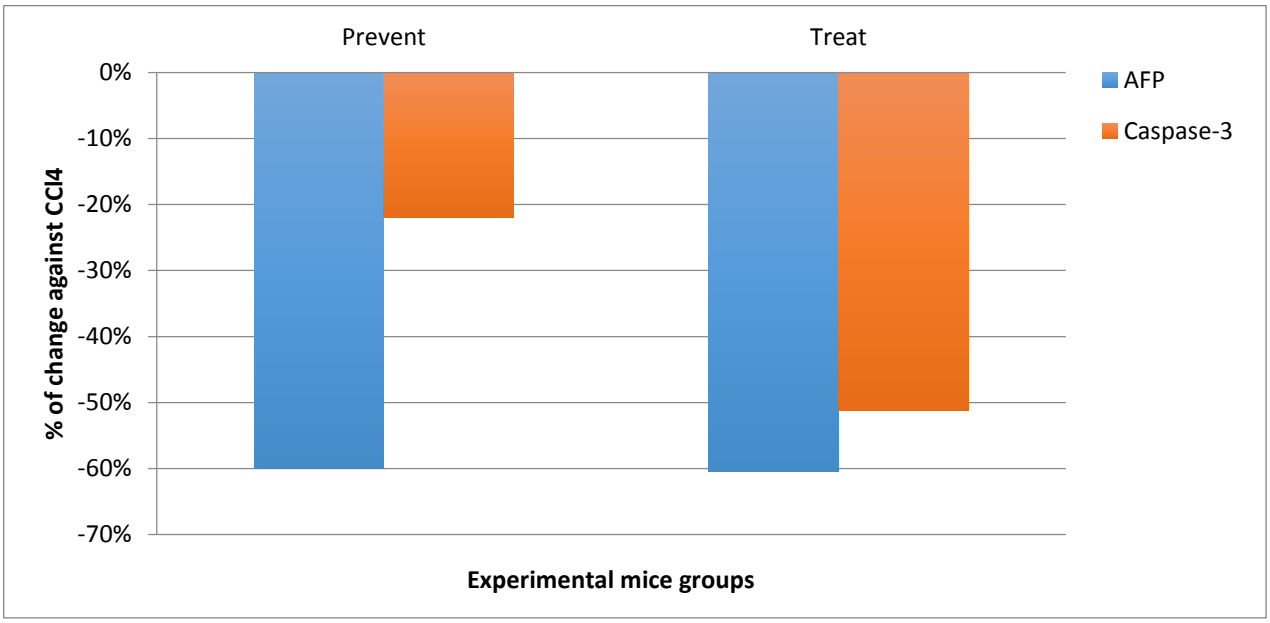

Figure 4 Percent of changes of AFP & Cas-3 in mice bearing CCl_4 treated with nano curcumin - chitosan mixture (Cur+Chito NP) against (CCl_4) group.

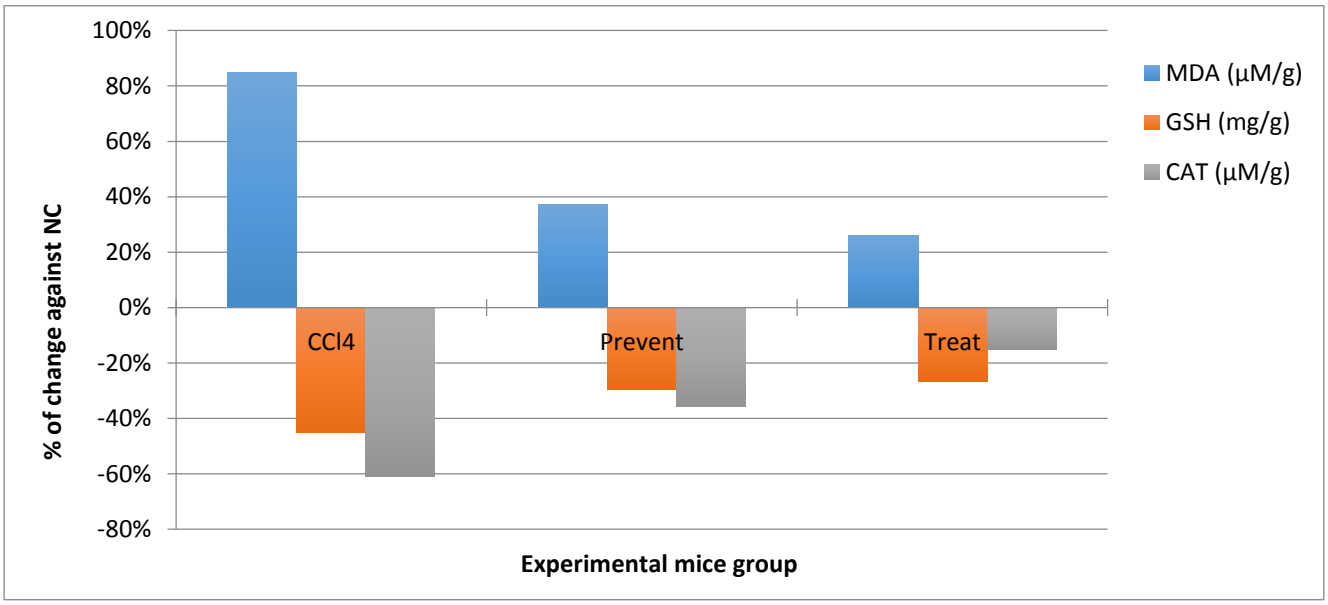

Figure 5 Percent of changes of MDA, GSH & CAT in mice bearing CCl_4 treated with nano curcumin - chitosan mixture (Cur+Chito NP) against normal control (NC) group.

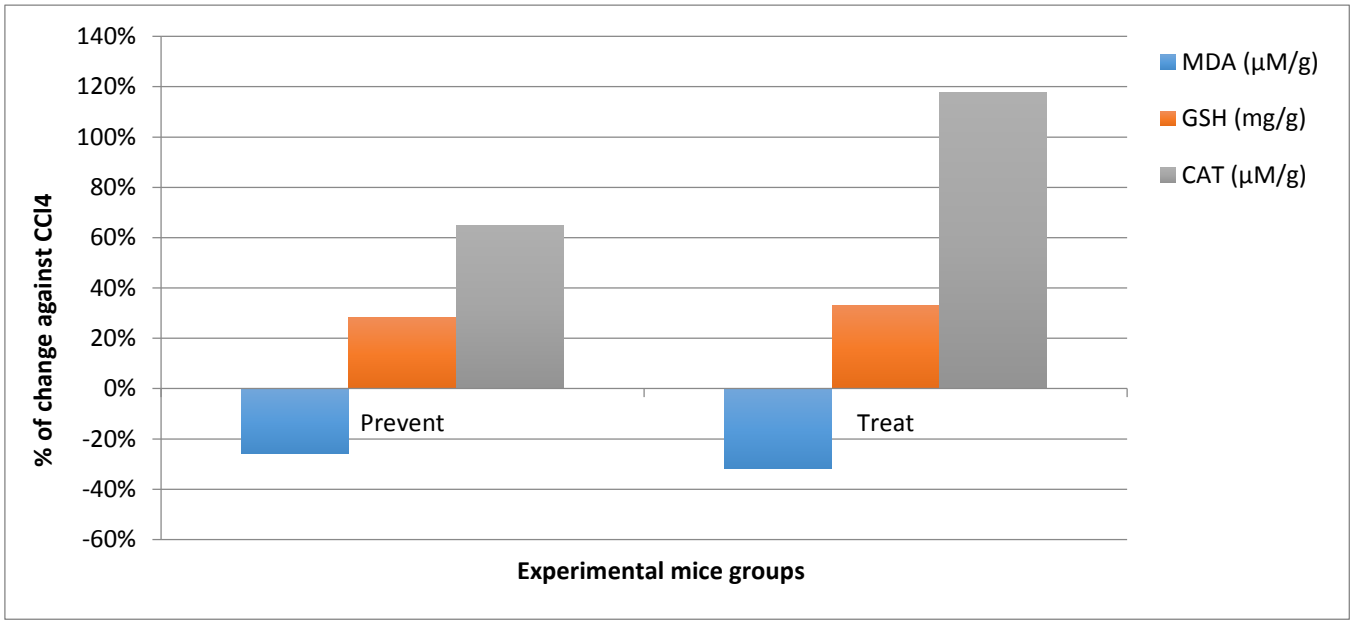

Figure 6 Percent of changes of MDA, GSH & CAT in mice bearing CCl_4 treated with nano curcumin - chitosan mixture (Cur+Chito NP) against (CCl_4) group.

Histopathological study

Hematoxylin & eosin (H&E) stain:

Regarding to Figure 7 A&B: A photomicrograph of a liver section of a control mice showing the general appearance of the hepatocytes, the central vein (CV), the portal area with branch of portal vein (PV), and a branch of bile duct.

C&D: Microphotographs of mouse liver sections in +ve group treated peritoneal with CCl_4 three times a week for 4 weeks showing congestion in the portal tract with congested hepatic artery and inflammatory cellular infiltrations (→). Also, focal areas of hepatic necrosis (▲) were detected.

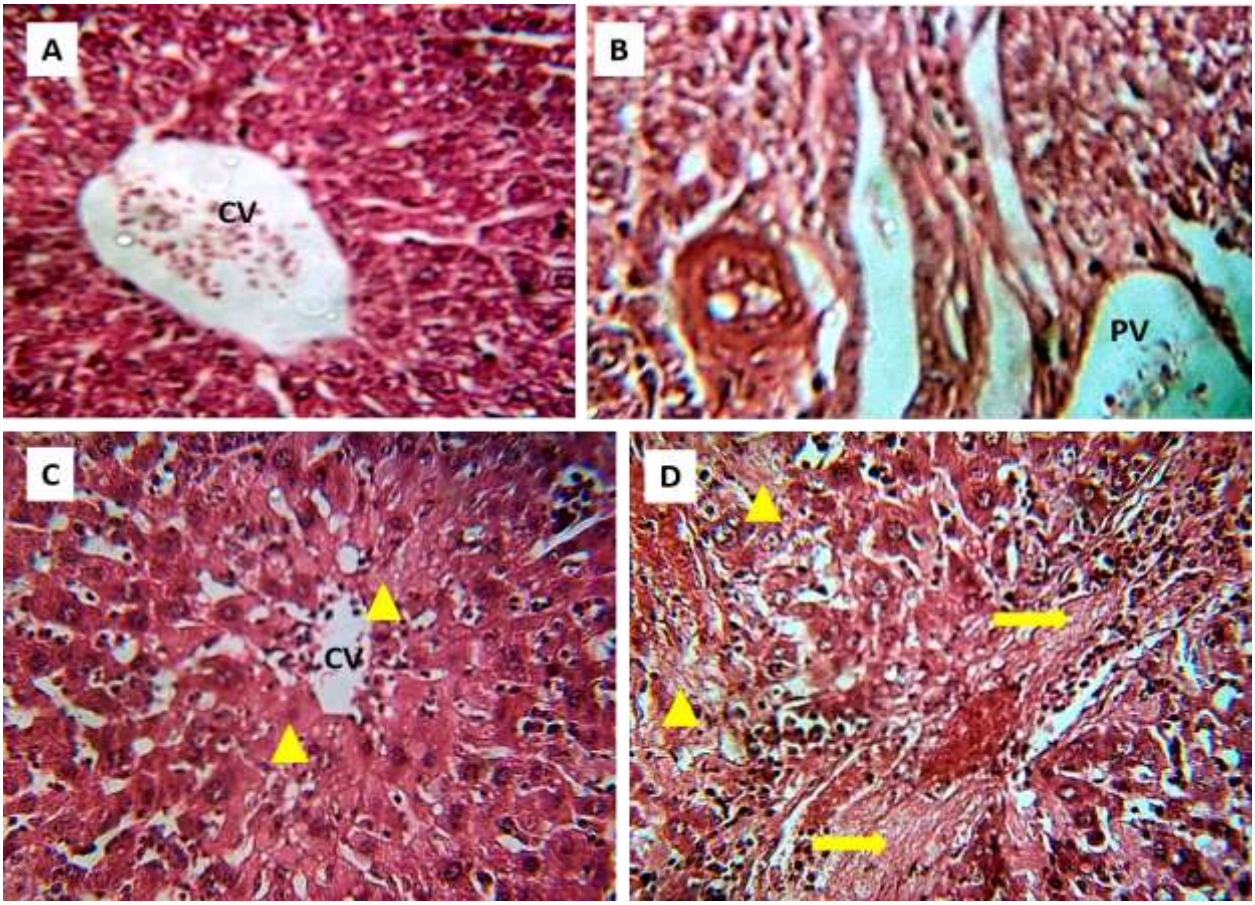

Figure 7: photomicrograph of a liver section where A & B: normal control (NC) mice. C & D: (CCl_4) mice. H & E stain.

According to Figure 8 E&F: Microphotographs of mouse liver sections in prev. group treated peritoneal with CCl_4 three times a week and orally administrated Cur-Chito NP five times a week for 4 weeks demonstrated the normal appearance of liver section architecture.

G&H: Microphotographs of mouse liver sections in trt. group treated peritoneal with CCl_4 three times a week for 4 weeks and orally administrated with Cur-Chito NP five times a week for another four weeks at the beginning of the fourth week of CCl_4 administration distinct a less congestion in the portal vein (PV) and very less inflammatory cellular infiltrations.

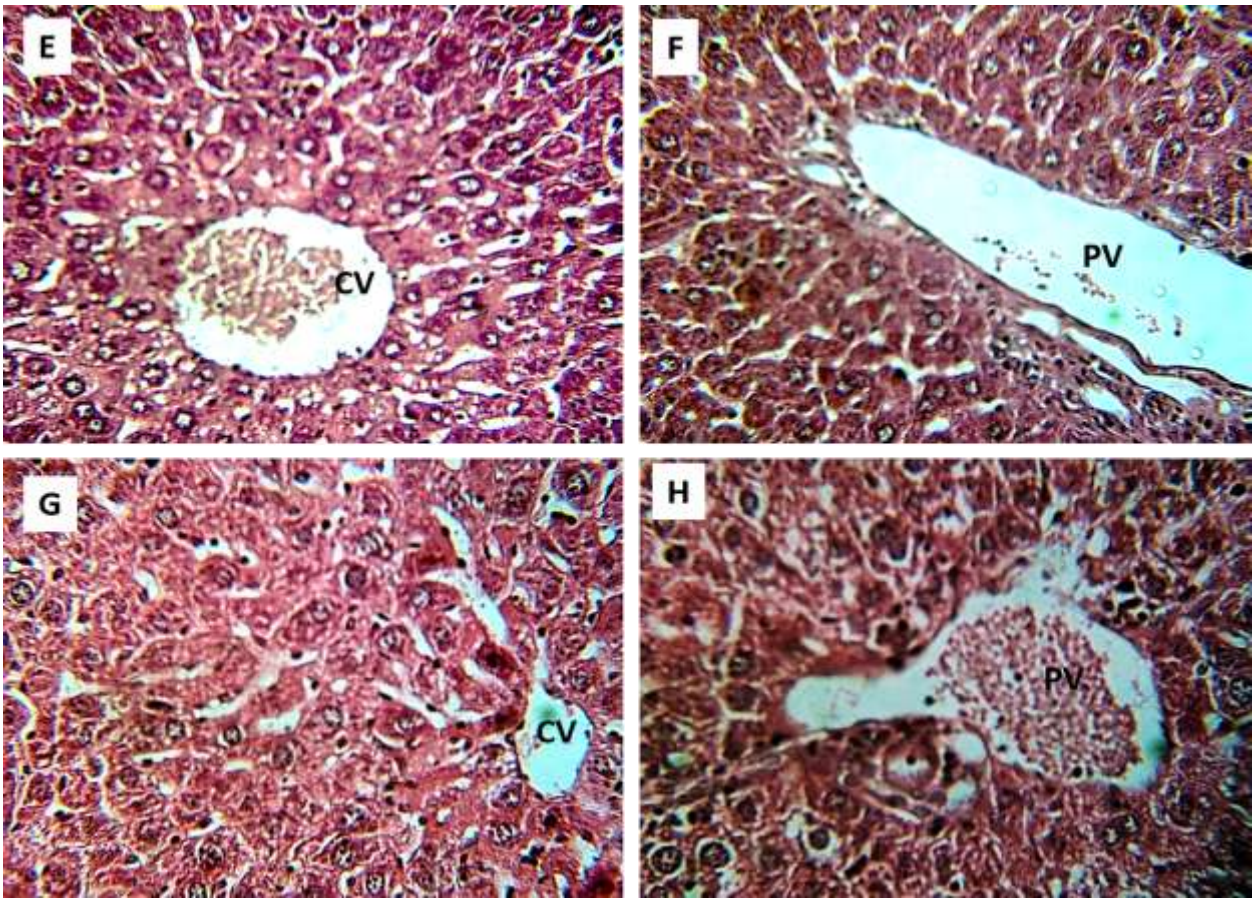

Figure 8 photomicrograph of a liver section where E & F: prevention (Prevent) mice. G & H: treatment (Treat) mice. H & E stain.

Masson's trichrome stain

According to Figure 9 A & B: Microphotographs of control mouse showing normal distribution of collagen fibers around the central vein, portal vein and hepatocytes.

C, D & E: Microphotographs of mouse liver sections in +ve group treated peritoneal with CCl_4 three times a week for 4 weeks developed extensive fibrosis in the periportal areas

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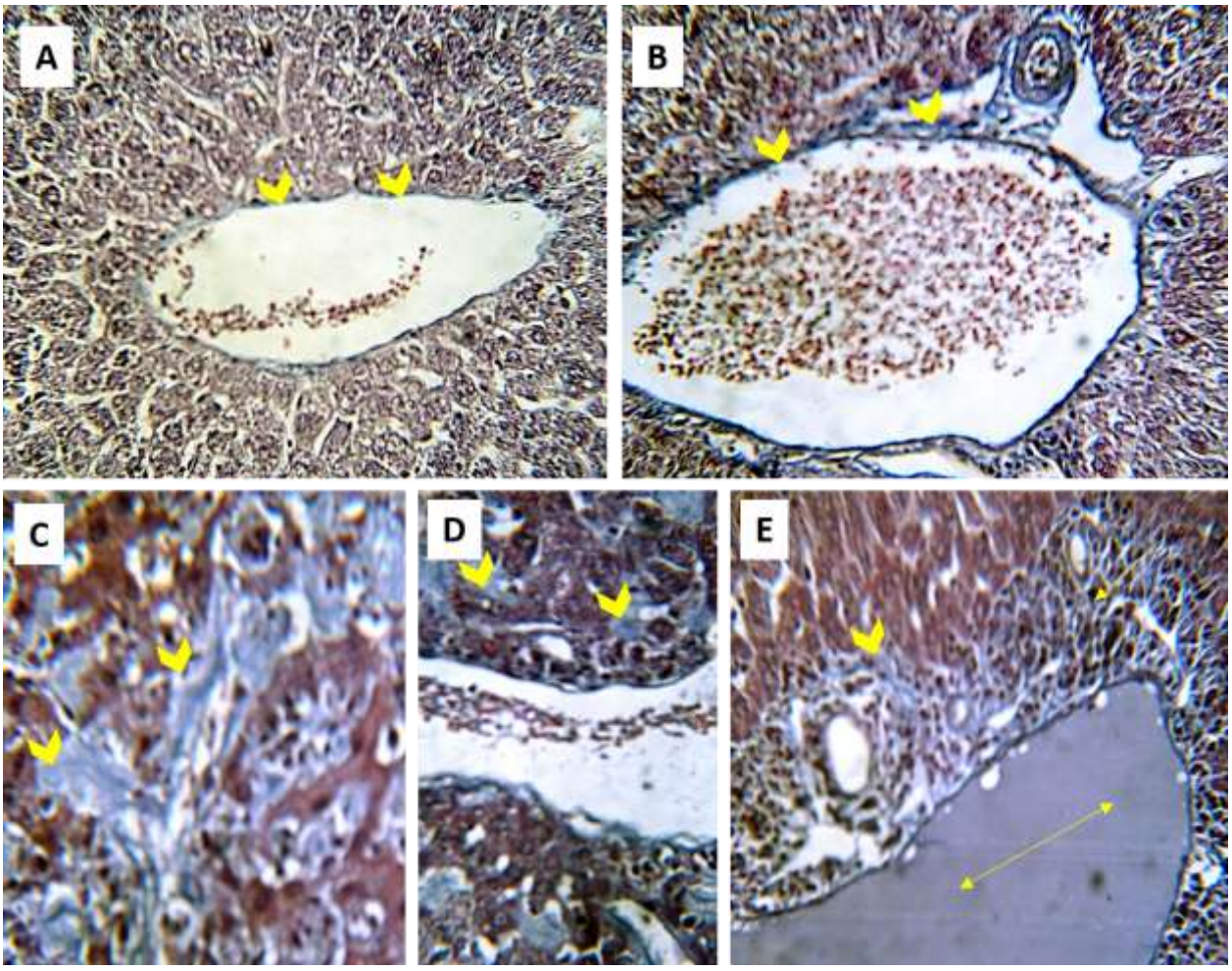


Figure 9 photomicrograph of a liver section where A & B: normal control (NC) mice. C, D & E: (CCl_4) mice. Masson's trichrome stain.

Regarding to Figure 11 F&G: Microphotographs of mouse liver sections in prev. group treated peritoneal with CCl_4 three times a week and orally administrated Cur-Chito NP five times a week for 4 weeks demonstrated a very less deposition of collagen fibers around the central vein, portal vein and hepatocytes.

H&I: Microphotographs of mouse liver sections in trt. group treated peritoneal with CCl_4 three times a week for 4 weeks and orally administrated with Cur-Chito NP five times a week for another four weeks at the beginning of the fourth week of CCl_4 administration distinct a very less deposition of collagen fibers around the central vein, portal vein and hepatocytes.

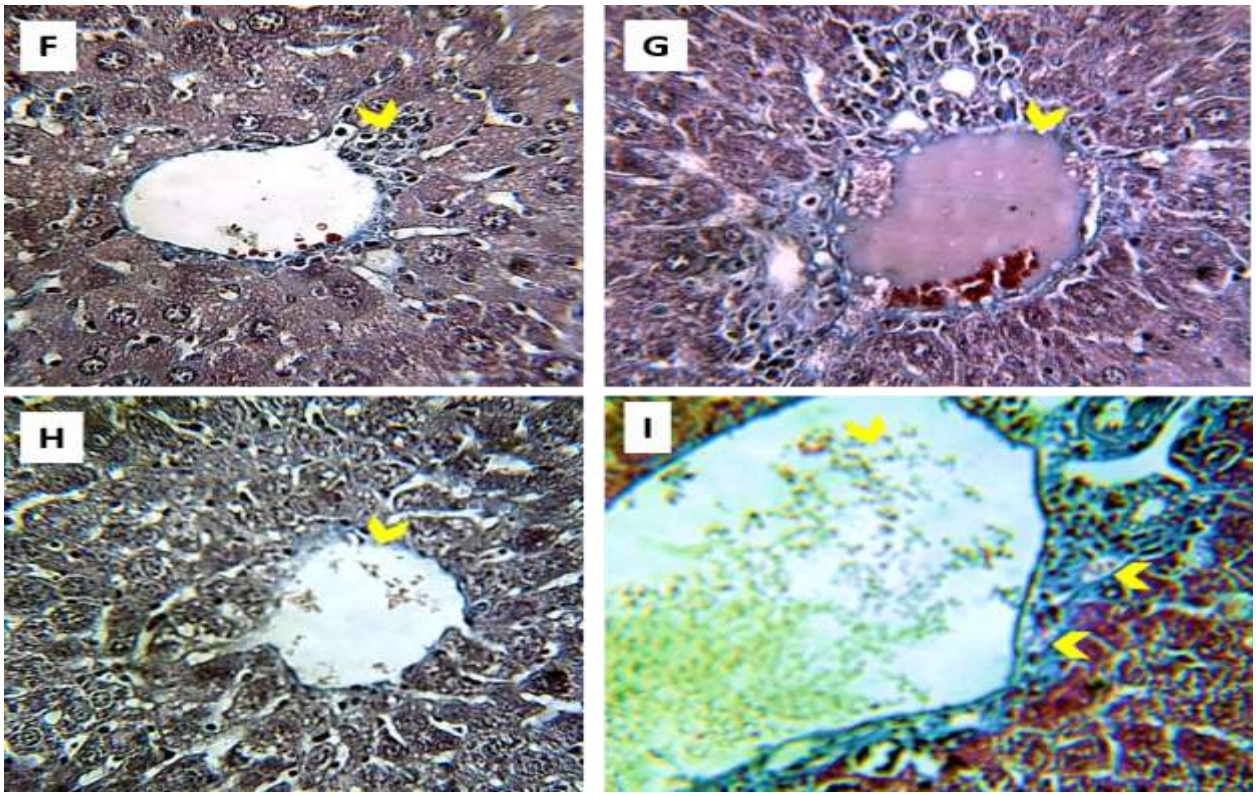
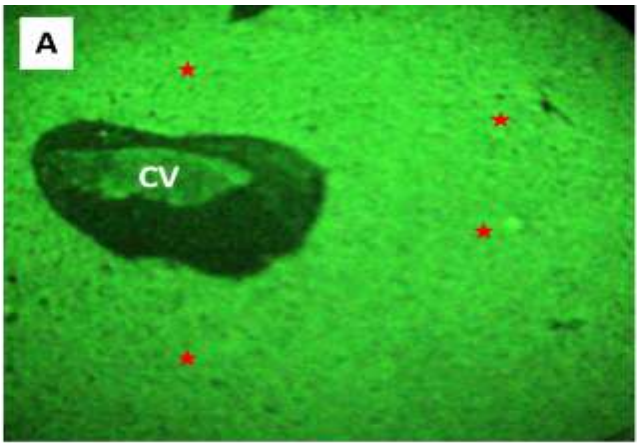


Figure 11 photomicrograph of a liver section where F & G: prevention (Prevent) mice. H & I: treatment (Treat) mice. Masson's trichrome stain.



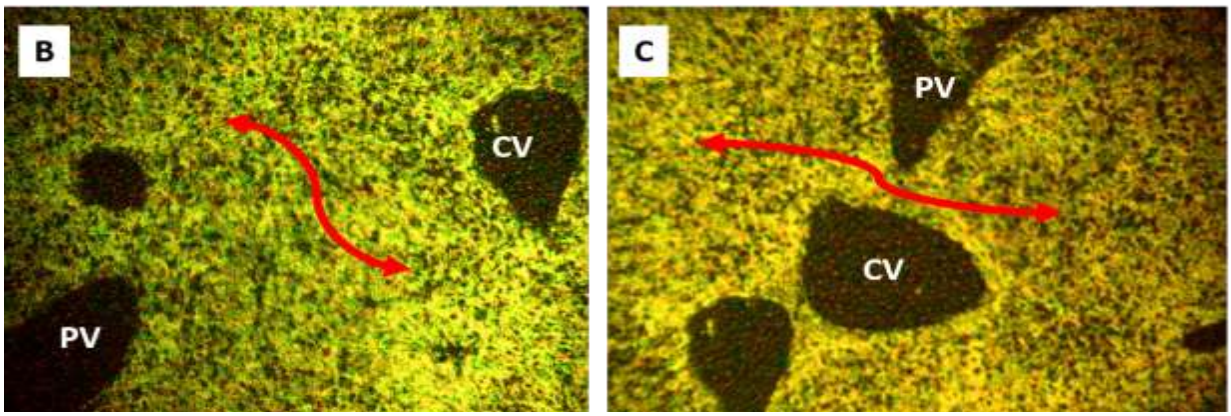


Figure 10 photomicrograph of a liver section where A: normal control (NC) mice. B & C: (CCl_4) mice. Acridine orange / propidium iodide stain.

Acridine orange / propidium iodide stain

In the Figure 10 A: Section in normal mouse liver. B&C: sections in liver of mouse of +ve group treated peritoneal with CCl_4 three times a week for 4 weeks. propidium iodide (living cells, *green*), acridine orange (dead cells, yellow and red, broken arrow). (All magnifications x 200).

Regarding to Figure 12 D&E: sections in liver of mouse of in prev. group treated peritoneal with CCl_4 three times a week and orally administrated Cur-Chito NP five times a week for 4 weeks

F: mouse liver sections in trt. group treated peritoneal with CCl_4 three times a week for 4 weeks and orally administrated with Cur-Chito NP five times a week for another four weeks at the beginning of the fourth week of CCl_4 administration. (living cells, green ↔), acridine orange (dead cells, yellow and red, ↔).

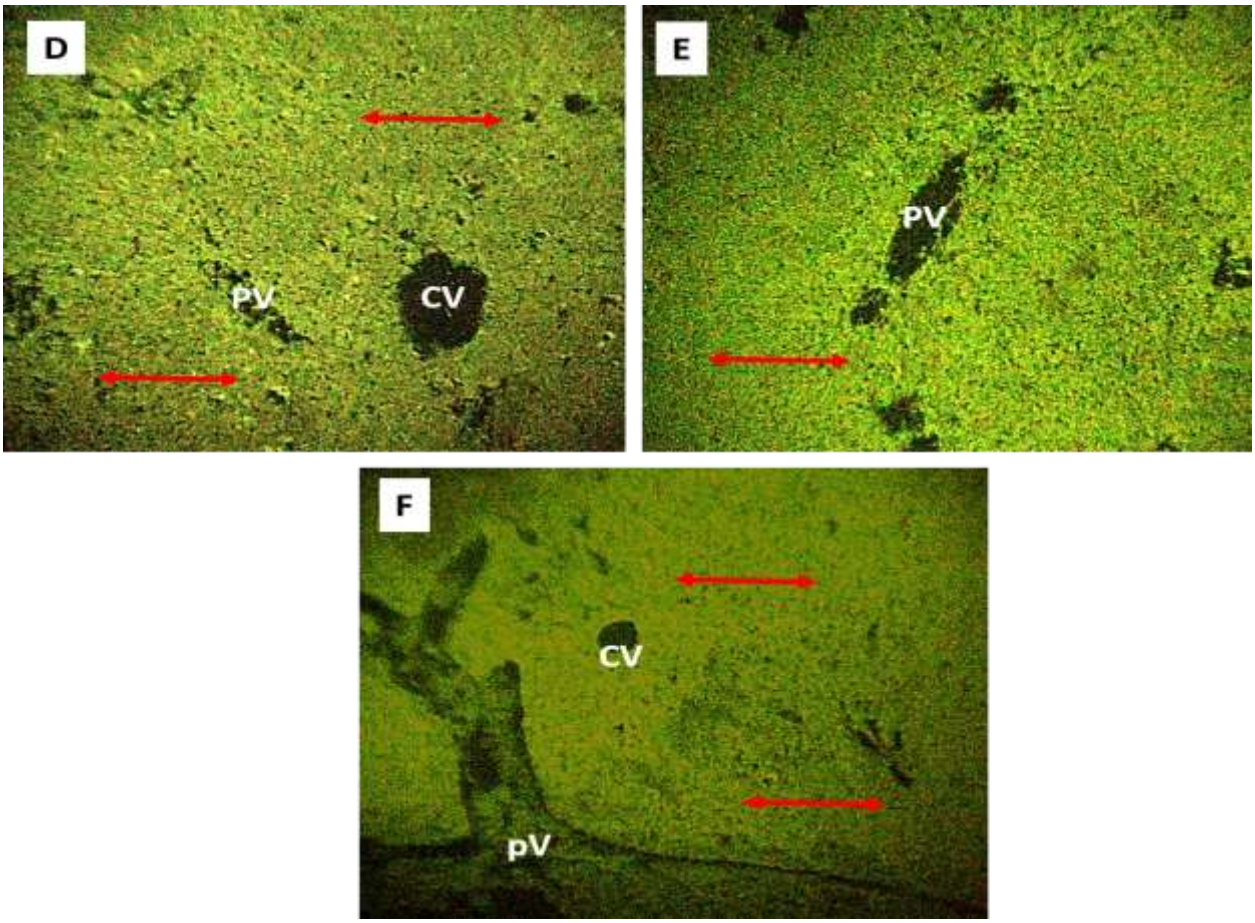


Figure 12 photomicrograph of a liver section where D & E: prevention (Prevent) mice. F: treatment (Treat) mice. Acridine orange / propidium iodide stain.

4. DISCUSSION

The main objective of the current study was to verify the in vivo hepatoprotective potential of nano-mixture of curcumin and chitosan (Cur - Chito NP) in prevention and treatment techniques against liver fibrosis in mice induced by CCl_4. So, we can study the cellular mechanisms behind oxidative damage and study histopathological alteration in experimental animals.

The acceleration of lipid peroxidation and the formation of free radicals by CCl_4 metabolism in the liver produces hepatocyte necrosis, inflammation, and accelerates the advancement of hepatic fibrogenesis. Steatosis and lipid peroxidation, which target and degrade polyunsaturated fatty acids, can occur when lipid metabolism is disrupted. In hepatic carcinoma, the reaction with DNA is considered the first stage. The

radical has a significant impact on the mitochondrial endoplasmic reticulum (MER) and plasma membrane, causing cellular damage [32,33]. The oxidative injury caused by CCl_4 broke the hepatic plasma membrane, and enzymes that are normally found in the cytosol were released into the bloodstream as a result of the previous mechanism. Hepatotoxicity is caused by the trichloromethyl radical ($CCl3^{\bullet}$), which alkylates cellular proteins and other macromolecules while simultaneously attacking polyunsaturated fatty acids to form lipid peroxides in the presence of oxygen, resulting in liver damage.

It was also discovered that cytochrome P-450 is the only enzyme that activates CCl_4, resulting in reactive metabolites such as trichloromethyl radical ($CCl_3^{\bullet}$) and dichloromethyl radical ($CHCl_2^{\bullet}$). These metabolites have the ability to increase triglyceride accumulation in hepatocytes and thus lipid peroxidation, resulting in hepatotoxicity. [14,33,34,] stated that cytochrome P-450 mainly located in endoplasmic reticulum (ER) of hepatic cell to participate in xenobiotic transformation and other important substances metabolism such as: vitamins, cholesterol, bile acids and steroid hormones, where microsomal cytochrome P-450 makes a strong partnership with electron donner NADPH.

Hepatic stellate cells are found between hepatocytes and sinusoidal endothelial cells and are dormant, are important players in the fibrotic process, where their initiation, activation, and transformation into myofibroblasts cause fibrogenesis through the overproduction of type I and III collagens. Various cytokines and chemokines, such as PDGF, EGF, and the fibrogenic factor TGF, generated by Kupffer cells and active HSC, stimulate the activation of HSC. Furthermore, standard antifibrotic medications have relatively limited therapeutic effects because there is no particular treatment for the liver that targets the fibrotic tissue [35,36].

Nanotechnology-based medicines have recently emerged as a possible alternative to traditional therapy. Nanotechnology is a promising discipline of research that focuses on the use of materials with sizes ranging from 1 to 500 nanometers. Nanoparticles are currently being made out of biocompatible materials, and they are putting a lot of work into drug delivery, not only by optimising the physical and chemical properties of the medication, but also by targeting the drug to the site of action and reducing side effects. As a result of their capacity to overcome several biological, biophysical, and biomedical constraints, NPs can be changed for effective and targeted medication delivery. [37] declared that curcumin as a chemical compound suffers from a spontaneous transformation process called autoxidation, where it's converted into bicyclopentadione. Here, chemical instability of curcumin is a crystal clear fact; it degrades quickly at physiological pH but more slowly when incubated in the presence of serum. Protein especially albumin increases the half-life of curcumin from a few minutes to 1 – 2 hours. On the other hand, there are worthwhile factors to make in consideration for curcumin stability in vivo: (a) presence of minor curcuminoids such as demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) enhance the curcumin stability [38]. (b) albumin increases the stability of curcumin against the autoxidation process. (c) glucuronidation of curcumin at the phenolic hydroxyl is a major metabolic pathway in vivo. Glucuronidation contributes to enhancing the stability of curcumin by blocking the free hydroxyl required for autoxidation.

[39] reported on the effect of liposomal curcumin (lcc) on acetaminophen (apap) hepatotoxicity, stating that there was not only a significant decrease in oxidative stress and matrix metalloproteinases (mmp-2 & mmp-9), but also an increase in antioxidant levels in groups treated with liposomal curcumin (lcc) compared to silymarin-treated animals. the histological investigation, fortunately, confirmed the findings.

In another study belongs to [40], showed that curcumin has great anti-fibrotic potential against APAP with liver fibrotic action in mice. Bulku and his team elucidated that under the dominant influence of APAP, caspase-3 activates the cell death signals probably through the mitochondrial pathway. This suggests that the caspase-3-activated apoptotic pathway may have a high rate of apoptosis. Curcumin, on the other hand, was found to be capable of blocking p53-mediated death pathways in hepatocytes. Despite the presence of

APAP, the Bulku study found that p53 protein levels are considerably lower in CUR-exposed livers. CUR has also driven hepatocytes to avoid programmed cell death. CUR suppresses APAP-induced apoptosis in hepatocytes via a p53-dependent mechanism, which is supported by the decrease in caspase-3 expression and subsequent suppression of DNA fragmentation.

Curcumin has poor bioavailability, similar to other natural medicines used in traditional therapeutics, due to poor oral absorption and fast metabolism in the liver and intestines [41]. To solve the shortcomings of bulk curcumin and increase its bioavailability, delivery system, and controlled drug release, the current work utilised curumin as nanoparticles in combination with nano chitosan.

Due to the positive charges on chitosan, it can interact with the negative component of a cell's membrane, facilitating drug delivery across cellular barriers and transiently causing the opening of tight junctions between epithelial cells, explaining the polysaccharide's permeation boosting characteristic. One of these studies; [42] that demonstrated the importance of positively charged chitosan in enabling interactions with negatively charged bacterium membranes. The positively charged AuNPs can interact with both gram-positive and gram-negative bacteria's anionic cell membranes, resulting in AuNP aggregation on the bacterial cell membrane and bacterial cell lysis.

[43] stated that the natural biopolymer chitosan holds some immunological functions including inhibition of pro-inflammatory cytokines. So, chitosan submit its utility as an anti-inflammatory and wound healing accelerant.

If the case of continual development of liver disease, it can move from simple steatosis to serious pathological diseases such as hepatitis, fibrosis, cirrhosis, and hepatocellular cancer. Collagen is the most abundant protein in the extracellular matrix, and HSCs are the primary source of collagen in the liver. Fibrosis is characterised by excessive collagen deposition between hepatocytes and sinusoids. Chronic tissue injury, reactive oxygen species (ROS), inflammatory cytokines, and apoptotic signals all activate the HSC. Furthermore, oxidative stress causes HSCs to become more active. If the imbalance between collagen synthesis and degradation persists, fibrosis will progress to cirrhosis. Cirrhosis, on the other hand, is the final stage of increasing fibrosis, marked by the degeneration of hepatic lobule structures and blood flow failure [44].

The present study has figured out an important observation related to liver efficiency; throughout different groups' animal sacrifice, mice of (CCl_4) group were with crystal clear ascetic discharges. While, mice of prevention (Prev) & treatment (Treat) groups were mostly with normal healthy peritoneal cavity like negative control (NC) mice. Thus could be related to liver malfunction in (CCl_4) group to produce albumin which plays key role in maintain colloid osmotic pressure. Where circulating blood tends to force fluid out of the blood vessels and into the tissues, where it results in edema so, preserve the fluids within capillaries and not let it to diffuse into tissues and then accumulate through peritoneal cavity causing edema as shown in (CCl_4) group mice.

According to [45]; copper produced lipid damage and thus lipid peroxidation, as evidenced by a significant reduction in hepatic CAT, SOD, and GSH in the $CuSO_4$ treated group. The antioxidant defense systems CAT, SOD, and GSH play an important role in oxidative stress prevention. Curcumin's potential to prevent liver dysfunction and changes in antioxidant parameters may be attributed to its free radical scavenging activities and antioxidant activity. Curcumin's protective properties may be attributed to their ability to chelate copper by forming complexes. Lipid peroxidation produces MDA as a byproduct. MDA levels in the liver were higher in the $CuSO_4$-treated group and lower in the curcumin-treated group. Other antioxidants such as CAT, SOD, and GSH also restored to normal levels, with a statistically significant increase when compared to the $CuSO_4$-treated group.

Selvam et al., conducted an experimental study, estimated the in vitro antioxidant potential of synthesized pyrazole and isoxazole analogs of curcumin, demonstrated all the compounds with methoxy phenolic compounds had high antioxidant effect comparable to curcumin. However, compounds with two methoxy groups, such as curcumin (compound 1), pyrazole analogue of curcumin (compound 4) and isoxazole analogue of curcumin (compound 7) showed higher antioxidant activity than the other curcumin analogues, demonstrating that the ortho-methoxy substitution improved the phenoxy radical's stability. Furthermore, compound 4 outperformed the other two compounds in terms of scavenging activity, which could be attributed to the presence of pyrazole NH [46].

5. CONCLUSION

By the present study, it is a crystal clear result that nano curcumin – chitosan mixture demonstrated significant hepatoprotective activity reflected by regulation of liver enzymes (ALT, AST & ALP), AFP, Caspase-3, oxidative stress biomarker (MDA), antioxidant biomarkers (GSH & CAT) and approved by histopathological study.

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