Slov Vet Res 2023; 60 (Suppl 25): 93–109
DOI 10.26873/SVR-1568-2022

Original Research Article
Veterinary Medicine and The One Health Concept

PROPOLIS LOADED POLYVINYL ALCOHOL ATTENUATES CCL4 INDUCED HEPATIC FIBROSIS VIA MODULATION OF LET-7B/TGF-B/SMAD SIGNALING PATHWAY

Saydat Saad[1], Doaa M. Abdel-Fattah[1], Aya M. El-Sobky[1]*, Tarek Khamis[2]

[1]Biochemistry department, [2]Pharmacology department, Faculty of veterinary medicine, Zagazig University, 44519 Zagazig, Egypt

*Corresponding author, E-mail: ayaelsobky93@yahoo.com

Abstract: Chronic liver disorders are a serious global health issue due to their widespread incidence. Nephropathy described the deterioration of kidney function. Safe drug delivery by nanoparticles is a rapidly developing field with promising applications in the treatment of a wide variety of diseases. The current study aimed to evaluate the use of propolis nanoparticles for managing carbon tetrachloride (CCl4)-induced hepato-nephropathy on rats. Seventy adult males Spargue Dawley rats were allocated into 7 equal groups 10 rats of each. Control, CCl4, CCl4 +Silymarin, CCl4 + propolis, CCl4+Nano-propolis, CCl4 +Silymarin +Propolis, CCl4 +Silymarin +Nanopropolis. Hepato- nephropathy was induced with oral administration of CCl4 dissolved in olive oil at dose of (1gm/kg) for 4 weeks. Silymarin, propolis and nanopropolis were orally administrated at a dose of (200mg /kg), (100 mg/kg) and (30 mg /kg) respectively for 4 weeks post hepato-nephropathy onset. Biochemical, molecular analysis, histological assessment of liver and kidney and serum oxidative stress were done. CCl4 caused a marked deterioration in biochemical, oxidative stress markers (MDA, TAC, CAT), serum TNF-α, IgM, molecular markers (SMAD-2, SMAD-3, SMAD-7, MMP-9, Desmin, TGF-β1, and let-7b), and the histopathological pictures of both liver and kidney. The above-mentioned parameters were restored with administration of silymarim + Nano-propolis, silymarin + propolis, silymarin, Nano-propolis, and propolis in order. Based on the previous findings we could speculated that combined therapy of nano-propolis and silymarin could be implicated in managing hepato-nepheropathy since it improves both liver and kideny function by targeting let-7b/TGF-β/Smad Pathway.

Key words: gene expression; hepatic fibrosis; nanoparticles; nephropathy; propolis; CCl4

Introduction

Hepatic fibrosis is the body's wound-healing response to liver injury from many causes. Cirrhosis is the most progressive form of fibrosis, meaning not just fibrosis but also liver parenchyma distortion, formation of septae and nodules, altered blood flow, and the likelihood of liver failure (1). Many medicines with strong antifibrotic properties *in vitro* have only moderate effects *in vivo* because insufficient quantities accumulate surrounding the target cell, causing detrimental effects in non-target cells. Hepatic stellate cells are the target cells of antifibrotic therapy because they play a key role in hepatic fibrogenesis (2).

The decline in renal function was termed nephropathy. End-stage renal disease, often known as ESRD, is the terminal stage of nephropathy. Reduced glomerular filtration rate, elevated albuminuria with time, high arterial blood pressure, and fluid restriction are the results of three types of nephropathies: nodular glomerulosclerosis, thickened glomerular basement membranes, and mesangial expansion. The two forms of nephropathies are diabetic nephropathy (DN) and hypertensive nephropathy. Hypertensive nephropathy is a kidney disease caused by a history of high blood pressure. It is

Received: 5 September 2022
Accepted for publication: 19 October 2022

a chronic disorder that poses a significant risk of developing ESRD (3).

Nanotechnology is the study and application of the unique chemical and physical properties of objects smaller than 100 nm in size, including enhanced chemical and physical reactivity and solubility. Because of its rapid and accurate motions, as well as its superior bioavailability and biodegradability, it offers a scientific advantage. The output of livestock animals, economic losses, and the creation of nutritious food and feed are all greatly impacted by all of these positives (4).

The chemical molecule CCl4 is a colorless and volatile liquid. Hepatotoxicity and nephrotoxicity are side effects. Hepatic fibrosis and nephropathy are two of the world's leading causes of death (5).

Honeybees create propolis, a natural resinous mixture made up of components collected from plant parts, buds, and exudates. Propolis is currently used as an antibacterial, anti-inflammatory, antiviral, anti-oxidant, anti-protozoal, anesthetic, anti-tumoral, anti-hepatotoxic, etc (6).

Propolis samples from all over the world were analysed, and researchers found that the major component varied between tropical and temperate regions. Despite the widespread availability of propolis in the Americas, the vast majority of research has focused on Brazilian green propolis. Liquidritigenin, formononetin, and biochanin A are the main components of red propolis, and they are extracted mostly from Dalbergia ecastophyllum. Flavonoids, phenolic acids, and their derivatives like caffeic acid phenethyl ester (CAPE), pinocembrin, and naringenin make up the bulk of Mexican poplar propolis. Acids like abietic acid, isopimaric acid, cycloartenol, and isocupressic acid can be found in the Mediterranean propolis that is harvested in Turkey, Egypt, and Greece (7).

The phenolic acids and polyphenols like flavonoids, are responsible for propolis biological actions. These substances have a low water solubility and bioavailability. To circumvent the physiological limits of propolis nanocarriers were mostly utilized as drug delivery methods. Furthermore, propolis in nanoform has been found to be more effective than propolis in terms of antibacterial and antifungal (8).

Milk thistle seeds, or silymarin, have been used to treat liver problems for undreds of years. The protective effects of silymarin on healthy liver cells or cells that have not yet been irrevocably damaged have been suggested by preclinical research (9).

This work aimed to evaluate the anti-inflammatory and apoptotic effects of propolis nanoparticles to that of the medication silymarin in a rat model of carbon tetrachloride (CCl4)-induced hepatic fibrosis and nephropathy. Biochemical and molecular analyses of blood and tissue samples were performed to evaluate hepatotoxicity, nephrotoxicity, and the results of treatment.

Material and methods

Materials and reagents

The propolis was purchased from Sigma Aldrich. Propolis nanoparticles, were prepared at NanoTech Egypt for photo-electronics (Dreamland,El-Wahaat Road, 6th October, Giza, Egypt, Email; sales@nanotecheg.com). Silymarin was purchased as powder from SEDICO-Egypt. All other chemicals used in this experiment were purchased from sigma Aldrich (St. Louis, MO).

Preparation of propolis nanoparticles

The emulsion diffusion technique was used to create propolis nanoparticles. Both the polyvinyl alcohol (PVA) (Fisher Scientific, UK) and the propolis solutions were dissolved in 10 ml of distilled water and then swirled for 30 minutes on a magnetic stirrer. The propolis solution was then dropped per drop onto aqueous PVA, stirred for 30 minutes, then centrifuged for 30 minutes at 2000 rpm. A transmission electron microscope was used to examine the clear supernatant containing propolis nanoparticles ([10]). TEM was carried out on a JEOL JEM-2100 high resolution transmission electron microscope with a 200 kV accelerating voltage.

Animal model and study protocols

Male albino rats (n = 70) weighing 200 to 250 g, obtained from the Animal House of the Faculty of Veterinary Medicine, Zagazig University were housed in a specific pathogen free animal laboratory and permitted free access to food and water. After a week of adaptation, rats were randomly divided into 7 groups each group containing 10 rats. Negative control group 1 in which rats have not received any treatment. Positive control group 2

in which rats were received orally CCl4 1 gm /kg (50% in olive oil) twice a week for 4 weeks. Group 3 CCl4-silymarin group in which rats were treated orally with silymarin (200 mg/kg) per day (11). Group 4 CCl4-propolis group in which rats were treated orally with propolis (100 mg/kg) per day (8). Group 5 CCl4-nanopropolis group in which rats were treated orally with nanopropolis (30 mg/kg) per day (8). Group 6 CCl4-silymarin and propolis group in which rats were treated orally with silymarin (200 mg/kg) and with propolis (100 mg/kg) per day which called combination group. Group 7 CCl4- silymarin and nanopropolis group in which rats were treated orally with silymarin (200 mg/kg) and with nanopropolis (30 mg/kg) per day which called combination group. The experimental work was approved by the Institutional Animal Care and Use Committees Zagazig University (ZU-IACUC) with approval No. ZU-IACUC/2/F/69/2022.

Sample collection and processing

Overnight, the rats were kept without food or water before being given ketamine/xylazine to put them to sleep. The rats were sacrificed, and their tissues were removed, after blood samples were taken from the orbital venous plexus, allowed to clot, and centrifuged at 3,000 rpm for 15 minutes. The serum samples were stored at -20 °C. Rats were sacrificed so that their organs could be harvested for study. When performing real-time PCR analysis, we snap-froze a sample of tissue by enclosing it in aluminium foil and placing it in a container of liquid nitrogen to stop the activity of endogenous RNase. Histological analysis was performed on another sample of tumour tissue after it was fixed in a 10% buffered formalin solution for 24 hours at room temperature.

Molecular determinations of liver and kidney tissues

The PureLink® RNA Mini Kit (Catalog number: 12183018A) from Ambion by life technologies by Thermo Scientific was used to extract total RNA. The purity of RNA samples was determined using a NanoDrop® ND-1000 Spectrophotometer from NanoDrop Technologies in Wilmington, Delaware, USA.To create cDNA, we used a Thermo Scientific High-Capacity cDNA Reverse Transcription Kit (catalogue number 4374966). To measure the gene expression of small mothers against decapentaplegic protein SMAD-2, SMAD-3, SMAD-7, Matrix Metallopeptidase 9 (MMP-9), let-7b miRNA, Desmin and TGF-β1, we used a Maxima SYBR Green qPCR Master Mix (2X) kit obtained from Thermo Scientific (cataloug #K0251). GADPH was used to standardize gene expression. The $2^{-\Delta\Delta ct}$ formula was used to calculate the amount of target gene expression levels. The following primers were used described in (Table 1).

Table 1: Primer sequence for genes used in this experiment

Gene	Sequence	Gene bank accession No.
SMAD-2	F 5′- CAAACGTGCACAGGTGACAG-3′ R 5‘- GACTGGCGTTGGAAGAAGGA-3‘	NM_001277450.1
SMAD-3	F 5′- CTGGGCAAGTTCTCCAGAGTT-3′ R 5‘- AAGGGCAGGATGGACGACAT-3‘	NM_013095.3
SMAD-7	F 5′- GAGTCTCGGAGGAAGAGGCT-3′ R 5‘- CTGCTCGCATAAGCTGCTGG-3‘	NM_030858.2
MMP-9	F 5′- GATCCCCAGAGCGTTACTCG-3‘ R 5‘- GTTGTGGAAACTCACACGCC-3‘	NM_031055.2
Desmin	F 5′- ATCTGCGGGAGTACCAGGAT -3′ R 5‘- GCAGAGAAGGTCTGGATCGG -3‘	NM_022531.2
TGF-β1	F 5′- AGGGCTACCATGCCAACTTC -3′ R 5‘- CCACGTAGTAGACGATGGGC -3‘	NM_021578.2
let-7b	F 5′- AACACGTGTGAGGTAGTAGGTT -3′ R 5‘- GTCGTATCCAGTGCAGGGT -3‘	MI_0000063
GADPH	F 5′- GCATCTTCTTGTGCAGTGCC -3′ R 5‘- GGTAACCAGGCGTCCGATAC -3‘	NM_017008.4

Determination of liver functions as markers of hepatic injury

Enzymatic colorimetric kits from Spinreact, Girona, Spain were used for quantitative detection of Alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST), and Serum lactate dehydrogenase (LDH) as inflammatory marker was assessed by using enzymatic colorimetric kits from Spinreact, Girona, Spain according to manufacture instruction as described by Reitman and Frankel (12).

Determination of kidney function as marker of kidney injury in Serum

Serum creatinine was quantitatively assessed colorimetrically, using enzymatic colorimetric kits from Spinreact, Girona, Spain.

Determination of IgM, TAC, MDA, CAT, TNF-a

The serum Immunoglobulin M (IgM) levels were evaluated by using the IgM ELISA Kit (rat) (CusaBio; Catalog No: CSB-E07978r-1), the serum total antioxidant capacity (TAC) levels were determined using OxiSelect™ TAC Assay Kit (Cell Biolabs, Inc, Catalog No: STA-360), the serum Malondialdehyde (MDA) levels were determined using Rat Malondialdehyde ELISA Kit (MyBioSource, Catalog No: MBS738685), and the serum catalase (CAT) levels were evaluated by using Rat catalase ELISA Kit (MyBioSource,, catalogue number: MBS701908). The serum tumor necrosis factor alpha (TNF-α) levels were evaluated by using Rat TNF-α ELISA kit (CusaBio; Catalog No: CSB-E11987r), according to the manufacturer's directions.

Histopathology

The tissues of the kidney and liver of rats were obtained and preserved with formaldehyde solution (10 % w/v) and processed in an automated tissue processor. Following that, follow-up techniques were used to do histological study of these tissues. The tissues were then fixed in paraffin blocks at the end of the operation. They were then cut into 5–6 m thick paraffin sections and blemished with haematoxylin–eosin.

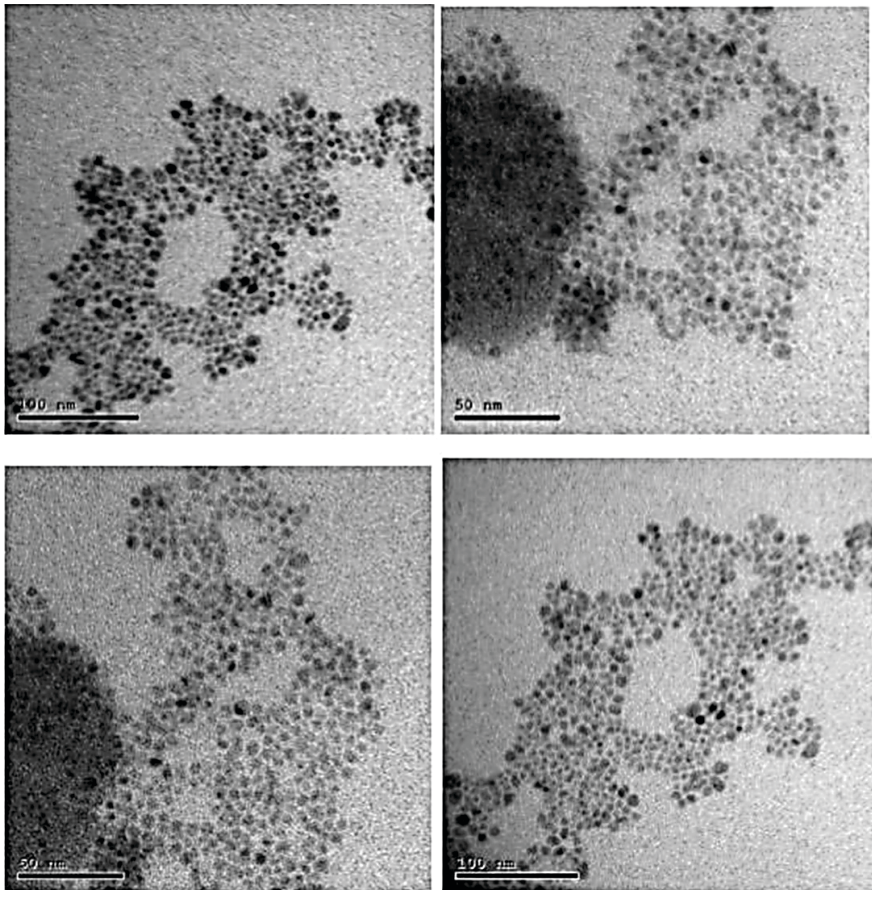

Figure 1: Size of Nano Propolis revealed by TEM

Statistical analysis

Standard deviations and means were used to summarise the data. The Shapiro-Wilk test establishes whether or not a distribution is normally distributed, the boxplot identifies outliers, and the Levene test evaluates for homogeneity. To compare group means, we used one-way analysis of variance (ANOVA), and to determine whether there were significant differences between groups, we used the Duncan's multiple range test. Having a P-value of less than 0.05 indicates statistical significance. Statistical analysis was performed using SPSS version 25. (Armonk, NY: IBM Corp).

Results

Transmission electron microscopy (TEM) validated the nanoparticle size and surface shape of the propolis (Figure 1). Nanoparticles were measured and determined to be spherical, with an average particle size of 10 nm and a standard deviation of 3 nm. Using a JEOL JEM-2100 high resolution transmission electron microscope and 200 kV accelerating voltage, we performed the real physical measurement.

Effect of silymarin (200 mg/kg/b.wt), propolis (100 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on hepatic gene expression mRNA

The mRNA levels of a MMP-9 in male albino rats which exposed to silymarin, propolis and nanopropolis for 1 month were analyzed using real-time PCR. The normalization of target gene expression data was done using housekeeping gene. The GADPH gene is found in all cells and is one of the most widely used in gene expression data comparisons The results demonstrated that there was highly statistically different among groups in MMP-9 values (Figure 2 A). Negative control group revealed the lowest MMP-9 reading and the highest significant elevation of MMP-9 in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant downregulation of MMP-9 values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

In our study, the expression SMAD-3 gene in the negative control group had revealed the lowest reading while it was significantly increasing in positive control group. The level of SMAD-3 gene expression was significantly downregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 2 B).

In reverse SMAD-7 gene expression was significantly the highest reading in the negative control group while in the CCl4 positive group showed the lowest reading. The level of SMAD-7 gene expression was significantly upregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 2 C).

Also let-7b gene expression was significantly the highest reading in the negative control group while in CCl4 positive group showed the lowest reading that was non-significant with CCl4+ propolis. The level of let-7b gene expression was significantly upregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 2 D).

It was revealed that there was highly statistically different among groups in TGF-β1 values. Negative control group revealed the lowest TGF-β1 reading and the highest significant elevation of TGF-β1 in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant downregulation of TGF-β1 values in comparison

with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group. This means that the group treated with CCl4+nanopropolis+silymarin can downregulate the TGF-β1 gene expression than other treatment groups (Figure 2 E).

Effect of silymarin (200 mg/kg/b.wt), propolis (100 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on renal gene expression mRNA

It was revealed that there was highly statistically different among groups in TGF-β1 values. Negative control group revealed the lowest TGF-β1 reading and the highest significant elevation of TGF-β1 in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant downregulation of TGF-β1 values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 A).

In our study, the expression SMAD-2 gene in the negative control group had revealed the lowest reading while it was significantly increasing in CCl4 positive control group. The level of SMAD-2 gene expression was significantly downregulated in the group

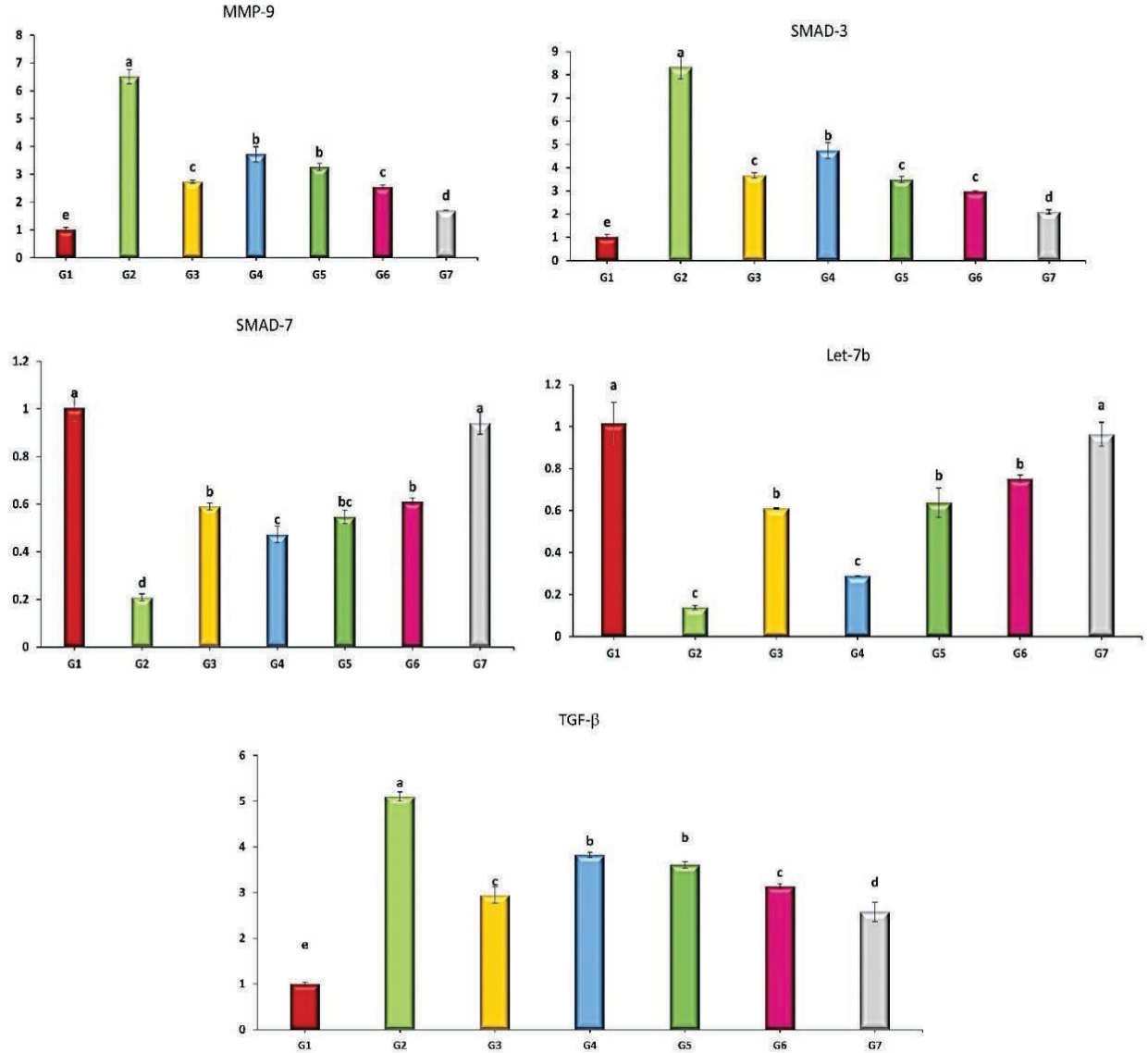

Figure 2: Effect of propolis (100 mg/kg/b.wt), silymarin (200 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on hepat-ic gene expression mRNA. Small mothers against decapentaplegic protein (SMAD-3, SMAD-7), Matrix Metallopeptidase 9 (MMP-9), Transforming growth factor β (TGF-β)

treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 B).

The SMAD-7 gene expression was significantly the highest reading in the negative control group while the CCl4 positive control group showed the lowest reading. The level of SMAD-7 gene expression was significantly upregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 C).

The let-7b gene expression was significantly the highest reading in the negative control group while the CCl4 positive control group showed the lowest reading. The level of let-7b gene expression was significantly upregulated in the group treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 D).

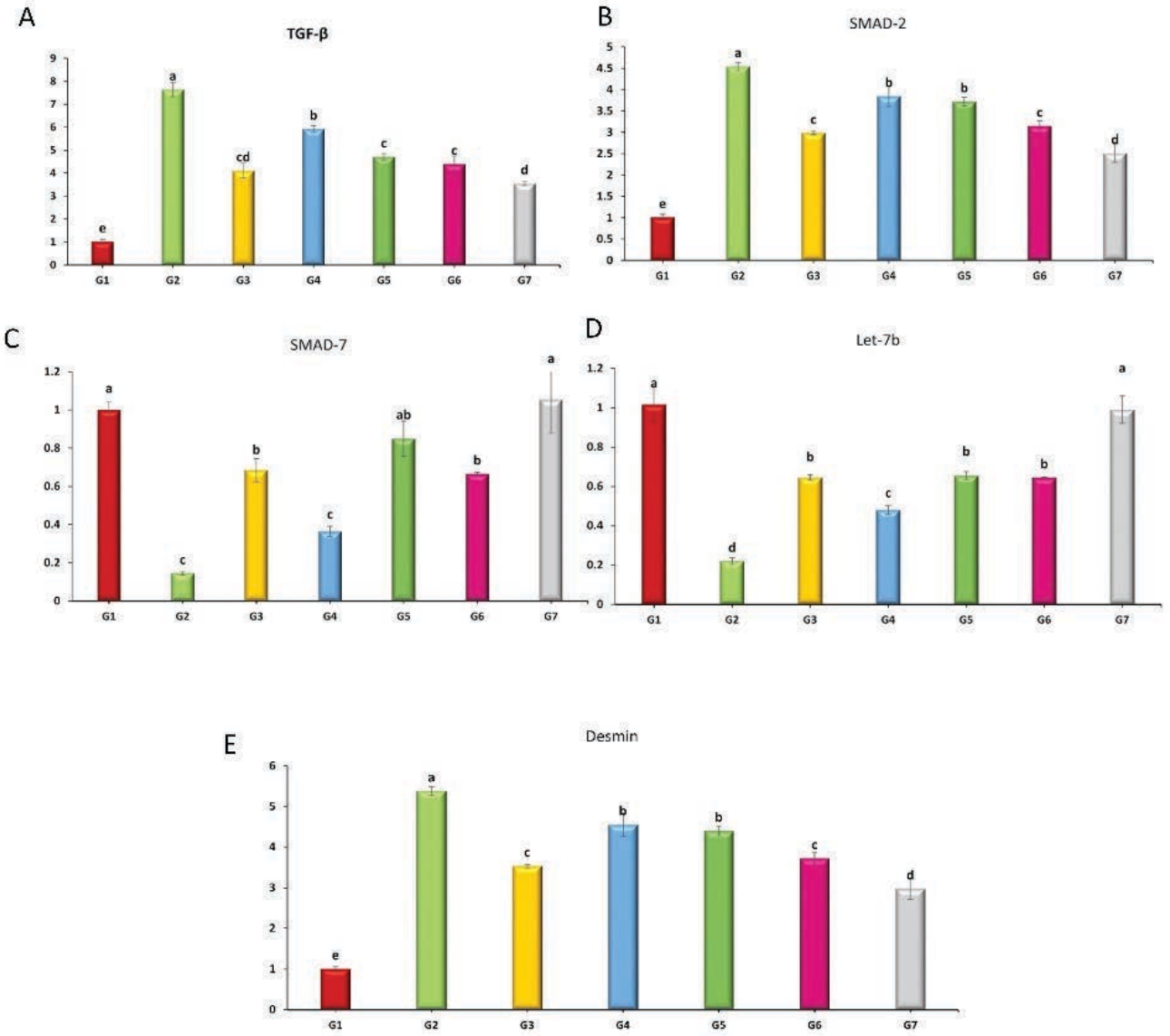

Figure 3: Effect of propolis (100 mg/kg/b.wt), silymarin (200 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on renal gene expression mRNA. Small mothers against decapentaplegic protein (SMAD-2, SMAD-7), Transforming growth factor β (TGF-β)

The results demonstrated that there was highly statistically different among groups in Desmin values. Negative control group revealed the lowest Desmin reading and the highest significant elevation of Desmin in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant downregulation of Desmin values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group (Figure 3 E).

Effect of silymarin (200 mg/kg/b.wt), propolis (100 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on kidney function, LDH and liver functions

One-Way ANOVA results revealed that levels of the parameters of liver function test showed high significant differences among groups $p < 0.0001$***. The highest levels of ALT, AST, ALP, LDH, and creatinine were recorded in CCl4 positive control group. that showed significant difference with group treated with other groups. The lowest levels of ALT, AST, and LDH were in control negative group, CCl4+ nanopropolis +sylimarin

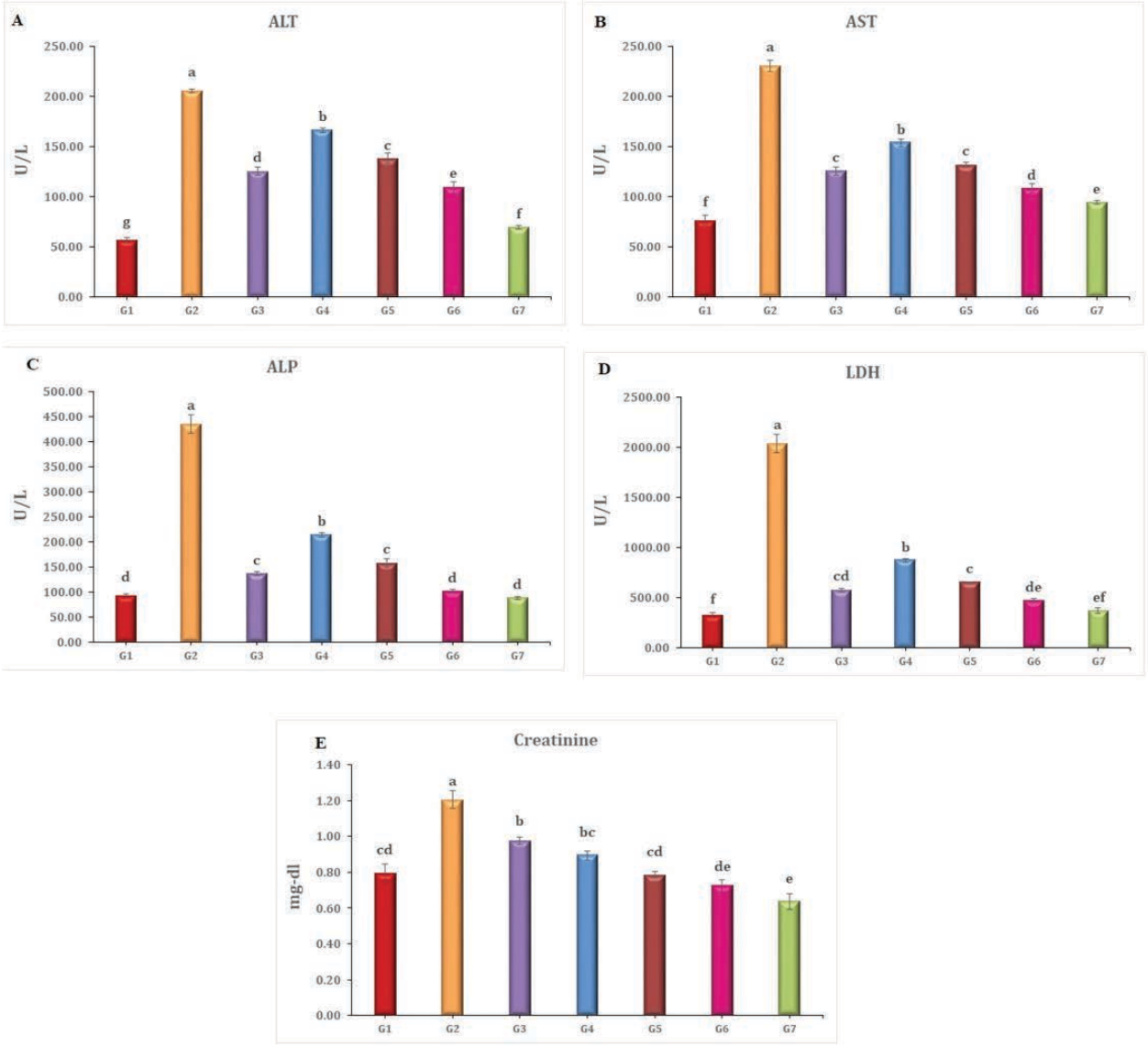

Figure 4: Effect of propolis (100 mg/kg/b.wt), silymarin (200 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on the mean value of kidney function, LDH and liver functions of rats in different treated groups. Alkaline phospha-tase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), serum lactate dehydrogenase (LDH)

treatment showed a significant up regulation in the levels of ALT, AST, ALP, LDH, and creatinine in comparison with CCl4 positive control group. These results revealed that the group of CCl4+ nanopropolis +sylimarin showed improvements in several markers than other groups (Figure 4).

Estimation of the mean value of oxidative stress markers

The result revealed that means with different superscript were statistically significant in CAT (Figure 5 A). The lowest significant value was revealed in CCl4 positive control group, and the highest significant value was found in control negative group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant increase of CAT values in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

Our data demonstrated that there was highly statistically different among groups in TAC levels (Figure 5 B). The lowest significant value was found in CCl4 positive control group, and the highest significant value was revealed in control negative group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+

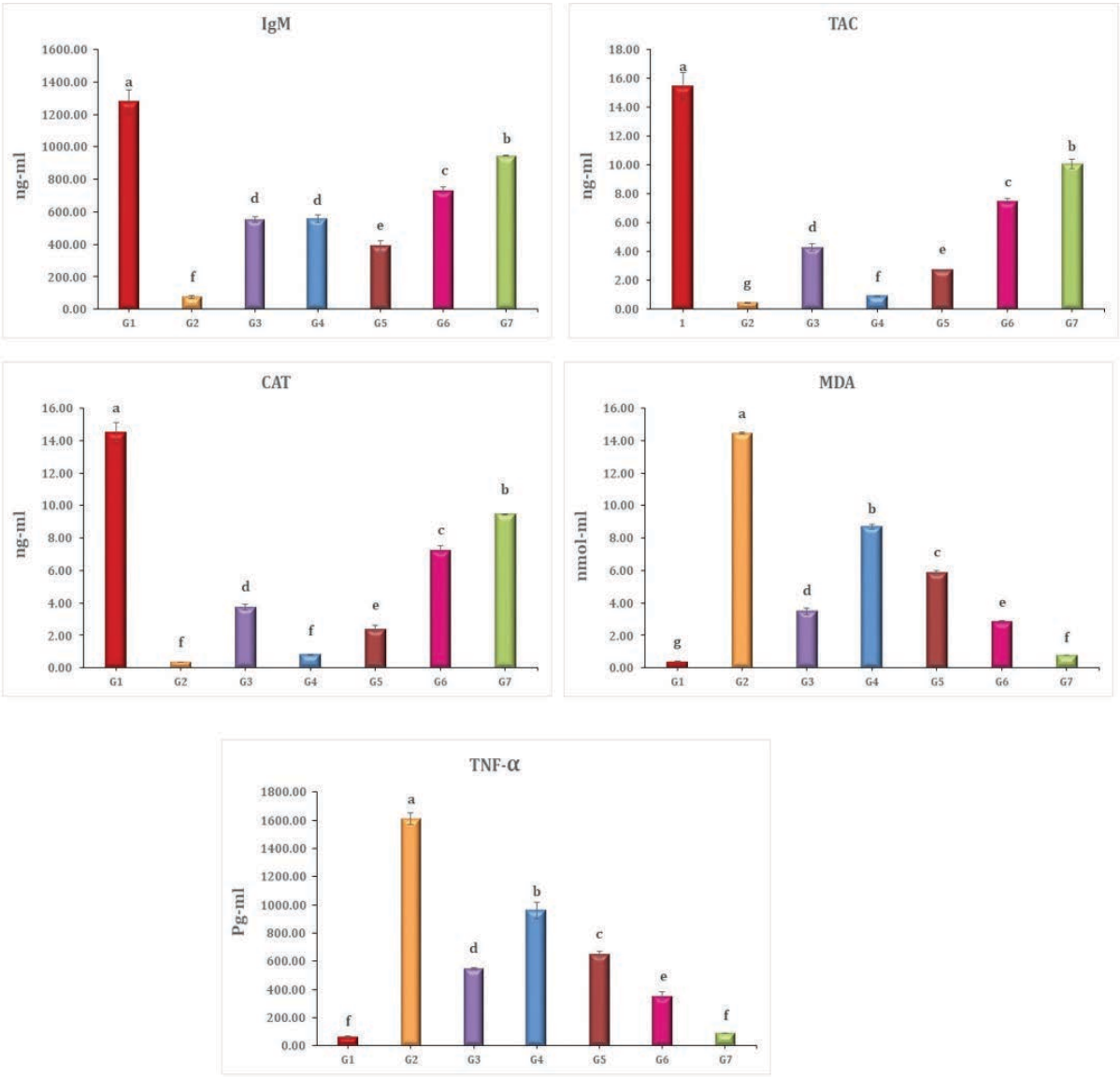

Figure 5: Effect of propolis (100 mg/kg/b.wt), silymarin (200 mg/kg/b.wt) and nanopropolis (30 mg/kg/b.wt) on the mean value of oxidative stress markers of rats in different treated groups. Immunoglobulin M (IgM), total antioxidant capacity (TAC) Malondialdehyde (MDA), catalase (CAT), tumor necrosis factor alpha (TNF-α).

nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant increase of TAC values in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

There was highly statistically different among groups in IgM levels (Figure 5 C). The highest significant value was found in control negative group, and the lowest significant value was revealed in CCl4 positive control group. The treating rats with CCl4+sylimarin and the group treated with CCl4+ propolis showed non-significant difference to each other and showed a significant increase in IgM level in comparison with CCl4 positive control group. The rats treated with CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin were effective in increase of IgM value in comparison with CCl4 positive control group. The highest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

We also explored the influence of propolis and nanopropolis on MDA activity (Figure 5 D). The results revealed that the lowest significant value was found in control negative group, and the highest significant value was demonstrated in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+propolis+silymarin and CCl4+nanopropolis+silymarin showed significant decrease of MDA values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

One-Way ANOVA results revealed that levels of the parameters of TNF-α showed high significant differences among groups $p < 0..0001$*** (Figure 5 E). The results revealed that the lowest significant value was found in control negative group, and the highest significant value was demonstrated in CCl4 positive control group. The rats treated with CCl4+sylimarin, the group treated with CCl4+ propolis, the CCl4+ nanopropolis, CCl4+ propolis+ silymarin and CCl4+ nanopropolis+ silymarin showed significant decrease of TNF-α values in comparison with CCl4 positive control group. The lowest value was revealed in CCl4+nanopropolis+silymarin treatment group after control negative group.

Histopathological Findings

Negative control group (G1): Liver and kidney sections from control group showed normal structures. Neither inflammatory, nor degenerative or apoptotic changes were recorded in any of the examined parts (Figure 6 A, B).

Positive control (CCl4 intoxicated group, G2): Identical feature of CCl4 toxicity were represented by moderate periportal hepatocytes, degenerative and necrotic changes accompanied by intense portal inflammatory reaction followed by fibroplastic hyperplasia with formation of fine fibrous strands enclosing hepatic lobules. Hepatocellular pathognomonic ballooning degeneration and necrosis with eccentrically situated pyknotic nuclei was pronounced. The portal blood vessels were dilated with occasional perivascular and interstitial hemorrhages (Figure 6 C). Renal sections demonstrated nephrotoxic changes represented by moderately dilated renal blood vessels, focal collecting tubular dilatation, moderate perivascular edema, hemorrhage and round cells infiltration beside tubular epithelial degenerative changes, glomerular lobulation and or atrophy (Figure 6 D).

CCl4-Silymarin treated group (G3): liver sections, demonstrated a comparatively moderate enhancing protective effect of the used compound as demonstrated by regenerative changes in the previously damaged hepatocytes with cytoplasmic basophilia and large hyperchromatic nuclei. Some hepatocytes were degenerated and or apoptotic (Figure 6 E). Renal pathology of this group gone parallel with the hepatic changes as a residual CCl4 toxic effects were seen and emphasized by perivascular edema, tubular epithelial degeneration, cystic dilatation and glomerular lobulation (Figure 6 F).

CCl4-Propolis treated group (G4): Sections from liver, showed a comparatively enhancing protective effect of the used compound as demonstrated by regenerative changes in the previously damaged hepatocytes with cytoplasmic basophilia and large hyperchromatic nuclei. Neither necrotic nor hemorrhagic or fibroblastic changes were recorded (Figure 7 A). Mild nephrotic changes represented by residual perivascular edema, a few dilated collecting tubules, tubular epithelial degeneration and lobulated glomerular tufts were recorded (Figure 7 B).

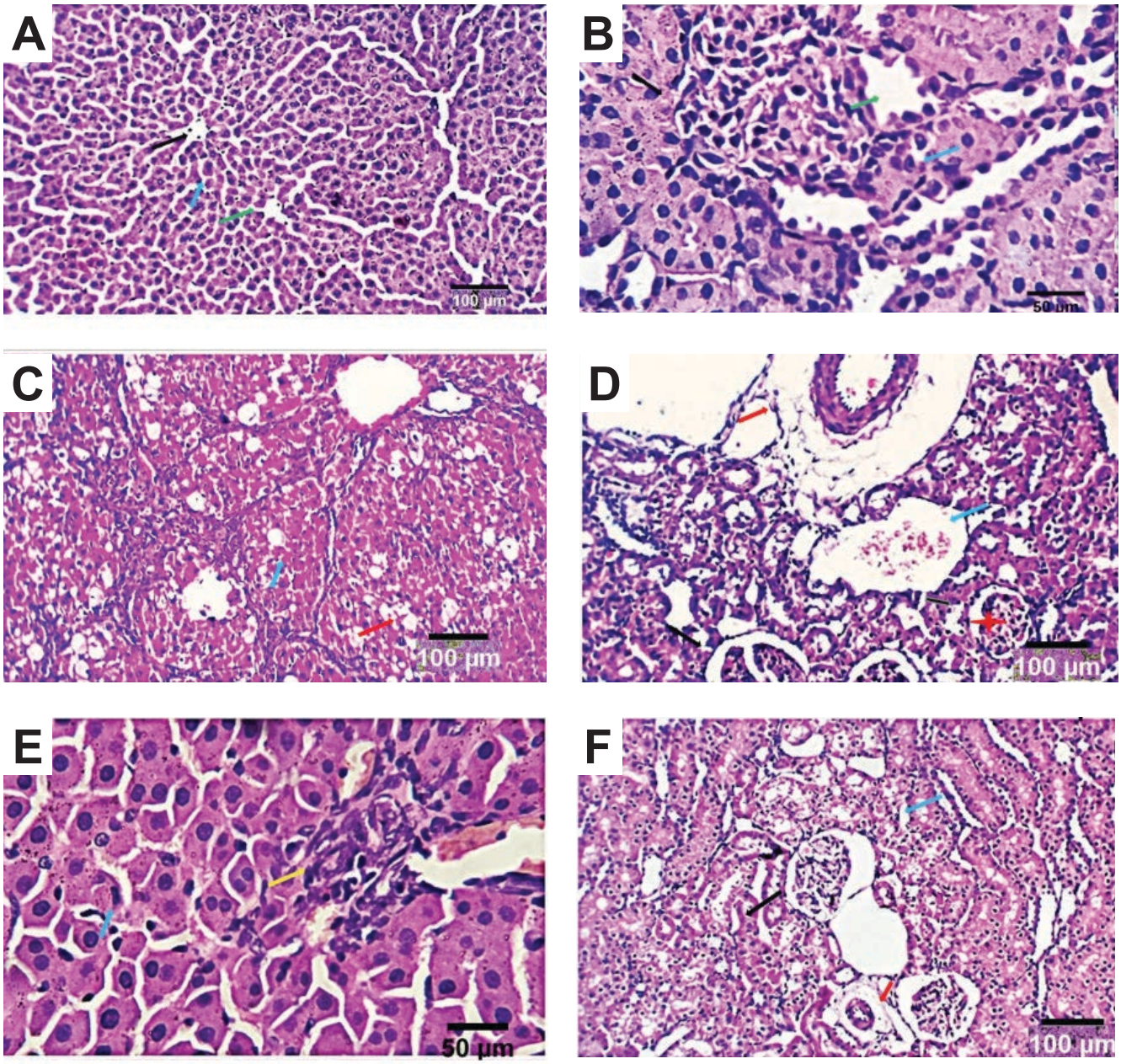

Figure 6: Photomicrograph from rat's liver (A), showing preserved hepatic cords, (blue arrows) portal triad's structures, vascular tributaries, and Von kupffer's cells (green arrows), and kidney of control negative group (B) showed apparently proximal and distal tubules besides loops of Henle (blue and green arrows). Photomicrograph from rat's liver (C) showed, moderate periportal hepatocytes, degenerative and necrotic changes accompanied by intense portal inflammatory reaction (blue arrow), hepatocellular pathognomonic ballooning degeneration and necrosis with eccentrically situated pyknotic nuclei is seen (red arrows), and Kidney (D) of control positive, CCl4-intoxicated group, nephrotoxic changes represents by moderately dilated renal blood vessels (red arrow), glomerular lobulation and or atrophy (red star). Photomicrograph from rat's liver (E), showed regenerative changes in the previously damaged hepatocytes with cytoplasmic basophilia and large hyperchromatic nuclei (blue arrow). Remnant of CCL4 toxic effects are seen and represents by periportal biliary proliferation and round cells infiltration (yellow arrow). and Kidney (F) of CCl4-Silymarin treated group (G3), renal pathology is seen as tubular epithelial degeneration (gray arrow) and glomerular lobulation (red star) (H&E X 200, 400)

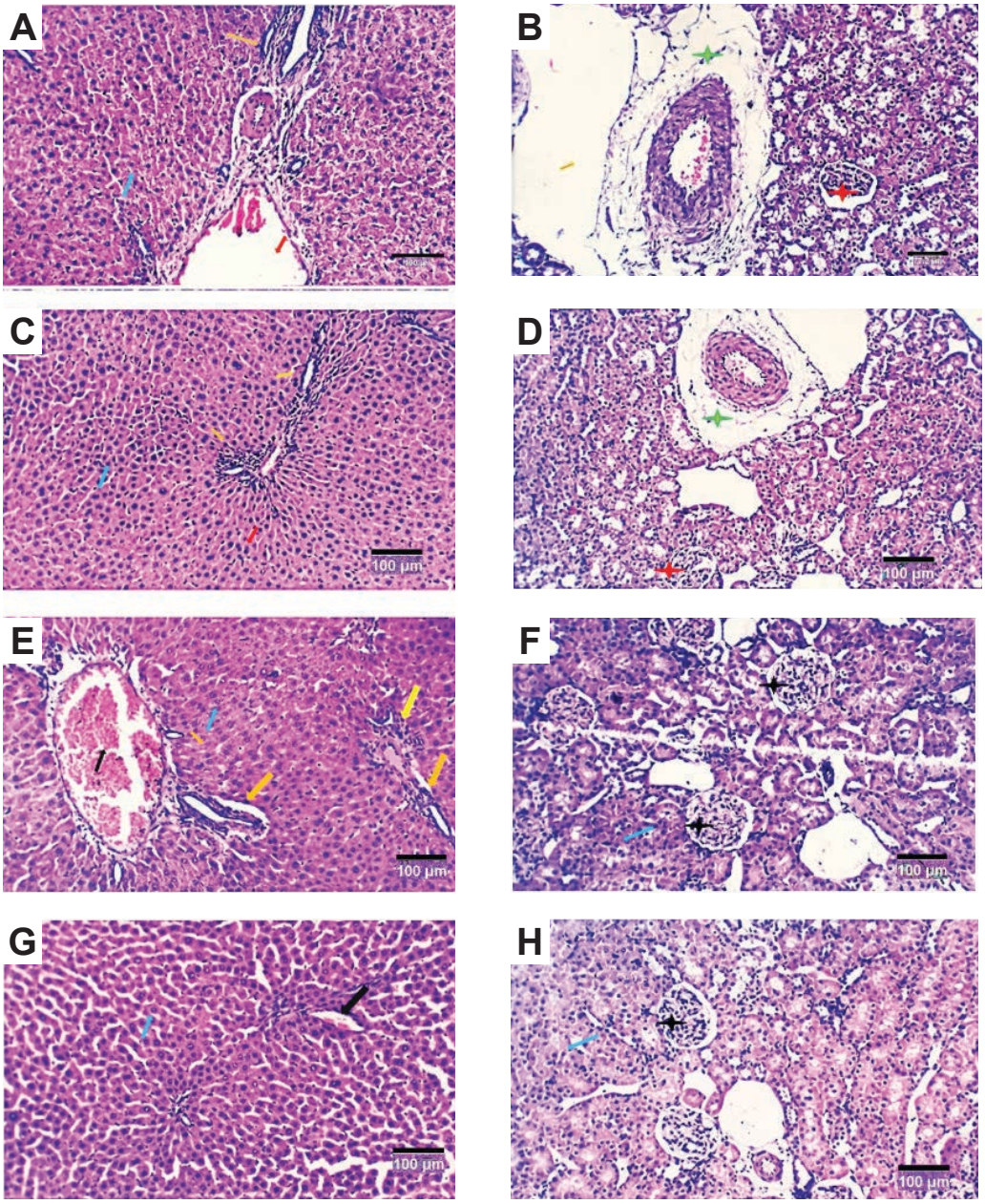

Figure 7: Photomicrograph from rat's liver (A) showed regenerative changes in the previously damaged hepatocytes with cytoplasmic basophilia and large hyperchromatic nuclei (blue arrow), portal vascular dilatation (red arrow), mild biliary proliferation and lymphocytic infiltration (orange arrows), and Kidney (B) of CCl4-Propolis treated group (G4), mild nephrotic changes represent by residual perivascular edema (green star), and lobulated glo-merular tufts (red star) were seen. Photomicrograph from rat's liver (C), showed apparently normal hepatic structures with preserved hepatic cords, some hepatocytes appeared with cytoplasmic basophilia and large hyperchromatic nuclei as a positively reacted anabolic ribosomal reactivities (blue arrow). A very few rats demonstrate residual portal round cells infiltration and biliary proliferation (orange arrows), and Kidney (D) of CCl4–Nano Propolis treated group (G5), showed an apparently normal nephron unit, excretory ducts, papillae and pelvis apart of residual focal perivascular edema and glomerular tufts lobulation (green and red arrows). Photomicrograph from rat's liver (E), showed normally arranged hepatic cords with active, occasionally binu-cleated cells (blue arrow). The portal triads structures, blood vessels and stroma were in a good histologic configuration. Mild portal blood vessels dilatation and blood engorgement (black arrow) biliary proliferation and round cells infiltration (orange and yellow arrows) are seen, and Kidney (F) of CCl4-Silymarin and propolis treated group (G6), showed normal histological structures of nephron units (black stars). Photomicrograph from rat's liver (G), showed hepatic tissue with normally arranged cords, active, occasionally binucleated cells (blue arrows), and Kidney (H) of CCl4-Silymarin and Nano propolis treated group (G7), showed absolutely normal histological structures of nephron units, collecting duct, papillae and pelvis beside normal stromal cells (blue arrows and black stars) (H&E X 100, 400)

CCl4-Nano-propolis treated group (G5): Sections from liver, showed apparently normal hepatic structures with preserved hepatic cords, portal triad's structures, vascular tributaries, biliary system, central veins, sinusoids. Some hepatocytes appeared with cytoplasmic basophilia and large hyperchromatic nuclei as positively reacted anabolic ribosomal reactivities. A few sections of the examined cases (10-15%) demonstrated residual portal round cells infiltration and biliary proliferation (Figure 7 C). Renal tissues of the same group declared a potent protective effect of the used compound as most of the examined sections showed apparently normal nephron units, excretory ducts, papillae and pelvis apart of residual focal perivascular edema and mild glomerular tufts lobulation (Figure 7 D).

CCl4-Silymarin and propolis treated group (G6): Hepatic tissue appeared with normally arranged cords with active, occasionally binucleated cells. The sinusoids comprised active phagocytic hypertrophic Von-Kupffer cells. The portal triads structures, blood vessels and stroma were in a good histologic configuration. A very few sections (8-10%) of the examined cases revealed mild portal blood vessels dilatation and blood engorgement, biliary proliferation and round cells infiltration (Figure 7 E). Renal sections demonstrated normal histological structures. A few renal blood vessels and collecting tubules were abnormally dilated. The latter showed atrophy of their lining epithelium. A few of the proximal and distal tubular epithelia were degenerated (cloudy swelling and hydropic degeneration) (Figure 7 F).

CCl4-Silymarin and Nano-propolis treated group (G7): The best protective effect was observed in this group. Hepatic tissue appeared with normally arranged cords and active, occasionally binucleated cells. The sinusoids comprise active phagocytic hypertrophic Von-Kupffer cells. The portal triads structures, blood vessels and stroma were in a good histologic configuration. A few residual round cells infiltration and fibroblasts were seen some portal areas (Figure 7 G). Renal sections demonstrated absolutely normal histological structures of nephron units, collecting duct, papillae and pelvis beside normal stromal cells (Figure 7 H).

Discussion

Nanocarriers are a new type of vehicle that is being developed to increase the solubility of medicinal compounds. Different nanonization procedures have increased the disintegration rates and bioavailability of certain medications by shrinking their size (13). Propolis nanoparticles had a substantial effect on the treatment of hepatic fibrosis; ALT, AST, LDH and creatinine levels gradually improved in the Propolis nanoparticles and silymarin group compared to CCl4 control positive group rats. The levels of ALT, AST, ALP, LDH and creatinine in the propolis nanoparticles and silymarin group had been restored to levels that were very near to control values. The recovery of hepatobiliary damage is indicated by lower level of ALP. In addition, no histological changes could be seen when compared to the control group. Our study indicated the beneficial effects of Propolis nanoparticles restoring on liver function and structure.

Our results show that propolis protects rats' livers from D-galactosamine and Lipopolysaccharide-induced toxicity, which is in line with previous studies that have linked propolis' hepatoprotective effect to its high concentration of phenolic components and their antioxidant, anti-inflammatory, and anti-apoptotic properties (14). Propolis protected rats' kidney from CCl4 damage, as shown by significantly lower serum creatinine activity in CCl4-injected rats. The antioxidant, anti-inflammatory, anti-hyperlipemic, and anti-hypertrophic properties of propolis may help to reduce cholestasis-related liver injury (15).

One more study examined at the haematological and biochemical effects of red propolis nanoparticles in dogs. Capsules containing 50 mg of polymeric nanoparticles loaded with 20% red propolis extract were orally administered once daily to eight otherwise healthy adult canines. Red propolis polymeric supplementation given orally once a day had no negative effects on hematologic, renal, or hepatic parameters in this study of healthy adult dogs. There was some evidence that nano propolis had a positive effect on the liver (16).

In a previous study utilizing propolis and nanopropolis, it was discovered that when oxidative stress was induced with alcohol, the levels of ALP and AST in male rats in the propolis-treated group and its and NP reduced dramatically (8). Another

study examined the effect of propolis and its nanoparticles on biochemical variables in the liver of tilapian fish. In comparison to the control groups, the meal containing *M. aeruginosa* cells induced significant increases in AST, ALT, ALP, creatinine, and urea. Co-administration of propolis and nanopropolis, results in significant reductions in these parameters. Propolis in nano form was more effective as a hepatoprotective agent and in competing with *M. aeruginosa* toxicity (17).

The antioxidant effect of propolis and its nano form was demonstrated in our study. The results approved with the subsequent researches described below. The highest values of IgM, TAC and CAT were revealed in CCl4+nanopropolis+silymarin treatment group after control negative group. While the lowest values of MDA and TNF-α were revealed in CCl4+nanopropolis+silymarin treatment group than other treatment groups and this confirms our theory that nanopropolis improved the effects of silymarin when administrated with it. Overall, these results indicated that nanopropolis has a significant inhibitory role in MDA and TNF-α levels.

Studies comparing propolis nanosuspension (PRO-NS) to free propolis have shown that the nano version is more effective at killing off Ehrlich ascites cancer (EAC) in female Swiss albino mice. PRO-NS inhibited the increase in blood AST and ALT activity, IgM, and creatinine and urea levels after EAC cells were implanted. In liver and EAC cells, PRO-NS boosted SOD activity and glutathione content. Their research revealed that PRO-NS significantly inhibits tumour growth through mechanisms that may include protection against oxidative damage, immune system activation, and apoptotic induction (18).

In Non-Alcoholic Fatty Liver Disease (NAFLD) mice, a similar pattern was observed; propolis alleviated symptoms by lowering liver lipids, enzymes, and advanced glycation end products. TNF-α and IL-6, pro-inflammatory cytokines are likewise reduced in NAFLD liver tissue by propolis (19). Propolis reduced liver damage in mice with diabetes and NAFLD in an experimental study as it lowered the levels of ALP, ALT, AST, LDH, and MDA and improved the protective antioxidant status of diabetic mice by upregulating antioxidant enzymes such as SOD, CAT, GPx, GST, and GR, as well as increasing glutathione levels and hepatic total antioxidant capacity. Propolis increases specific IgM and IgG titers while reducing serum IFN-γ, IL-1, and IL-6 cytokine levels in rats infected with *Toxoplasma gondii* (20).

In the current study we reported that nanopropolis improved the silymarin treatment through the inhibitory impact on the rat liver and kidney homogenate the gene expression of TGF-β1, MMP 9, SMAD-3, SMAD-2 and Desmin, while increased the expression of SMAD-7 and let-7b miRNA in comparison with other treatment groups. It was found that silymarin mediated reduction of CCl4-induced production of IL-17, TNF-α, and TGF-β was dosage dependent, and that levels were higher in CCl4-treated groups in on hepatic fibrosis and renal damage generated by CCl4 (21).

Hepatic stellate cells (HSCs) are active and display various markers, therefore the researchers used immunohistochemistry to look at the expression of α-SMA, vimentin, desmin, and MMP 9 in rat liver tissue to corroborate the fibrosis-related alteration of HSCs. CC14-administered group rats had greater expression of the above markers.Relative to the CC14 group, the expression of the aforementioned ECM proteins was reduced in the liver tissue of the rats given Silymarin (100 mg/kg) (22).

One of the most important epigenetic regulatory categories is microRNAs (miRNAs). They are non-coding, endogenous RNA molecules with a length of 20-23 nucleotides that play a function in either physiological or pathological situations. For example, one of the first miRNAs found was Lethal-7 (let-7). A member of the let-7 protein family, let-7b contributes to the onset of viral infection, alcoholic liver damage, and hepatocellular cancer. There is evidence that let-7b is down-regulated in a number of diseases. Let-7b-5p functions as a tumour suppressor in multiple myeloma by negatively regulating insulin-like growth factor receptor 1 at the post-transcriptional level (23). Let-7b's ability to increase p21 levels is an additional mechanism by which it suppresses HCC cell growth. Let-7b also goes after transforming growth factor-β1 (24).

In animal models of advanced chronic kidney disease, propolis inhibited pro-inflammatory signaling pathways in the TGF-β family signaling cascades, such as SMAD 2/3-dependent and SMAD-independent JNK/ERK activation, which have been associated to the development of tubulointerstitial fibrosis (25). Reduced oxidative stress and fibrosis, as well as co-inhibition of the

JAK2/STAT3/SOCS1 and TGF-β/Smad signalling pathways, are how silymarin nanoliposomes prevent STZ-induced kidney injury in diabetic rats (26).

Our histopathological findings appeared that groups treated with nano-propolis alone or with silymarin and nano-propolis restored the deterioration made by CCl4 in rat renal and hepatic tissues and the following results confirmed our data. Propolis protected the kidney from ischemic-reperfusion acute renal injury by reducing oxidative stress and increasing endothelial nitric oxide synthase and heme-oxygenase. After ischemic–reperfusion, propolis-treated renal tissue exhibited a considerably reduced tubular necrosis score, according to histological examination (27).

Bhadauria (28) discovered that propolis improved the kidney histoarchitecture, reduced glomerulus swelling, and created more consistent space between the glomerulus and capsule wall in CCl4-damaged mouse renal tissue. When administered to renal tissues of diabetic rats, propolis maintained glomerular basement membrane thickness. Diabetic rats that were not treated showed a considerable rise in the thickness of their glomerular basement membrane. Histopathological examinations showed that propolis protected the liver from further damage caused by diabetes. Propolis treatment reduced inflammation and immune cell infiltration in diabetic mice, and reduced the number of vacuolized cells (29).

Typical central vein, sinusoidal area, and portal triad sections were present in the liver tissue of negative rats. Neutrophil infiltration, sinusoidal congestion, and portal inflammation were observed in the liver tissue of the CCl4 group, while clear sinusoidal space and substantial regeneration were observed in the silymarin group (22).

CCl4's effect on kidney biochemical indicators is described by El-Haskoury et al. (30). Renal tubule dilatation and atrophy were observed on histology, showing that CCl4 interferes with glomerular function and causes acute nephropathy. Similarly, Yoshioka et al. (31) found an immediate histological alteration in the renal tissues of injected rats, which is consistent with our findings. The results we got line up with those from Wang et al (32). High fibrotic tissue, larger Kupffer cells, and a blubbing hepatocytes membrane were observed histologically in CCl4-injected rats, corroborating the biochemical result.

In another studies it was suggested that the histopathological findings, elevation of liver transaminases, elevation of lipid peroxidation (thiobarbituric acid reactive substances, TBARS), depletion of reduced glutathione (GSH), and reductions in the activities of antioxidant enzymes CAT, SOD, glutathione peroxidase (GPX), and glutathione-s-transferase (GST) were all indicative of CCL4 toxicity and this was in agreement with our study (33- 36).

Conclusion

Bees collect propolis, a resinous plant compound, which they combine with digestive enzymes and, on occasion, beeswax to create a sticky substance called propolis. Propolis possesses powerful anti-inflammatory, antiviral, anti-oxidant, anti-protozoal, anaesthetic, anti-tumoral, and anti-hepatotoxic qualities, and it also aids in the protection of the bee colony. Multiple in vitro, animal, and most importantly human clinical investigations have demonstrated the safety and usefulness of propolis. In conclusion, our research confirmed the importance of employing propolis nanoparticles for the management of hepatic fibrosis and nephropathy. Propolis nanoparticles boosted hepatocyte regeneration and alleviated hepatobiliary injury. The propolis nanoparticle acted as an anti-inflammatory by altering the relative expressions of SMAD-2, SMAD-3, SMAD-7, MMP-9, let-7b miRNA, Desmin, and TGF-β1 mRNA in liver and kidney tissues. Propolis nanoparticles were crucial in the treatment of nephropathy in rats given CCl4.Healing of renal tissue was aided by the nanoparticles of propolis in a definite and obvious fashion. It was shown that propolis nanoparticles regulate the apoptotic cascade by acting as antioxidants, protecting cells by altering the levels of IgM, TAC, MDA, CAT, and TNF-α. In addition, we proposed combining nanopropolis with other commercial medications for hepatic fibrosis and nephropathy.

Acknowledgments

The authors received no financial support for this research.

All authors contributed equally.

The authors declare no conflict of interests.

The experimental work was approved by the Institutional Animal Care and Use Committees Zagazig University (ZU-IACUC) with approval No. ZU-IACUC/2/F/69/2022.

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