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Original Paper

The Therapeutic Mechanisms of Propolis Against CCl_4-Mediated Liver Injury by Mediating Apoptosis of Activated Hepatic Stellate Cells and Improving the Hepatic Architecture through PI3K/AKT/mTOR, TGF-β/Smad2, Bcl2/BAX/P53 and iNOS Signaling Pathways

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Key Words
Antioxidants • Inflammation • Liver fibrosis • Oxidative stress • Propolis

Abstract
Background/Aims: Propolis is one of the most promising natural products, exhibiting not only therapeutic but also prophylactic actions. Propolis has several biological and pharmacological properties, including hepatoprotective activities. The present study aimed to investigate the underlying molecular mechanisms of propolis against CCl_4-mediated liver fibrosis. ***Methods:*** Three groups of male BALB/c mice (n=15/ group) were used: group 1 comprised control mice; groups 2 and 3 were injected with CCl_4 for the induction of liver fibrosis. Group 3 was then orally supplemented with propolis (100 mg/kg body weight) for four weeks. Different techniques were used to monitor the antifibrotic effects of propolis, including histopathological investigations using H&E, Masson's trichrome and Sirius red staining; Western blotting; flow cytometry; and ELISA. ***Results:*** We found that the induction of liver fibrosis by CCl_4 was associated with a significant increase in hepatic collagen and α–smooth muscle actin (α-SMA) expression. Moreover, CCl_4-treated mice also exhibited histopathological alterations in the liver architecture. Additionally, the liver of CCl_4-treated mice exhibited a marked increase

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in proinflammatory signals, such as increased expression of HSP70 and increased levels of proinflammatory cytokines and ROS. Mechanistically, the liver of CCl_4-treated mice exhibited a significant increase in the phosphorylation of AKT and mTOR; upregulation of the expression of BAX and cytochrome C; downregulation of the expression of Bcl2; a significant elevation in the levels of TGF-β followed by increased phosphorylation of SMAD2; and a marked increase in the expression of P53 and iNOS. Interestingly, oral supplementation of CCl_4-treated mice with propolis significantly abolished hepatic collagen deposition, abrogated inflammatory signals and oxidative stress, restored CCl_4-mediated alterations in the signaling cascades, and hence repaired the hepatic architecture nearly to the normal architecture observed in the control mice. ***Conclusion:*** Our findings revealed the therapeutic potential and the underlying mechanisms of propolis against liver fibrosis.

Introduction

Liver fibrosis is an injury-mending process that happens in response to chronic liver injury from an assortment of etiologies and eventually progresses to liver cirrhosis following constant inflammation and fibrogenesis [1, 2]. Incessant viral hepatitis B and C, alcoholic liver disease, nonalcoholic fatty liver disease, parasitic infections, immune system dysfunction, and less often, adverse drug reactions are the most widely recognized reasons for hepatic fibrosis [3]. Fibrosis is the overaggregation of extracellular matrix (ECM) proteins in the liver as an outcome of chronic hepatic damage of any etiology [4]. Hepatic stellate cells (HSCs), the primary hepatic cells involved in the aggregation of ECM proteins, are activated by a few cytokines, for example, interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and transforming growth factor beta (TGF-β), as well as reactive oxygen species (ROS) [5]. Despite the fact that HSCs are the primary source of myofibroblasts in the liver [6], other cell types add to the pool of fibrogenic myofibroblasts in liver disease. The accumulation of HSCs is induced by injured and apoptotic hepatocytes through two principle pathways: the arrival of injury-related ROS and other fibrogenic mediators [7] and the recruitment of immune cells, which thus intervene in HSC activation and stimulate collagen production through cytokine and chemokine release [8]. Following the underlying activation of HSCs, cytokines released by HSCs in an autocrine manner, similarly to normal cell-determined cytokines, produce signals that maintain HSC activation and survival and the related ECM statement [9]. Accordingly, an endless loop arises, in which common activation among specialized fibrogenic cells drive hepatic fibrogenesis [10]. Carbon tetrachloride (CCl_4) is a xenobiotic that has been found to cause intense and constant tissue wounds and is an entrenched hepatotoxin; therefore, it has been utilized broadly to study hepatotoxicity in animal models by inducing lipid peroxidation and liver pathogenesis [11]. CCl_4 has been shown to cause intense hepatotoxicity with necrotic and apoptotic hepatocellular damage and debilitation of the liver capacity [12]. CCl_4 is utilized by the cytochrome P450 framework (CYP2E1) to shape trichloromethyl free radicals (CCl3•) and trichloromethyl peroxy radicals (CCl3OO•), which initiate layer lipid peroxidation [13] and exacerbate Ca2+ homeostasis to cause hepatocellular damage [14]. Hepatic macrophages are activated, which induces inflammatory mediators, promoting liver damage [15]. A few lines of evidence recommend that liver injury might be alleviated by restraining oxidative pressure and inflammatory reactions [16]. Natural products have a variety of regular components for healing purposes [17]. One of the normal components regularly utilized in customary drugs without appropriate thought of its safety is honeybee propolis [18]. Propolis is a natural glue material collected from plants by honeybees that is used to seal cracks in beehives and to protect the bee population from infections. Propolis has a long history of medicinal use as a natural remedy [19]. It has therapeutic properties, for example, antimicrobial, antioxidant, cell reinforcement, immunomodulatory and antitumour activities [20]. Studies have likewise shown that the ethanolic concentrate of propolis has mitigating properties against both interminable and intense inflammation and immunological effects against hepatotoxicity

[21]. Propolis contains many antioxidative components, such as caffeic acid, ferulic acid and caffeic acid phenethyl ester [22]. The mechanistic role associated with the modulatory and anti-inflammatory effects of this characteristic compound involves limiting the inhibition of T cell activation mainly by influencing IL-2, NF-κB, MAP, STAT3, and IL-6 [23]. Consequently, researchers are considering searching for new restorative uses. However, the molecular mechanisms underlying the antifibrotic effects of propolis against liver fibrosis are not fully clarified. Therefore, the present study focused on investigating the underlying molecular mechanisms of propolis against CCl_4-mediated liver fibrosis in a mouse model.

Materials and Methods

Preparation of propolis

Propolis was collected and characterized at our laboratory using high-speed countercurrent chromatography and off-line atmospheric pressure chemical ionization mass-spectrometry injection as previously described [24]. Briefly, propolis extracts were prepared in three stages: drying, extraction, and evaporation. The drying process began by washing the sample and cutting it into small pieces, which were placed in an oven at 40–60°C for 1 hour. Prior to the extraction process, the samples were dried and then crushed using a blender. One hundred grams of dry sample were placed in a 1-L Erlenmeyer flask and soaked in 1 L of ethanol. The sample in ethanol was stirred for approximately 30 minutes and was allowed to stand overnight to settle. Then, the solution containing the active substance was filtered with filter paper. The soaking process was repeated three times. The final stage was evaporation. The extraction solvent (ethanol) was added to a 1-L evaporation flask. Then, a water bath was filled with water up to a full circuit, installed according to the manufacturer's instructions, and set to a temperature of 90°C. Ethanol was allowed to drip into the flask (approximately 1.5–2 hours/flask containing approximately 900 mL). The extraction yielded roughly one-tenth of the dried natural materials (10 g extract/100 g sample). The final solutions were stored in hermetically sealed brown glass bottles at room temperature (RT). Previous studies have shown that propolis extract prepared using this method is stable for 6 months, maintaining its antimicrobial and antioxidant activities over this period [24]. Abundant data from our laboratory obtained using various animal models indicated that a 50–250 mg/kg body weight daily dose of the ethanol-soluble derivative of propolis does not elicit toxic effects. Therefore, this dose is considered safe, and we used an optimal concentration (100 mg/kg body weight) of ethanol-soluble derivative of propolis for the treatment of normal and diabetic mice.

Carbon tetrachloride

Carbon tetrachloride (CCl_4) was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). The CCl_4 was dissolved in olive oil [1:9 (v/v)]; thereafter, 10% CCl_4 was prepared by dissolving 1 ml of CCl_4 in 9 ml olive oil. For the induction of chronic liver fibrosis, CCl_4 was intraperitoneally injected twice a week for six weeks (1 µl of 10% CCl_4 /g body weight). Mice were weighed before injection with CCl_4, and if the body weight of an animal was 25 g, it would be injected with 25 µl of 10% CCl_4 twice a week.

Experimental design and doses

Forty-five adult male BALB/c mice weighing 25-30 g were used in this study. The mice were purchased from the Institute of Theodor Bilharz (Cairo, Egypt). Mice were housed in cages in a room with a controlled temperature (25 ± 5°C) and a 12-h light/12-h dark cycle [25]. Mice had *ad libitum* access to a pelleted diet and water for 1 week for acclimatization. After 1 week of acclimatization, mice were randomly categorized into three main groups (15 mice each): control group (group I), CCl_4-treated group (group II) and CCl_4-treated group that were then orally supplemented with propolis (group III; CCl_4+propolis-treated group). Chronic liver fibrosis was induced in mice of groups II and III (n = 30) by intraperitoneal (i.p.) injection of CCl_4 (1 µl of 10% CCl_4/g body weight, twice a week, for six weeks). Mice in the control group were injected with vehicle alone (1 ml of olive oil/kg body weight, twice a week, for six weeks). After the induction period of chronic liver fibrosis, mice of group III were then orally supplemented with 100 µl of 50% ethanol-soluble derivative of propolis (100 mg/kg body weight/day) for another four weeks. However, mice in groups I and II received 100 µl of 50% ethanol daily (as a vehicle) via oral gavage for four weeks.

Blood collection and measurement of liver enzymes

Whole blood was collected from the abdominal aorta and immediately transferred to heparinized tubes as previously described [26]. The heparinized and nonheparinized blood was centrifuged at 3, 000 × *g* for 20 minutes using a bench top centrifuge (Anke TGL-16B) to remove blood cells and recover plasma and serum, which were immediately stored at -20°C until further use. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels of the different animal groups were detected by an automated chemistry analyzer (Beckman Coulter).

Histological evaluation

Mouse liver tissues were fixed with formal alcohol, dehydrated, and further embedded with paraffin. Paraffin-embedded liver samples were sectioned into 5 μm-thin slices, which were stained with hematoxylin and eosin (H&E), Masson's trichrome and Sirius red according to standard protocols. Sections with H&E staining were examined for liver fibrosis under light microscopy by an experienced pathologist in a blinded fashion.

Electron microscopic examinations

For the electron microscopic examination, small liver pieces (1 × 1 mm) from the five animals per group were quickly removed and fixed in 5% cold glutaraldehyde buffer for one week. The specimens were then washed with PBS (pH 7.2) for 15 minutes 4 times with slow shaking and post-fixed in 1% osmium tetroxide for 2 hours. The slides were washed, dehydrated and embedded in propylene oxide for 30 minutes to remove any alcohol remnants. The samples were embedded in propylene oxide plus Epon 812 (1:1, v/v) for 30 minutes and then embedded in Epon 812 for 4 hours. The samples were finally embedded into capsules containing the embedding mixture, and the tissue blocks were polymerized in an oven for 2 days at 60°C. Semithin sections were prepared using a standard protocol, and then, ultrathin sections were prepared accordingly. The sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Jeol, 100 CXII), which was operated at 80 KV in the Electron Microscopic Center, Assiut University. Electron micrographs were captured and examined for normal liver architecture and pathological alterations.

Western blot analysis

Whole-tissue lysates were prepared from liver tissues isolated from control mice, CCl_4-treated mice, and CCl_4-treated mice that were then orally supplemented with propolis. Liver tissues were incubated in RIPA buffer (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10% glycerol, 1 mM EDTA, and 1% protease inhibitor cocktail (Roche)) for 30 minutes. Following centrifugation at 16, 000 g for 15 minutes at 4°C, the protein concentration of each supernatant was determined using a protein assay kit (Bio-Rad, Hercules, CA), and the lysates were then stored at -20°C for later Western blot and ELISA analysis. An immortalized rat hepatic stellate cell line (HSC-T6) was also used to confirm the therapeutic effect of propolis against liver fibrosis. HSC-T6 cells were cultured in DMEM supplemented with 10% FBS on uncoated plastic overnight with medium (control), TGF-β (4 ng/ml) or TGF-β plus propolis (5 μg/ml). Cell lysates were then prepared using RIPA buffer as previously described and then stored at -20°C for later Western blot analysis. For Western blot analysis, equal amounts of each whole-cell protein lysate (50 μg) were mixed with reducing sample buffer (0.92 M Tris-HCl, pH 8.8, 1.5% SDS, 4% glycerol, and 280 mM 2-mercaptoethanol) and separated by discontinuous SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes using a Bio-Rad Trans-Blot electrophoretic transfer device. Next, the membranes were blocked for 1 hour at room temperature with 1% BSA or 5% skim milk dissolved in TBS (20 mM Tris-HCl, pH 7.4, and 150 mM NaCl) supplemented with 0.1% Tween20 and then incubated in the same blocking buffer with primary antibodies including anti-iNOS, anti-Bcl-2, anti-BAX, anti-P53, anti-cytochrome C, anti-phospho-PKB/AKT, anti-total AKT, anti-phospho-mTOR, anti-total mTOR, anti-phospho-SMAD2, anti-α-SMA, or β-actin antibodies (1:1, 000; Santa Cruz Biotechnology). The blots were thoroughly rinsed and then incubated with an HRP-labeled species-matched secondary antibody for 1 hour. Protein bands were detected by enhanced chemiluminescence (ECL, Super Signal West Pico Chemiluminescent Substrate, Perbio, Bezons, France), and the ECL signals were recorded on Hyperfilm ECL. To quantify the protein band intensities, the films were scanned, saved as TIFF files, and analyzed using NIH ImageJ software, which helps to quantify the protein band intensities as previously described [27-29].

ELISA

The levels of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and TGF-β were measured in liver tissue lysates by ELISA as previously described [30] using mouse ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The results of the ELISA assays of the tumor tissues are expressed in pg/mg.

Measuring the levels of reactive oxygen species (ROS) and nitric oxide (NO)

ROS levels in the liver tissue lysate were measured using 2, 7-dichlorodihydrofluorescein in diacetate (H2DCF-DA; Beyotime Institute of Biotechnology, Haimen, China) as previously described [31]. Nitrite (NO2-) concentration in peritoneal exudate was determined by the Griess reaction as an indicator of nitric oxide production. Briefly, 100 µl of liver lysates and 100 µl of Griess reagent (mix of 2% sulfanilamide in 5% phosphoric acid and 0.2% N-(1-naphthyl) ethylenediamine hydrochloride-NEED) were mixed in a 96-well ELISA plate. Absorbance was measured at 550 nm, and the levels of NO2- were determined using a standard curve of NaNO2. The results are expressed in µM of NO2- per cavity.

Estimation of glutathione levels and antioxidant enzyme activities

Liver tissue lysates were subjected to a GSH assay kit (Cayman Chemical, # 703002, Ann Arbor, MI, USA) and a GSH Px assay kit (Abcam ab102530, USA) according to the manufacturer's instructions as previously described [31]. To determine the manganese superoxide dismutase (MnSOD) activity, aliquots of liver lysates were treated with CuZnSOD inhibitor and then subjected to a commercial superoxide dismutase (SOD) assay kit (Cayman Chemical, # 706002, Ann Arbor, MI, USA) according to the manufacturer's instructions. Catalase activity was measured using a commercial catalase activity assay kit (Cayman Chemical, # 707002, Ann Arbor, MI, USA) according to the manufacturer's instructions as previously described [31].

Apoptosis detection and flow cytometry analysis

HSC-T6 cells were cultured in DMEM supplemented with 10% FBS and were treated with propolis or vehicle overnight. Dead cells were identified using the trypan blue exclusion test. However, to distinguish between viable, early apoptotic and late apoptotic cells, the cells were washed and incubated in PBS containing 30% human AB serum (4°C for 30 minutes) prior to staining with Annexin V-FITC and propidium iodide (PI) (15 minutes at 25°C) using a commercial kit according to the manufacturer's instructions (Abcam, Canada). The cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD-Pharmingen) within 1 hour of staining, and the percentage of cells undergoing apoptosis was determined as previously described [32, 33]. The activity levels of caspase-3 and caspase-9 were evaluated with a fluorometric protease assay kit (MBL, Aichi, Japan) according to the manufacturer's instructions.

Statistical analysis

The data were tested for normality using Anderson-Darling tests and variance homogeneity prior to further statistical analysis. The data were normally distributed and are expressed as the means ± standard errors of the means (SEMs). Significant differences between groups were analyzed using one-way analysis of variance (for more than two groups) followed by Tukey's post hoc test using Graph Pad Prism software version 5. Differences with *P* values of less than 0.05 were considered statistically significant.

Results

Oral supplementation of CCl_4-treated mice with propolis improved the pathological alterations and collagen deposition in the liver

After induction of liver fibrosis by CCl_4, we monitored the histopathological changes in the liver sections of the three animal groups using H&E, Masson's trichrome and Sirius Red staining methods. Using the H&E staining method, pictures were taken at x200 magnification from the control (Fig. 1A), CCl_4-treated (Fig. 1B) and CCl_4+propolis-treated (Fig. 1C) groups, and one representative picture from mice in each group is shown. The liver sections of control animals displayed normal histological features of classical hepatic cells and normal central veins (Fig. 1A). However, liver sections of the CCl_4-treated group showed massive, diffuse,

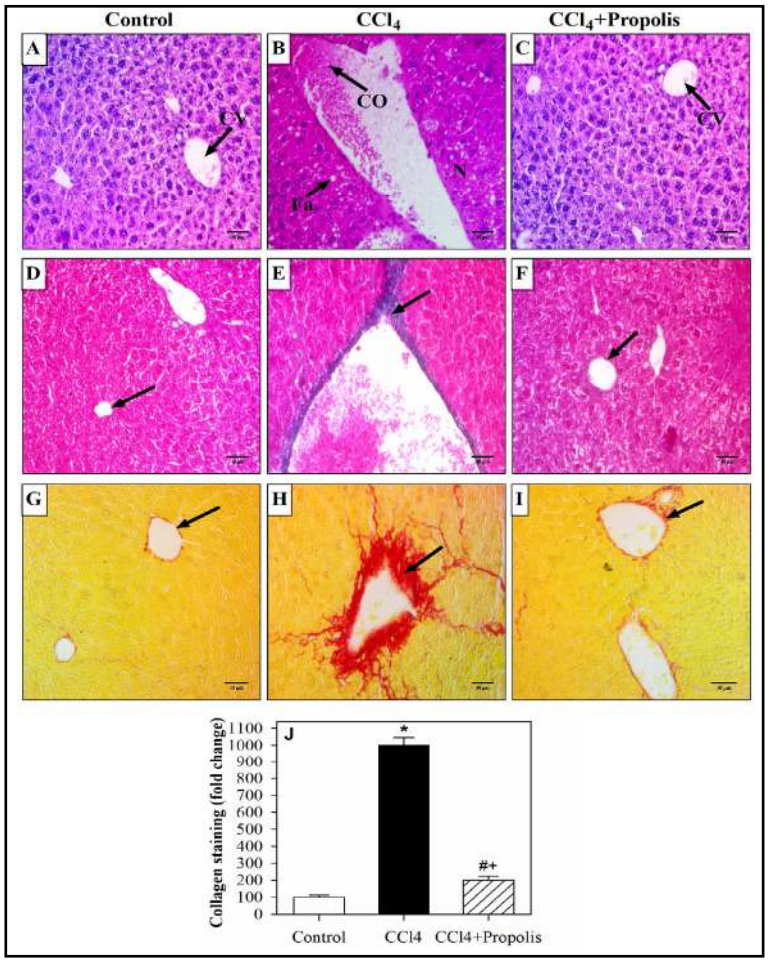

Fig. 1. Induction of a fibrotic mouse model by CCl4 and the effect of propolis on the liver architecture. Histological changes were assessed by H&E, Masson's trichrome and Sirius red staining standard protocols. All images are at x200 magnification and are representative of samples from mice in each group. Photomicrographs of liver sections from the control, CCl4-treated and CCl4+propolis-treated groups stained with H&E (A-C), Masson's trichrome (D-F), or Sirius red for collagen deposition (G-I). Quantification of collagen staining in the liver sections from three animals per group was analyzed using NIH ImageJ software (J). The collagen level of the control group was set as the basal level. Data are expressed as the mean ± SEM. *P<0.05 for CCl4-treated mice versus control mice. #P<0.05 for CCl4+propolis-treated mice versus CCl4-treated mice. +P<0.05 for CCl4+propolis-treated mice versus control mice (ANOVA with Tukey's post-test).

progressive histological alterations. Loss of architecture with massive mononuclear cellular infiltration was commonly observed in association with vacuolar degeneration and necrosis of hepatocytes, fatty changes in hepatocytes, sinusoidal dilatation and dilated congested central veins together with marked fibrosis (Fig. 1B). The CCl_4+propolis-treated group showed the disappearance of most of the histological alterations to be more or less similar to that of the control group (Fig. 1C). Because it is difficult to monitor collagen deposition in the liver sections of the CCl_4-treated group using the H&E staining method, we then used the Masson trichrome and Sirius Red staining method, and one representative photomicrograph of liver sections from the three animal groups was taken at x200 magnification. Compared to the control group, the CCl_4-treated group exhibited abundant collagen fibers surrounding the central vein (Fig. 1D, E). In the CCl_4+propolis-treated group, the liver sections exhibited a marked decrease in the collagen fibers surrounding the central vein and an obvious improvement (Fig. 1F) compared to the CCl_4-treated group. Photomicrographs of liver sections from the three animal groups were taken at x200 magnification and stained with Sirius red. The results of Sirius red staining showed that only a small amount of collagen was present in the area of the portal and central veins in normal mice (red color) (Fig. 1G). However, collagen fibers were steadily enhanced in the liver sections of the CCl_4-treated group (Fig. 1H) compared to those of the control group. Interestingly, supplementation of the CCl_4-treated group with propolis yielded a marked decrease in the collagen fibers surrounding the central vein (Fig. 1I) compared to that of the CCl_4-treated group. Staining of collagen fibers in the liver sections from three animals per group was quantified using NIH

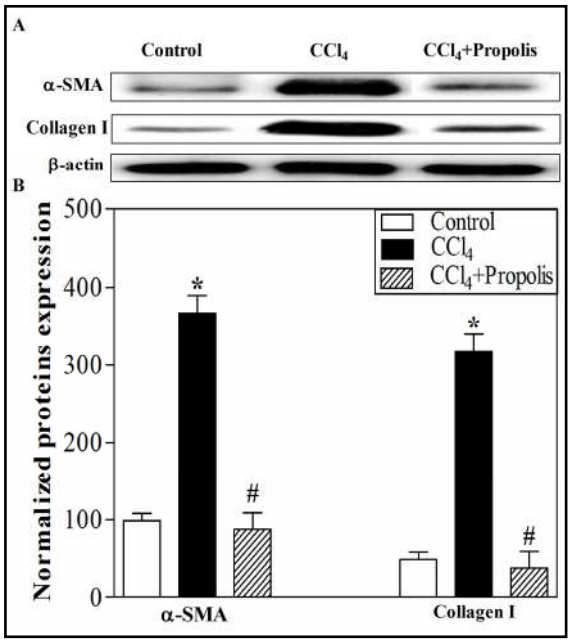

Fig. 2. Oral supplementation with propolis abrogated liver fibrosis mediated by CCl_4. Liver tissue lysates were subjected to Western blotting analysis using antibodies recognizing α-SMA, collagen type I and β-actin. The protein bands from one representative experiment are shown (A). β-actin was used as a loading control. The expression of α-SMA and collagen type I was normalized to total β-actin protein levels. The results are expressed as the means ± SEM of the normalized values of α-SMA and collagen type I. The accumulated data of five mice from each group are shown in (B) for the expression of normalized α-SMA from control mice (open bar), CCl_4-treated mice (closed black bar), and CCl_4+propolis-treated mice (hatched bar). $^*P<0.05$ for CCl_4-treated mice versus control mice. $^\#P<0.05$ for CCl_4+propolis-treated mice versus CCl_4-treated mice (ANOVA with Tukey's post-test).

ImageJ software (Fig. 1J). The collagen level of the control group was set as the basal level. Quantification of the collagen staining showed that collagen levels vigorously increased in the CCl_4-treated group, reaching 9-fold above basal values in the control group. Treatment of the CCl_4-treated group with propolis significantly reduced the increase in collagen, which was decreased by 8-fold compared to the CCl_4-treated group.

Activated hepatic stellate cells (HSCs) are the major source of collagen deposition in the diseased liver; hence, we evaluated the expression of α-SMA, an indicator of HSC activation, by Western blot analysis in the liver specimens of the three animal groups (Fig. 2). Using Western blot analysis, immunoblots for α-SMA, collagen type I and β-actin (loading control) in the liver lysates of control, CCl_4-treated mice, and CCl_4+propolis-treated mice were obtained (Fig. 2A). We observed that CCl_4-treated mice exhibited a marked increase in the expression of α-SMA and collagen type I compared to that in the control mice. When CCl_4-treated mice were then orally supplemented with propolis, we found that propolis induced a marked reduction in the expression of α-SMA and collagen type I nearly to the normal expression in the control mice. The expression levels of α-SMA and collagen type I were normalized to total β-actin protein levels. The results are expressed as the means ± SEM of the normalized values of α-SMA and collagen type I. The accumulated data from five individual mice from each group are shown in Fig. 2B. The CCl_4-treated mice exhibited a significant elevation in the expression level of α-SMA and collagen type I compared to that in the control mice ($^*P<0.05$). Interestingly, when CCl_4-treated mice were then orally supplemented with propolis, they exhibited a significant downregulation of α-SMA and collagen type I expression compared to that in CCl_4-treated mice ($^\#P<0.05$).

Induction of liver fibrosis by CCl_4-mediated proinflammatory signals that are dampened by propolis treatment

It has been established that CCl_4 is quickly metabolized by liver cytochrome P450 enzymes into trichloromethyl free radicals that initiate a lipid peroxidation chain reaction leading to hepatocyte death and liver damage [34]. Necrotic hepatocytes release damage-associated molecular pattern (DAMP) signaling molecules, including HSP70, that induce the activation, proliferation, and recruitment of inflammatory cells [35], thus amplifying liver injury and establishing chronic inflammation. Therefore, we investigated the expression of HSP70 in the liver sections of the three animal groups using Western blot analysis and measured the levels of proinflammatory cytokines in the liver lysates (Fig. 3). Immunoblots for HSP70 and

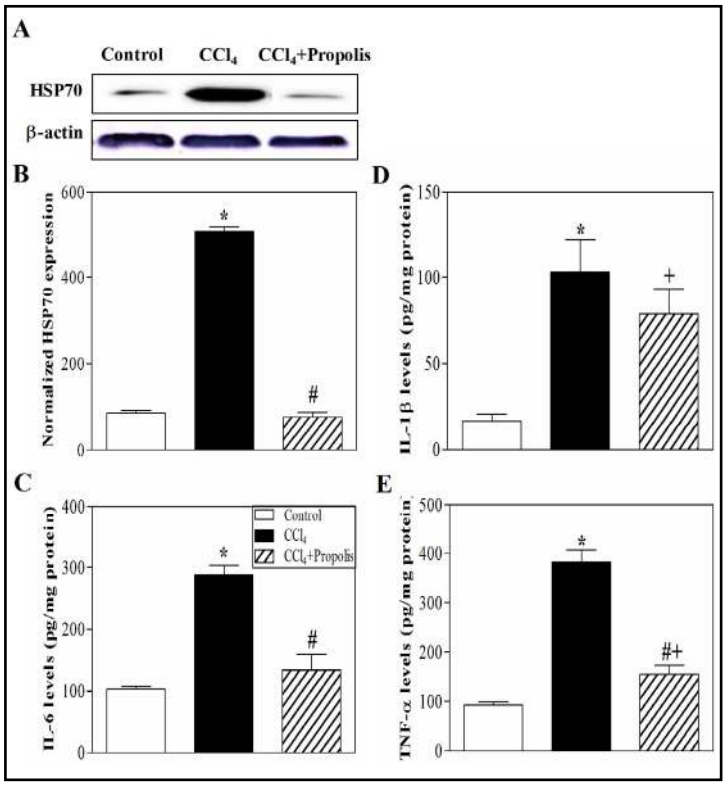

Fig. 3. Effects of CCl_4 and propolis on the proinflammatory markers in liver tissues. The expression of HSP70 was detected in the liver lysates using Western blot analysis. The protein bands from one representative experiment are shown (A). β-actin was used as a loading control. The expression of HSP70 was normalized to total β-actin protein levels. The results are expressed as the means ± SEM of the normalized values of HSP70. The accumulated data of five mice from each group are shown in (B) for the expression of normalized HSP70 from control mice (open bars), CCl_4-treated mice (closed black bars), and CCl_4+propolis-treated mice (hatched bars). The levels of the proinflammatory cytokines IL-6 (C), IL-1β (D) and TNF-α (E) were measured in three groups of mice using ELISA. The results are presented as cytokine levels (pg) per mg of liver lysate in control (open bars), CCl_4-treated (closed black bars), and CCl_4+propolis-treated (hatched bars) animals. Data are expressed as the mean ± SEM (n = 5). $^{*}P<0.05$ for CCl_4-treated mice versus control mice. $^{\#}P<0.05$ for CCl_4+propolis-treated mice versus CCl_4-treated mice. $^{+}P<0.05$ for CCl_4+propolis-treated mice versus control mice (ANOVA with Tukey's post-test).

β-actin in the liver lysates of control, CCl_4-treated mice, and CCl_4+propolis-treated mice are shown (Fig. 3A). We demonstrated that CCl_4-treated mice exhibited a marked increase in the expression of HSP70 compared to that in control mice. When CCl_4-treated mice were orally supplemented with propolis, we found that propolis induced a marked reduction in the expression level of HSP70. The expression of HSP70 was normalized to total β-actin protein levels, and the results are expressed as the means ± SEM of the normalized values of HSP70. Accumulated data from five individual mice from each group are shown in Fig. 3B. In this context, the CCl_4-treated mice exhibited a significant upregulation in the expression level of HSP70 compared to that in the control mice ($^{*}P<0.05$). Additionally, the levels of the proinflammatory cytokines (IL-1β, IL-6 and TNF-α) were measured in the liver lysates of the three animal groups using ELISA (Fig. 3C-E). Accumulated data from five individual mice from each group revealed that CCl_4 induced a significant increase in the levels of these proinflammatory cytokines compared to those in control animals. Supplementation of CCl_4-treated mice with propolis significantly decreased the levels of proinflammatory cytokines.

Propolis improved the chronic oxidative stress state mediated in the hepatic tissue during fibrogenesis

We then measured the activities of some antioxidant enzymes (GSH Px, MnSOD and catalase) and the levels of GSH and ROS in the liver lysates of the three animal groups using ELISA. Accumulated data from five individual mice from each group are expressed as the mean value ± SEM (Fig. 4). We found that the activities of GSH Px (Fig. 4A), catalase (Fig. 4B) and MnSOD (Fig. 4C) in the liver lysates (expressed as U/mg protein) were significantly ($^{*}P < 0.05$) decreased in the CCl_4-treated mice compared to the control mice. Interestingly,

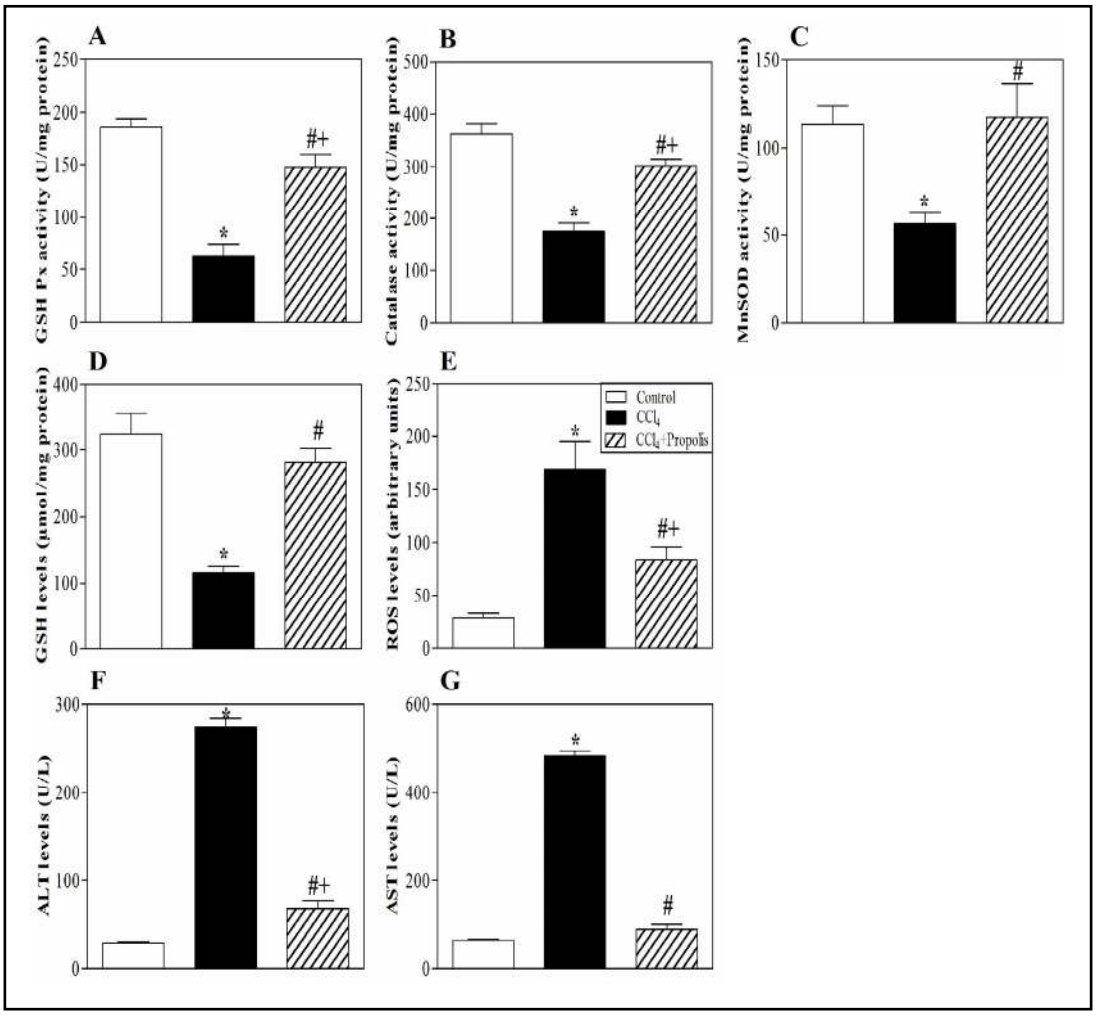

Fig. 4. Influence of propolis on the activities of antioxidant enzymes, levels of GSH and ROS and on the liver functions (ALT and AST levels) in CCl_4-treated mice. The activities of antioxidant enzymes and the levels of GSH and ROS were measured in the liver lysates, but the levels of ALT and AST were measured in the serum of control (open bars), CCl_4-treated (closed black bars), and CCl_4+propolis-treated (hatched bars) animals. The activities of GSH Px (A), catalase (B) and MnSOD (C) were measured in the liver lysates of the three animal groups, and the results were expressed as U per mg of liver lysate proteins as written in the materials and methods section. The levels of GSH (D) and ROS (E) were also measured in the liver lysates of mice in the three animal groups. The serum levels of ALT (F) and AST (G) were detected in the three animal groups. Data are expressed as the mean ± SEM (n = 5). $^{*}P<0.05$ for CCl_4-treated mice versus control mice. $^{\#}P<0.05$ for CCl_4+propolis-treated mice versus CCl_4-treated mice. $^{+}P<0.05$ for CCl_4+propolis-treated mice versus control mice (ANOVA with Tukey's post-test).

supplementation of CCl_4-treated mice with propolis significantly ($^{\#}P < 0.05$) restored the activities of GSH Px, MnSOD and catalase in the liver tissue lysates compared to those in CCl_4-treated mice. Similarly, the level of GSH was significantly decreased in the CCl_4-treated mice compared to the control mice (Fig. 4D). In contrast, the level of ROS was significantly increased in the liver tissue lysates of CCl_4-treated mice compared to the control mice (Fig. 4E). Most interestingly, supplementation of CCl_4-treated mice with propolis significantly restored the chronic oxidative stress state that was mediated by CCl_4 by increasing the level of GSH and decreasing the level of ROS in the liver tissue lysates. As we previously showed that the induction of liver fibrogenesis by CCl_4 was accompanied by hepatic inflammation and a chronic oxidative stress state, we determined whether hepatic inflammation and chronic

oxidative stress would affect liver function. The serum levels of ALT and AST were measured in the three animal groups. Accumulated data from five individual mice from each group are expressed as the mean levels of ALT and AST ± SEM. We demonstrated that the levels of ALT (Fig. 4F) and AST (Fig. 4G) were significantly increased in CCl_4-treated mice compared to the control mice. However, when these CCl_4-treated mice were orally supplemented with propolis, the levels of ALT and AST were significantly restored to the normal values observed in the control group.

Propolis improved the liver architecture postfibrogenesis induction

Induction of hepatic fibrogenesis by CCl_4 was accompanied by hepatic inflammation and a chronic oxidative stress state, leading to an aberrant elevation in the levels of ALT and AST. We therefore examined the impact of propolis on the ultrastructure of liver tissue after the induction of liver fibrogenesis by CCl_4 using transmission electron microscopy (TEM). Ultrastructure examination of the liver sections of the control group showed normal euchromatic nuclei, large stacks of rough endoplasmic reticulum (RER), abundant mitochondria and normal structure of nucleus of Kupffer cells (Fig. 5A, D, G, & J). The CCl_4-

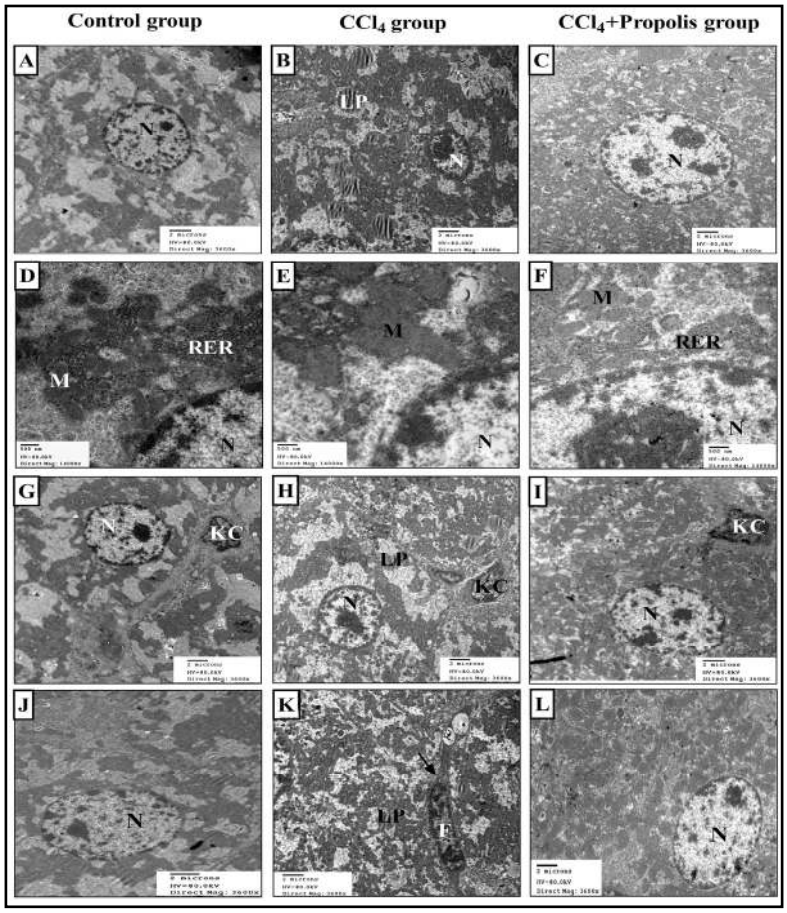

Fig. 5. Oral supplementation with propolis mitigated CCl_4-mediated distortion of the hepatic architecture. Electron micrographs of liver sections from the three animal groups. Control group showing hepatocytes with a normal architecture of the nucleus (N) (A), CCl_4-treated group showing hepatocytes with heterochromatic nucleus (B), and the presence of a large number of lipid droplets (LP). CCl_4+propolis group showing hepatocytes with euchromatic nuclei (N) (3600X) (C). Control group showing part of a nucleus (N), rough endoplasmic reticulum (RER), and mitochondria (M) (D), CCl_4-treated group showing part of a nucleus (N), devastated mitochondria (M) and fragmented rough endoplasmic reticulum (RER) (E), CCl_4+propolis group showing part of the nucleus (N), with mitochondria (M), and rough endoplasmic reticulum (RER) localization near the nuclear envelope or scattered into the cytoplasm (14000X) (F). Control group showing hepatocytes with a normal nucleus (N), Kupffer cell (KC) (G), CCl_4-treated group showing a nucleus (N) with increased heterochromatin patches, lipid droplets (LP), and Kupffer cell (KC) (H), CCl_4+propolis group showing a nucleus (N) with normal chromatin content, and a Kupffer cell (KC) (3600X) (I). Control group showing a normal nucleus (N) (J), CCl_4-treated group showing an elongated nucleus of fibroblast cell (F), some lipid droplets (LP), and collagen fibers (arrow) (K). CCl_4+propolis group showing a normal-looking nucleus (N) of an hepatocyte (3600X) (L).

treated group exhibited extensive cellular damage, and the nuclei were shrunken and pyknotic or displayed extensive chromatin condensation. The hepatocytes showed lysis of the cytoplasm where most of the cell organelles were absent and abnormal structure of the nucleus of Kupffer cells. We also observed a large number of lipid droplets and the presence of active fibroblasts in some areas (Fig. 5B, E, H, & K). While mice of the CCl_4+propolis group exhibited an improvement and restoration of the normal hepatic cell architecture, including a normal appearance of the nuclei shape with centrally located euchromatin and heterochromatin aggregates at the periphery of islands; reduced damage in hepatocytes, including the organelles and cytoplasm structures, which were widely protected against the effects of CCl_4; and partial restoration in the architecture of Kupffer cells similar to those of the control group (Fig. 5C, F, I, & L).

Propolis decreased the expression of myofibroblastic markers and mediated inactivation and apoptosis of the HSC-T6 cell line in a caspase-dependent manner

It has been clarified that myofibroblasts are one of the key cellular components involved in liver fibrosis mediated through the activation of HSCs; therefore, the majority of anti-fibrotic therapies are designed to inhibit the activation, proliferation, or synthetic products of HSCs [36]. Because activated HSCs are the major source of collagen deposition during liver fibrogenesis, we monitored the impact of propolis on the expression of α-SMA and collagen type I in HSC-T6 cells that were first activated by TGF-β. The cells were first treated with medium (control), TGF-β and TGF-β+propolis and cultured overnight. Cell lysates were prepared using RIPA buffer as described in the materials and methods. Immunoblots for α-SMA, collagen type I and GAPDH (loading control) in the cell lysates are shown (Fig. 6A). The results demonstrated that TGF-β mediated the overexpression of α-SMA and collagen type I, which are necessary for hepatic fibrogenesis. Treatment of HSC-T6 cells with propolis (5 μg/ml) markedly downregulated the expression of α-SMA and collagen type I. The expression levels of α-SMA and collagen type I were normalized to GAPDH protein levels, and accumulated data from ten independent experiments are expressed as the means ± SEM of the normalized values. The results revealed that activation of HSC-T6 cells by TGF-β induced significant overexpression of α-SMA and collagen type I compared to that in medium-treated cells (Fig. 6B). When these cells were cotreated with TGF-β and propolis (5 μg/ml), the activating profibrotic effect of TGF-β was significantly abrogated by propolis (Fig. 6B). We then monitored the impact of propolis on apoptosis induction in HSC-T6 cells. HSC-T6 cells were stained with a PI/Annexin V double stain and analyzed by flow cytometry to determine the percentage of viable cells (lower left quadrant), early apoptotic cells (lower right quadrant) and late apoptotic cells (upper right quadrant). The data from one representative experiment are presented in the dot plot (Fig. 6C). The percentage of apoptotic lymphocytes (early and late apoptotic) was 11% in the control (medium-treated cells) and was markedly increased to 77% in the propolis-treated cells. The pooled data from ten independent experiments revealed that treatment of HSC-T6 cells with propolis significantly induced apoptosis compared to medium-treated cells (Fig. 6D). Additionally, the caspase-3 and caspase-9 activity levels were evaluated in HSC-T6 cell lysates using a fluorometric protease assay (Fig. 6E). The data from 10 independent experiments are presented as the mean ± SEM. The results showed that treatment of HSC-T6 cells with propolis significantly increased the caspase-3 and caspase-9 activity levels by 8- and 7-fold compared to medium-treated cells.

Propolis abrogated liver fibrogenesis through downregulation of TGF-β levels, phospho-SMAD2 and the expression of P53 and iNOS

It has been established that liver macrophages, including Kupffer cells, produce TGF-β for activating HSCs, which are, in turn, considered key regulators of liver fibrosis [37]. Additionally, it has also been shown that P53 is necessary for the induction of liver fibrogenesis [38]. We therefore investigated the impact of propolis on key regulators and signatures of liver fibrogenesis, including the levels of TGF-β and the phosphorylation of

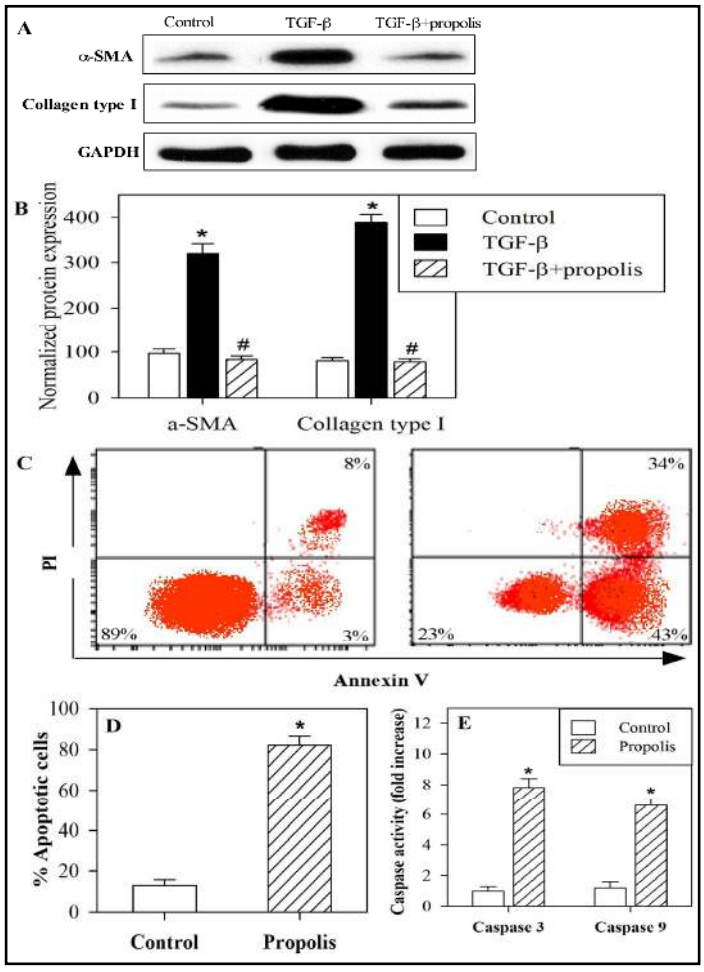

Fig. 6. Propolis decreased the expression of myofibroblastic markers in hepatic stellate cell-T6. Expression of α-SMA and collagen type I was detected in the HSC-T6 cell line using Western blot analysis. Cells were treated with medium (control), TGF-β and TGF-β+ propolis overnight, and cell lysates were prepared using RIPA buffer. The protein bands from one representative experiment are shown for the expression of α-SMA, collagen type I and GAPDH (loading control) (A). The expression of α-SMA and collagen type I was normalized to GAPDH protein levels. The results are expressed as the means ± SEM of the normalized values of α-SMA and collagen type I. The accumulated data of ten mice from each group are shown from medium-treated cells (open bars), TGF-β-treated cells (closed black bars) and TGF-β+propolis-treated mice (hatched bars) (B). *P<0.05 for TGF-β-treated cells versus control. #P<0.05 for TGF-β+propolis-treated cells versus TGF-β-treated cells. (ANOVA with Tukey's post-test). The potential of propolis to induce the apoptosis or necrosis of HSC-T6 cells was determined by flow cytometry based on their PI/Annexin V staining patterns. A dot plot of one representative data set from 10 independent experiments (n=10) is shown (C). Accumulated data from ten mice from each group are expressed as the mean percentage of apoptotic cells ± SEM for TSC-T6 cells treated with medium (control) (open bar) and propolis-treated cells (hatched bar) (D). The caspase-3 and caspase-9 activity levels were evaluated in TSC-T6 cell lysates using a fluorometric protease assay (E). The data of ten mice from each group are presented as the mean ± SEM. *P<0.05 for propolis-treated cells versus control cells (ANOVA with Tukey's post-test).

its downstream SMAD2, the expression of iNOS, the expression of P53 and the levels of NO in the liver lysates from the three animal groups. Immunoblots for phospho-SMAD2, the expression of iNOS, P53 and total β-actin (loading control) in the liver lysates are shown (Fig. 7A). The results demonstrated that CCl_4-treated animals exhibited a marked increase in the phosphorylation of SMAD2 and an obvious increase in the expression of iNOS and P53 compared to that in control animals. Supplementation of CCl_4-treated animals with propolis obviously decreased the phosphorylation level of SMAD2 and the expression of iNOS and P53. The phosphorylation of SMAD2 and the expression of iNOS and P53 were normalized to the total β-actin protein levels, and accumulated data from five individual mice from each group are expressed as the means ± SEM of the normalized value. Our data revealed that the CCl_4-treated group exhibited a significant increase in the phosphorylation of SMAD2 and in the expression of iNOS and P53 compared to those in the control group (Fig. 7B). When the CCl_4-treated group was supplemented with propolis, the phosphorylation level of SMAD2 and the expression levels of iNOS and P53 were significantly restored. The levels of TGF-β were measured in the liver lysates using ELISA. Accumulated data from five individual

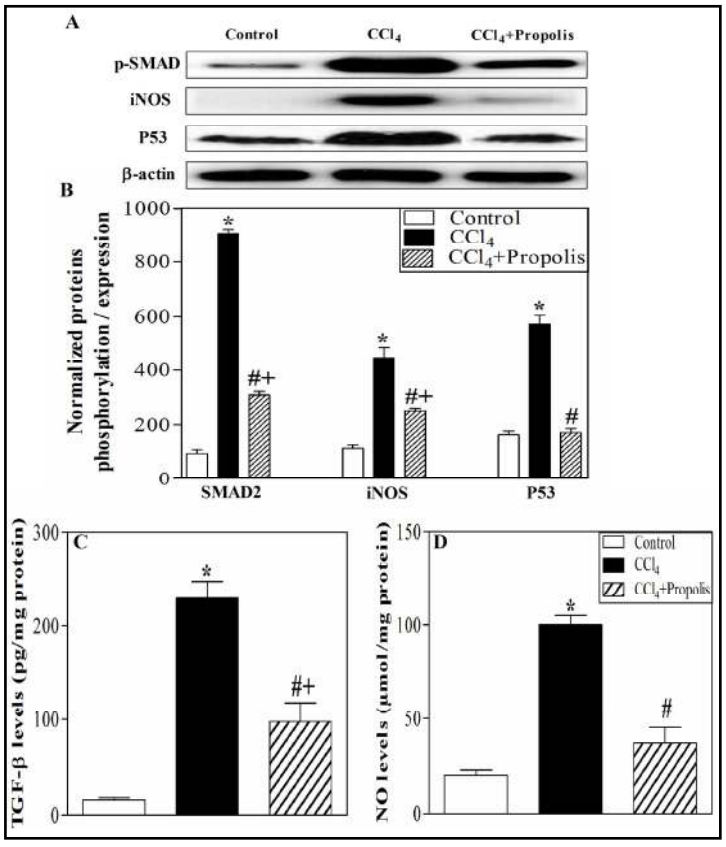

Fig. 7. Induction of liver fibrosis was associated with alterations in TGF-β, P53 and iNOS signaling. Liver lysates were prepared from hepatic tissues of mice from each group. The liver lysates were then subjected to Western blotting using antibodies recognizing pSMAD2, P53, iNOS and β-actin. The protein bands from one representative experiment are shown for the phosphorylation of SMAD2 and the expression of iNOS, P53 and β-actin (A). The phosphorylated SMAD2 and the expression of iNOS and P53 were normalized to the total β-actin protein levels. The results are expressed as the means ± SEM of the normalized value of p-SMAD2 in the three animal groups, (n=5) (B). The levels of TGF-β were measured in the liver lysates from control (open bar), CCl_4-treated (closed back bar) and CCl_4+propolis-treated (hatched bar) groups by ELISA. The results of five individual mice from each group are expressed as the mean level of TGF-β ± SEM (C). The levels of NO were also measured in the liver lysates of five mice from each group, and the results are expressed as the mean level of NO ± SEM (D).

mice from each group are expressed as the mean level of TGF-β (pg/mg protein) ± SEM. The results demonstrated that the level of TGF-β was significantly increased in CCl_4-treated mice compared to the control group (Fig. 7C). Supplementation of CCl_4-treated mice with propolis significantly restored the level of TGF-β. Because iNOS is responsible for NO generation, we also measured the levels of NO in the liver lysates from the three animal groups. Accumulated data from five individual mice from each group are expressed as the mean level of NO ± SEM (Fig. 7D). Similarly, the results demonstrated that the level of NO was significantly increased in CCl_4-treated mice compared to the control group. Supplementation of CCl_4-treated mice with propolis significantly restored the level of NO to the normal value observed in the control mice.

Propolis abolished liver fibrogenesis through the PI3K/AKT/mTOR signaling pathway and by targeting Bcl-2 family members and cytochrome C

It has been recently shown that targeting AKT/mTOR significantly attenuates the fibrogenic response of hepatic stellate cells *in vivo* and *in vitro,* suggesting the central role of this signaling pathway during liver fibrosis [39]. Subsequently, we investigated the effect of propolis supplementation on the phosphorylation of AKT and mTOR post-induction of liver fibrosis using Western blot analysis. Immunoblots for phosphorylated AKT (phospho-AKT), total AKT (pan-AKT), phospho-mTOR and total mTOR in the liver lysates are shown (Fig. 8A & B). The results revealed that the CCl_4-treated group exhibited an obvious increase in the phosphorylation of AKT and mTOR compared to that in the control animals. Interestingly, the supplementation of CCl_4-treated animals with propolis decreased the phosphorylation of AKT and mTOR. The phosphorylated levels of AKT and mTOR were normalized to the total AKT and mTOR protein levels, respectively. Accumulated data from five individual mice

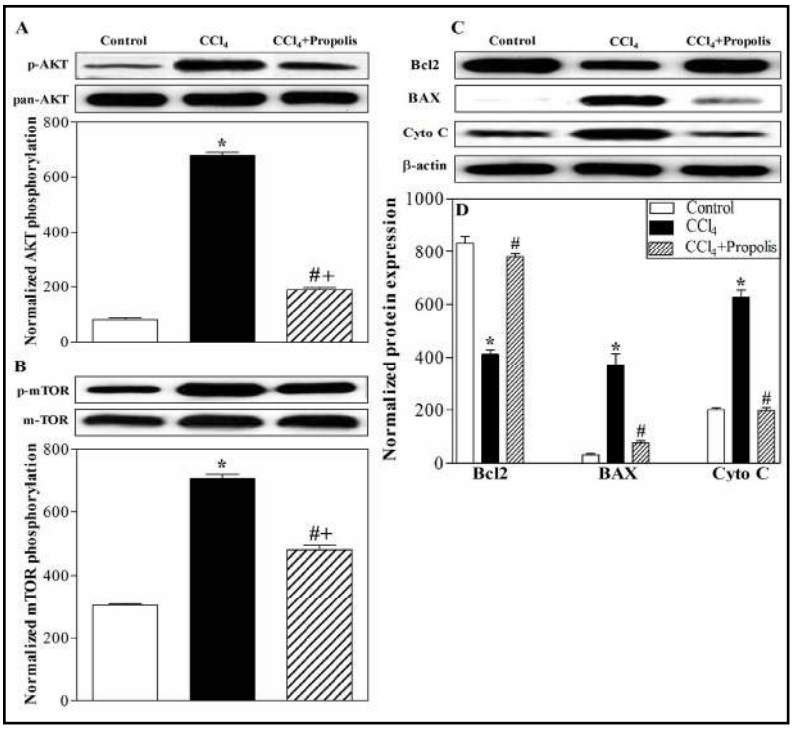

Fig. 8. Propolis repaired the liver architecture via PI3K/AKT/mTOR and Bcl2 family members. The liver lysates of the three animal groups were subjected to Western blotting using antibodies recognizing p-AKT, pan-AKT, p-mTOR, total-mTOR, Bcl2, BAX, cytochrome C and β-actin. The protein bands from one representative experiment are shown for the phosphorylation of AKT, phosphorylation of mTOR, the expression of Bcl2, the expression of BAX and the expression of cytochrome C. The levels of phosphorylated AKT (A) and mTOR (B) were normalized to total AKT and mTOR protein levels, respectively. The protein bands from one representative experiment for the expression of Bcl2, BAX, cytochrome C and β-actin are shown (C). The expression of Bcl2, BAX and cytochrome C was normalized to the total β-actin protein levels and accumulated data of five mice from the control group (open bars), CCl_4-treated (closed black bars), and CCl_4+propolis-treated (hatched bars) groups are expressed as the means ± SEM of the normalized value of each parameter, (n=5) (D). $^{*}P<0.05$ for CCl_4-treated mice versus control mice. $^{\#}P<0.05$ for CCl_4+propolis-treated mice versus CCl_4-treated mice. $^{+}P<0.05$ for CCl_4+propolis-treated mice versus control mice (ANOVA with Tukey's post-test).

from each group are expressed as the means ± SEM of the normalized values. Our results showed that the CCl_4-treated group exhibited a significant increase in the phosphorylation of AKT and mTOR compared to that in the control group. When the CCl_4-treated group was supplemented with propolis, the phosphorylation of AKT and mTOR was significantly decreased compared to that in the CCl_4-treated group.

Normally, the balance between the pro-apoptotic and anti-apoptotic members of the Bcl2 family is an important factor in cellular damage and tumorigenesis. While overexpression of pro-apoptotic proteins, such as BAX, has been implicated in liver injury, high levels of antiapoptotic proteins, such as Bcl2, could be targeted for antifibrotic drug therapy. In this context, we used Western blot analysis to monitor the expression of Bcl2, BAX and cytochrome C in the liver lysates from the three groups of animals. Immunoblots for the expression of Bcl2, BAX and cytochrome C in the liver lysates are shown (Fig. 8C). The results indicated that the CCl_4-treated group exhibited an obvious decrease in the expression of Bcl2 and a marked increase in the expression of BAX and cytochrome C compared to that in the control animals. Interestingly, supplementation of CCl_4-treated animals with propolis increased the expression of Bcl2 and downregulated the expression of BAX and cytochrome C compared to that in CCl_4-treated animals. The expression levels of Bcl2, BAX and cytochrome C were normalized to the total β-actin protein levels. Accumulated data from five individual mice from each group are expressed as the means ± SEM of the normalized values. Our results demonstrated that the CCl_4-treated group exhibited a significant reduction in the expression levels of Bcl2 and a significant increase in the expression levels of BAX and cytochrome C compared to those in the control group (Fig. 8C). When the CCl_4-treated group was

supplemented with propolis, the expression level of Bcl2 was significantly increased, and the expression levels of BAX and cytochrome C were significantly decreased compared to those in the CCl_4-treated group.

Discussion

Antioxidants play a crucial role in protecting against infectious and degenerative diseases such as liver fibrosis, which ultimately leads to liver cirrhosis and organ failure [40]. Propolis is a natural product collected by honeybees that is used to seal cracks in beehives and to protect the bee population from infections. The multiple biological properties are related to variations in their chemical compositions. Collection of propolis and determination of its contents were performed in our laboratory using high-speed countercurrent chromatography and off-line atmospheric pressure chemical ionization mass-spectrometry injection as previously described [41, 42, 24]. The present study demonstrated that liver sections of the CCl_4-treated group showed massive diffuse progressive histological alterations, loss of architecture with vacuolar degeneration and necrosis of hepatocytes, fatty changes in hepatocytes, sinusoidal dilatation and dilated congested central vein together with marked fibrosis. Similarly, ultrastructure examination of the liver sections revealed extensive cellular damage, and the nuclei were shrunken and pyknotic or displayed extensive chromatin condensation. The hepatocytes showed lysis of the cytoplasm, from which most of the cell organelles were absent, and abnormal structure of the nucleus of Kupffer cells. We also observed a large number of lipid droplets and the presence of active fibroblasts in some areas. These results were in agreement with [11, 43]. The recorded pathological alterations can be attributed to the CCl_4 effect on mitochondrial function, with loss of $Ca+^2$ from the mitochondria and endoplasmic reticulum and elevation of its cytosolic concentration. Such elevation facilitates the destruction of the cytoskeletal structure and increases the activation of a number of hydrolytic and catabolic enzymes, such as proteases, endonuclease and phospholipase, which in turn contribute to and enhance cellular necrosis [44, 45].

Propolis is a natural antioxidant product found in plant materials and is processed by worker bees. Propolis supplementation successfully and partially mitigated both macroscopic and microscopic pathological changes induced by CCl_4 injection [46] to be more or less similar to the condition of the control group. As a process of liver fibrosis, HSCs proliferate and transform to a myofibroblast-like phenotype. Activated HSCs express α–smooth muscle actin (α-SMA) and are known to be the major source of collagens and other matrix proteins that are deposited in fibrosis [46]. Our results additionally showed that CCl_4-treated mice that were then supplemented with propolis showed a reduction in chronic inflammation, a decrease in the deposition of types I and III collagen [47] and low expression of α-SMA in liver lysates. Heat shock proteins (HSPs) are intracellular proteins that maintain immune and cellular homeostasis [48]. The synthesis of heat shock proteins (HSPs) is highly upregulated following environmental, physiological and pathophysiological stress [49]. Our data demonstrated that CCl_4 promoted the up-regulation of HSP70, which may induce the observed increase in the levels of proinflammatory cytokines. However, HSP70 that is released into the extracellular space (eHSP70) acts as a proinflammatory factor and plays a crucial role in several proinflammatory pathways when secreted by activated immune cells or released from injured cells [50, 51]. The results of the present study suggest that CCl_4-induced upregulation of HSP70 contributes to the increased levels of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β. Increased inflammatory cytokine activity is a phenomenon in many forms of experimental and clinical liver injury [52, 53].

Our results showed that the activities of GSH Px, catalase and MnSOD in the liver lysates were decreased in the CCl_4-treated mice compared to control mice. Propolis has free radical scavenging properties that can induce the activation of the antioxidant enzyme system; therefore, supplementation of CCl_4-treated mice with propolis restored the activities of GSH Px, MnSOD and catalase in the liver tissue lysates compared to those in CCl_4-treated mice

[54, 55]. Similarly, there was a depletion of hepatic GSH in CCl_4-treated mice compared to the control mice [56]. GSH is a redox regulator that eliminates reactive toxic metabolites and acts as a preventive agent for many diseases, including liver disease [57-59]. Most interestingly, supplementation of CCl_4-treated mice with propolis significantly restored the chronic oxidative stress state that was mediated by CCl_4 by increasing the level of GSH and decreasing the level of ROS in the liver tissue lysates. The ability of propolis to reduce oxidative stress and to inhibit free radical formation due to its high content of phenolics [60, 61] and the ability of its phenolic compounds to donate hydrogen ions that can attack free radicals to prevent oxidation reactions in the cell [62]. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are reliable markers of liver function, so their levels were estimated. The levels of ALT and AST were significantly increased in CCl_4-treated mice compared to the control mice, indicating the induction of liver injury [63, 64]. However, oral supplementation of CCl_4-treated mice with propolis significantly restored the levels of ALT and AST to their normal values observed in the control group, as previously shown [65, 66]. The results of the most recent studies indicated that flavonoids are biologically active compounds that have a significant effect on reducing the serum levels of ALT and AST [67].

In this context, several molecular targets or pathways have been linked to liver fibrosis. Among these, transforming growth factor beta (TGF-β) has been recognized as one of most powerful profibrogenic mediators, promoting the activation of hepatic stellate cells and the accumulation of extracellular matrix proteins [68]. Our results showed that supplementation of CCl_4-treated mice with propolis significantly restored the level of TGF-β. This result is in accordance with the findings of other investigators, who reported that propolis might exert a modulatory effect on cytokine TGF-β1-induced fibrosis [69]. Studies have shown that TGF-β/Smad signaling pathway inactivation may serve as a promising therapeutic target against hepatic fibrosis. The present study demonstrated increased expression of TGF-β and phospho-Smad3 in CCl_4-treated mice. Supplementation of CCl_4-treated animals with propolis obviously decreased the phosphorylation level of Smad2 [70]. Moreover, it has been shown that P53 is necessary for the induction of liver fibrogenesis [38]. Our data showed overexpression of P53 in CCl_4-treated animals, whereas supplementation of CCl_4-treated animals with propolis has been found to attenuate P53 expression [71-73].

In this regard, CCl_4 initiated the release of proinflammatory mediators, including inducible NO synthase (iNOS), TNF-α, IL-1β, and IL-6. The overproduction of NO can cause hepatic injury, and the inhibition of NO can reduce inflammatory damage [74]. The current results demonstrated that CCl_4 increased the expression of iNOS, which suggests that iNOS-derived NO could regulate proinflammatory gene expression, contributing to inflammatory liver injury [75, 76]. Supplementation of CCl_4-treated animals with propolis clearly decreased the expression level of iNOS. In agreement with our results [77], we observed the inhibitory effect of an ethanolic extract of propolis on iNOS gene expression [78]. Free radicals induced by CCl_4 can also induce cell apoptosis and necrosis [79]. Over-production of ROS causes mitochondrial pore oxidation, thus depolarizing the potential of the mitochondrial membrane and thereby releasing cytochrome C [80]. When cytochrome C enters the cytosol, it binds to apoptosomes, thus initiating the caspase cascade leading to caspase-3 [81], which is essential for apoptosis induction in liver injury. Our results showed that the CCl_4-treated group exhibited an obvious decrease in the expression of Bcl2 and a marked increase in the expression of BAX and cytochrome C, which is in agreement with previously published data [82]. Supplementation of CCl_4-treated animals with propolis increased the expression of Bcl2 and downregulated the expression of BAX and cytochrome C compared to that in CCl_4-treated animals. Propolis was reported to improve oxidative stress and attenuate hepatic dysfunction and apoptosis via restored levels of P53 and Bcl2 [83].

The PI3K/AKT/mammalian target of rapamycin (mTOR) signaling pathway is a key regulator of cellular survival, proliferation, and apoptosis [84]. PI3K pathways were demonstrated to be a requirement for both the antioxidant response and activation of hepatic stellate cells [85]. AKT is sensitized by PI3K, while the phosphorylation level of AKT is associated with HSC proliferation and collagen I expression [86, 87]. Compared to the

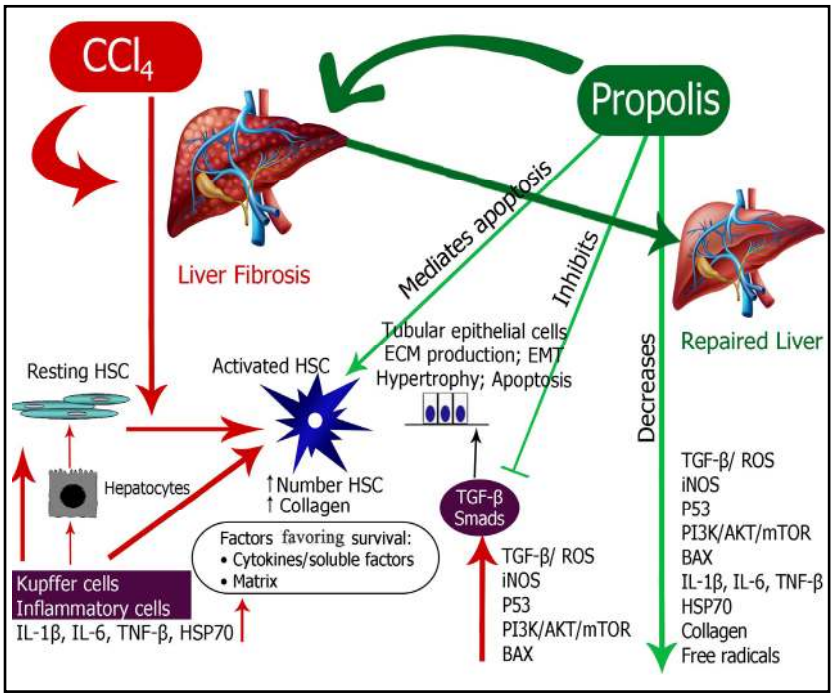

Fig. 9. The antifibrotic effect of propolis and the associated underlying molecular mechanisms. This diagram illustrates and summarizes the molecular mechanisms of propolis in reversing liver fibrosis mediated by CCl_4.

control group, the CCl_4-treated group exhibited an obvious increase in the phosphorylation of AKT and mTOR. Interestingly, our results showed that supplementation of CCl_4-treated animals with propolis significantly attenuated the impairment of the PI3K/AKT/mTOR pathway via decreased phosphorylation of AKT and mTOR. In agreement with our findings, propolis has been shown to inhibit TGF-β1-induced Smad2 and AKT phosphorylation [88].

It has been demonstrated that reduced CCl_4-induced liver fibrosis is mediated by repressing the initiation of HSCs through the PI3K/AKT/mTOR and ERK pathways [39]. Nonetheless, in our study, we found that propolis mediated antifibrotic effects through PI3K/AKT/mTOR without influencing ERK phosphorylation. Additionally, curcumin hindered CCl_4-mediated liver fibrosis by repressing Kupffer cell activation through TGF-β flagging and iNOS [37]. In particular, it has recently been shown that the H19/miR-148a/USP4 axis encourages liver fibrosis by improving TGF-β signaling in both hepatic stellate cells and hepatocytes [89], suggesting that the TGF-β/Smad pathway is an objective signaling pathway for the treatment of liver fibrosis. In addition, it has been demonstrated that esomeprazole enhances CCl_4-induced liver fibrosis through TGF-β and Bcl2/BAX [90]. In this unique circumstance, it has likewise been demonstrated that P53-subordinate acceptance of ferroptosis is required for artemether to ameliorate CCl_4-initiated liver fibrosis and hepatic stellate cell activation [38].

Conclusion

In our current work, we investigated the impact of propolis on PI3K/AKT/mTOR, ERK, TGF-β/Smad2, Bcl2/BAX, P38, P53 and iNOS. We found that the antifibrotic effects of propolis against CCl_4-mediated liver fibrosis, as summarized in Fig. 9, were dependent on PI3K/AKT/mTOR, TGF-β/Smad2/Bcl2/BAX, P53 and iNOS but not on ERK and P38 (data not shown).

Abbreviations

ALT (alanine aminotransferase); AST (aspartate aminotransferase); CCl_4 (carbon tetrachloride); GSH (glutathione); HSCs (hepatic stellate cells); HSP70 (including heat shock protein70); IL (interleukin); MnSOD (manganese superoxide dismutase); ROS (reactive oxygen species); α-SMA (α-smooth muscle actin); TGF-β (transforming growth factor β); TNF- α (tumor necrosis factor-α).

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All experimental animal protocols were performed according to regulations set by the Institutional Animal Care and Use Committee and were approved by Assiut University. We made every effort to minimize animal distress and reduce the number of animals used in this study.

Disclosure Statement

The authors declare no conflicts of interest, state that the manuscript has not been published or submitted elsewhere, state that the work complies with the Ethical Policies of the Journal and state that the work has been conducted under internationally accepted ethical standards after relevant ethical review.

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