



Original article

Induction of liver fibrosis by CCl₄ mediates pathological alterations in the spleen and lymph nodes: The potential therapeutic role of propolis



Eman A. Sayed^{a,b}, Gamal Badr^{a,b,*}, Khadiga Abdel-Hameed Hassan^a, Hanan Waly^a, Betul Ozdemir^c, Mohamed H. Mahmoud^d, Salman Alamery^d

^aZoology Department, Faculty of Science, Assiut University, Assiut, Egypt

^bLaboratory of Immunology, Zoology Department, Faculty of Science, Assiut University, Assiut, Egypt

^cDepartment of Cardiology, Faculty Medicine, Niğde Ömer Halisdemir University, Niğde, Turkey

^dDepartment of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia

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ABSTRACT

In an animal models, carbon tetrachloride (CCl₄) is a carcinogenic agent that causes liver fibrosis. The current study aims to investigate whether induction in liver-fibrosis by CCl₄ in the mouse model could promote the initiation of fibrosis in lymph node and spleen due to sustained increase of inflammatory signals and also aimed to clarify the protective therapeutic effects of propolis. The male mice (BALB/c) were categorized into three experimental sets and each group involved 15 mice. Control group falls into first group; group-II and group-III were injected with CCl₄ to induce liver-fibrosis and oral supplementation with propolis was provided in group-III for 4-weeks. A major improvement with hepatic collagen and α -smooth muscle actin (α -SMA) production was aligned with the activation of liver fibrosis from CCl₄. Mice treated with CCl₄ exhibited collagen deposition towards liver sections, pathological alterations in spleen and lymph node architectures, and a significantly increase the circulation of both T&B cells in secondary lymphoid organs. Mechanically, the secondary lymphoid organs treated with CCl₄ in mice exposed a positive growth in α -SMA and collagen expression, increased in proinflammatory cytokine levels and a significant increase in TGF- β , NO and ROS levels. A manifest intensification in the expression of Nrf2, COX-2, and eNOS and upregulation of ASK1 and P38 phosphorylation. Interestingly, addition of propolis-treated CCl₄ mice, substantially suppressed deposition of liver collagen, repealed inflammatory signals and resorted CCl₄-mediated alterations in signaling cascades, thereby repairing the architectures of the secondary lymphoid organs. Our findings revealed benefits of propolis against fibrotic complications and enhancing secondary lymphoid organ architecture.

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1. Introduction

The immune system defines the molecules, cells, and tissues involved in host defenses that are crucial in defending animals from external non-self-invaders. This vital system comprises lymphoid organs, barriers, leukocytes, and proteins, such as antibodies and complement components (Blach-Olszewska and Leszek, 2007).

The liver controls immune homeostasis by couple of mechanisms. Initially, it plays a key role in immune response, protecting by its dual blood supply against blood-borne pathogens, ultimately eliminating the pervasive transmission of nutritional antigens and microbes from the gut (Albillos et al., 2014). Secondly, a proliferation of simple compounds essential for an adequate immune response leads to homeostasis of the immune response (Racaneli and Rehmann, 2006). The liver executes its antimicrobial-monitoring role across various coordinated inhabitants by antigen-presenting cells (APCs) with lymphocytes that specifically screen for general and gut-derived pathogen. The APCs involves both Kupffer and sinusoidal endothelial cells from liver and dendritic cells (Gregory et al., 2002). Furthermore, liver encompasses T and B-cells of lymphocytes via the inhabitant and transiting population by the adaptive immune response of parenchyma and portal tracts. However, liver is abundant in NK-cells and unusual

* Corresponding author.

E-mail address: gamal.badr@aun.edu.eg (G. Badr).

URL: http://www.aun.edu.eg/membercv.php?M_ID=393 (G. Badr).

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lymphocytes that are involved in the liver's innate immune responses (Schildberg et al., 2008). Bone-marrow, lymph-nodes, appendix, spleen and thymus are some of the immune system organs (Blach-Olszewska and Leszek, 2007). The spleen is the major organs of secondary-lymphoid gland which has a wide range of immunological activities, including hematopoiesis roles and consent of RBCs. Spleens physical structure enables the filtration of pathogens and abnormal cells from the blood and promotes low-probability interactions between APCs and lymphocytes. Spleen-specific APCs control the response of T and B cells to these blood antigen targets (Lewis et al., 2019). Lymph nodes are controlled by lymphoid organs that contain lymphocytes within a fine reticular stroma. Lymph nodes act as tissue filters and are sites of lymphocyte origin and development for normal physiological functions (Elmore and Bouknight, 2017).

Carbon tetrachloride (CCl₄) is a poly-chlorinated hydro-carbon and an ozone-depleting substance which is used as a liquid-solvent for decades as an intermediate material. CCl₄ exposure damages many organs. While in most animal species the primary-organ for CCl₄-induced liver toxicity, evidence indicates the affect of immune system (Guo et al., 2000). Propolis is a natural adhesive agent produced from plants by honeybees. Closing splits in bees is used and preventing diseases for the bee population. Propolis has a strong tradition in natural medicine and possesses antimicrobial, antioxidant, cellular-strengthening, immunomodulatory, and antitumor activity (Badr et al., 2019). The current study aims to explore the defensive effect of propolis against CCl₄-mediated immunomodulation in a mouse model.

2. Materials and methodologies

2.1. Propolis preparation

Propolis honey was purchased since Etman Hives, Shabshir Al Hissah, Al Gharbiyah, Egypt and prepared in our laboratory (Badr et al., 2019). Abundant evidence in our lab was obtained through various animal-studies, showed the regular dosage of 50–250 mg/kg of ethanol-soluble propolis derivatives did not cause noxious-effects and appropriate conc. of 100 mg/g of body-weight in the ethanol-soluble bee propolis derivatives to the fibrotic mice in our experiment.

2.2. CCl₄

Sigma chemicals has provided the CCl₄ to induce chronic liver-fibrosis in the mice. Using the olive oil, CCl₄ was liquified in 1:9 ratio to obtain a 10% CCl₄ solution that was injected intraperitoneally into mice twice a week for 6 weeks.

2.3. Experimental design and dosages

We bought mostly from the Theodor Bilharz research institute in Egypt, a group of 45 male adult BALB/C mice weighing between 25 and 30 g. The mice were caged around 25 °C under 12 h of each in day and night (Badr et al., 2011). Mice were acclimatized for one week, with access to a pelleted towards diet cum water before being bifurcated in three-categories. Fifteen mice were categorized into each group. Group-I is a control-group of mice; Group-II is treated with CCl₄ and Group-III is treated with both CCl₄ and orally supplemented with propolis (group III; CCl₄ + propolis-treated group). The CCl₄ (1 μ by 10% of CCl₄/g body weight, injected twice a week for six weeks) cause intraperitoneal liver fibrosis in Groups II & III.

The control group mice were vaccinated intraperitoneally with the vehicle only (1 ml of olive oil/kg body-weight, twice a week for

about six-weeks). After the induction of liver-fibrosis (using CCl₄ for six weeks), mice in Group-III received oral supplementation with 100 μl of 50% ethanol-soluble propolis derivative (100 mg/kg body weight/day) for additional for four weeks, and the mice in groups I and II received 100 μl of 50% ethanol per day (as a vehicle) through oral gavage for additional four weeks.

All research was completed in agreement through internationally accepted principles care of lab-animals (based on US and Europe guidelines). Ethical approval and protocol procedure were obtained from Assiut_s university.

2.4. Histological evaluation

Mice tissues of the liver, spleen, and lymph nodes were fixed with formal alcohol, embedded and desiccated, into five-μm of paraffin-blocks, blemished with hematoxylin and eosin of Sirius-red according to standard protocols.

2.5. Immunohistochemistry

The paraffin units of both spleen and lymph nodes were immobile complete-night in a freshly prepared alcohol solution (Mohany et al., 2012). The dehydrated samples were arranged as paraffin-blocks and sections were blemished for IHC using main antibodies anti-CD3 and anti-CD20 to identify T&B-cells. Tissue samples were washed in xylene, rehydrated in 70% ethanol, submerged in distilled water for 5 mins. Then, they were boiled in citrate-buffer for 2/3rd hour to unmask epitopes. Further stage, the slides were cleaned in water, tracked by phosphate-buffered saline (PBS). The IHC technique were performed as per the Badr et al. (2019) studies.

2.6. Measuring the reactive oxygen species (ROS) levels

The ROS levels in spleen and lymph node tissue-lysates were determined using H₂DCFDA, where the oxidation of H₂DCFDA to 2'-7-dichlorofluorescein (DCF) was used to quantify the levels of H₂O₂. Briefly, following the cellular absorption of H₂DCFDA and its acetomethyl ester, non-specific cellular-esterase's eliminate the lipophilic groups and produce an electric composite that is trapped within cell. ROS-oxidation of H₂DCFDA transforms the molecule into extremely fluorescent DCF. The DCF fluorescence is determined at 498 nm and 522 nm for excitation and emission, respectively (Badr et al., 2013).

2.7. Measuring nitric oxide (NO) levels

The concentration of NO₂⁻ in peritoneal exudate was calculated by the Griess reaction as an indication of NO development. Briefly, 100 μl of spleen and lymph node tissue lysates and 100 μl of Griess substance (2% mixture of sulfanilamide in 5% phosphoric acid and 0.2% N-(1-naphthyl) ethylenediamine hydrochloride) were assorted into the 96-well plate of enzyme-linked immunosorbent assay (ELISA).

2.8. ELISA

Proinflammatory cytokines levels such as IL-1β, TGF-β and IL-6 were measured by ELISA in spleen and lymph nodes tissue lysates (Sayed et al., 2010).

2.9. Western blot analysis

The complete tissue-lysates were organized via radioimmuno-precipitation assay-buffered liver, spleen, and lymph node tissues isolated after control mice. The lysates were centrifuged around 15mins at 4 °C for 16 kg. Protein assay kit was used in this study

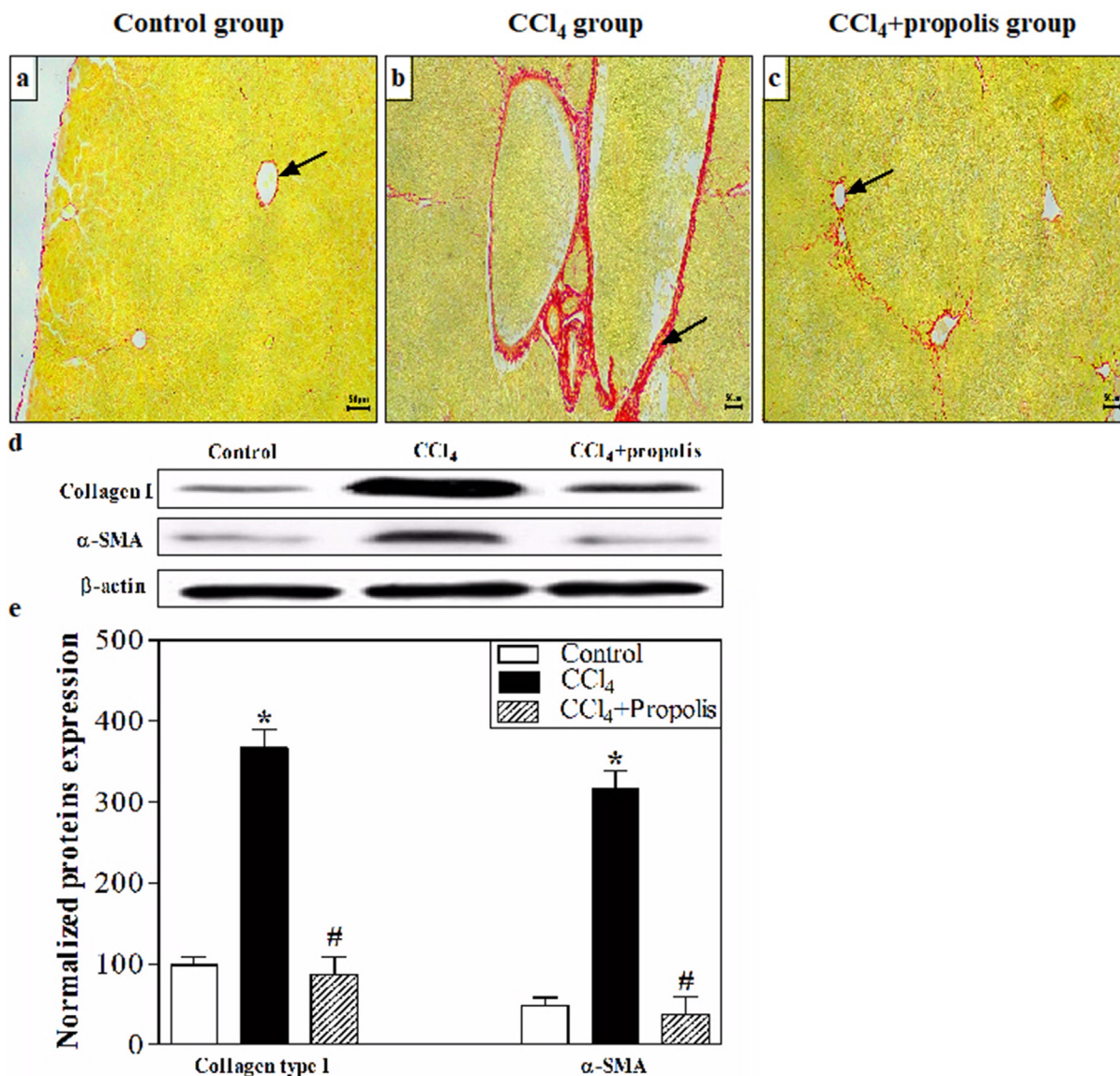


Fig. 1. Propolis treatment mitigated hepatic fibrosis induced by CCl₄ in our mouse model. Histological changes in collagen distribution were assessed by staining liver sections with Sirius red according to a standard protocol. (A–C). All images were taken at 100× magnification and are representative of samples from mice in each group. The liver sections of the control group showed normal collagen fibers distribution (A). The CCl₄-treated group showed extensive collagen deposition around the central vein (arrow) (B). The collagen fibers in the CCl₄ + propolis-treated group were small and delicate, suggesting that the liver had repaired itself. Western blotting analysis of the liver tissue lysates was performed using antibodies against collagen type I, α-SMA, and β-actin (loading control). The protein bands from one representative experiment are shown (D). The expression of collagen type I and α-SMA was normalized to total protein levels of β-actin and expressed as the means ± SEM of the collagen type I- and α-SMA-normalized values. The accumulated data of five mice from each group are shown for the expression of normalized collagen type I and α-SMA from control mice (open bar), CCl₄-treated mice (closed black bar), and CCl₄ + propolis-treated mice (hatched bar) (E). *P < 0.05 for CCl₄-treated mice vs. control mice. #P < 0.05 for CCl₄ + propolis-treated mice vs. CCl₄-treated mice. *P < 0.05 for CCl₄ + propolis-treated mice vs. control mice (ANOVA with Tukey’s post-hoc test).

and lysates was stored for further western blot analysis. The SDS-PAGE analysis was done based on Khan et al. (2016) studies. The western blot analysis was performed as described previously (Badr et al., 2013, 2012, 2010).

2.10. Statistical analysis

Normality using the Anderson-Darling test and homogenous variances were tested for results. The statistical data were expressed as mean and standard deviation and Anova analysis was performed (Khan et al., 2019). P value is confirmed as < 0.05. Statistical analysis was carried out using GraphPad Prism v5 statistical software (Graph Pad Software, CA, USA).

3. Results

3.1. Treatment of fibrotic mice with propolis repaired the pathological alterations of liver and collagen deposition

The key cause of collagen deposition in the diseases liver is activated hepatic stellate cells (HSCs). Following CCl₄-induced liver fibrosis, collagen deposition in the three groups was monitored by staining liver sections with the collagen stain Sirius red. Only a small amount of collagen was present in the portal area and central veins (Fig. 1A). Compared with the control group, abundant collagen fibers surrounded the central vein in the CCl₄-preserved assembly (Fig. 1B). Interestingly, a great change

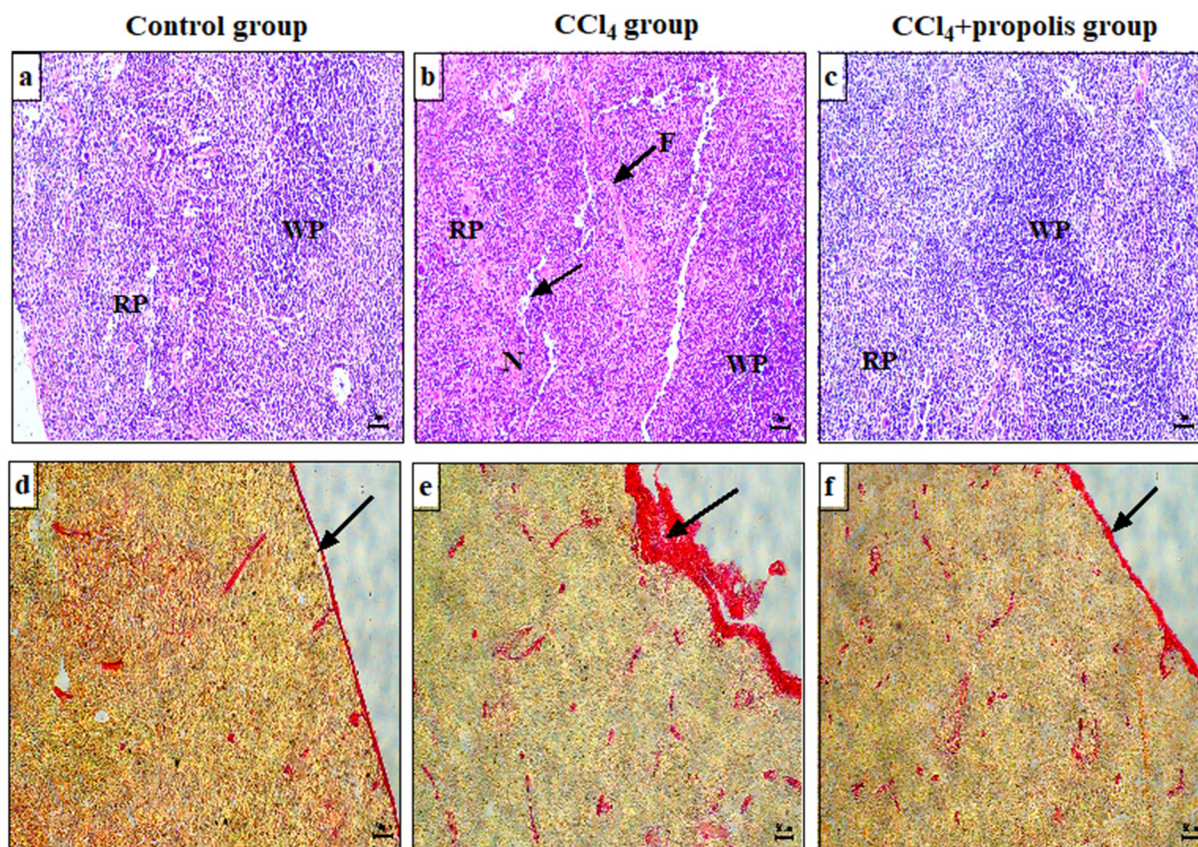


Fig. 2. Oral propolis supplementation improved the spleen histoarchitecture in fibrotic mice. Histopathological changes in spleen tissue sections were assessed following staining with H&E and Sirius red using standard protocols. All images were taken at 100 \times magnification and are representative samples from mice in each group. Spleen sections from the control group, CCl₄-treated group, and CCl₄ + propolis group stained with H&E (A–C, respectively) and Sirius red (D–F, respectively) for collagen deposition. RP: red pulp; WP: white pulp; F: fibrosis; N: necrosis.

in histoarchitecture was detected in CCl₄ + propolis-treated groups (Fig. 1C) linked with the CCl₄-treated groups. We also used western blotting to assess the expression of α -SMA (an HSC activation predictor), collagen type I, and β -actin (loading control) in liver samples from the three groups (Fig. 1D). Mice treated with CCl₄ has demonstrated a substantial growth in expressing type-I collagen and α -SMA in comparison. When CCl₄-treated mice were orally supplemented with propolis, there was a significant reduction in the appearance of collagen type-I and α -SMA to almost the same closes as that of the control mice. Fig. 1E shows the accumulated data from five mice in each group. The treated mice thru CCl₄ disclosed a substantial growth in collagen type-I and α -SMA expression relative to the control mice ($P < 0.05$). Fascinatingly, when CCl₄-treated via the mice were orally supplemented with propolis, they displayed an important downregulation of collagen type-I and α -SMA expression associated with the CCl₄-treated mice ($P < 0.05$).

3.2. Treatment of CCl₄-induced fibrosis in mice with oral propolis supplementation decreased pathogenic changes in spleen tissue

We used H&E and Sirius red staining to evaluate the histopathological alterations in spleen sections (as a secondary immune organ) from the three mouse groups after inducing fibrosis by CCl₄. The spleen sections of the control group showed a normal histological appearance. The white pulp was made up of concentrated lymphocytes and central arteries, and the red pulp contained splenic cords and sinusoids (Fig. 2A). The spleen sections in the CCl₄-treated group showed degenerative changes characterized by fibrosis, lymphocytic necrosis, and lymphocyte depletion and marked fibrosis in the red and white pulp (Fig. 2B). The CCl₄ +

propolis-treated group showed partial restoration of the white and red pulp to typical splenic histoarchitecture similar to that of the control group (Fig. 2C). We used a Sirius red staining method to identify collagen fiber deposition in the spleen sections of the CCl₄-treated groups. The collagen fibers in the spleen capsule sections in the control group were normally distributed (Fig. 2D). Collagen deposition and the establishment of fibrosis were observed in the spleen capsule in the CCl₄-treated group (Fig. 2E). Interestingly, the CCl₄ + propolis-treated group showed less collagen deposition in the spleen capsule, and the structure was preserved, similar to the control group (Fig. 2F).

3.3. Propolis supplementation enhanced the toxic effects of CCl₄ on lymph node histology

We investigated the effect of propolis on lymph nodes (secondary immune organs). Microscopic examination of lymph node sections stained with H&E from the control group revealed a normal histological appearance of the cortex, divided into the outer cortex and inner cortex (paracortex), and medulla and normal lymphocyte populations in the outer cortex, paracortex, and medulla (Fig. 3A). The lymph node sections from the CCl₄-treated group displayed degenerative changes, reflected by necrosis and a decreased lymphocyte population in the cortex and medulla compared with the control group (Fig. 3B). The CCl₄ + propolis group displayed a relatively regular outer cortex, paracortex, and medulla, with a decreased lymphocyte population in the cortex and medulla compared with the CCl₄-treated group (Fig. 3C). The lymph node sections were also stained with Sirius red for the selective staining of collagen fibers. The lymph node sections of the control group

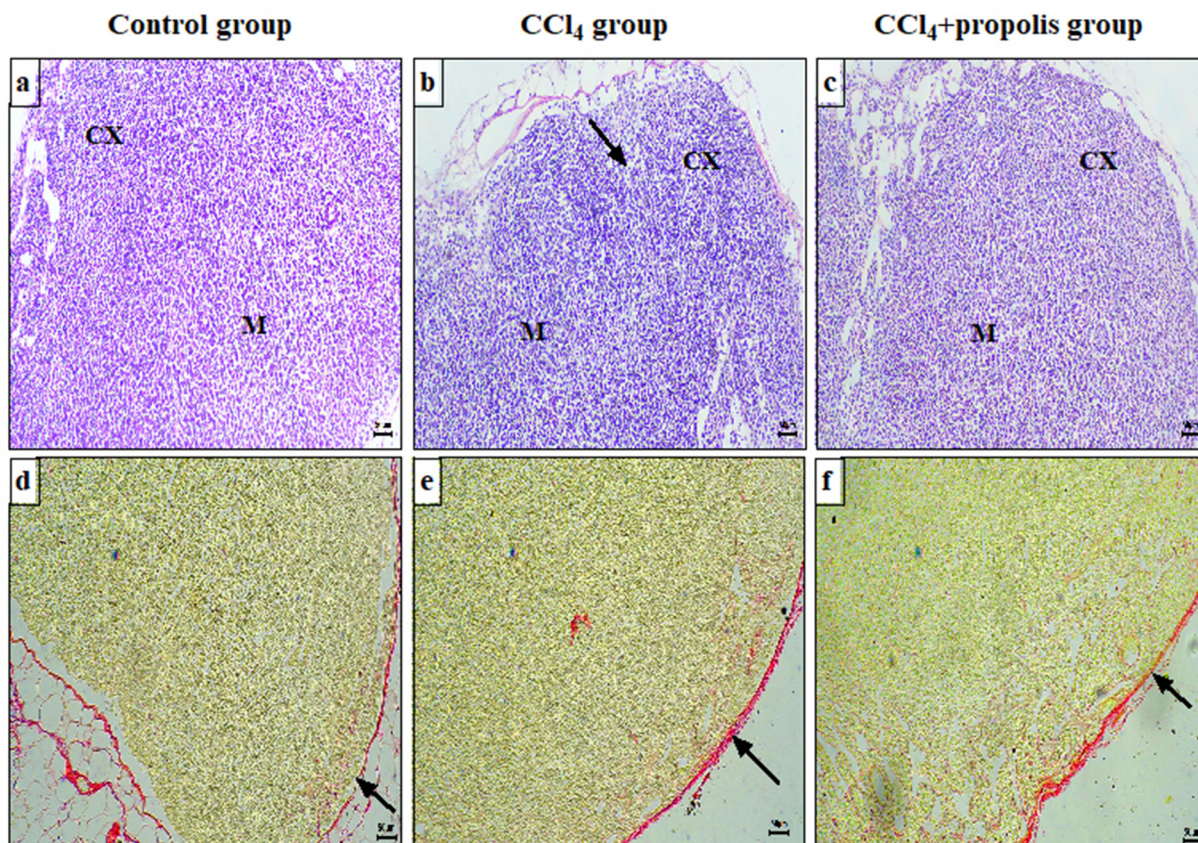


Fig. 3. The effect of CCl_4 and propolis on lymph node architecture in our fibrotic mouse model. Lymph node sections from the three mouse groups were stained with H&E and Sirius red, which selectively stains collagen fibers red. All images were taken at $100\times$ magnification and are representative of samples from mice in each group. Lymph node sections from the control, CCl_4 -treated, and CCl_4 + propolis-treated groups stained with H&E for the investigation of histopathological alterations (A–C, respectively) and Sirius red for collagen deposition (D–F, respectively). CX: cortex; M: medulla.

showed marginal collagen fiber content in the capsule (Fig. 3D). Those of the CCl_4 -treated group showed a relative increase in capsule collagen fibers (Fig. 3E). Interestingly, the CCl_4 + propolis group showed less collagen deposition in the lymph node capsules compared with the CCl_4 -treated group (Fig. 3F).

3.4. Propolis reduced myofibroblastic marker expression in secondary lymphoid organs of fibrotic mice

Most antifibrotic therapies prevent the activation, proliferation, or synthetic products of HSCs. Activated HSCs are the main source of collagen deposition during fibrogenesis. We used western blot analysis of tissue lysates of the secondary lymphoid organs (spleen and lymph nodes) to monitor the influence of propolis on the expression of α -SMA and collagen type I (Fig. 4A). The results indicated that CCl_4 mediated the overexpression of α -SMA and collagen type I in the spleen and lymph nodes. The CCl_4 + propolis-treated group showed a significant reduction in α -SMA and Type I collagen expression. The CCl_4 -treated group displayed significant overexpression of α -SMA and collagen type I compared with the control group. Interestingly, the CCl_4 + propolis-treated group demonstrated a significant decrease in the expression of collagen type I and α -SMA relative to the CCl_4 -treated group (Fig. 4B).

3.5. Propolis supplementation enhanced the altered distribution of CCl_4 -mediated T and B cells in spleen and lymph node tissues

We examined the effect of CCl_4 on the distribution of T and B cells in the spleen and lymph nodes (secondary lymphoid organs) of the three mouse groups using anti-CD3 and anti-CD20 antibodies

to monitor T and B cells, respectively. The spleen sections from the control mice showed the moderate distribution of T cells between the red pulp and the white pulp periarterial lymphatic sheaths (Fig. 5A), while the CCl_4 -treated mice showed a marked increase in the number of T cells in both regions (Fig. 5B). The T cell distribution in the red pulp and the periarterial lymph sheaths of the white pulp in the CCl_4 + propolis-treated mice was significantly restored to close to that observed in the control mice (Fig. 5C). The B cells tended to be clustered in the red pulp and marginally scattered throughout the white pulp of the control mice spleen sections (Fig. 5D), whereas there was increased clustering of B cells in the white pulp and widespread dispersion of these cells throughout the red pulp in the CCl_4 -treated group (Fig. 5E). The CCl_4 + propolis-treated mice showed the restored distribution of B cells in the white and red pulp that was close to that observed in the control mice (Fig. 5F). Moderate amounts of T cells were observed in the lymph node tissue from the control group, with the T cells dispersed in the outer cortex lymphoid follicles with normal distribution in the inner cortex (paracortical zone) (Fig. 5G). There was a significant increase in the distribution of T cells in the outer cortex lymphoid follicles and in paracortical lymph node region in the CCl_4 -treated group (Fig. 5H) compared with the control mice. The lymph node sections of the CCl_4 + propolis-treated group showed an apparent restoration of T cell distribution in the outer cortex lymphoid follicles and the paracortical zone, with patterns identical to those observed in the control group (Fig. 5I). Furthermore, the B cells in the control lymph node sections were normally distributed in the outer cortex lymphoid follicles (Fig. 5J). The lymph node sections prepared from the CCl_4 -treated group showed a marked reduction in the B cell distri-

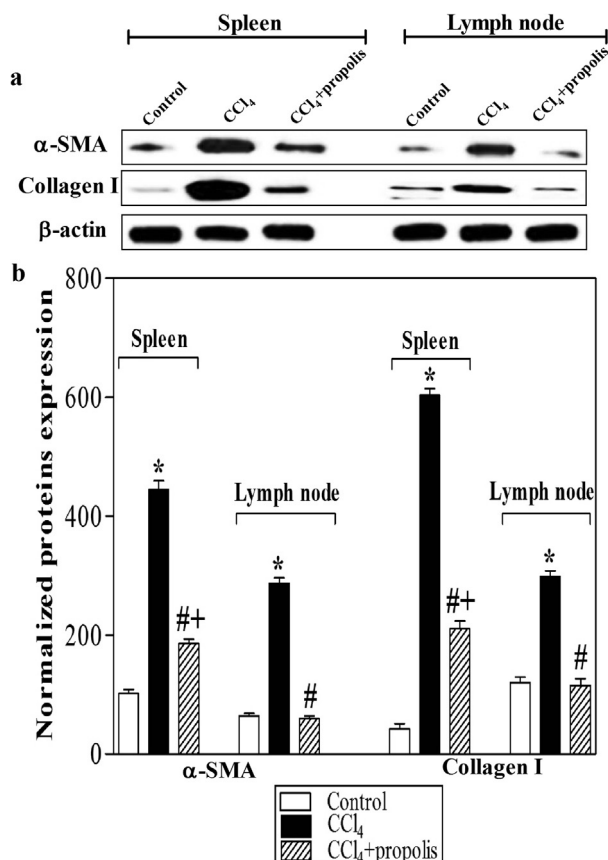


Fig. 4. Propolis reduced myofibroblastic marker expression in secondary immune organs. Lysates of spleen and lymph node tissues were subjected to western blotting analysis using antibodies against α -SMA, collagen type I, and β -actin. The protein bands from one representative experiment are shown for the expression of α -SMA, collagen type I, and β -actin (loading control) (A). α -SMA and collagen type I expression was normalized to the total protein levels of β -actin. The results are expressed as the means \pm 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 and collagen type I from the control mice (open bar), CCl₄-treated mice (closed black bar), and CCl₄ + propolis-treated mice (hatched bar). * $P < 0.05$ for CCl₄-treated mice vs. control mice. # $P < 0.05$ for CCl₄ + propolis-treated mice vs. CCl₄-treated mice (ANOVA with Tukey's post-hoc test).

tribution in the outer cortex lymphoid follicles (Fig. 5K). Interestingly, the B cell distribution in the lymph nodes of the CCl₄ + propolis-treated group was relatively normal in the outer cortex lymphoid follicles (Fig. 5L).

3.6. Oral propolis supplementation recovered Nrf2, COX-2, and eNOS expression in fibrotic mice

Nrf2, COX-2, and eNOS are the major regulators of the cellular protection mechanism against oxidative stress. Thus, we used western blot analysis of spleen and lymph node lysates to investigate the impact of propolis supplementation on the expression of Nrf2, COX-2, and eNOS in mice with CCl₄-induced liver fibrosis (Fig. 6A). The CCl₄-treated group demonstrated increased expression of Nrf2, COX-2, and eNOS relative to that of the control group. Interestingly, the CCl₄-treated mice that received propolis supplementation showed decreased levels of Nrf2, COX-2, and eNOS expression (Fig. 6B). The CCl₄-treated group showed a marked increase in Nrf2, COX-2, and eNOS expression compared with the control group. The CCl₄ + propolis-treated group showed significantly decreased expression levels of Nrf2, COX-2, and eNOS compared with the CCl₄-treated group.

3.7. Treatment of fibrotic mice with propolis restored the levels of TGF- β , proinflammatory cytokines, ROS, and NO in lymphoid tissues

Induction of liver fibrosis by CCl₄ resulted in the inflammation of secondary lymphoid organs and a chronic oxidative stress condition. CCl₄ is a commonly used hepatotoxin in animal models of liver fibrosis. It is activated by CYP450 in the liver to form trichloromethyl and trichloromethyl peroxy radicals. CCl₄ may also induce Kupffer cells to generate ROS and then destroy liver cells, resulting in an aberrant elevation of TGF- β , proinflammatory cytokines (IL-1 β and IL-6), ROS, and NO levels. Liver macrophages, including Kupffer cells, generate TGF- β to activate HSCs, which are important regulators of liver fibrosis (Fararh et al., 2016). Therefore, we used ELISA to examine the effect of propolis on TGF- β levels after CCl₄-induced liver fibrogenesis in lysates of the spleen and lymph nodes. The TGF- β level in the CCl₄-treated group was significantly increased relative to the control group (Fig. 7A). Supplementing the CCl₄-treated group with propolis significantly reduced the TGF- β levels. Additionally, ELISA was implemented in spleen and lymph node lysates from the three animal groups (Fig. 7B, C). Amassed data from five mice in each group showed that CCl₄ induced a substantial growth in the levels of these proinflammatory cytokines relative to the control group. The supplementation of CCl₄-treated mice with propolis considerably decreased the levels of proinflammatory cytokines. We also used ELISA to determine the ROS levels in spleen and lymph node lysates from the three mouse groups (Fig. 7D). The ROS levels in the liver tissue lysates from CCl₄-treated mice were significantly increased compared with the control mice. Most interestingly, the liver tissue lysates from the CCl₄-treated mice that received propolis supplementation showed significant restoration of the chronic oxidative stress condition alterations induced by CCl₄ by decreasing the ROS levels (Fig. 7E) found in the control group.

3.8. Propolis inhibits ASK1 and P38 phosphorylation induced by CCl₄ treatment

Liver fibrosis is a specific disorder resulting from inflammation. HSCs are the focal point of the fibrogenic response, which results in the expression of different inflammatory factors, including COX-2, IL-1, IL-6, tumor necrosis factor- α (TNF- α), and TGF- β . In turn, these factors activate HSCs in a process based on p38 mitogen-activated protein kinases (MAPKs). ASK1 is one of several mitogen-activated protein kinase kinases (MAP3Ks) that are activated in response to proinflammatory stimuli, ROS, and other cellular stresses. ASK1 then activates the c-Jun N-terminal kinase (JNK) and p38 MAPK pathway. Activation of these pathways induces cellular responses such as apoptosis, differentiation, cell survival, and production of inflammatory cytokines. ASK1-deficient mice have reduced CCl₄-induced hepatic fibrosis, and TGF- β expression, which is responsible for hepatic fibrosis. The analysis of ASK1-deficient mice has also demonstrated the requirement of the ASK1-p38 pathway for immune responses. Liver fibrosis may be caused by inflammatory and stress factors. Thus, we used western blot analysis to investigate the impact of propolis supplementation on ASK1 and P38 phosphorylation following CCl₄ induction of liver fibrosis. We used antibodies that recognized phosphorylated ASK1 (p-ASK1), total ASK1 (T-ASK1), phosphorylated P38 (p-P38), and total P38 (T-P38) (Fig. 8A). The CCl₄-treated group showed a significant increase in p-ASK1 and p-P38 compared with the control group. Interestingly, the supplementation of propolis to CCl₄-treated mice decreased p-ASK1 and p-P38. The levels of p-ASK1 and p-P38 were normalized to the levels of T-ASK1 and T-P38, respectively (Fig. 8B). The CCl₄-treated group exhibited a significant increase in p-ASK1 and p-P38 relative to the control group. When propolis was administered to the CCl₄-

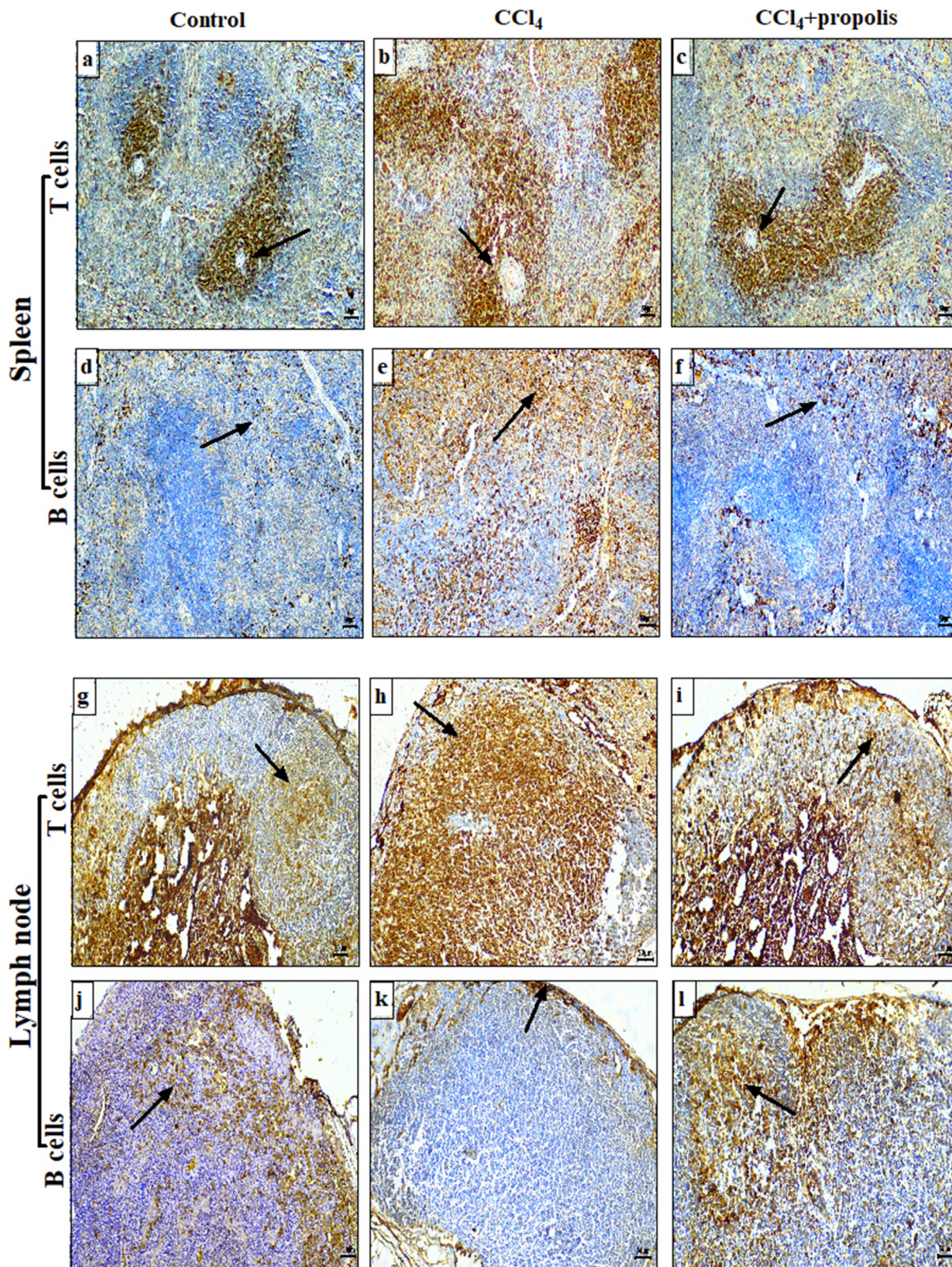


Fig. 5. Effects of CCl₄ and propolis on T and B cell distribution in the spleen and lymph nodes of mice with CCl₄-induced hepatic fibrosis. Spleen sections from the control, CCl₄-treated, and CCl₄ + propolis-treated groups were immunohistochemically stained with anti-CD3 (A–C, respectively) and anti-CD20 (D–F, respectively) and then detected by immunohistochemical analysis (Immunoperoxidase × 100). Lymph node sections from the control, CCl₄-treated, and CCl₄ + propolis-treated groups were immunohistochemically stained with anti-CD3 (G–I, respectively) and anti-CD20 (J–L, respectively) and then detected by immunohistochemical analysis (Immunoperoxidase × 100).

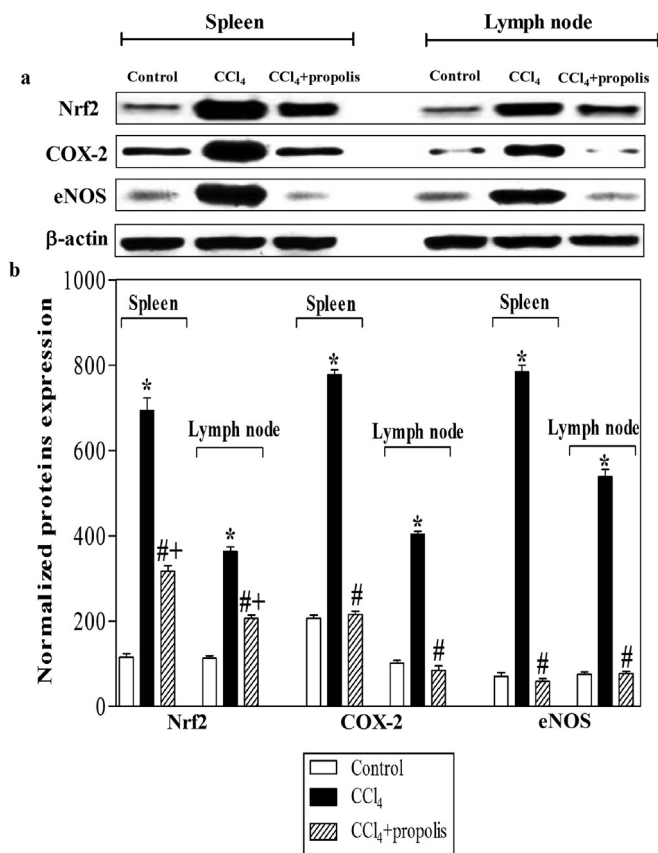


Fig. 6. Impact of propolis treatment on the expression of Nrf2, COX-2, and eNOS in secondary immune organs of mice with CCl₄-induced hepatic fibrosis. The spleen and lymph node lysates of the three mouse groups were prepared for western blotting using antibodies against Nrf2, COX-2, eNOS, and β-actin (A). The protein bands from one representative experiment are shown for the expression of Nrf2, COX-2, eNOS, and β-actin (A). The expression of Nrf2, COX-2, and eNOS was normalized to the total protein levels of β-actin. The accumulated data of five mice from the control group (open bars), CCl₄-treated (closed black bars), and CCl₄ + propolis-treated (hatched bars) groups, respectively, are expressed as the means ± SEM of the normalized value of each parameter (B). *P < 0.05 for CCl₄-treated mice vs. control mice. #P < 0.05 for CCl₄ + propolis-treated mice vs. CCl₄-treated mice. ##P < 0.05 for CCl₄ + propolis-treated mice vs. control mice (ANOVA with Tukey's post-hoc test).

treated group, the levels of p-ASK1 and p-P38 in the CCl₄ + propolis group were significantly reduced compared with the CCl₄-treated group.

4. Discussion

Liver is one of the largest organs in the human body which performs many vital functions, such as eliminating damaged red blood cells from the blood in conjunction with the spleen, processing bile, synthesizing coagulation factors, and storing vitamins, minerals, proteins, fats, and dietary glucose (Fararh et al., 2016). Liver fibrosis is a natural process which ensues in response to hepatic injuries such as toxicity or drug exposure. Studies of liver fibrosis over the past 2 decades have improved knowledge of the condition's molecular pathogenesis, which enabled the identification of potentially effective drugs to treat liver fibrosis. CCl₄ is widely used as hepatotoxin in hepatopathy studies (Geetha et al., 2008) and causes liver cirrhosis in animals that is similar to human liver cirrhosis (Lee et al., 2007). This study investigated the immunotherapy properties of propolis against CCl₄-mediated immune toxicity. The current study results confirmed CCl₄ as persuaded histological alterations in liver architecture, characterized

by the abundant deposition of collagen fibers surrounding the central vein, which was in agreement with Bataller and Brenner (Bataller and Brenner, 2005), who reported that the production of extracellular matrix by the liver increased approximately 6-fold in the advanced stages of fibrosis. Hepatic injury results in HSC propagation and revolution into a myofibroblast-like phenotype in a process called activation. The activated HSCs express α-SMA and procollagen-I and are the main source of collagen I, III, and IV and other fibrosis-deposited matrix proteins (Friedman, 1993). Accordingly, most antifibrotic therapies are designed to prevent HSC activation, proliferation, or synthesis of products (Nguyen-Lefebvre et al., 2018). Our results disclosed lowered chronic inflammation, with a decrease in type I & III-collagen deposition (Montes and Junqueira, 1991), and decreased α-SMA expression in hepatic tissue in the CCl₄-treated group supplemented with propolis. Similarly, CCl₄ mediated the overexpression of α-SMA & type-I collagen in spleen and lymph nodes, while supplementing the CCl₄-treated groups with propolis markedly downregulated α-SMA and collagen type-I appearance. Likewise, CCl₄ treatment encouraged collagen deposition and spleen fibrosis, while propolis supplementation restored normal splenic histoarchitecture (Abraham and Wilfred, 1999). Lipid peroxidation can arise in tissues apart from liver, especially in kidney, testicles, spleen, and also in lungs (Casini and Benedetti, 1978). It was documented that CCl₄ radicals bound to cellular lipids in extrahepatic tissues of rats, albeit to a lesser extent than in the liver. Additionally, CCl₄ toxicity had been shown to alter the morphology of splenic macrophages by modulating macrophage differentiation (Chakraborty and Sengupta, 2012). Moreover, it has been shown that apitherapy had a beneficial effect on the histoarchitecture of the liver and spleen, in line with our findings (Andritoiu et al., 2014). In this context, it has been observed that the appearance of the spleen mostly returns to normal after propolis treatment (AlGabbani et al., 2017). The lymph node sections of the CCl₄-treated group in our study showed degenerative changes in the cortex and medulla characterized by necrosis and depletion of the lymphocyte population. Our results correlated with Doi et al. (1991) that might have been a secondary effect of induced hepatic injury (Faroon, 2005). Our study results showed that propolis supplementation restored the histoarchitecture and induced an increase in the lymphocyte populations in the splenic cortex and medulla. These results were in line with Sforcin et al. (2005), who conveyed flavonoids possessed anti-inflammatory activity and confirmed that the tissue and regenerative effect improved damage to immune system organs (Elshama et al., 2016). Our study results exhibited a growth clustering of B cells in white pulp and a large dispersion of B-cells in red pulp of spleen of mice treated with CCl₄. Similarly, there was a marked increase in the number of T and B cells in the outer cortex lymphoid follicles and the paracortical region. Such results were in line with Jirova et al. (1996), who found that T-cell based areas showed significant activation of lymphoid tissue. The morphological examination of spleen and lymph nodes after CCl₄ treatment showed stimulated B cell areas. Interestingly, propolis therapy recovered the circulation of T and B cells in the lymphoid follicles and white and red splenic pulps. Hayriye, 2018 reported that caffeic acid phenethyl ester, a propolis component, possessed immune-modulatory properties.

Though the accurate role of propolis action in immune-cells remain simple, flavonoids are capable of stimulating B and T-cells (Wolska et al., 2019).

Nrf2 mediates the activity of cleansing and antioxidant enzymes in CCl₄-induced liver fibrosis by regulating stage II. Thus, Nrf2 activation is crucial in treating hepatic fibrosis (Zou et al., 2017). COX-2 was recently identified as an immediate early gene linked to inflammation, cell formation, differentiation, apoptosis prevention, and tumorigenesis. Evidence suggests that NO pro-

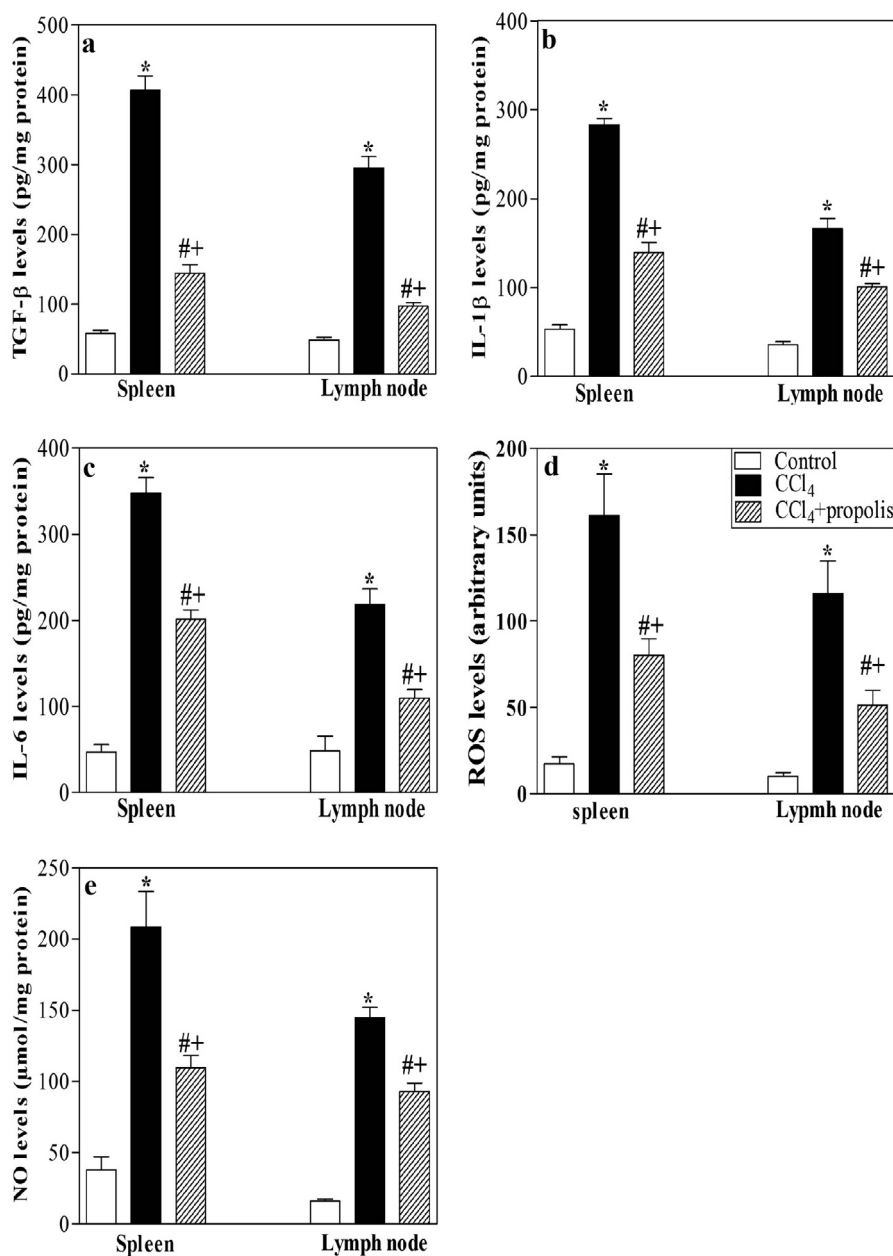


Fig. 7. Influence of propolis on the levels of TGF- β , IL-1 β , IL-6, ROS, and NO in secondary immune organs of fibrotic mice. The levels of TGF- β (A), IL-1 β (B), IL-6 (C), ROS (D), and NO (E) were measured in the spleen and lymph node lysates of the control (open bars), CCl₄-treated (closed black bars), and CCl₄ + propolis-treated (hatched bars) groups. The accumulated data from five mice of each group are expressed as the means \pm SEM and were analyzed by ANOVA, followed by Tukey's post-hoc test. Differences were considered statistically significant at * $P < 0.05$ for CCl₄-treated mice vs. control mice, # $P < 0.05$ for CCl₄ + propolis-treated mice vs. CCl₄-treated mice, and * $P < 0.05$ for CCl₄ + propolis-treated mice vs. control mice.

duced by eNOS improves COX-2 activity (Rahman et al., 2001). Our study results showed a significant increase in the expression of Nrf2, COX-2, and eNOS in the CCl₄-treated group, which was in accordance with Domitrović et al. (2012), who claimed that the presence of COX-2 and eNOS in the nucleus indicated their involvement in the control of certain nuclear functions. Our analysis was also in contract with Yang et al. (2014), who showed the upregulation of Keap1 and Nrf2 mRNA and CCl₄-induced liver fibrosis in rats. However, our results were against the results by Said et al. (2018), who reported that CCl₄ significantly downregulated Nrf2 expression. Our study results showed that the supplementation of CCl₄-treated animals with propolis decreased COX-2 and eNOS expression. Furthermore, propolis administration also contributes to decreasing the amount of nitric oxide synthetase. The CCl₄-

treated animals who received propolis supplementation in our study showed decreased Nrf2 expression, which was in disagreement with Mujica et al. (2017), who reported that propolis could activate Nrf2 and improve the antioxidant ability of the cells.

TGF- β signaling plays a major role in maintaining liver homeostasis, terminal differentiation, and hepatocyte apoptosis. TGF- β is regulated under conditions of liver damage and controls parenchymal cells, inflammatory cells, and HSCs (Karkampouna et al., 2016). TGF- β 1 plays a crucial role in regulating immune homeostasis and inflammation within the immune system (Tamayo Revuelta et al., 2018). Our study results showed a significant increase in the level of TGF- β in the CCl₄-treated group. Supplementing the CCl₄-treated group with propolis significantly reduced the level of TGF- β in spleen and lymph node tissue lysates.

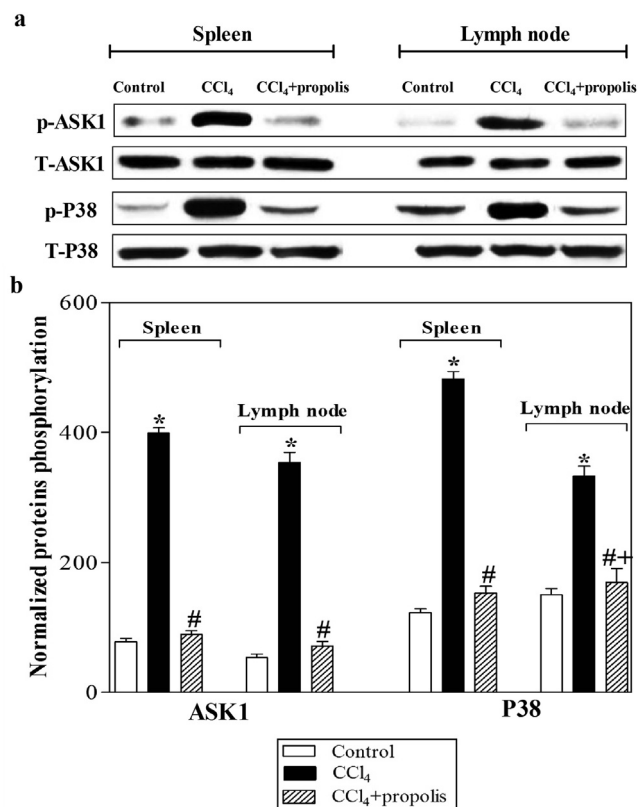


Fig. 8. Propolis enhances the phosphorylation of ASK1 and P38 in spleen and lymph node tissues of mice with CCl₄-induced hepatic fibrosis. Lysates of spleen and lymph node tissue samples collected from mice in each group were subjected to western blotting using antibodies recognizing p-ASK1 and p-P38 and the total relevant proteins T-ASK1 and T-P38. The protein bands from one representative experiment are shown (A). The phosphorylation of ASK1 and P38 was normalized to the total relevant protein levels, and accumulated data of five mice from the control (open bars), CCl₄-treated (closed black bars), and CCl₄ + propolis-treated (hatched bars) groups are expressed as the means ± SEM of the normalized value of each parameter (B). *P < 0.05 for CCl₄-treated mice vs. control mice. #P < 0.05 for CCl₄ + propolis-treated mice vs. CCl₄-treated mice. *P < 0.05 for CCl₄ + propolis-treated mice vs. control mice (ANOVA with Tukey's post-hoc test).

These results were in accordance with Said et al. (2018), who reported that treatment with pinocembrin (a flavanone abundant in honey and propolis) mitigated the increased levels of the prominent CCl₄-induced pro-fibrogenic cytokine TGF-β.

Cytokines are polypeptides with a broad range of regulatory, inflammatory, metabolic, hematopoietic, and immunological properties. The liver represents a major site for the synthesis and clearance of several cytokines (Rey et al., 2018). IL-1 is a proinflammatory cytokine that plays a crucial role in chronic and acute inflammation. IL-1 refers to two different cytokines, of which one is mainly secreted (IL-1β) and the other intracellular (IL-1α), that bind to the same receptor (Meier et al., 2019). Improving the clearance and signaling of hepatic IL-6 partially improves the liver function of patients with liver cirrhosis (Prystupa et al., 2015). The impaired hepatic elimination of IL-6 may explain the elevated levels of systemic IL-6 in patients with liver cirrhosis. The toxicity of CCl₄ is mediated by many signaling pathways, such as TGF-α and NO, leading to inflammation and apoptosis, while TGF-α and -β induce fibrosis (Weber et al., 2003). Our results showed that the NO level in the CCl₄-treated group was significantly increased. Oral supplementation of the CCl₄-treated mice with propolis improved the NO level to a normal value. The antioxidant and anti-inflammatory effects of propolis are due to its high content of polyphenols, flavonoids, and phenolic acids, which serve as reduc-

ing agents, inhibitors of lipid peroxidation, and free radical scavengers (Nna et al., 2018). Our results revealed that the CCl₄-treated group exhibited a growth in phosphorylation of ASK1 and P38, while supplementation of CCl₄-treated animals with propolis decreased.

5. Conclusion

Distinctive immune-cells are plentiful in the liver which is an important lymphoid organ. Liver-fibrosis is a chief challenge of global health and the level of fibrosis characterizes the foremost risk-factors for the progression of other diseases such as hepatocellular carcinoma (HCC). Anti-fibrotic therapies to inhibit deterioration of liver failure and HCC progression have also been tremendously unmet. Propolis exhibited anti-fibrotic therapeutic effects against liver fibrosis. Our results conclude that induction of liver fibrosis by CCl₄ mediated inflammatory and fibrotic signals which provoke disruption of physiological architectures of the spleen and lymph node. Supplementation of CCl₄-treated mice with-propolis significantly lowered the free-radical-levels and proinflammatory cytokines; the expression of α-SMA, collagen, Nrf2, COX-2, and eNOS; and the phosphorylation of ASK1 and P38. Hence, propolis abolished the pro-fibrogenic signals mediated by CCl₄ and inhibited the life-threatening complications of liver fibrosis.

CRedit authorship contribution statement

Eman Abdo Sayed: Methodology, Formal analysis, Investigation. **Gamal Badr:** Supervision, Validation, Visualization, writing original draft. **Khadiga Abdel-Hameed Hassan:** Validation, Supervision, Investigation. **Hanan Waly:** Methodology, Formal analysis, writing. **Betul Ozdemir:** Formal analysis, Data curation. **Mohamed Mahmoud:** Data curation, Writing-reviewing and editing, Funding. **Salman Alamery:** Project administration, Writing-reviewing and editing, Funding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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