

## Immunohistopathological Studies on Rats Injected with CCl<sub>4</sub> and Treated with Propolis and Honey Bee

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### ABSTRACT

**Background:** liver and kidney play a pivotal role in metabolism of nutrients, drugs, hormones, metabolic waste products and thereby maintaining body homeostasis. The present study was conducted to demonstrate the protective effects of honey and propolis on liver and kidney tissues of rats injected with CCl<sub>4</sub>.

**Material and method:** rats were divided into 4 groups (10 rats in each group). **Normal control group:** received standard food and water; **CCl<sub>4</sub> group:** injected with CCl<sub>4</sub> 0.5 ml/kg b. wt. mixed in olive oil(v/v) twice a week for six weeks; **CCl<sub>4</sub> & honey group:** injected with CCl<sub>4</sub> 0.5 ml/kg b. wt. mixed in olive oil(v/v) twice a week for six weeks and 10% honey in drinking water. **CCl<sub>4</sub> & Propolis:** injected with CCl<sub>4</sub> 0.5 ml/kg b. wt. mixed in olive oil(v/v) twice a week for six weeks and 200 ml/kg b.wt/rat/day of propolis. After 6 weeks, rats were anesthetized, then liver and kidney organs were collected, washed in normal saline, fixed in 10% formalin, then processed for the histopathological and immunohistochemical examinations.

**Results:** marked histopathological alterations were observed in CCl<sub>4</sub> group, the most common changes were cloudy swelling of hepatocytes, fatty changes, clear vacuolation of renal cells and congested blood vessels. Treatment with honey or propolis improved the histopathological changes induced by CCl<sub>4</sub> in liver and kidney tissues of rats.

**Conclusion:** the present study indicated that CCl<sub>4</sub> has a toxic effect on liver and kidney tissues, but administration of honey and propolis can protect rats against from the toxic effect of CCl<sub>4</sub>.

**Key words:** CCl<sub>4</sub>, histochemistry and immunohistochemistry, collagen fibres, honey bee and propolis.

### INTRODUCTION

Carbon tetrachloride is widely used as hepatotoxic compound in the experimental model systems. It has been reported that CCl<sub>4</sub>-induced hepatotoxicity is due to its hepatotoxic metabolites and trichloromethyl free radicals (•CCl<sub>3</sub>) which induce lipid peroxidation<sup>[1,2]</sup>. One of the therapeutic strategies against liver injury induced by CCl<sub>4</sub> is to find natural antioxidant compounds that are able to block liver injury through scavenging of trichloromethyl free radicals generated by CCl<sub>4</sub><sup>[2]</sup>.

Propolis is a resinous hive product that contains flavonoids, sugar and aliphatic acids<sup>[3,4,5]</sup>. Flavonoids are thought to be responsible for many biological and pharmacological activities including anticancer<sup>[6]</sup>, anti-inflammatory<sup>[7]</sup>, antioxidant effects<sup>[8,9,10]</sup>, antimicrobial<sup>[11]</sup>, antiparasitic and immune modularity and immune stimulant effects<sup>[12]</sup>. Propolis increases the percentage of protected animals suggesting its use in vaccines as an adjuvant<sup>[13,14]</sup>.

Also, honey is bee's products which are used in medicine in many cultures since ancient times. Honey is known to exhibit a broad spectrum of activities including antiviral, antibacterial and immunostimulant<sup>[15,16]</sup>. It has antioxidant activity due to its high content of flavonoids<sup>[17,18]</sup>. The aim of the present study is to evaluate the histopathological changes in liver and kidney tissues of rats induced by CCl<sub>4</sub> toxicity and the possible protective role of honey bee and propolis for CCl<sub>4</sub> treated rats.

### MATERIALS AND METHODS

#### Animals

Forty female Albino Wister rats weighing 120-140 gm were purchased from Helwan animal station, Ministry of Health, Egypt. Animals were allowed to adapt for two weeks and housed in animal house of Zoology Department, Faculty of Science, Damietta University, Egypt. CCl<sub>4</sub> was purchased from Modern Lab. Co. for Chemicals, Egypt. Honey

was purchased from general markets in New Damietta City, Damietta, Egypt. Propolis was purchased from local pharmacy.

#### **Experimental design:**

Rats were divided into 4 groups each of 10 rats. **Normal control group:** fed on standard food and water. **CCl<sub>4</sub> group (CC):** injected i.p. with 0.5mg/kg b.wt. of CCl<sub>4</sub> mixed with olive oil v/v twice weekly for six weeks. **Honey group(CC+H):** injected i.p. with 0.5mg/kg b. wt. of CCl<sub>4</sub> mixed in olive oil v/v twice weekly for six weeks and treated with 10% honey bee in drinking water daily. **Propolis group(CC+P):** injected i.p. with 0.5mg/kg of b. wt. of CCl<sub>4</sub> twice weekly for six weeks and treated with propolis, 200ml/kg/rat orally day after day for six weeks.

#### **Preparation of propolis:**

The method was done according to the method of **Orsolio and Basic** [19]. Briefly, the propolis was prepared by dissolving 16.8 mg in 10ml of distilled water. After shaking for 10 minutes, the water extract was centrifuged at 1000 rpm for 10 minutes. The supernatant of the water extract was used for the treatment of rats.

#### **Methods**

##### **H&E stain:**

Liver tissue specimens were fixed in 10% formalin and embedded in paraffin wax and 5 micrometer sections were obtained and stained with H&E stain according to the method of **Drury and Wallington**. [20]

##### **Masson's trichrome stain**

Collagen fibres were stained according to the method of **Carson** [21].

##### **Silver stain**

Two methods of silver staining have been developed and classified into: 1-silver amine or alkaline method and 2- silver nitrate or acidic method [22,23].

##### **Immunohistochemistry**

5µm paraffin sections were dewaxed in xylene and rehydrated in descending grades of alcohol. Microwave antigen retrieval in 10 mM sodium citrate at pH 6 was carried out for 20 min. and the sections were subsequently covered with 3% hydrogen peroxide for 10min. Sections were blocked by 100µl of PBS containing 10% normal swine serum for 1hour in humidified chamber to reduce unspecific background staining. 100 µl of rabbit anti-laminin (Muri Lab. Prep.) with dilution 1:50 in blocking buffer was applied to slides overnight

at 4°C in humidified chamber and then sections washed in PBS 3 times. 100 µl of biotinylated swine anti-rabbit antibody diluted 1:500 in blocking buffer were applied for 2hrs in humidified chamber. After washing in PBS, 100µl of Avidin-Biotin complex was added for two hrs in humidified chamber. 100 µl of freshly prepared diaminobezidine-HCl (Sigma) were added to develop color for 12min. Sections were washed in running water to stop color development and then dehydrated, counter stained and cover slip in Permount [24].

## **RESULTS**

### **The histopathological and histochemical observations :**

#### **1.The histopathological studies:-**

##### **1.1. Hematoxylin and eosin staining:**

Liver sections from the untreated rats (control group) showed hepatocytes with acidophilic cytoplasm and single central rounded vesicular nuclei and some of the cells are binucleated (Fig.1a). In CCl<sub>4</sub> group, most of the hepatocytes contained multiple large cytoplasmic vacuoles and some hepatocytes had deeply acidophilic cytoplasm and deeply stained nuclei (pyknosis) (Fig.1b). After treatment of rats injected with CCl<sub>4</sub> with honey or propolis, remarkable improvement in the hepatocytes was noticed as they appeared somewhat similar to that of the control rats. Only few hepatocytes appeared with slight vacuolated cytoplasm (Figs. 1c and 1d).

In normal kidney sections (Fig. 2a) there was normal structure, normal glomeruli. CCl<sub>4</sub> injected rats (Fig.2b), showed marked deleterious histological changes with significant glomerular and tubular degenerations varying from, glomerular basement thickening, interstitial inflammation, tubular cell swelling, pyknotic nuclei, medullary congestion and moderate to severe necrosis. The kidney sections of CCl<sub>4</sub> injected rats and treated with honey showed normal glomeruli, and tubule-interstitial cells (Fig 2c). In CCl<sub>4</sub> injected rats and treated with propolis normal morphology of the kidney and detected with normal architecture of the kidney (Fig. 2d).

##### **1.2. Masson's trichrome stain**

In the liver tissue of the control group, there was very delicate meshwork of few collagenous fibres surrounding the central veins (Fig. 3a). Sections of CCl<sub>4</sub> group, showed an

apparent increase in the collagen fibres around the central veins in the portal areas (Fig. 3b). In rats injected with CCl<sub>4</sub> and treated with honey and propolis respectively, few collagen fibres were detected and the collagen deposition was sharply decreased compared with the CCl<sub>4</sub> group and no fibrotic septa could be observed (Fig.3c, fig. 3d).

Normal control kidney sections, showed normal distribution of collagen fibres in the renal tubules and glomeruli (Fig. 4a). The kidney sections of CCl<sub>4</sub> treated showed extensive collagen fibres deposition around the glomeruli (Fig. 4b). In CCl<sub>4</sub> injected rats and treated with honey and propolis the deposition of collagen fibres was significantly reduced (Figs 4c and 4d).

## **2.The histochemical studies (Liver and kidney tissues) :-**

### **2.1. Silver stain**

In silver nitrate stained sections of liver sections of normal control group showed slight reticulin stain (Fig. 5a).The liver sections of CCl<sub>4</sub> injected rats showed dark black stained reticular fibers (Fig. 5b). However after treatment with honey and propolis, the deposition of reticulin fibers was decreased (Figs 5c and 5d).

Kidney tissue of the control rats showed black stained reticular fibres in the glomeruli the (Fig. 6a).The kidney sections of CCl<sub>4</sub> treated rats showed increased dark black staining affinity in the reticulin fibres supporting walls of the blood vessel in and around the glomeruli (Fig. 6b). Honey and propolis treatment for CCl<sub>4</sub> injected rats showed reduced reticulin fibres in the kidney cortex(Figs 6c, 6d).

### **3. Immunohistochemical examination:**

Immunohistochemistry of the control liver tissue stained with anti-laminin showed negative staining affinity (Fig. 7a). In CCl<sub>4</sub> injected rats, hepatic tissue showed necrosis with diffusely stained anti-laminin antibody (Fig. 7b). Hepatic tissue stained with anti-laminin in CCl<sub>4</sub> injected rats and treated with honey bee showed significant repress in laminin expression (Fig. 7c). Liver sections of CCl<sub>4</sub> injected rats and treated with propolis showed mild staining affinity in hepatocytes and in non-parenchymal cells along the sinusoids (Fig. 7d).

## **DISCUSSION**

The present study showed that, CCl<sub>4</sub> administration for sex weeks, resulted in loss of

the usual hepatic architecture, many vacuoles with dark stained nuclei in most of the liver cells. These findings agree with the study of **Tasci *et al*** <sup>[25]</sup> who reported that structural damage was due to edema of the organelles. Also, **Gomaa *et al.***<sup>[26]</sup> found that histopathological examination of CCl<sub>4</sub> liver of rats revealed congestion of the central and portal veins with hydrotrophic degeneration of the hepatocytes.

Liver fibrosis was clearly evidenced in the present work by a significant increase in area percentage of the collagen fibres in Masson trichrome stained sections. It was explained that hepatic fibrosis is usually initiated by hepatocyte damage leading to activation of Kupffer cells and subsequent release of inflammatory cytokines and growth factors. These factors activate HSCs which proliferate and transform into myofibroblasts-like cells that deposit large amounts of connective tissue components <sup>[27]</sup>. Moreover, CCl<sub>4</sub> causes oxidative stress that activates HSCs <sup>[28]</sup>.

Histological examination also demonstrated a large number of inflammatory cells infiltrated into the intralobular and interlobular regions and there were more collagen fibres in CCl<sub>4</sub> - treated rats compared to normal rats <sup>[29]</sup>. Honey bee exhibits a significant protection against hepatotoxicity as evidenced from the histopathology changes which were detected due to CCl<sub>4</sub> <sup>[30]</sup>. The histopathological studies in the liver of rats also supported that honey and propolis extract markedly reduced the toxicity of CCl<sub>4</sub> and preserved the histo- architecture of the liver and kidney tissues to somewhat normal appearance. Results of the present study showed many histopathological changes which indicating liver damage after CCl<sub>4</sub> administration. It has been reported by previous findings that CCl<sub>4</sub> causes necrosis <sup>[31,32]</sup>, fibrosis <sup>[32,33,34]</sup>, mononuclear cell infiltration<sup>[34]</sup>, steatosis and degeneration of hepatocytes, increase in mitotic activity <sup>[35]</sup> and cirrhosis <sup>[32]</sup> in liver. It has also been reported that CCl<sub>4</sub> causes apoptosis in liver. <sup>[31,36]</sup>

Our results reveal that honey has a marked protective effect on renal tubular toxicity, these results are in agreement with the study of **Mousa**<sup>[37]</sup>.

One of the structural glycoproteins is a high molecular weight molecule called laminin <sup>[38]</sup>. It is well known from previous immunohistochemical studies that laminin and type IV collagen are permanent constituents of

the basement membrane<sup>[39,40,41]</sup>. It has also been reported that basement membrane components such as type IV collagen and laminin can be synthesized by trophoblastic cells<sup>[42]</sup>.

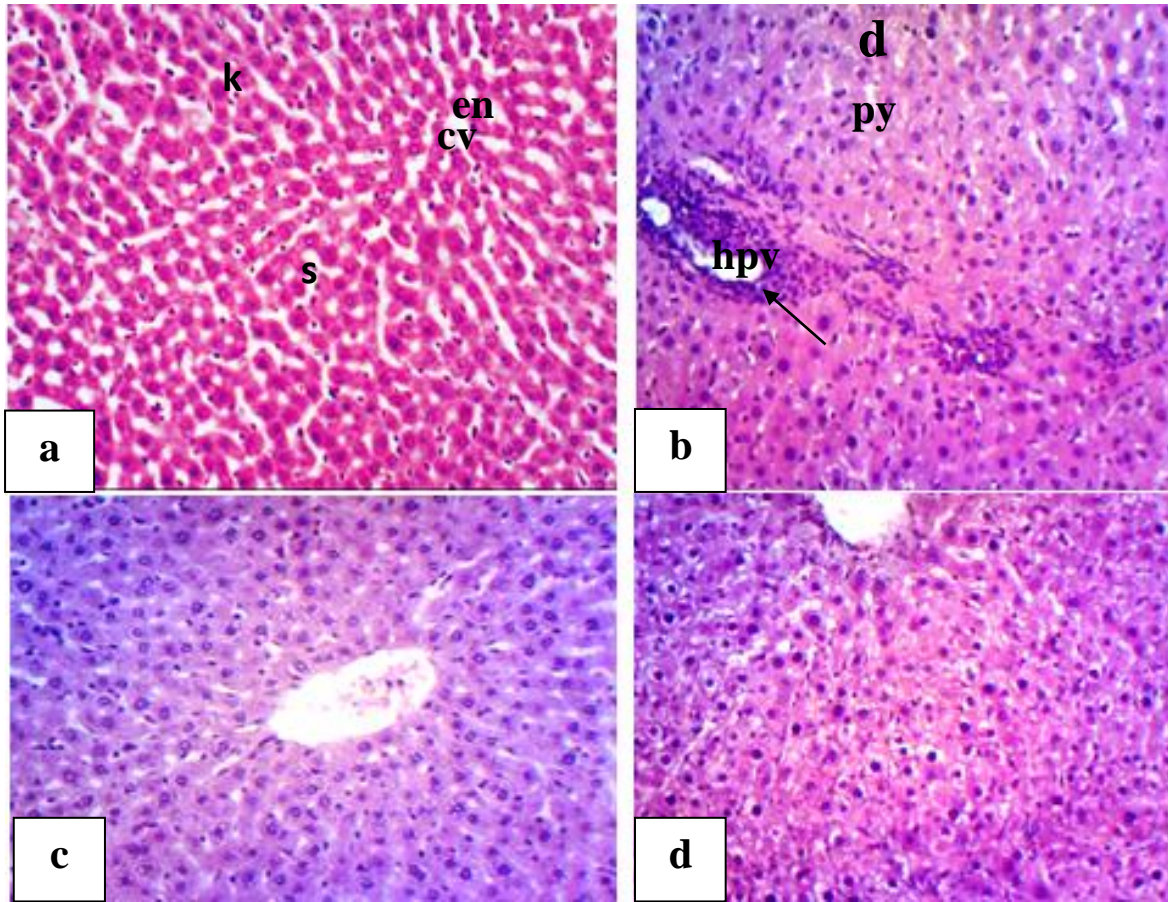
In the present study it was showed that the positivity of laminin protein stained with anti-laminin antibody was higher in CCl<sub>4</sub> group and decreased on the other side after treatment with honey and propolis.

The basement membranes may control selective permeability for macromolecules as well as providing structural tissue support. Laminin, which binds to itself, to type IV collagen, to heparin and to cell surface receptors, is able to promote the adhesion and growth of various epithelial cells and tumor cells<sup>[43,44]</sup>, as well as the outgrowth of neuritis<sup>[45]</sup>. The interactions of cells with various tissue matrices may result in quite different cellular responses, and modification of the extra cellular matrix structure is likely to play an important role in the regulation of cell behaviour during developmental processes. In conclusion, the results of this study indicated that honey and propolis are effective for the prevention of CCl<sub>4</sub>-induced nephrohepatic damage in rats. Further studies are required to recommend the use of honey and it's therapeutic potential in human.

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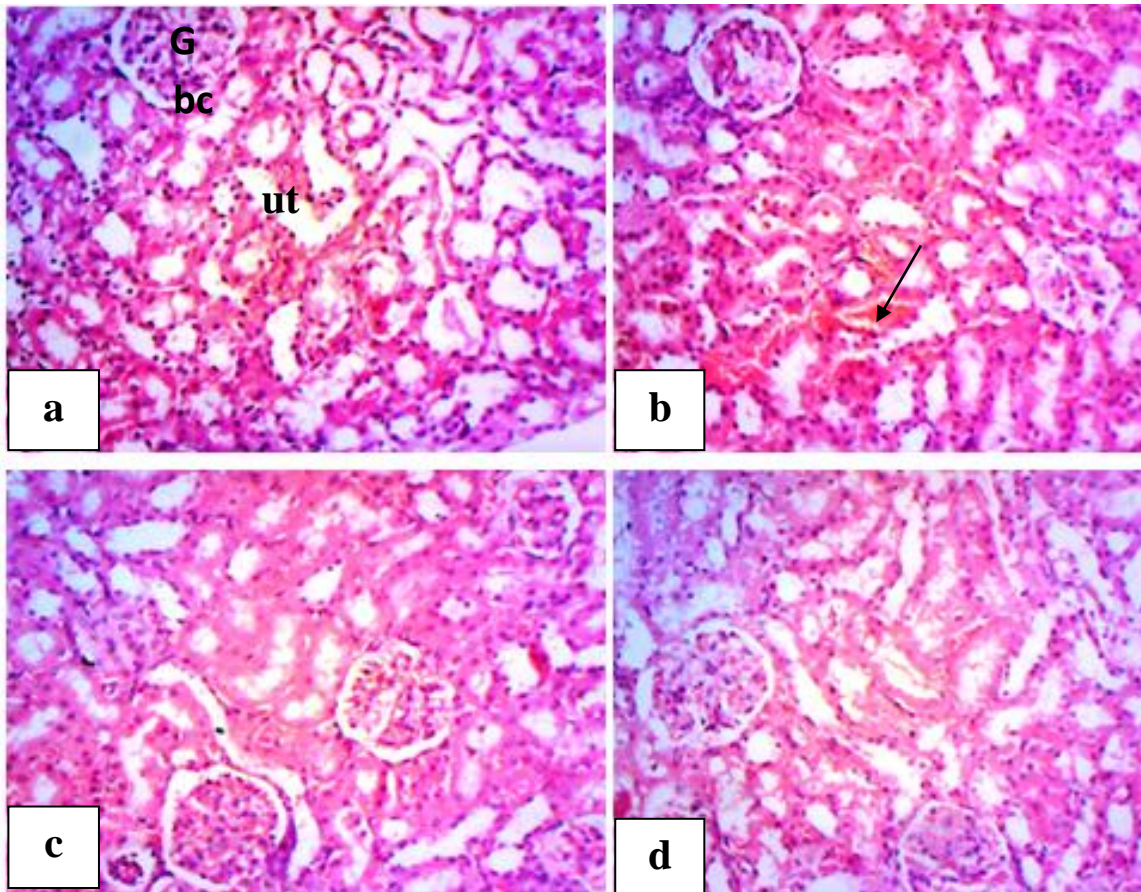
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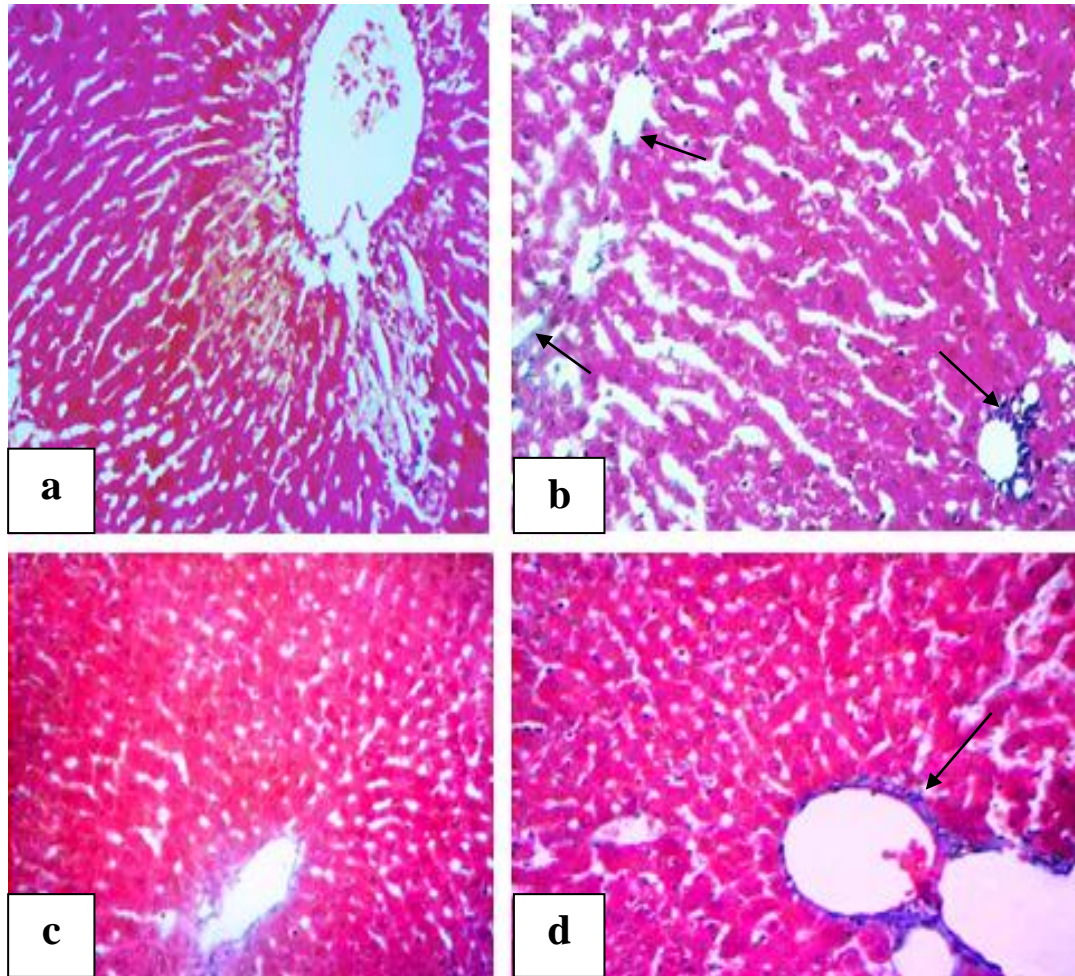


**Figure 1.**Light photomicrograph of liver section of the control and treated groups showing:

1-a. showing liver tissue of the control untreated rat that displaying the central vein (CV) lined by endothelial cells (en), hepatic cells , kupffer cells (K) and narrow sinusoids (S); 1-b. Liver section of 6 weeks  $\text{CCl}_4$  injected rat showing inflammatory cells around the hepatic portal vein (hpv) with numerous pyknotic nuclei (py) and hepatocyte degeneration (d); 1-c. 6 weeks  $\text{CCl}_4$  injected rat and treated with honey bee showing improvement of hepatic architecture; 1-d. showing the liver section of 6 weeks  $\text{CCl}_4$  injected rat and treated with propolis displaying normal lobular architecture , central vein and hepatocytes (H&E stain X100).



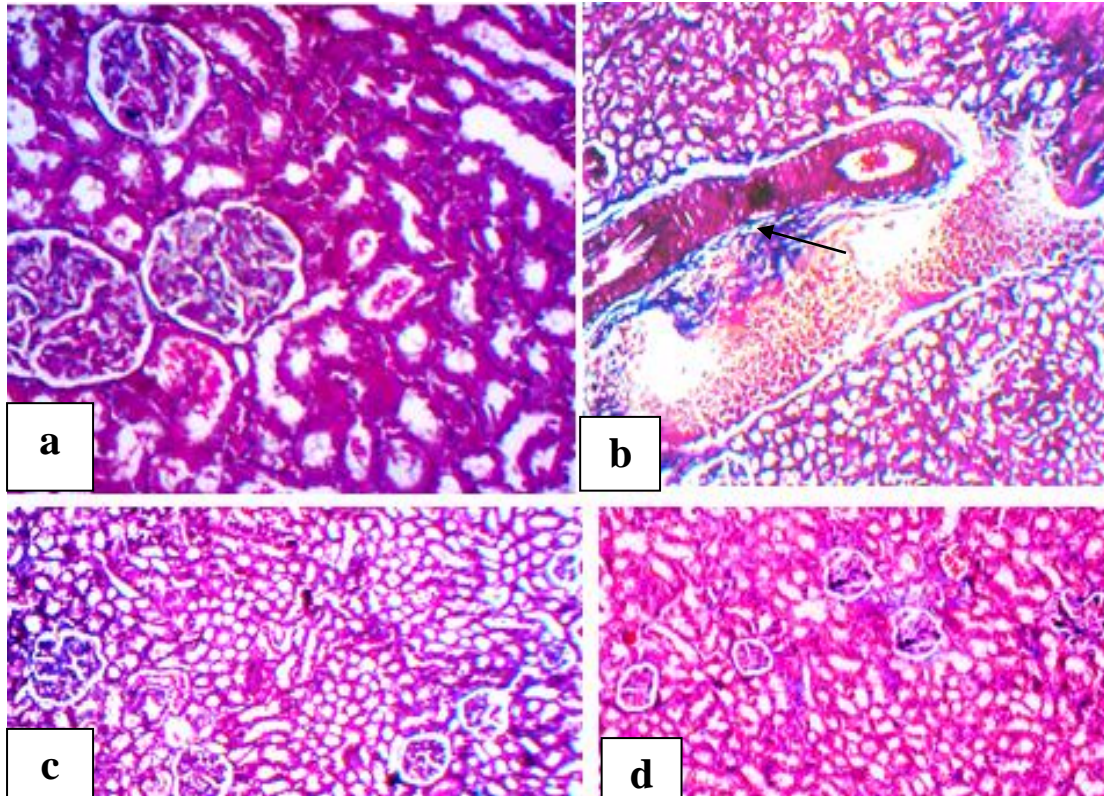
**Figure 2.** Light photomicrograph of the kidney section of the control and treated groups showing: 2-a. Showing kidney the control untreated rat displaying the normal structure of the glomerulus (G), Bowman's capsule (bc) and uriniferous tubules (ut); 2-b. Showing kidney cortex of 6 weeks CCl<sub>4</sub> injected rat. Notice clear vacuolation of cells and congested blood vessels (arrow) ; 2-c. Showing almost normal morphology and architecture of kidney; 2-d. showing the liver section of 6 weeks CCl<sub>4</sub> injected rat and treated with propolis administration showing improvement of general kidney architecture (H&E stain X100).



**Figure 3.** Photomicrograph showing distribution of collagen fibres in the liver of the control and treated groups:

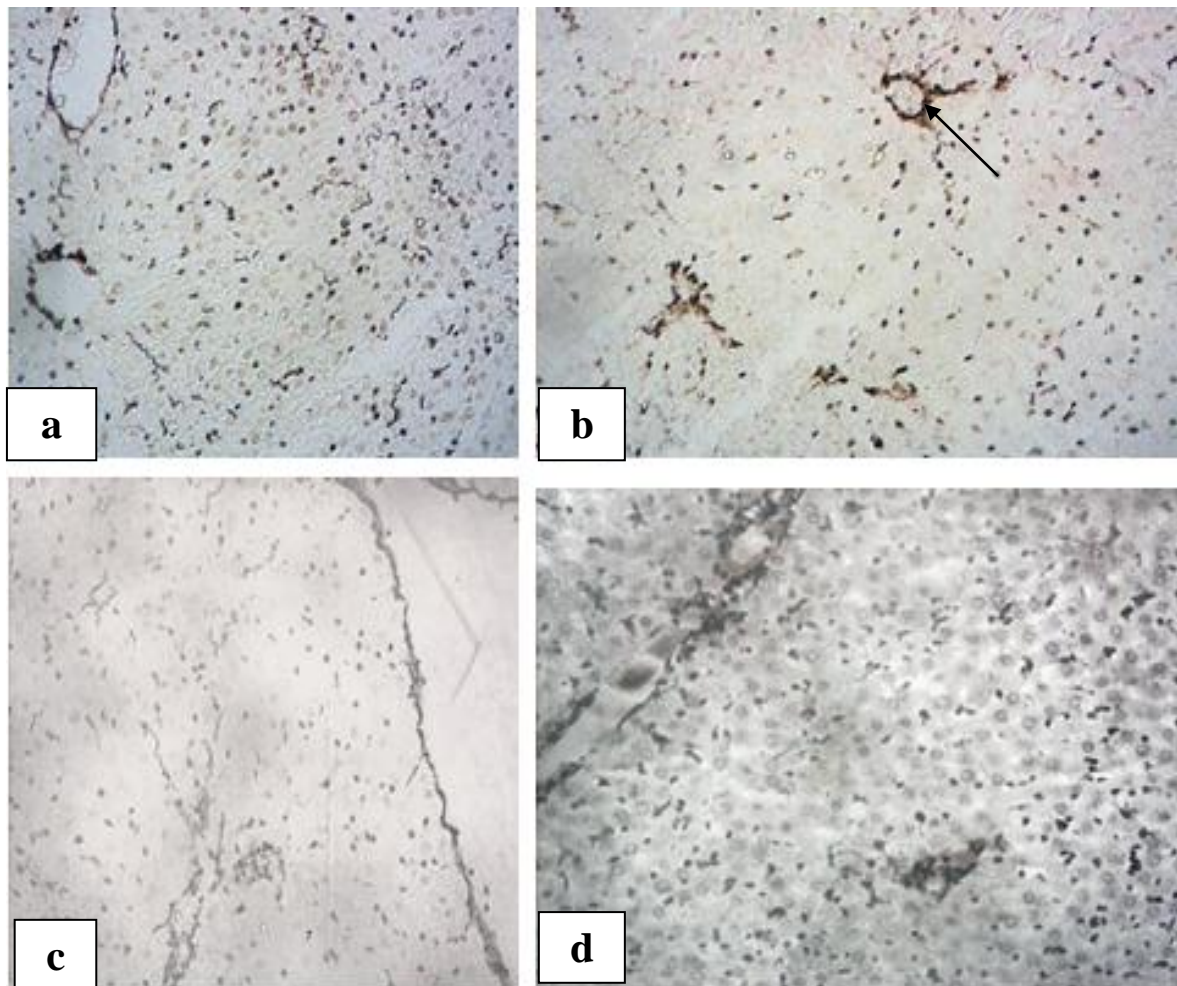
3-a. Showing liver tissue of the control untreated rat displaying normal distribution of collagen fibers; 3-b. Showing the liver section of 6 weeks  $\text{CCl}_4$  injected rat showing increased collagen fibres which are stained blue (arrow) especially in wall of the hepatic portal vein ; 3-c. Showing the liver section of 6 weeks  $\text{CCl}_4$  injected rat and treated with honey bee .notice reduced collagen deposition compared to the control group ; 3-d. Showing liver section of 6 weeks  $\text{CCl}_4$  injected rat and treated with propolis showing thin bundles of collagen fibres supporting walls of the hepatic portal vein (arrow) (Masson's trichrome stain X100).





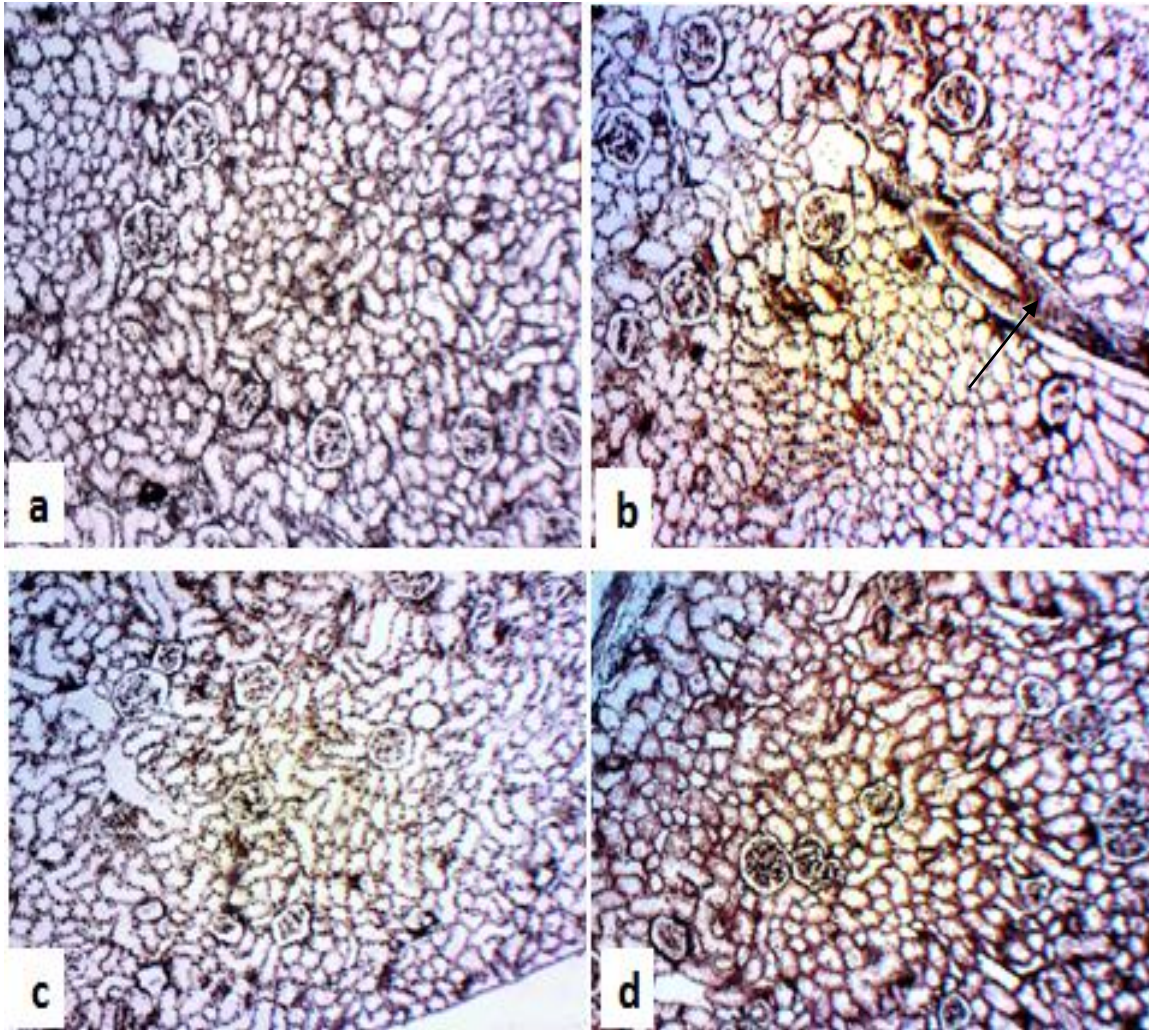
**Figure 4.** Photomicrograph showing distribution of collagen fibres in the kidney of the control and treated groups:

4.a- kidney section of a control untreated rat showing normal distribution of collagen fibers ; 4.b- Showing kidney section of 6 weeks CCl<sub>4</sub> injected rat, the kidney section showing interstitial fibrosis around the blood vessel (arrow) with highly increased collagen fibres ; 4.c- After 6 weeks CCl<sub>4</sub> injected rat and treated with honey bee administration showing reduced collagen deposition ; 4.d-Showing the kidney section of 6 weeks CCl<sub>4</sub> injected rat and treated with propolis administration .Notice a slight increase of collagen fibres in the glomeruli (Masson's trichrome stain X100)



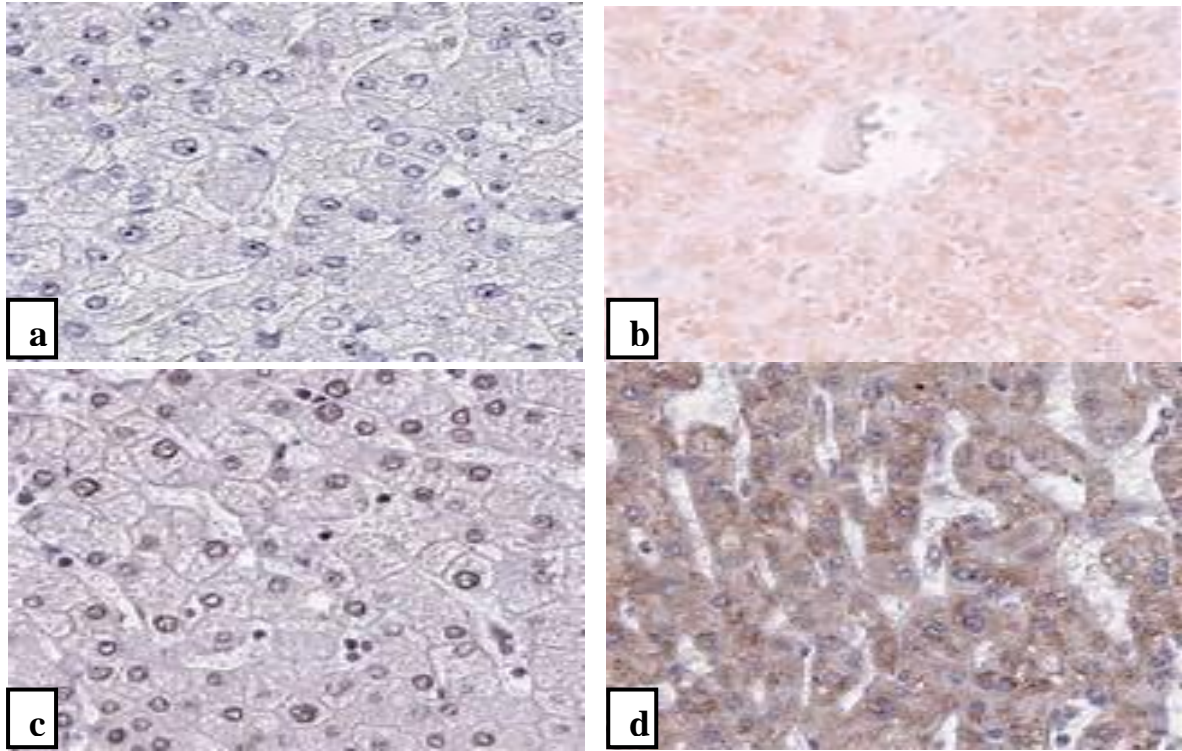
**Figure 5.** Photomicrograph showing distribution of reticular fibers in the control and treated groups:

5-a. Showing the liver section of a control untreated rat showing distribution of reticular fibres in the liver tissue ; 5-b. Showing the liver section of 6 weeks CCl<sub>4</sub> treated rat displaying increased dark black stained reticular fibers (arrow) ; 5-c. showing the liver section of 6 weeks CCl<sub>4</sub> injected rat and treated with honey bee showing reduced reticular fibres deposition compared to the control group ; 5-d. Showing the liver section of 6 weeks CCl<sub>4</sub> injected rat and treated with propolis showing somewhat normal appearance of reticular fibres (Silver nitrate stain X100).



**Figure 6:** photomicrograph showing distribution of reticular fibers in the control and treated groups:

6-a. Photomicrograph of a T.S in kidney section of control untreated rat showing black stained reticular fibers displaying the normal distribution ; 6-b. showing the kidney section of 6 weeks  $\text{CCl}_4$  injected rat displaying increased the dark black staining of the reticulin fibers supporting the wall of the blood vessel (arrow) .6-c. administration showing faintly staining of reticular fibers in group of  $\text{CCl}_4$  injected rat and treated with honey; 6-d. showing kidney section of 6 weeks  $\text{CCl}_4$  treated rats with propolis, administration showing improvement of reticulin staining (Silver stain X100)



**Figure 7.** Light micrograph showing immunohistochemical localization of laminin in liver section of rat by anti-laminin antibody: a) Liver section of the control group with negative immunoreactivity for laminin. b) Liver section of a rat treated with CCl<sub>4</sub> showing extensive hepatocellular necrosis and diffused reaction affinity of laminin. c) Liver section of a rat treated with CCl<sub>4</sub>+Bee honey showing significant repression in laminin expression. d) Liver section of a rat treated with CCl<sub>4</sub>+propolis showing mild reaction affinity of laminin (Mayer's hematoxylin x250).