

Study of the hepatoprotective activity of ethanol extract of local propolis in rabbits

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الخلاصة

أجريت هذه الدراسة لتقييم التأثير الواقي للكبد للمستخلص الايثانوليللعكبر المحلي في محافظة الديوانية ضد الكرب التأكسدي وعسر وظيفة الكبد في ذكور الارانب المهقاء . تم استخدام اربع وعشرون من ذكور الارانب والتي تم تقسيمها عشوائياً الى اربع مجاميع وبواقع ستة حيوانات في كل مجموعة . اعطيت المجموعة الاولى الماء المقطر بمقدار 2,5 مل عن طريق الفم يومياً ولمدة ثلاث اسابيع بعدها تم حقنها تحت الجلد بالماء المقطر بمقدار 0,25 مل لمرتين وبفارق زمني (36) ساعة بين الحقنتين وعدت كمجموعة سيطرة ، في حين جرعت المجموعة الثانية بالمستخلص الايثانوليللعكبر وبجرعة 75 ملغم / كغم من وزن الجسم يومياً ولمدة ثلاث اسابيع وبعد ذلك حقنت تحت الجلد بالماء المقطر بمقدار 0,25 مل لمرتين متتاليتين وبفارق زمني (36) ساعة بين الحقنتين . اعطيت المجموعة الثالثة المستخلص الايثانوليللعكبر وبجرعة 75 ملغم / كغم من وزن الجسم عن طريق الفم يومياً ولمدة ثلاث اسابيع قبل ان يتم حقنها تحت الجلد برباعي كلوريد الكربون وبجرعة 0,25 مل / كغم من وزن الجسم ولمرتين متتاليتين وبفارق زمني (36) ساعة بين الحقنتين . بينما جرعت المجموعة الاخيرة بالماء المقطر بمقدار 2.5 مل عن طريق الفم يومياً ولمدة ثلاث اسابيع بعد ذلك حقنت تحت الجلد برباعي كلوريد الكربون بمقدار 0,25 مل لمرتين متتاليتين وبفارق زمني (36) ساعة بين الحقنتين . في نهاية التجربة تم جمع عينات المصل من الارانب لتحديد مستوى الكلوتاثيون المختزل في المصل، بعد ذلك تم التضحية بالحيوانات حيث تم استخراج الكبد ليتم فحصه عياناً ومجهرياً . أدى إعطاء رباعي كلوريد الكربون الى انخفاض مستوى الكلوتاثيون المختزل في المصل وبشكل معنوي (أ $0,05 \geq$) تم رصد تغيرات مرضية عيانية ومجهرية واضحة بعد حقن رباعي كلوريد الكربون تميزت بتنخر واضح خلال نسيج الكبد . أدت المعاملة المسبقة بالمستخلص الايثانوليللعكبر قبل حقن رباعي كلوريد الكربون الى النجاح في إيقاف الانخفاض في مستوى الكلوتاثيون المختزل في المصل بالاضافة الى التحسن في البنيان النسيجي للكبد . يمكن الاستنتاج بأن المستخلص الايثانوليللعكبر له فعالية مضادة للأكسدة ، وكفاءة في وقاية الكبد من التأثيرات السمية.

Abstract

The present study has been conducted to evaluate the hepatoprotective effect of ethanol extract of local propolis at Al-Diwaniyah province against CCl_4 induce oxidative stress and liver dysfunction in white male rabbits .Twenty four male rabbits were randomly divided in four groups with (6) animal each , the first group was received distilled water (2.5 ml. orally) daily for three weeks then injected with distilled water at(2.5 ml. subcutaneous) two times at (36) hours interval and considered as control group , the second group was drenched with Ethanol Extract of Propolis (EEP) at dose of (75 mg/kg .BodyWight) daily for three weeks then rabbits were injected with distilled water(0.25 ml/kg. BodyWight. Subcutaneous) two times at (36) hours interval . The third group was pretreated with Ethanol Extract of Propolis at dose of (75 mg/kg BodyWight. orally) daily for three weeks then were injected with CCl_4 at dose of(0.25 ml/kg Body Wight .subcutaneous) two times at (36) hour interval , whereas the last group was received distilled water(2.5 ml. orally) daily for three weeks after that they were injected with CCl_4 at dose of (0.25 ml/kg. BodyWight. Subcutaneous) two times at (36) hours interval .At the end of experiment serum samples were collected from rabbits to determine the serum Glutathione (GSH) , then animals were sacrificed where the liver was excised to be examined macroscopically and microscopically.The serum GSH level was significantly (Propapilty \leq 0.05) decreased after CCl_4 injection . A remarkable macroscopic and microscopic pathological alterations were detected after CCl_4 administration

characterized by necrosis and degeneration of hepatic tissue . Pretreatment with EEP before CCl₄ injections significantly reverse the marked decrease of serum GSH also there is improvement in hepatic histoarchitecture. . It was concluded that EEP had the potential antioxidant activities as well as, hepatoprotective efficacy.

Introduction

Interest in free radical, began with the work of mosesGomberg⁽¹⁾, who in 1900 demonstrated the existence of the triphenylmethyl radical (Ph₃C[•]). A free radical, may in simple terms be defined as any chemical species capable of independent existence possessing one or more unpaired electrons ⁽²⁾. Excessive formation of free radicals lead to oxidative stress, which is a deleterious condition resulting from an imbalance between the generation of free radicals and the activity of antioxidant defense system in which oxidation predominates ⁽³⁾.

A deleterious process that can adversely alter lipids, proteins, and DNA and have been implicated in aging and number of human diseases. The most important diseases caused by oxidative stress are liver diseases, which are remain one of the major threats to public health and are a worldwide problem ⁽⁴⁾. It is a term for a collection of conditions diseases, and infections that affect the cells, tissues, structures, or function of the liver. About 20,000 deaths found every year due to the liver disorders ⁽⁵⁾.

Liver disorders are detectable by clinical signs (Jaundice, Swollen and tender liver) biochemical alteration(elevated levels of hepatic enzymes in the

blood, loss of enzymatic activities in the liver) or histological examination (fatty degeneration and necrosis of hepatocytes, destruction of intracellular organelles, fibrosis, cirrhosis). Using of drugs to treat liver diseases is usually associated with a lot of dangerous side effects . So there is increasing interest toward using of natural substance and their active ingredients to overcome this problem .one of the interesting natural substance is propolis, or " bee glue " which is a resinous substance collected by bees from the bunds of tree around the hives , is masticated by the bees mandible , mixing it with products of their salivary glands and bees wax was added , then applied to the combs and walls of the hive⁽⁶⁾.Its chemical composition is very complex and more than 300 constituents have identified in propolis⁽⁷⁾ . Its composition varies with the season and the vegetation in the areas in which it is collected.

It has then been resumed that a need to perform an investigation as an aim of the present study focusing on the using of ethanol extract of propolis (EEP) to decrease hepatotoxicity induced by CCl₄,awell known hepatotoxic compound.

Materials and methods

Propolis collection and preparation: Propolis sample were obtained from different parts of Al-diwanayah government (Diwanayah, Alseder, Saneya, and Shnaffia) by personal contact with beekeepers during the period of (February, March and April of 2012). The samples of crude propolis was cut in to small piece by using medical mortar then, grinded by using of

electrical grinder to a powder of suitable practical size following grinding, the powdered crude propolis was sifted to ensure the proper practical size, where in sieving the material is passed through a sieve of suitable mesh size giving two fractions. The fraction passing the sieve consist of particals with a size smaller than or corresponding to the mesh size. The remaining fraction consist of coarser

particals which are returned to the electrical grinder for further grinding. By Slightly modification for methods presented by ^(8,9). Fifty grams of crude powdered propolis was macerated in (1000) ml of 70 % ethanol (1:20W/V) for (6) days with mixing and shaking by thermo magnetic stirrer (300 rpm at 25c°) (4hour / day). The extract solution was stored overnight at (4c°) to obtain crystallization of dissolved waxes. The resultant solution was filtered through a whatmanfilter paper. Then the filtered

Statistical analysis

All the values were expressed as mean \pm standard error. Data were analyzed statistically by using of one way analysis of variance (ANOVA). Analyses were performed, probability value less than 0.05 was considered statistically significant .

Experimental Design

All rabbits were randomly divided into four groups , Six rabbits for each group . Animals of all groups were administered as follows :

Group (1) Control (intract rabbits): administered orally with distilled water by using of oral drencher , at dose of (2.5 ml / kg . BodyWight. orally daily) for three weeks then ingected with distilled water (0.25ml/kg, subcutaneous) two time at (36)hour interval.

Group (2) tretment1 (EEP treated Group) : drencher with EEP at dose of (75 mg / kg . BodyWight. orally) according to (Nader *etal* ;(2010)⁽¹⁰⁾ in average of (2.5 ml/kg. BodyWight. orally) from freshly prepared milky solution for three weeks , then injected with CCl₄ (0.25ml/kg, subcutaneous) two times at (36)hour interval .

was dried by using of over at (35C°) till complete dryness, giving a resinous brown products. Before using , (3) grams of propolis extract was dissolved in (4) ml of absolute ethanol by using of vortex mixer until complete dissolving occurred, then the volume was complete to 100 ml by adding of distilled water to obtain 3% (w/v) milking solution . The final concentration of ethanol in this milky solution didn't exceed 5% which had no effect on inviro and invitro experiment according to what stated by ⁽¹⁰⁾.

Group (3) treatment 2 (CCl₄ intoxicated group) : rabbits were received distilled water at (2.5 ml / kg . BodyWight. orally) for three weeks , after that they were received CCl₄ dissolved in liquid paraffin (1 : 1) at dose of (0.25 ml / kg . BodyWight. Subcutaneous) by using of an insulin injector two times at (36) hours interval.

Group (4) treatment 3 (EEP + CCl₄ treated group) : rabbits were pretreated with EEP at dose of (75 mg / kg . BodyWight. orally daily) for three weeks after that they were injected with CCl₄ dissolved in liquid paraffin (1 : 1) at dose of (0.25 ml / kg . BodyWight. Subcutaneous) two times at (36) hour interval

Twenty four days after the beginning of experiment , blood samples , about (15) ml , were obtained from each animal directly from the heart , by using of syringe , for assessment of serum GSH according to the method of (Burtiset *al*; 1999)⁽¹¹⁾. Then rabbits in all groups were sacrificed and the abdomen was cut open to excise the liver from each animal and was taken for weighting and examination of macroscopic changes.

Results

Effect of EEP on serum GSH level

CCl₄ injection in rabbits caused significant reduction ($P \leq 0.05$) in GSH mean levels in serum compared with mean values measured in serum samples from control animals .

The decrease in GSH levels was encountered by previously oral administration of EEP .

This encounterment showed significant elevation ($P \leq 0.05$) in GSH levels compared with results found in rabbits subcutaneously injected with CCl₄ only . Animals treated with EEP alone showed no significant difference ($P \geq 0.05$) in GSH level compared with control group.

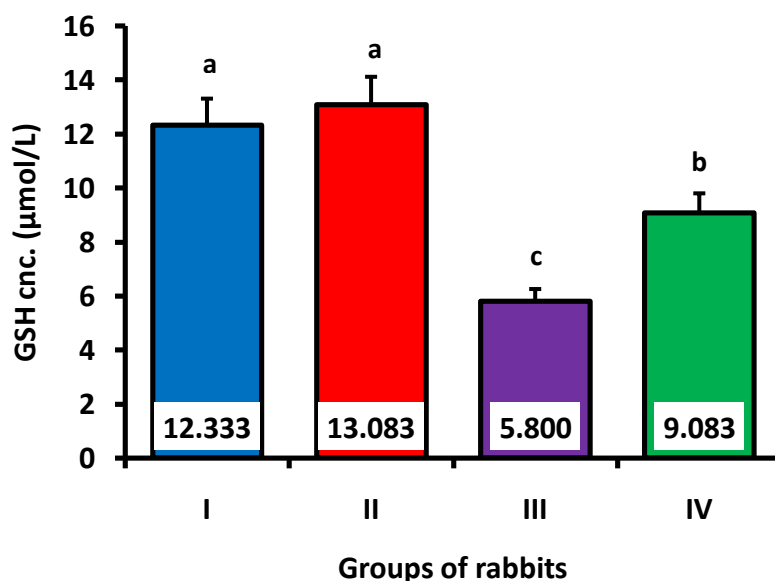


Figure (1): Effect of Ethanolic extract of propolis on serum GSH concentration ($\mu\text{mol/L}$) in CCl₄-injected male rabbits.

- □ Values represent mean \pm standard error.
- □ Different letters represent significant difference ($P < 0.05$) between groups.
- □ I: Control (Intact rabbits): male rabbits drenched with distilled water (5ml) for 3 weeks then injected with distilled water (0.25ml/kg, sc)
- □ II: Treatment 1: male rabbits drenched with EEP (75mg/kg) for 3 weeks then injected with distilled water (0.25ml, sc) two times for 3 days.
- □ III: Treatment 2: male rabbits drenched with DW (5ml) for 3 weeks then injected with CCl₄ (0.25ml/kg, sc) two times for 3 days.
- □ IV: Treatment 3: male rabbits drenched with EEP (75mg/kg) for 3 weeks, then injected with CCl₄ (0.25ml/kg, sc) two times for 3 days.

Discussion

CCl₄ , a well-known and most commonly used chemical , hepatotoxin , induced toxic action in the rabbits was found to be due to formation of active metabolite of CCl₄ , where CCl₄ is activated by cytochrome P40 (cyp_{2E1} , cyp_{2B1} or cyp_{2B2} and possibly cyp_{3A}) in the mixed function oxidase system of the liver endoplasmic reticulum , to form

trichloromethyl (CCl₃) radical . This radical can bind to cellular molecules (nucleic acid , proteins and lipids) impairing important cellular function^(12,13) . In contrast to the toxic activation of CCl₄ via metabolism (biotransformation) processes , the detoxification pathway also involves GSH conjugation to CCl₃⁽¹⁴⁾ leading to

gradual decline in GSH level . from the other hand , the excessive formed $\cdot\text{CCl}_3$ radical readily reacts with oxygen to form a trichloromethylperoxyl radical (CCl_3O_2)⁽¹³⁾ .This radical forms covalent bond with sulphhydryl groups of several membrane molecules like GSH leading to depletion of GSH . Therefore , the decrease of GSH level in serum of rabbits might be occurred due to increase its utilization by the cells in scavenging toxic free radicals of CCl_4 metabolites . It has been reported that most covalent binding of toxicant to hepatic protein occurs only after depletion of GSH and the severity of hepatic damage was directly related to degree of covalent binding with obvious proportionality⁽¹⁵⁾. So GSH represented the first line of defense against free radicals⁽¹⁶⁾ .

Furthermore , a previous study by⁽¹⁷⁾ on the mechanism of CCl_4 – induce hepatotoxicity showed that GSH plays a key role in detoxify the toxic metabolites of CCl_4 and the liver damage begins when GSH stores are markedly depleted .

Effect of EEP on histopathological changes:

The results of biochemical alterations were insured by both macroscopic and microscopic examination of rabbits liver, which revealed degenerative and necrotic changes. The destructive and degenerative changes that recorded after CCl_4 injection were including:

Macroscopic lesions: there is sever congestion and petechial hemorrhage with obvious hepatomegaly clear icterus and distention (enlargement) of gall bladder .

Microscopically there is sever fatty degeneration (distention of hepatocytes with vacuolated cytoplasm and peripheral nucleus, the cell appears signet- like shape, also there is compressed sinusoid and congestion of blood vessels. There is congestion of blood vessels and hepatic injury with massive hepatic necrosis, foamy degeneration and a prominent

The enhancement in the GSH level caused by EEP of propolis may be attributed to more than one mechanism may share this phenomenon . the first is through increasing of GSH synthesis

Firstly where CAPE , one of the propolis components , is known to increase the expression of γ - glutamyl cysteinesynthetase , an important enzyme in synthesis of GSH resulted in production of glutathione⁽¹⁸⁾.

Secondly , EEP may maintain the serum GSH level by directly neutralizing reactive oxidants resulted from CCl_4 due to its antioxidant nature of its components , such as flavonoids that act AS scavengers by reacting with peroxy radicals of polyunsaturated fatty acid resulted in breaking the chain of free radicals reaction⁽¹⁹⁾.

Third , EEP also may increases the capacity of other endogenous antioxidant defense of the body and thus increased the steady state of GSH.

infiltration of neutrophils,(figure;2). Liver. CCl_4 -treated animals showing congestion of blood vessels with presence of thrombus and hepatic injury with massive hepatic necrosis foamy degeneration also was detected,(figure;3). CCl_4 -treated rabbits showing sever injury with desquamation in the epithelial lining of bile duct and fibrosis in the wall of bile duct(figure;4).

The recorded pathological alterations were came in agreement with results of^(20,21) and that can be attributed to its effect on mitochondrial function with loss of Ca^{+2} from mitochondria and endoplasmic reticulum and elevation of its cytosolic concentration, such elevation help in destruction of cytoskeletal structure and increase in the activation of a number of hydrolytic and catabolic enzymes like proteases ,endonuclease and phospholipase which

in turn contributed to and enhance cellular necrosis⁽²²⁾.

The presence of necrotic foci showed in this study come in agreement with⁽²⁰⁾ who reported that the extent of necrosis was extended from 24 h (to be at maximum rate) to 72 h from treatment with CCl₄.

Treatment of rabbits by EEP as antioxidant before injection of CCl₄ successfully and partially mitigates the above pathological changes both macroscopic and microscopic changes induced by CCl₄ injection.

Macroscopically, there is mild degree of petechial hemorrhage, icterus and also there is significant reduction in hepatomegaly as compared with that recorded in CCl₄ treated animals, (figure;4)

Microscopically there is partial improvement of the histoarchitecture of hepatocytes with better portal vein. Also there is enhancement of the cellular structure of hepatocytes with more prominent and well-formed nucleated hepatocytes, few hepatocytes showed slightly vacuolated and obvious sinusoidal arrays, (figure;5). There is mild congestion with small thrombi within portal vein, mild recovery of cellular structure of hepatocytes. Also there is mild necrosis in the hepatic tissue with

slightly disturbed sinusoids, (figure;6). there is evidence of regeneration of hepatocytes where the nucleus appears divided, small, dense and picnotic within the hepatocyte, (figure;6) as well as therecovery and hyperplasia of .

Nevertheless, EEP succeeded to reduce necrotic hepatocytes which agreed with the findings of⁽²³⁾. The enhancement of both microscopic and macroscopic alterations after treatment with EEP may be through one or more of the followings:

First: by its ability to balance oxidant – antioxidant status via its effect as radical scavenger agent of various ROS.

Second: due to its ability to inhibit neutrophil infiltration.

Third: EEP included active substances have ability to regulate the releasing of inflammatory mediators (as athromboxane, prostaglandins, and leukotriene) by inhibition of both cyclooxygenase and lipooxygenase enzymes, where thromboxane B₂ is responsible for plasma membrane bleb which is the early event to oxidative stress⁽²³⁾

No macroscopic or microscopic changes were detected in both control and EEP treated groups (figure;7;8;9;10) indicating that EEP has not hepatotoxic activity.

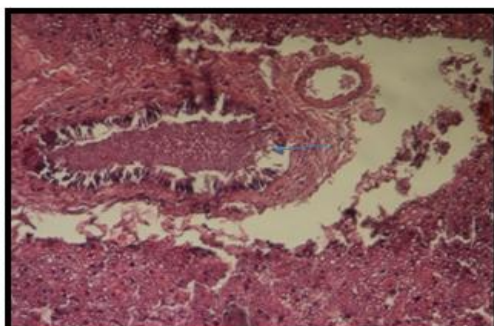
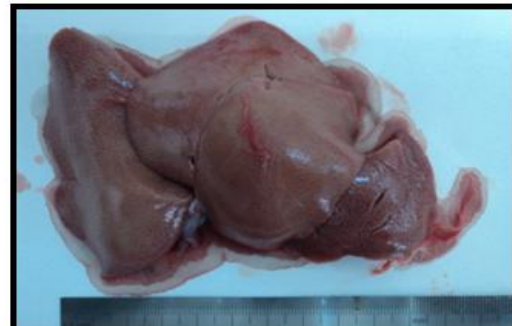


Figure (2): Liver. CCl₄-treated animals showing sever injury with desquamation in the epithelial lining of bile duct (long arrow) and fibrosis in the wall of bile duct (head of arrows). 50X H&E.



Figure(3):liver of rabbits, (gross appearance). pretreated with EEP before injection of CCl₄. There is mild degree of petechial hemorrhage, icterus and also there is significant reduction in hepatomegaly.

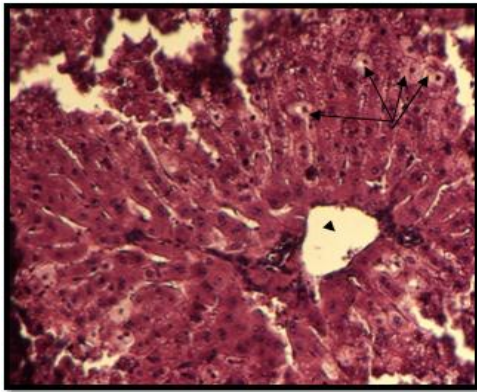


Figure (4): Liver. Rabbits pretreated with EEP before injection of CCl_4 Partially improvement of the histoarchetecture of hepatocytes with better portal vein (head of arrow). Also there is enhancement of the cellular structure of hepatocytes with more prominent and well formed nucleated hepatocytes, few hepatocytes showed slightly vacuolated (arrows) and obvious sinusoidal arrays. 50X H&E.

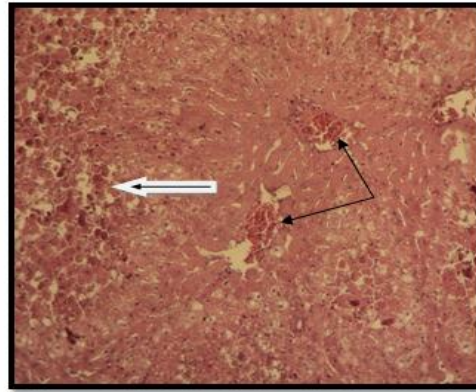


Figure (5): Liver. Show mild congestion with small thrombi within portal vein (arrows), mild recovery of cellular structure of hepatocytes. Also there is mild necrosis in the hepatic tissue particularly in the left side of figure (thick arrow) with slightly disturbed sinusoids. 50X H&E.

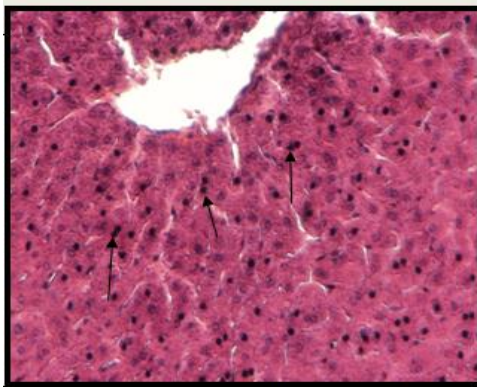


Figure (6): Liver. There is obvious regeneration of hepatocytes (the nucleus appears divided, small, dense and picnotic within the hepatocyte) (arrows). Normal portal vein and obvious sinusoidal arrays. 50X H&E.

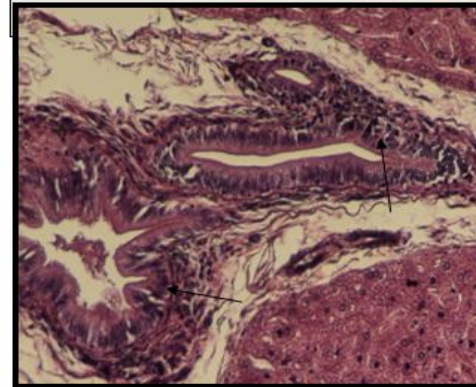


Figure (7): liver. There is recovery and hyperplasia of bile ducts, proliferation of epithelial lining of bile duct (arrows) and normal nucleated hepatocytes. 50X H&E.

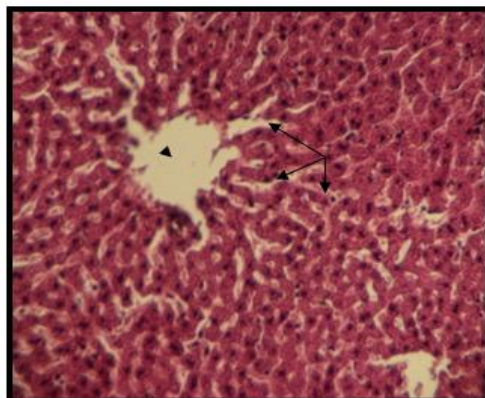


Figure (8): liver. Note normal histoarchetecture of the hepatocytes, better portal vein (head of arrows) with well prominent sinusoidal array (arrows) and well formed nucleated hepatocytes. 50X H&E.

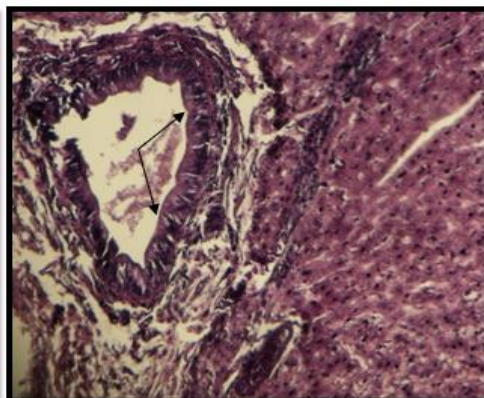


Figure (9): Liver. There is normal bile duct with normal columnar epithelium which covered the bile duct (arrows). Also there are normal hepatocytes with well prominent sinusoidal array. 50X H&E.

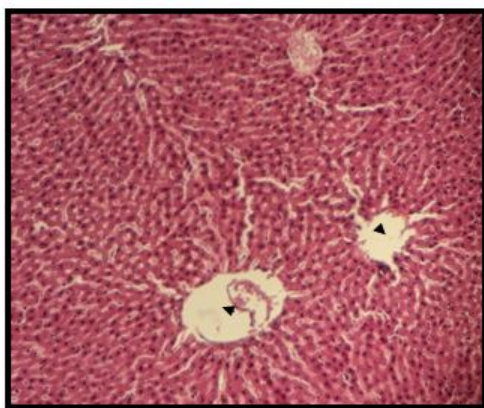


Figure (10): Liver. There are better portal veins (head of arrows) with well prominent sinusoidal array and well formed nucleated hepatocytes. 50X H&E.

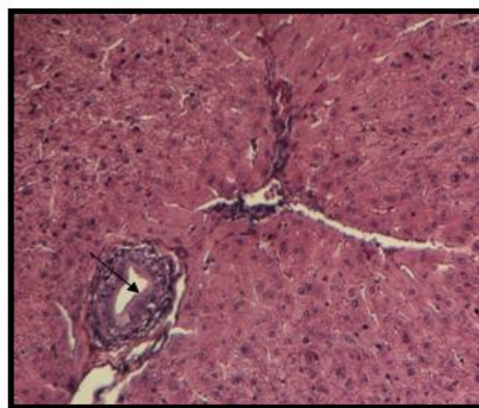


Figure (11): Liver. There is normal bile duct with normal columnar epithelium which covered it (arrow). Also there are normal nucleated hepatocytes with well prominent sinusoidal array (double arrows). 50X H&E.

Conclusions

1 -The present study shows that the local EEP contains enough biological compounds as flavonoids, phenols and other antioxidant compounds which exhibited the antioxidant activity and enhancement of antioxidant status in rabbits.

2-It can be concluded that EEP has a strong potential to provide protection against hepatotoxicity that was confirmed by improvement in both biochemical alteration and histoarchitectural change induced by CCl_4 .

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