Hepatoprotective Effect of *Echinops tenuisectus (Compositae*) on CCl_4 Induced Hepatic Damage in Rats

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Abstract

Flavonoids are known to play a vital role in the management of various liver disorders.They are a large family of compounds synthesized by plants; they belong to a group of natural substances with variable phenolic structures. In this study we aim to scan the types of flavonoids in a newly studied, wild Iraqi plant named *Echinops tenuisectus of Compositae* family. The medicinal importance of flavonoids on one hand, and the absence of any phytochemical investigation on tenuisectus species of Echinops genus on the other hand, acquired this study it's importance. Three flavonoids were identified in the seeds extract of this plant (Silymarin, Rutin, Quercetin) by two chromatographic methods, first Thin layer chromatography (TLC) using TLC ready made GF254 plates, UV detector at 254 nm, and two different solvent systems in which the R_f value of the standards (Silymarine, Rutin, Quercetin) matched with the R_f value of the Silymarin, Rutin and Quercetin found in the plant seed's extract. High pressure liquid chromatography (HPLC) was the other chromatographic method that used to identify the presence of these flavonoids in the plant seed. The plant seed's aqueous extract was evaluated for its efficacy in rats by inducing hepatotoxicity with CCl_4.Single oral dose of 250mg/kg of Seeds Extract was given to rats for 7 days. Serum activities of transaminases (ALT and AST) were used as the biochemical marker of hepatotoxicity. Histopathological changes in rats liver section were also examined. The results of the study indicated that, the pretreatment of rats with Echinops extract before the hepatotoxins agent (CCl_4) offered a hepato- protective action.

Key words: Echinops, Flavonoids

الخلاصة

الفلافينويدات تلعب دور مهم و حيوي في معالجة و تنظيم الكثير من امراض الكبد .الهدف من هذه الدراسة هو عمل مسح لمعرفة الأنواع المختلفة من الفلافينويدات في نبتة عراقية جديدة لم تدرس سابقا.نظرا للأهمية الطبية للفلافينويدات من جهة، وعدم وجود أي منشورات علمية تتناول المكونات الكيميائية لهذه النبتة، أخذت هذه الدراسة أهميتها .تم أكتشاف ثلاث أنواع من الفلافينويدات في مستخلص البذور للنبات)السليمارين، روتين، كوارستين(بواسطة طريقتين من طرق الكروماتوغرافيا، الأولى هي تقنية كروماتوغرافيا الطبقة الرقيقة (TLC) بأستخدام رقائق TLC ذات النوعية GF254 وكاشف الأشعة فوق البنفسجية U.V بالطول الموجي 254nm وثلاثة محاليل ناقلة مختلفة، حيث أن قيمة R_f للفلافينويدات القياسية طابقت قيمة R_f للفلافينويدات الموجودة في المستخلص النباتي .ثم طريقة كروماتوغرافيا تحت الضغط العالي (HPLC) التي أكدت وجود الفلافينويدات في المستخلص النباتي بتطابق retention times لكل من الفلافينويدات القياسية و الفلافينويدات في المستخلص النباتي .تم تقييم الفعالية العلاجية لهذه النبتة على كبد الفئران التي تم استحداثها بواسطة CCl_4 حيث تم اعطاء المستخلص المائي عن طريق الفم بجرعة قدرها 250 ملغم/كلغم ولمدة سبعة أيام وتم قياس مستوى الأنزيم ALT وAST الذي يبين الفعالية الوقائية والعلاجية لهذه النبتة ضد امراض الكبد .

Introduction

The *Echinops tenuisectus* belong to the *Family Compositae* (Fig1) is a wild, Iraqi plant first studied in Iraq. The Echinops genus consist of 100 spp.[1] which are widely distributed in Sharaban, Diyalah, Badrah ((Upper Tigris Plain))[2] . Preliminary investigation indicated that, this plant contain different types of flavonoids in accepted amount. Among these flavonoids: Silymarin (figure 2) which is a flavonolignan that has been introduced fairly recently as a hepatoprotective agent[3,4,5,6,7]. Silymarin has been found to provide hepatoprotection through its antioxidants properties (scavengers and regulators of the intracellular content of glutathione)[8,9,10] , as cell membrane stabilizers and permeability regulators that prevent hepatotoxic agents from entering hepatocytes[11,12]. Also as inhibitors of the transformation of satellite hepatocytes in to myofibroblasts, the process responsible for the deposition of collagen fibers leading to cirrhosis [13, 14, 15].

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Figure 1: Photography of *Echinops tenuisectus*

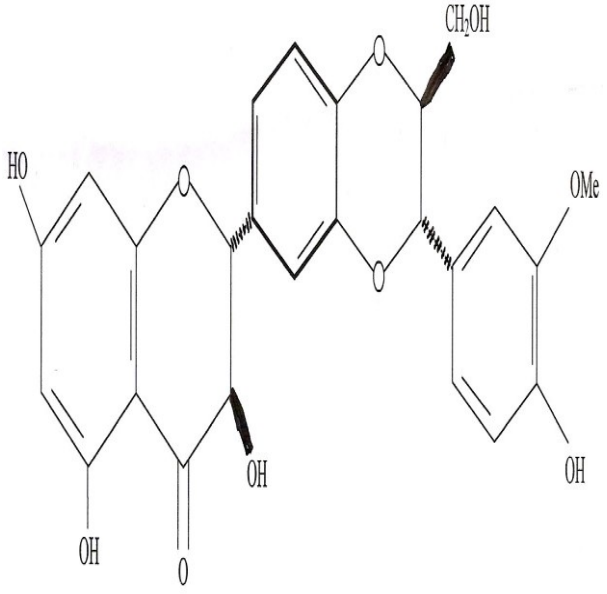


Figure 2: Structure of silybin (commercially called silymarin)

The other flavonoids found in this plant are the Quercetin and Rutin (Figure 3,4), both of them possess a powerful antioxidant activity which help to prevent free radical oxidative damage to cells, also help in the treatment and prevention of alcohol and chemical - induced hepatotoxicity by increase glutathione in the liver[16]. Glutathione responsible for detoxifying a wide range of hormones, drugs, and chemicals. High level of glutathione in the liver increases its capacity for detoxification. Quercetin and Rutin increase the level of the important antioxidant enzyme superoxide dismutase in the cell cultures [17]. In addition they stimulate protein synthesis in the liver, which results in an increase in the production of new liver cells to replace the damaged one [18]. Shoskes 1999 demonstrate that Quercetin and Rutin also inhibit the synthesis of leukotrienes (mediators of inflammation, which can result in psoriasis) [19].Recently, flavonoids can help in prevention of cancer through several pathway: inhibiting proliferation and inducing apotosis[20,21]or through inhibiting tumor invasion and angiogenesis[22,23]. This wide variety of beneficial health effects of these flavonoids acquired this study its importance in finding a new uninvestigated source of these important flavonoids , contained within *Echinops tenuisectus* of the Family *Compositae* and evaluate their possible protective effect against the experimentally- induced liver damage in rats by CCl_4 . Liver, the largest organ in vertebrate body, is the major site of intense metabolic activities. Liver injury caused by toxic chemicals and certain drugs has been recognized as a toxicological problem. Herbal drugs are playing an important role in health care programs world wide, and there is a resurgence of interest in herbal medicines for treatment of various ailments including hepatopathy[24]. CCl_4 is reported to produce free radicals which affect the cellular permeability of hepatocytes and it causes massive histopathological changes like necrosis, congested vessel and fatty changes (steatosis). So, the reverse of these phenomenon can be considered as the index of hepatoprotective[25].

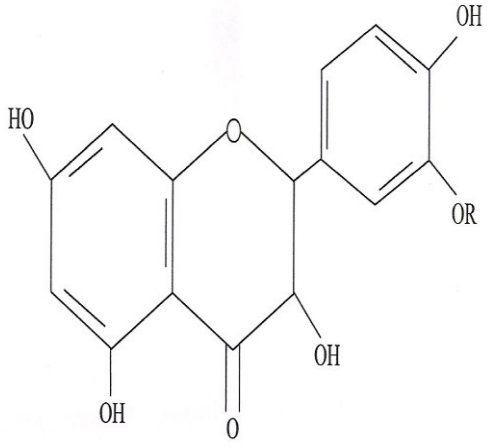


Figure 3: Quercetin; R = H
Figure 4: Rutin ; R= rhamno-glucosyl

Materials and Methods

A. Plant materials:

The plant material was collected during July 2005 From Sharaban,Dyala city. The plant was identified by the Department of Pharmacognosy, College of Pharmacy /University of Baghdad; and authenticated by the Herbarium of Baghdad University. Fifty grams of the powdered plant material (seed part) were first defatted by reflux with 100 ml of petroleum ether (60°-80°C) for one hour and filtered. The defatted dried plant material was then extracted by reflux using 100 ml of 70% ethanol for three hours.This step was repeated for four times, then the combined filtrates were evaporated under reduced pressure using Buchi rotatory evaporator attached to vacuum pump at 40°C, to a thick residue of ethanol extract (F1). This residue was then hydrolyzed with 2N HCl in aqueous methanol (1:1) under reflux for three hours; the resultant solution was then partitioned with 100 ml of ethyl acetate (F2). This fraction was evaporated under reduced pressure to dryness, as shown in the following diagram (Figure 5).

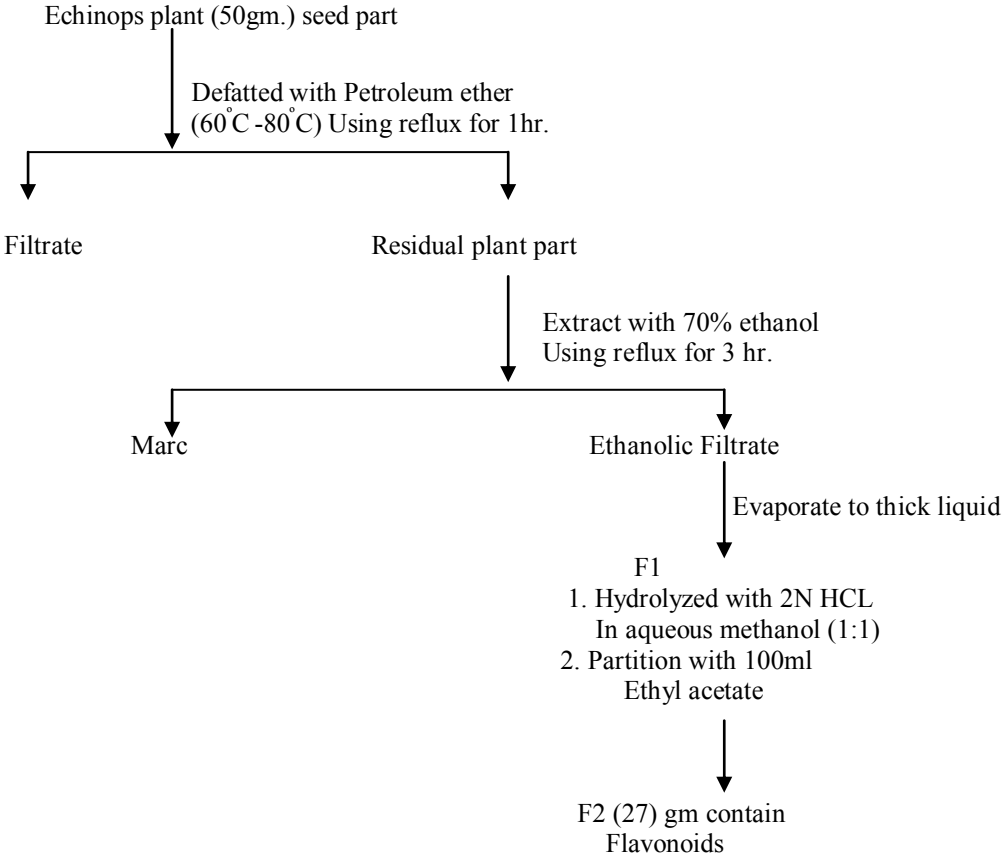


Figure-5 [Schematic representation of flavonoids extraction From *Echinops tenuisectus*]

F2 (ethyl acetate fraction) → evaporation to dryness under reduce pressure → black- greenish oily residue, TLC and HPLC indicated that this fraction contain three compounds which are silymarin, rutin and quercetin and by preparative thin layer chromatography and HPLC we can separate each one and calculate the percentage of each one by weighting.
F2 (oily residue fraction) → dissolved in water → suspention (ready for hepato- protective study)

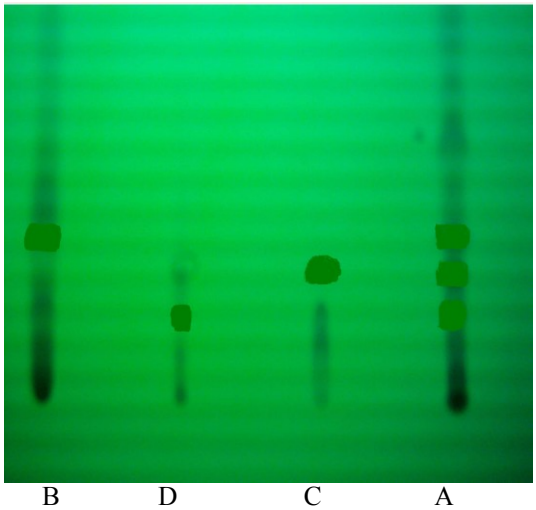
B. Identification of Silymarin,Quercetin and Rutin in the plant seed extract.

The Identification of these flavonoids in the seed extract was performed by:

1. Identification of Flavonoids by TLC:

Using TLC ready made Gf254 plates, UV detector at 254 nm, Standard flavonoids and two different solvent systems that were [26] :

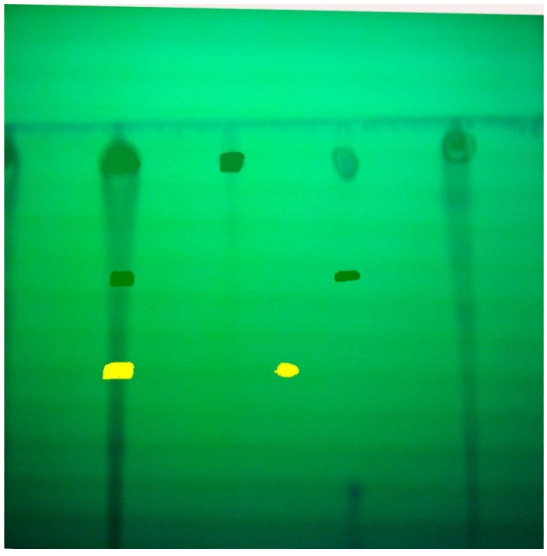
Solvent (1): chloroform: acetone: formic acid (75:16.5:8.5) (Figure 6)
Solvent (2): n.butanol: glacial acetic acid: water (40: 10:50) (Figure 7)
(Table-1) showed the R_f values of the standards Silymarin, Quercetin and Rutin, and the Rf value of plant seed part extract.



B D C A

Figure 6: TLC Gf254 plate of the seed extract and standards using S1 mobile phase.

A Plant seed extract C Quercetin standB Silymarin standard D Rutin standard



A C B D

Figure 7: TLC Gf254 plate of the seed extract and standards using S2 mobile Phase

A Plant seed extract C Quercetin standard
B Silymarin standard D Rutin standard

Table 1: R_f values of standard silymarin, rutin and quercetin and seed extract.

Solvent system	Standard silymarin	Standard Quercetin	Standard Rutin	Seed extract
S1	0.43	0.35	0.28	0.4,0.33,0.2
S2	0.2	0.81	0.56	0.21,0.8,0.5

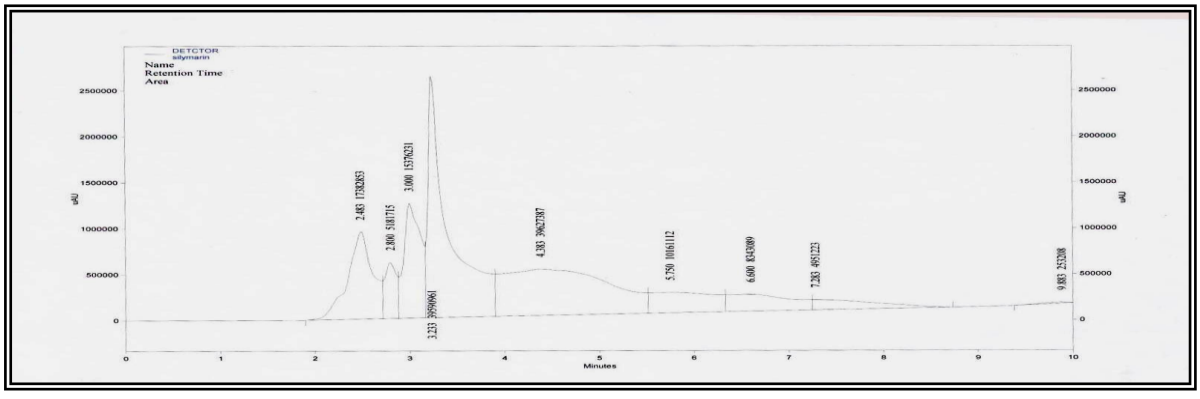


Figure 8: HPLC of plant seed extract of *Echinops tenuisectus.*

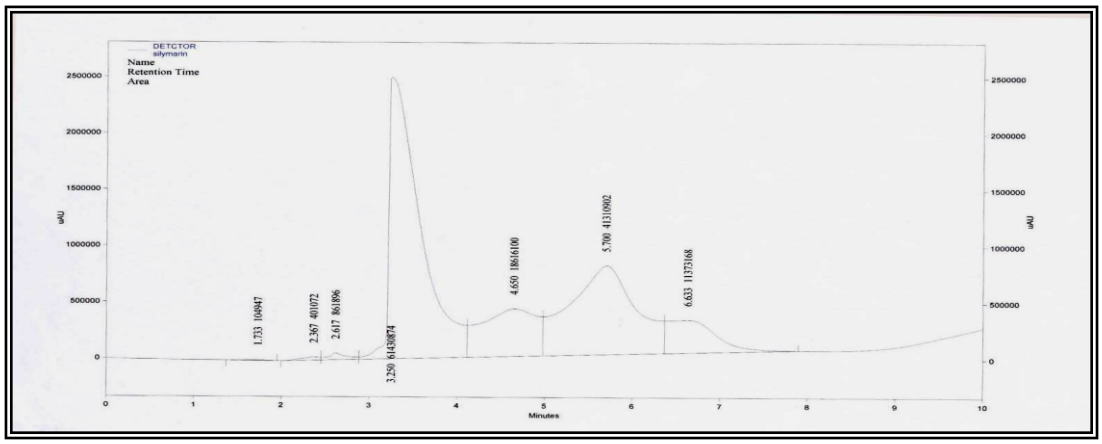


Figure 9: HPLC of standard Silymarin.

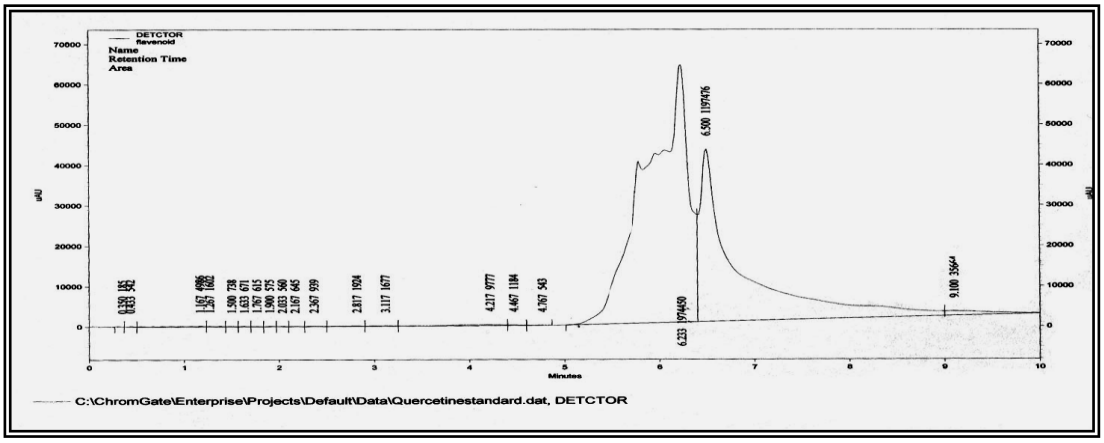


Figure 10: HPLC of standard Quercetin.

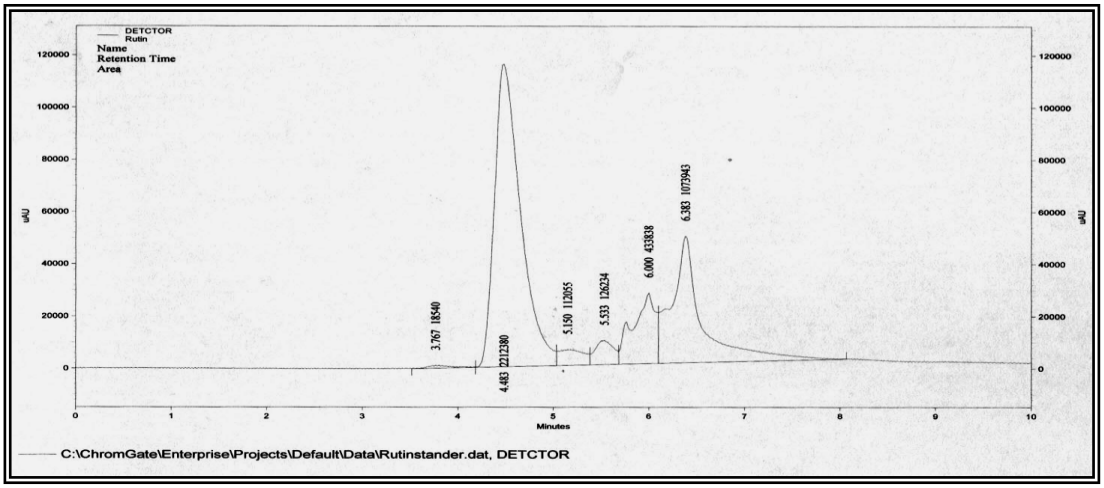


Figure 11: HPLC of standard Rutin.

2. Identification of Flavonoids by HPLC:
Silymarin, Quercetin and Rutin were authenticated by HPLC . (Figures 8-11) The HPLC conditions are listed in the following table. (Table-2)

Table 2: HPLC conditions.

HPLC Conditions	
Mobile phase	Methanol:water (50:50)
Column	C18 25cm
Flow rate	1ml/min
Detector	288 nm

C. Hepatoprotective studies:
1. Experimental animals:
Eighteen – Albino rats of both sexes weighting 150-200 gm (both sex) were used in this study. Animals were kept in the animal house of the College of Pharmacy/ University of Baghdad, under standardized condition (12 hr light dark cycle at room temperature). The animals were fed standard chow and given water ad libitum.

2. Experimental design:
The animals were divided in to three groups (each group have 6 animals) and treated as follows:

Group (1): Six rats received normal saline for 7 day orally and secreted at along 7, saved as control

Group (2): Six rats received single oral dose of CCl_4 (1mg/kg) diluted by corn oil in ratio of 1:1 v/v for the induction of liver damage and animals were sacrificed after 24 hr of CCl_4 administration.

Group (3): Six rats received oral dose of the seed extract of *Echinops tenuisectus* Plant in amount equivalent to 250mg/kg by gavages tube for 7 days, befor CCl_4 (1mg/Kg diluted by corn oil in a ratio of 1:1 v/v), then the rats were sacrificed after 24 hr, after CCl_4 administration.

3. Biochemical estimation:
Serum was prepared from the collected blood and subjected to biochemical estimation of ALT and AST.

4. Histopathology:
Portion of liver tissue in each group was fixed in 10% formalin (Formalin diluted to 10% with normal saline) and proceeded for histopathology. After paraffin embedding and block making, serial section of 5µ thickness were made, stained with Haematoxylin and Eosin and examined under microscope.

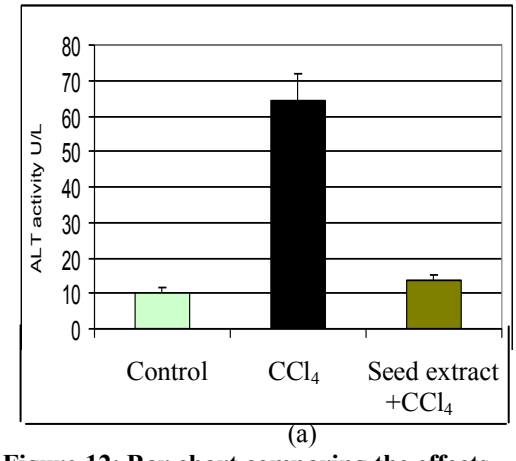
5. Statistical analysis:
The significance of difference between the mean values was calculated using unpaired student's t-test. P-value less than 0.05 were considered significant for all data showed in our results.

6. Results:
A) Biochemical parameters:
Table-3 showed a significant elevation in the activities of both ALT and AST in CCl_4-treated rats compared to control group. Pre-treatment rats with seed extract of *Echinops tenuisectus* (250mg/kg) showed a significant decline in the activities of ALT and AST compared with CCl_4 treated rats (Table 3 , Figure 12 and 13).

Table 3: Effects of seed extract of *Echinops tenuisectus* on the activities of serum ALT and AST in rats treated with CCl_4.

Treatment	Serum ALT U/L	Serum AST U/L
Control N=6	10.24±1.21	45±3.8
CCl_4-treated N=6	64.4±7.53[a]	68.6±1.67[a]
Seed extract + CCl_4 N=6	13.6±1.34	54.4±3.28[b]

- Each value represents Mean ± standard deviation.
- Values with non=identical superscripts (a,b) within each parameter are significantly different (P< 0.05)
- N= Number of animals.



(a)

Figure 12: Bar chart comparing the effects of seed extract of *Echinops tenuisectus* pre-treated with CCl_4 on serum ALT activity.

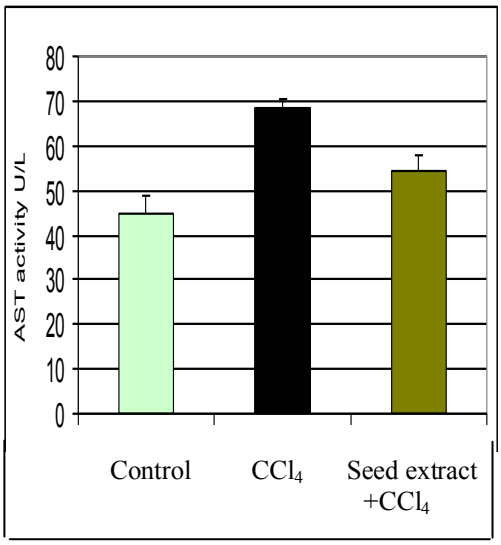


Figure 13: Bar chart comparing the effects of seed extract of *Echinops tenuisectus* pre-treated with CCl_4 on serum AST activity.

***B) Histological examination*:**

Histological examination of rats' liver treated with CCl_4 showed that, there was centrilobular hemorrhage, with heavy inflammation and necrosis. In addition to steatosis and individual necrosis were observed compared with control (Figure 14 and 15). Pre-treatment of rats with seed extract of *Echinops tenuisectus* before CCl_4, exhibit variable degrees of recovery with slight centrilobular congestion, marked reduction in inflammatory reaction. Furthermore, neither necrosis nor steatosis was observed in rat's liver section (Figure 16).

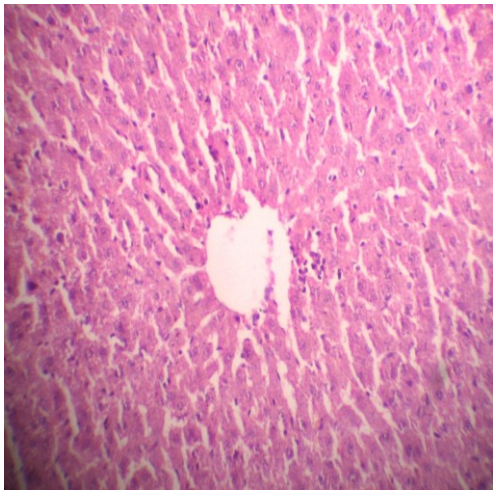


Figure 14: Section showing normal rat's liver. Magnification: 40X, staining: haematoxylline and eosin.

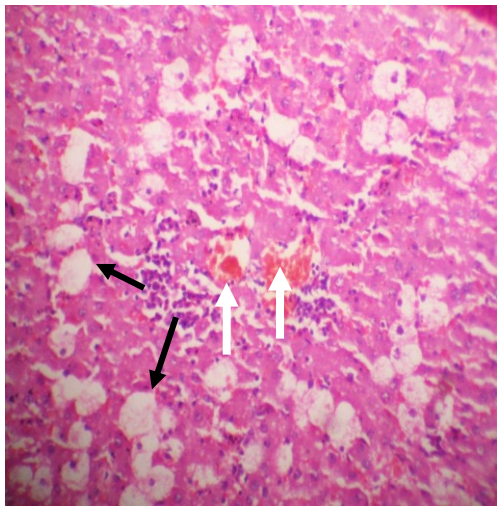


Figure 15: Section showing morphological alteration of liver from CCl_4-treated rats. Black arrow represents fatty changes (steatosis), white arrow represent haemorrhage. Magnification: 40X, staining: haematoxylin and eosin.

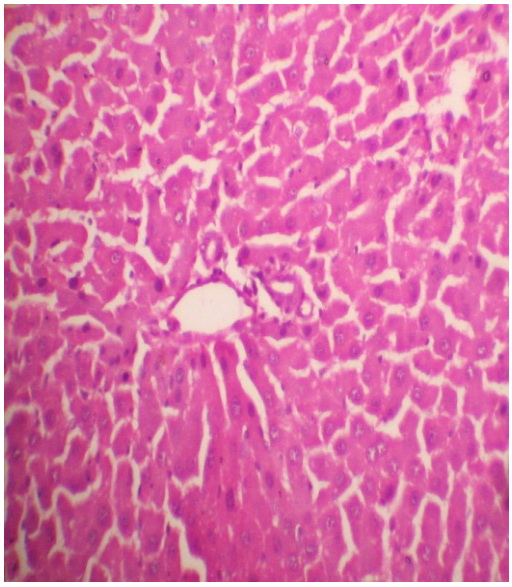


Figure 16: Section showing the administration of seed extract of *Echinops tenuisectus* improved CCl_4-induced hepatic damage. Magnification: 40X, staining haematoxylin and eosin.

Discussion:

Many compounds exhibit hepatoprotective activity, demonstrated either by decreasing the harmful effect of hepatotoxic compound or by maintaining the normal hepatic physiology. The present study showed that, the seed extract of *Echinops tenuisectus* have good hepatoprotective effect against CCl_4-induced hepatotoxicity in rat manifested by attenuating the increases in the serum activities of ALT

and AST (Table 3, Figure 12and13) and by reversing the histological damage induced by CCl_4, this was attributed to the presence of flavonoids, especially the silymarin, rutin and quercetine which possess antioxidant properties [8,16] which can improve the normal physiology of hepatocyte [17,18,19].

Conclusion

The present study showed that, seed extract of *Echinops tenuisectus* improve the hepatic damage and steatosis induced by CCl_4 toxicity.

Acknowledgment

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References:

1. Trease and Evans "Pharmacognosy" fifteenth Edn University of Nottingham, U.K. (2002) pp.36, 415,246, 247.
2. K. H. Rechinger "Flora of Lowland Iraq" (1964) pp. 637.
3. Morazzoni P, Bombardelli E. "Silybum marianum (Carduus marianus)". *Fitoterapia* 1995; LXVI: 3-42
4. Farghali H, Kamenikova L, Hynie S, et al. "Silymarin effects of intracellular calcium and cytotoxicity" a study in perfused rat hepatocytes after oxidative stress injury. *Pharmacol Res* 2000; 41: 231-237
5. Jane Higdon,Roderick H. Dashwood "Flavonoids" Sciences Centre Oregon State University, 2005 Linus Pauling Institute.
6. Muriel P, Mourelle M. "Prevention by silymarin of membrane alterations in acute CCl4 liver damage." J Appl Toxicol 1990; **10**: 275-279
7. Muriel P , Garciapi´a T, Perez-Alvarez V, et al. "Silymarin protects against paracetamol-induced lipid peroxidation and liver damage." J Appl Toxicol 1992; 12: 439-442
8. Chun OK, Kim DO, Lee CY. "Superoxide radical scavenging activity of the major polyphenols in fresh plums". J Agric Food Chem.2003; 51(27):8067-8072.(PubMed).
9. Comoglio A., Leonarduzzi G., Carini R., Busolin D., Basaga H., Albano E., Tomasi A., Poli G., Morazzoni P., Magistretti M. J. "Studies on the antioxidant and free radical scavenging properties of IdB1016 a new flavanolignan complex. Free Radical Res. Commun.", 1990; **11***:* 109-115.[Medline]
10. Valenzuela A, Guerra R. "Differential effect of silybin on the Fe2+ -ADP and t-butyl hydroperoxide-induced microsomal lipid peroxidation". Experientia 1986; 42: 139-141
11. Mourelle M. "Prevention by silymarin of membrane alterations in acute CCl4 liver damage." J Appl Toxicol 1990; 10: 275-279
12. Farghali H, Kamenikova L, Hynie S, et al. "Silymarin effects of intracellular calcium and cytotoxicity": a study in perfused rat hepatocytes after oxidative stress injury. Pharmacol Res 2000; 41: 231-237
13. Fuchs EC, Weyhenmeyer R, Weiner OH. "Effects of silibinin and of a synthetic analogue on isolated rat hepatic stellate cells and myofibroblasts." Arzneimittelforschung 1997; 47: 1383-1387
14. Boigk G, Stroedter L, Herbst H, et al. "Silymarin retards collagen accumulation in early and advanced biliary fibrosis secondary to complete bile duct obliteration in rats." Hepatology 1997; **26**: 643-649
15. Favari L, Perez-Alvarez V. "Comparative effects of colchicine and silymarin on CCl4 chronic liver damage in rats." Arch Med Res 1997; 28: 11-17
16. Valenzuela A, et al. Selectivity of flavonoids on the increase of the glutathione content in different tissues of the rat. *Planta Medica* 1989; 55, 420-422.
17. *Hungarica* 78, 3-9. Muzes G, et al. Effect of the bioflavonoids on the in vitro activity and expression of super oxide dismutase (SOD) enzyme. 1991; *Acta Physiol*
18. Ray Sahelian, M.D., Reduction of rat prostate weight by combined Quercetin – finasteride treatment is associated with cell cycle deregulation. J Endocrinol. 2004 Jun; 181(3): 493-507.
19. Shoskes DA, Zeitlin SI, Shahed A, Rajfer J. Quercetin in men with category III chronic prostatitis : a preliminary prospective double- blind, placebo-controlled trial. Urology. 1999; **54**: 960-963.
20. Sah JF,Balasubramanian S, Eckert RL, Rorke EA."Epigallocatechin-3- gallate inhibits epidermal growth factor receptor signaling pathway. Evidence for direct inhibition of ERK/2 and AKT kinases".J Biol Chem.2004; 279(13):12755-12762. (PubMed).
21. Kavangh KT, Hafer LJ, Kim DW, et al."Green tea extracts decrease carcinogen-induced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture".J Cell Biochem.2001;82(3):387-398.(PubMed).

22. Bagli E, Stefaniotou M, Morbidelli L, et al."Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phospha-tidylinositol 3'-kinase activity".Cancer Res.2003.

23. Kim MH."Flavonoids inhibit VEGF/bFGF-induced angiogenesis in vitro by inhibiting the matrix- degrading proteases".J Cell Biochem. 2003 ; 89(3) :529-538.

24. M.R Venukumar, M.S. Latha. Hepatoprotective effect of the methanolic extract of *Curculigo Orchioides* in CCl_4-treated male rats. Indian Journal of Pharmacology. 2002;**34**; 269-275.

25. P. Jayasekhar, P.V. Mohanan, and K.Rathinam. Hepatoprotectvie activity of ethyl acetate extract of *Acacia Catechu*. Indian Journal of Pharmacology. 1997; 426-428.

26. Merck and CO., Inc. Rahway, NJ. The merck index 8th Ed U.S.A (1966), Pp 8216.