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The Biological Effect of Grape Leaves on Liver Disease Rats Induced by Carbon Tetrachloride (CCL_4)

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Abstract
The aim of this study was to investigate the effect of grape leaves (VitisVinifera L.) on rats suffering from acute liver disease. Male albino rats of the Sprague Dawely Strain (24 rats), weighing (195 ±10gm.), were fed on basal diet for one week for adaptation. The rats were then divided into two main groups as follows. The first main group of six rats was fed on basal diet (as a control negative group). The second main group of 18 rats was treated with CCl4, in paraffin oil (50 % v/v 4 ml/kg) subcutaneous injection once a week for one week to induced acute damage in the liver. Then the second main group (18 rats) was divided into three sub-groups; each subgroup consisted of six rats and was fed on diets for (one month) as follows: 1) Rats were fed on a basal diet as control positive group; 2) Rats were fed on a diet containing 2.5% grape leaves; 3) Rats were fed on a diet containing 5% grape leaves. The present findings suggested that regular intake from 5% grape leaves may be useful in improving liver functions and may protect against (CCL4) induced acute damage of the liver disease in rats.

Key Words
(C -CL4); Grape Leaves; Liver Enzymes; Liver Disease

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Introduction

Hepatitis is mostly caused by viral and toxic agents. Deemed chronic when persisting for longer than six months, hepatitis triggers an ongoing inflammation that often leads to fibrosis and eventually cirrhosis, with a concomitant increased risk of hepatocellular carcinoma (Centers for Disease Control and Prevention, 1998).

Grape (*VitisVinifera L.*) is widely distributed in incident times. They help in the treatment of many serious diseases like diabetes mellitus, arthrosclerosis, hyperlipidemia, and hypertension (Srivastava *et al.*, 2003).

Grape leaves (*Vitis ViniferaL.*) have been used in folk medicine for their biological activities since ancient times. The leaves of the plant, which have astringent and haemostatic properties, are used in the treatment of diarrhea, hemorrhage, varicose veins, hemorrhoids, inflammatory disorder, pain, and free radical related diseases (Lardos and Kreuter, 2000).

The leaves of *V. vinifera* are used in the formulation of dietary antioxidant supplements (Monaga *et al.*, 2006). A many number of in vivo and in vitro studies have been conducted on the plant material and have revealed that *V. vinifera* leaves exert various biological activities, including hepatoprotective, spasmolytic, hypoglycemic, and verso-relaxant effects (Orhan *et al.*, 2006)

This study was carried out to study the effect of Grape leaves on rats suffering from acute liver disease.

Materials and Methods

Materials

- Casein, vitamins, minerals, cellulose and carbon tetrachloride (CCL_4) were obtained

from El-Gomhorya Company, Cairo, Egypt.
- Grape leaves (*Vitis Vinifera L.*) were obtained from fields in El- Giza, Egypt.
- Starch and soy oil were obtained from a local market in Cairo, Egypt.
- Normal male albino rats (Sprague - Dawely Strain) (n=42) weighing 195 ± 10g were purchased from Helwan Experimental Animals station.

Methods:

Preparation of Grape Leaves Powder

The leaves from healthy plants were washed. The leaves dried for three days by solar energy and were ground to fine powder in an electric mixer.

Determination of Phenolic Compounds in Leaves

Phenolic compounds of grape leaves were estimated at Food Technology Research Institute, Giza, Egypt, according to Pascale *et al.* (1999).

Preparation of Basal Diet

The basal diet consists of Casein (14%) ≥80% protein, soy oil (4%), Cellulose (5%), vitamin mixture (1%), salt mixture (3.5%), and Choline chloride (0.25%). The remainder is corn starch (72.25%) (Reeves *et al.*, 1993).

Experimental Design

Male albino rats of the Sprague Dawely Strain (24 rats), weighing (195±10gm.), were used. All rats were housed in well aerated cages under hygienic conditions and fed on a basal diet for one week for adaptation in the animal house lab of Faculty of Home Economics, Helwan University.

After a week (for adaptation), the rats were divided into two main groups as follows:

The first main group, six rats, were fed on a basal diet (as a control negative group).

The second main group, 18 rats, were treated with CCl_4 in paraffin oil (50 % v/v 4 ml/kg) subcutaneous injection, one a week for one week to induce acute damage in the liver (Jayasekhar *et al.*, 1997).

Then the second main group (18 rats) was divided into three sub-groups. Each sub-group consisted of six rats and was fed on diets for one month, as follows:

1. Rats were fed on a basal diet as a control positive group.
2. Rats were fed on a diet containing 2.5% grape leaves.
3. Rats were fed on a diet containing 5% grape leaves.

During the experimental period (one month), the diet consumed was recorded every day, and the body weight was recorded every week.

At the end of the experimental period, the rats were fasted overnight before sacrificing. Blood samples were collected from the aorta of each rat in a dry, clean centrifuge tube and left for 15 minutes to clot at room temperature. The samples were then centrifuged for 15 minutes at 3000 rpm to separate the serum.
Serum was carefully separated and transferred into dry, clean Ebendorf tubes and kept frozen at -20°C till analysis.

Liver, kidneys and spleen were removed, cleaned, weighed and kept in formalin solution 10% until histo-pathological examination of the liver.

Chemical Analysis of Serum

The following determinations were carried out for all serum samples:

Determination of total cholesterol:

Total cholesterol in the serum was determined according to the method described by Allain *et al.* (1974).

Determination of the low-density lipoprotein (LDL) cholesterol:

The concentration of LDL was estimated according to the equation of Friedewald *et al.* (1972), as follows:
LDL cholesterol (mg / dL) = Total cholesterol – HDL cholesterol – VLDL cholesterol

Determination of very low-density lipoprotein (VLDL) cholesterol:

The concentration of VLDL was estimated according to the equation of Friedewald *et al.* (1972), as follows:

$$\text{VLDL cholesterol (mg / dL)} = \frac{\text{triglycerides}}{5}$$

Determination of high-density lipoprotein (HDL) cholesterol:
HDL-cholesterol was determined according to the method described by Burstein (1970).

Determination of serum transaminases:
Aspartate amine transaminase (AST) and Alanine amine transaminases (ALT) activities were measured according to the method described by Reitman and Frankel (1957).

Determination of uric acid:
Serum uric acid was determined according to the method described by Fossati *et al.* (1980).

Determination of urea nitrogen:
Serum urea was determined according to the method described by Patton and Crouch (1977).

Determination of serum creatinine:
Serum creatinine was determined according to the method described by Bohmer (1971).

Determination of serum glucose:
Glucose was determined in serum according to the method described by Trinder (1959).

Statistical analysis:
The statistical analysis was carried out using SPSS, PC statistical software (Version 20 SPSS Inc., Chicago, USA).

The results were expressed as mean ± SD. Data were analyzed by one-way analysis variance (ANOVA). The differences between means were tested for significance using least significant difference.

(LSD) test at ($P < 0.05$) (Steel and Torri, 1980).

Results and Discussion

Chemical Analysis (Phenolic compounds) of Grape Leaves

Data in Table 1 showed levels of some Phenolic compounds (mg/100g) for grape leaves. The results of chemical analysis for these leaves revealed that the value of Phenolic compounds from grape leaves were Pyrogallol, followed by Vanillic, represented (17.93 and 0.64, respectively).

Table 1: Phenolic Compounds (mg/100g) of Grape Leaves

Items	Phenolic Compounds (mg/100g)
	Grape Leaves
Gallic	0.70
Protocatechuic	10.71
Pyrogallol	17.93
P -Coumaric	- -
Catechin	1.31
Caffeic	--
Vanillic	0.64
Salicylic	--
Ferulic	1.63
Coumarin	0.51

According to Pascale et al. (1999).

Effect of grape leaves on feed intake and body weight gain % of rats suffering acute liver disease

Feed Intake (FI):

The mean value of feed intake of acute liver disease rats (control positive group) was 10.70g, while the mean value of feed intake of healthy rats (control negative group) was 15.11g/day. Feed intake in the negative control group increased by about 4.41g compared to the positive control group. The mean value of feed intake in all treated groups increased than the positive control group. On the other hand, all treated groups showed a decrease in the mean value of feed intake when compared with the negative control group. The lowest mean value of feed intake was observed in the group treated with grape leaves.

*Values are expressed as mean ± SD.
Significance at $p<0.05$
*Values which do not share the same letter in each column are significantly different.
*GL: Groups of rats fed on a diet containing grape leaves.

Table 2: Effect of grape leaves on feed intake and body weight gain % (BWG%) of rats suffering from acute liver disease

Parameters / Groups	Mean of feed intake (g/day for each rat)	Body weight gain % (BWG%)
Control(-)	15.11	8.1329±1.60788[a]
Control(+)	10.70	1.5271±.03988 [c]
2.5%GL	11.89	2.0657±.68709 [bc]
5% GL	12.31	3.3371±.96757[b]

Body Weight Gain % (BWG %):
Body weight gain % of the positive control group decreased significantly ($p< 0.05$) compared to all groups.
All groups treated with grape leaves achieved a significant increase in body weight gain % compared to the positive control group.

Effect of grape leaves on organs weight / body weight % of rats suffering from acute liver disease
Mean value of organs weight such as liver, kidney and spleen relative to body weight percent of acute liver disease rats fed on grape leaves is summarized in Table 3.

Statistical analysis in our results in one month (experiment period) indicated that all organs weight / body weight % of acute liver disease group (control positive group) showed significant increase $p<0.05$ than that of the negative control group (healthy rats).

On the other side, the mean values of liver, kidneys and spleen weight / body weight % for all treated groups of rats demonstrated significant decrease $p<0.05$ compared to the positive control group, while a clear significant increase $p<0.05$ than that of negative control group (healthy rats).

Effect of grape leaves on serum cholesterol and triglycerides of rats suffering from acute liver disease
Table 4 illustrates the effect of grape leaves on serum cholesterol (mg/dl) and triglycerides (mg/dl) of rats suffering from acute liver disease.

Total serum cholesterol (mg/dl):
In regard to the group of rats in the positive control group, it could be observed that the total serum cholesterol level significantly increased $p< 0.05$ compared to the negative control group fed on a basal diet. Total serum cholesterol in groups treated with diets containing different ratios of grape leaves significantly decreased $p< 0.05$ when compared to the positive control group, while they had non-significant differences compared to the negative control group.

In this respect, Gray and Flatt (1998) showed that dietary fiber might play a corrective role in liver function, either by reducing the blood glucose level or by some either mechanisms which, in turn, reduces the level of triglyceride and total cholesterol in blood plasma of diabetic animals.

Table 3: Effect of grape leaves on organs weight / body weight % of rats suffering from acute liver disease

Parameters / Groups	Kidney weight/ body weight %	Liver weight/ body weight %	Spleen weight/ body weight %
Control(-)	0.6657±0.06604 [c]	2.1086±0.16866 [d]	0.2314±0.02478 [d]
Control(+)	0.9743±0.13011 [a]	2.9443±0.06901[a]	0.3443±0.01134 [a]
2.5%GL	0.7471±0.02059[b]	2.7829±0.21922[ab]	0.3071±0.03729[bc]
5% GL	0.7214±0.03848[bc]	2.6129±0.15261[bc]	0.2914±0.02340[cd]

*Values are expressed as mean ± SD. Significance at $p<0.05$
*Values which do not share the same letter in each column are significantly different.

Table 4: Effect of grape leaves on serum cholesterol and triglycerides of rats suffering from acute liver disease

Parameters / Groups	mg/dl	
	Cholesterol	TG
Control(-)	83.6714±2.27072[c]	45.8429±3.13255[c]
Control(+)	103.9143±1.79028[a]	67.8857±1.84721[a]
2.5%GL	88.3000±0.72121[b]	50.6000±5.03984[b]
5% GL	85.3714±1.87236[c]	46.8714±1.37321[c]

*Values are expressed as mean ± SD. Significance at $p<0.05$
*Values which do not share the same letter in each column are significantly different.

Effect of grape leaves on serum cholesterol and triglycerides of rats suffering from acute liver disease

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Total serum cholesterol (mg/dl):

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In this respect, Gray and Flatt (1998) showed that dietary fiber might play a corrective role in liver function, either by reducing the blood glucose level or by some either mechanisms which, in turn, reduces the level of triglyceride and total cholesterol in blood plasma of diabetic animals.

Serum triglycerides (mg/ dl):

As indicated in Table 4, the positive control group showed that triglyceride level significantly increased $p< 0.05$ compared to the negative control group (67.8857±1.84701 vs. 45.8429±3.13255, respectively).

Levels of serum triglycerides between all acute liver disease groups after treatments with different ratios of grape leaves showed significantly decreased $p< 0.05$, especially in groups fed on 5% GL, when compared to the positive control group.

Arvil and Bodi (1995) showed that the substantial decrease in triglyceride and total cholesterol level in the diabetic animals by dietary fiber reinforces its hypoglycemic and hypolipidemic potential. Serum cholesterol and low-density lipoprotein cholesterol (LDL-c) levels were significantly reduced in the dietary fiber treated rat groups.

Daiki *et al.* (2003) and Park *et al.* (2002) reported that resveratrol reduced the serum triglyceride levels by increased excretion of bile acids into feces in hepatoma-bearing rats.

Orhan *et al.* (2007) showed that mainly condensed tannins and flavonoids were suggested to contribute to the anti-diabetic activity and prevention of lipid peroxidation of vitis vinifera leaves.

Effect of grape leaves on serum lipoproteins of rats suffering from acute liver disease

The effects of both different kinds and levels of grape and mulberry leaves on high-density lipoprotein HDL-c, low-density lipoprotein LDL-c, and very low-density lipoprotein cholesterol VLDL-c of acute liver disease rats are discussed in Table 5. During the experiment, feeding rats on a basal diet (negative control group) showed significant variations: the serum HDL-c increased significantly, while the LDL-c and VLDL-c decreased significantly, compared to the positive control group (injected with ccl4), which recorded a significant decrease in serum HDL-c, while LDL-c and VLDL-c increased significantly.

Table 5: Effect of grape leaves on serum cholesterol and triglycerides of rats suffering from acute liver disease

Parameters / Groups	mg/dl		
	HDL-c	LDL-c	VLDL-c
Control(-)	65.2714±0.90315[a]	9.2314±1.14128[e]	9.1686±0.62251[c]
Control(+)	54.5857±2.34835[c]	35.7514±3.76138[a]	13.5771±0.36940[a]
2.5%GL	63.7857±1.69355[ab]	14.3943±1.24073[bc]	10.1200±1.00797[b]
5% GL	63.2143±0.84742[b]	12.7829±2.64982[c]	9.3743±0.27464[c]

*Values are expressed as mean ± SD. Significance at $p<0.05$
*Values which do not share the same letter in each column are significantly different.

Leelavinothan and Arumugam (2008) suggested that grape leaves extract exerts its protective effect by decreasing the lipid peroxidation and improving antioxidant status, thus proving itself as an effective antioxidant in alcohol-induced oxidative damage in rats.

Orhan *et al.* (2007) showed that mainly condensed tannins and flavonoids were suggested to contribute to the anti-diabetic activity and prevention lipid peroxidation of *V. vinifera* leaves (grape leaves).

The levels of serum VLDL-c for all treated groups showed significant decrease ($p< 0.05$) compared to the positive control group (+), while groups receiving the diet containing 5% GL showed non-significant differences in the mean values of serum VLDL-c compared to the control negative group (-).

The best results for the mean values of lipoproteins were for the groups of rats which received a diet containing 5% GL compared to the positive control group.

Effect of grape leaves on serum kidney functions of rats suffering from acute liver disease.

The data confirmed that, after one month from the experiment period, the rats of serum uric acid, urea nitrogen and creatinine were significant increase ($p< 0.05$) in control positive group compared to the negative control group (healthy rats) fed on a basal diet (4.9286±0.13801, 27.800±2.60768 and 0.8571±0.05345 vs. 2.7286±0.11127, 16.5714±0.52825 and 0.5429± 0.05345, respectively).

The mean values ± SD of serum uric acid, urea nitrogen and creatinine in the negative control group cleared non-significant differences compared to those of the groups that received a diet containing 5% GL in the mean value of serum uric acid and creatinine only. However, in urea nitrogen there was a significant increase in the same groups (received diet containing 5% GL) compared to the control negative group ($p< 0.05$).

There was a significant decrease in the levels of serum uric acid, urea nitrogen and creatinine for all groups which fed on different ratios of grape leaves ($p< 0.05$) compared to the positive control group (injected with ccl4).

Results indicated that the group of rats fed on 5% GL recorded the lowest mean value.

Vikas and Kan (2006) affirmed that resveratrol exerts its protective effect through nitric oxide release, along with the anti-oxidative effect in glycerol induced acute renal failure. Resveratrol (phenol substance occurring in plant leaves) can have a significant effect on the inflammatory process seen in glycerol-induced renal injury.

Joseph *et al.* (2011) showed that resveratrol given at six, 12 and 18 hours significantly improved survival. Hence, resveratrol may have a dual mechanism of action to restore the renal microcirculation and scavenge reactive nitrogen species, thus protecting the tubular epithelium even when administered after the onset of sepsis.

Table 6: Effect of grape leaves on serum kidney functions of rats suffering from acute liver disease

Parameters / Groups	mg/dl		
	Uric acid	Urea nitrogen	Creatinine
Control(-)	2.7286±0.11127^{cd}	16.5714±0.52825^{d}	0.5429± 0.05344^{d}
Control(+)	4.9286±0.13800^{a}	27.8000±2.60768^{a}	0.8571±0.05345^{a}
2.5%GL	3.0143±0.06902^{bc}	20.7286±1.31494^{bc}	0.6714±0.07557^{bc}
5% GL	2.8857±0.08995^{c}	18.5714±0.37289^{c}	0.6429±0.05345^{c}

*Values are expressed as mean ± SD. Significance at $p<0.05$
*Values which do not share the same letter in each column are significantly different.

Effect of grape leaves on serum liver functions and glucose of rats suffering from acute liver disease

Effect of grape leaves on some liver enzymes, aspartate amine transferase (AST), alanine amine transferase (ALT), and glucose of rats suffering from acute liver disease are presented in Table 7.

After one month, in the acute liver disease rats (control positive group), a significant increase in the mean value for both AST and ALT enzymes levels and also glucose level was noticed, compared to healthy rats (control negative group).

The mean value of AST and ALT enzymes in acute liver disease rats fed on a diet containing different ratios of grape leaves showed significant decrease ($p< 0.05$) compared to the positive control group, while the mean value of glucose in acute liver disease rats fed on a diet containing different ratios of grape leaves decreased significantly $p< 0.05$ compared to the control positive group.

Groups fed on 5% GL showed non-significant changes compared to the control negative group in the serum level of ALT.

The mean value ± SD of serum glucose in groups fed on different ratios showed significant increase $p< 0.05$ compared to the control negative group, but revealed a significant decrease compared to the control positive group.

On the other hand, the mean value ± SD of serum glucose levels in all treated groups with different ratios of grape leaves showed significant differences between all groups, but the best mean values were for the groups that received 5% GL.

In this respect, Heibatollah *et al.* (2009) showed that the hydro alcoholic extract of *Vitis vinifera L.* at a dose of 800mg/kg exhibited a significant liver protective effect by lowering the serum levels of AST and ALT, decreasing the sleeping time and resulting in less pronounced destruction of the liver architecture. There was no fibrosis or inflammation.

Livia *et al.* (2011) and Orhan *et al.* (2007) demonstrated that ethanol extracts of *Vitis vinifera L.* were able to induce a hepatoprotective action on carbon tetrachloride induced acute liver damage in rats, which was attributed to the polyphenolic compounds. The ethanolic extract of *Vitis vinifera L.* at 250mg/kg dose was found effective to protect liver and kidney from the oxidative damage and high anti-diabetic.
Orhan *et al.* (2007) emphasize that mainly condensed tannins and flavonoids were suggested to contribute to the anti-diabetic activity and prevention lipid peroxidation of *Vitis vinifera L.*

Orhan *et al.* (2007) also showed that ethanol extracts of *Vitis vinifera L.* were able to induce a hepato-protective action on carbon tetra chloride-induced acute liver damage in rats which was attributed to the polyphenolic compounds.

Table 7: Effect of grape leaves on serum liver enzymes and glucose of rats suffering from acute liver disease

Parameters / Groups	Glucose (mg/dl)	U/l	
		AST	ALT
Control(-)	125.18 ±3.56[d]	122.14±1.51[d]	33.17±2.08[c]
Control(+)	200.72±2.02[a]	195.27±3.785[a]	50.07±1.61[a]
2.5%GL	183.35±2.46[b]	170.42±1.55[b]	39.38±1.39[b]
5% GL	157.11±3.61[c]	132.91±2.60[c]	32.81±3.001[c]

*Values are expressed as mean ± SD. Significance at $p<0.05$
*Values which do not share the same letter in each column are significantly different.

Histopathology Examinations of Liver:
Microscopically, the liver of rats from control negative group revealed the normal histological structure of hepatic parenchyma (Photo 1). On the other hand, the liver of rats from control positive group revealed steatosis of hepatocytes, focal hepatocellular necrosis associated with mononuclear inflammatory cells infiltration, and fibroplasia in the portal triad (Photo 2). The liver of rats from the group fed on 2.5% GL showed hydropic degeneration of focal hepatocytes (Photo 3). The liver of rats from the group fed on 5% GL showed slight activation of Kupffer cells (Photo 4).

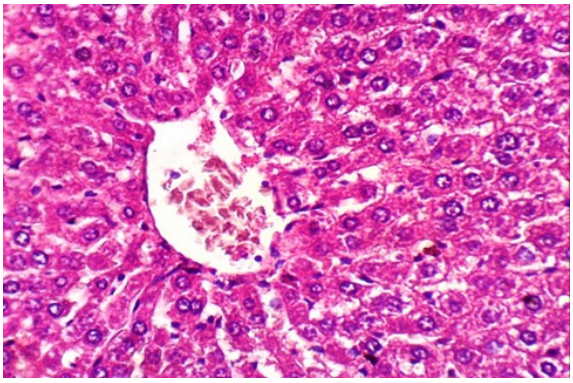

Photo 1: Liver of a rat from the control negative group showing the normal histological structure of hepatic parenchyma (H & E X 400).

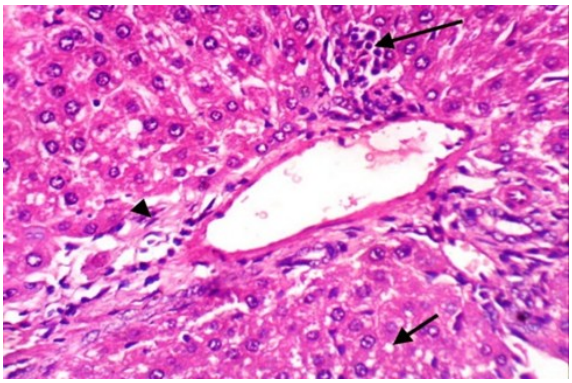

Photo 2: Liver of a rat from the control positive group showing steatosis of hepatocytes (short arrow), focal hepatocellular necrosis associated with mononuclear inflammatory cells infiltration (long arrow), and fibroplasia in the portal triad (arrow head) (H & E X 400).

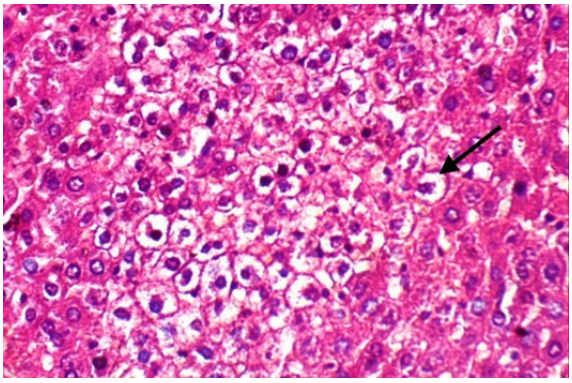

Photo 3: Liver of a rat from the group fed on 2.5% GL showing hydropic degeneration of focal hepatocytes (H & E X 400).

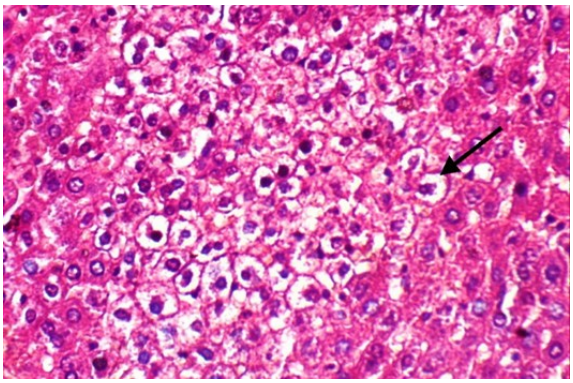

Photo 4: Liver of a rat from the group fed on 5% GL showing slight activation of Kupffer cells (H & E X 400).

Conclusion:

In this article, it appears that Grape Leaves may be useful in improving liver functions and may protect against CCL4-induced acute damage of the liver disease in rats. Ternary resveratrol complex with cyclodextrin and lecithin may be a good alternative medicine for the treatment of liver damage (Gehan F Balata et al., 2017).

In addition, the group of herbs that contain similar flavonoids have a potent effect on different systems in male and female rats (ElSawy, N. A. et al., 2014; ElSawy, N.A. et al., 2014; El-Sayed H Bakr and N. A. ElSawy, 2014; ElSawy, N. A. et al., 2019).

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