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Silymarin, the antioxidant component and *Silybum marianum* extracts prevent liver damage

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ABSTRACT

Liver disorders are one of the common recent problems affects on the human health. These disorders due to many environmental polluted sources. Many herbal, medicinal and pharmaceutical plants and their extracts are widely studied by many researchers. *Silybum marianum* got a bright reputation in relieve the liver diseases, and that might be for the potent silymarin mixture. Mechanism of action for silymarin conducted mainly to the antiradical and anticarcinogenic roles. Ethyl acetate (100 mg/kg bw) and ethanol seed extracts for *S. marianum* (100 mg/kg bw) were tested against the injection (i.p.) by carbon tetrachloride (2 ml/kg bw) the inducer of liver damage. Their activity were compared with standard hepatic drug hepaticum (100 mg/kg bw) for 10 days. Ethanolic extract showed the most significantly decrease in the liver enzymes. For the oxidative experiments, ethyl acetate showed the most increase for glutathione level and the risk factor HDL/LDL significantly. Hepaticum was the most powerful group for the significant decreasing for malondialdehyde and fucosidase activity. Some equal improvements were noticed in the histopathological studies for the protective groups.

1. Introduction

The liver regulates many important metabolic functions, so the hepatic injury is associated with distortion of these metabolic functions (Wolf, 1999). Liver is the key organ of metabolism and excretion is continuously and variedly exposed to xenobiotics because of its strategic placement in the body. Toxins absorb from the intestinal tract gain access first to the liver resulting in a variety of liver ailment. Thus liver diseases remain one of the serious health problems. Liver damage ranges from acute hepatitis to hepatocellular carcinoma, through apoptosis, necrosis, inflammation, immune response, fibrosis, ischemia, altered gene expression and regeneration. Loguercio and Federico (2003) stated that all processes that involve hepatocyte, Kupffer, stellate and endothelial cells which induce liver disease are related to the crucial role of reactive oxygen and nitrogen species. The main sources of free radicals are represented by hepatocyte mitochondria and citochrome *P450* enzymes, by endotoxin-activated macrophages (Kupffer cells) and by neutrophils.

The extracts of the flowers and leaves of *Silybum marianum* (St. Mary's thistle, milk thistle) have been used for centuries to treat liver, spleen and gallbladder disorders (Rainone, 2005). In the 1960s the biologically active principles of the seed and fruit extracts were studied, and the chemical structures were elucidated. Sonnenbichler et al. (1999) isolated a mixture named silymarin, and it was with flavonolignan mixture that most clinical studies were carried out. The most constituents are silibinin, isosilibinin, silicristin and silidianin. One of the important issues about plant *S. marianum* is that it may be accepted as a safe herbal product, since no health hazards or side effects are known in conjunction with the proper administration of designed therapeutic dosages (Med. Economic Company, 2000). Recently oxidized derivatives of silybin (the major component forming 70–80% of silymarin) and their antiradical and antioxidant activity was studied by Gazak et al. (2004). Katiyar (2005) studied the antioxidant, anti-inflammatory and anticarcinogenic properties were demonstrated in the studies conducted with silymarin against oxidative stress, inflammatory responses and benzoil peroxide-induced tumor promotion in mice.

Recent studies have focused on mechanistic studies regarding possible molecular targets of silymarin for cancer prevention (Ramasamy and Agarwal, 2008). Silymarin modulates imbalance between cell survival and apoptosis through interference with the expressions of cell cycle regulators and proteins involved in apoptosis. For the importance of silymarin in many fields, our research studied the role of silymarin and *S. marianum* extracts as protective agent against CCl_4 causing liver fibrosis and necrosis. The study concentrated on the antioxidant effect, antitumor factor and anti-inflammatory efficiency of silymarin and plant content.

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2. Materials and methods

Plant was identified by the taxonomist of Botany Department of Minia University. Seeds of *S. marianum* were fine separated from the plant fruits, dried in the shade, chopped and extracted with ethyl acetate EAE and ethanol EE. Solvents were removed under reduced pressure to obtain the dried extract (2.5% and 2.1% yield, w/w for EAE and EE, respectively). An aqueous suspension contain the dried extract was prepared and administered to the animals orally. Silymarin as standard protection was administered to the rats through the Hepaticum drug (Medical Union Pharmaceuticals Company). Carbon tetrachloride was obtained from Merck Limited, India.

Thirty male Sprague–Dawley rats between 165 and 180 g were purchased from Agricultural Research Center, Giza, Egypt. Upon arrival, the animals were given 2 weeks acclimation period, during which they were fed a standard rat pellet diet and water *ad libitum*, with alternated 12-h dark/light cycle, and the ambient temperature was held constant between 21 and 25 °C. They were housed individually in cages for specific five groups, and the mean weight of each group was 174 ± 2 g. The food was withdrawn on the day before the injection but free access of water was allowed. The six rats per treatment were randomly assigned up to the mean weight distribution, as follows:

(1) Normal control group fed the basel diet and injected only with vehicle on the third day.
(2) Positive control group was fed the same basel diet for control group. Toxicity was induced on the third day by carbon tetrachloride (single administration 2 ml/kg bw i.p.) (Janbaz and Gilani, 1995). Equal amount of liquid paraffin (1:1) was administered as vehicle.
(3) The same injected treatment as positive control, with oral 100 mg standard drug silymarin/kg daily.
(4) The same injected treatment as positive control, with oral 100 mg ethyl acetate seed extract/kg daily.
(5) The same injected treatment as positive control, with oral 100 mg ethanol seed extract/kg daily.

Carbon tetrachloride injections for groups 3, 4 and 5 were followed the oral protection treatments by 2 h.

Food consumption and body weight were determined and the biological experiment was lasted for 10 days. The animals were sacrificed, the blood was collected in the end of the biological experiment from the orbital plexus. Blood was allowed to clot and then centrifuged at 3000 rpm for 15 min, and serum kept at −20 °C until required.

2.1. Biochemical analysis methods

Only, reduced glutathione GSH was determined on fresh heparinized blood according to the method of Beutler et al. (1963). Malondialdehyde MDA (Ohkawa et al., 1979), and α-L-fucosidase (Bukofzer et al., 1989) were colorimetrically examined for rat serum. Glutamic-oxaloacetic transaminase (AST), glutamic-pyruvic transaminase (ALT) (Reitman and Frankel, 1957) and alkaline phosphatase (Belfield and Goldberg, 1971) were colorimetrical measured. Triglycerides TG, cholesterol CHL and HDL were enzymatic colorimetrically determined in rat serum using the enzymatic colorimetric (Richmond, 1973; Fassati and Prencipe, 1982). LDL was calculated (Friedewald et al., 1972) (mg/dl) as follows:

$$\text{LDL} = \text{Total CHL} - \text{HDL} - (\text{TG}/5)$$

2.2. Histopathological examination

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% formol saline for 24 h. Washing was done in tap water then serial dilutions of alcohol absolute ethyl) were used for dehydration. Specimens were cleared in xylene embedded in paraffin at 56° in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stain for histopathological examination through the light microscope (Banchroft et al., 1996). Histopathological examinations have been done and explained by Prof. Dr. A. Khlosy, Pathology Dept., Cairo Univ.

Table 1
Effect of CCl_4 and protective treatments at weight (g ± SD), diet consumed per rat (g), food efficiency and liver weight (g).

Group	Weight (g/d)	Diet (g/d)	Food efficiency	Liver (g/bw)
Control	$4.167^{d} \pm 0.36$	15	27.8	$6.55^{ab} \pm 0.53$
Positive con.	$2.45^{ab} \pm 11.6$	15	16.3	$6.89^{bc} \pm 0.27$
Hepaticum	$3.5^{cd} \pm 7.7$	14.73	23.7	$7.49^{d} \pm 0.43$
Ethyl acetate ext.	$1.6^{a} \pm 2.3$	13.6	11.7	$6.12^{a} \pm 0.12$
Ethanol ext.	$2.6^{bc} \pm 9.6$	15	17.3	$7.29^{cd} \pm 0.59$

Table 2
Effect of CCl_4 and protective treatments at SGOT, SGPT and ALP (IU/L).

Group	GOT (IU/L)	GPT (IU/L)	ALP (IU/L)
Control	$102^{bc} \pm 18.1$	$41.33^{a} \pm 4.0$	$204^{ab} \pm 15.5$
Positive con.	$120.7^{c} \pm 20.5$	$51.75^{b} \pm 3.6$	$221.75^{b} \pm 18.9$
Hepaticum	$107^{bc} \pm 10.3$	$49^{b} \pm 2.9$	$221.5^{b} \pm 28.8$
Ethyl acetate ext.	$88^{a} \pm 1.3$	$43^{a} \pm 1.7$	$197^{a} \pm 5.4$
Ethanol ext.	$81.7^{a} \pm 7.9$	$41.5^{a} \pm 3.5$	$192^{a} \pm 6.2$
F	11.29^{**}	12.82^{**}	2.24

** Significance at $p < 0.01$.

Table 3
Effect of CCl_4 and protective treatments at GSH (mg/dl), and MDA (nmol/ml).

Group	GSH (mg/dl)	MDA (nmol/ml)
Control	$16.22^{d} \pm 0.57$	$0.98^{a} \pm 0.15$
Positive con.	$5.7^{abc} \pm 3.0$	$6.64^{c} \pm 1.18$
Hepaticum	$3.21^{ab} \pm 1.7$	$2.4^{a} \pm 1.65$
Ethyl acetate ext.	$8.17^{c} \pm 4.4$	$4.3^{b} \pm 2.03$
Ethanol ext.	$6.9^{bc} \pm 6.6$	$5.01^{bc} \pm 1.75$
F	12.21^{**}	12.77^{**}

** Significance at $p < 0.01$.

2.3. Statistical analysis

Mean data for six rats were calculated and SD was measured for each mean number. The obtained data were statistically analyzed using (ANOVA) procedure (SPSS, 1990). The levels of significance was accepted with $p < 0.05$.

3. Results and discussion

Liver damage caused by alcoholic cirrhosis or carbon tetrachloride is causing fatty infiltration in the liver cells. David et al. (1981) concluded that ethanol leads to fat accumulation in the liver (hyperlipidemia) and ultimately liver cirrhosis. Liver damage has been determined by rat weight, food efficiency, liver enzymes, tumor marker, oxidative status and lipid profile.

Table 1 shows a remarkable logical significance decreasing in mean daily rat weight and feed efficiency value for positive control comparing to normal control. While, the highest daily body weight and food efficiency was for the standard drug hepaticum treatment comparing with other protective extract groups. Liver enlargement was noticed for positive control comparing with the negative control. While, hepaticum as well showed a significant increase for mean liver weight comparing with the protective groups.

One of the most sensitive and dramatic indicators of hepatocyte injury is the release of intracellular enzymes, such as transaminases and serum alkaline phosphatase in the circulation after CCl_4 administration. Rajesh and Latha (2004) stated that elevated

Table 4
Effect of CCl_4 and protective treatments at FS (U/L).

Group	Fucosidase (U/L)
Control	$1.48^{b} \pm 0.21$
Positive con.	$1.54^{b} \pm 0.24$
Hepaticum	$0.57^{a} \pm 0.37$
Ethyl acetate ext.	$1.52^{b} \pm 0.35$
Ethanol ext.	$1.51^{b} \pm 0.59$
F	6.69^{**}

** Significance at $p < 0.01$.

Table 5a
Effect of CCl_4 and protective treatments at lipid profile TG, chlosterol and HDL (mg/dl).

Group	TG (mg/dl)	Chl (mg/dl)	HDL (mg/dl)
Control	$72.8^{c} \pm 3.4$	$29.6^{a} \pm 0.37$	$6.12^{b} \pm 1.7$
Positive con.	$86.9^{d} \pm 11.5$	$84.7^{d} \pm 28.7$	$4.4^{a} \pm 10.3$
Hepaticum	$87.5^{d} \pm 14.6$	$58.8^{bc} \pm 16.8$	$7.38^{de} \pm 4.5$
Ethyl acetate ext.	$57^{b} \pm 1.7$	$41.8^{ab} \pm 5.3$	$6.33^{bc} \pm 10.8$
Ethanol ext.	$37.3^{a} \pm 7.3$	$66.6^{c} \pm 21$	$7.04^{cd} \pm 4.4$
F	27.8**	11.6**	21.2**

** Significance at $p < 0.01$.

Table 5b
Effect of CCl_4 and protective treatments at calculated LDL and risk factor.

Group	LDL (mg/dl)	LDL/HDL ratio
Control	8.92	1.46
Positive con.	62.92	14.3
Hepaticum	33.92	4.6
Ethyl acetate ext.	24.07	3.8
Ethanol ext.	52.1	7.4

activities of these enzymes are indicative of cellular leakage and loss of the functional integrity of liver cell membranes. Liver functions showed significance increase for SGPT, and insignificance increases in SGOT and serum alkaline phosphatase. Ethanolic plant extract is showing insignificance decreases in liver functions as has been shown in Table 2. In agreement to our data, Raja et al. (2007) found significant rise in levels of SGOT, SGPT and ALP. On the other hand, plant extract and standard drug significantly decreased enzymes levels. The stabilization of these enzymes by crude extract is a clear indication of the improvement of the functional status of the liver.

Indices of free radical-mediated damage as increase of malondialdehyde and decrease of glutathione and vitamins E, C have been

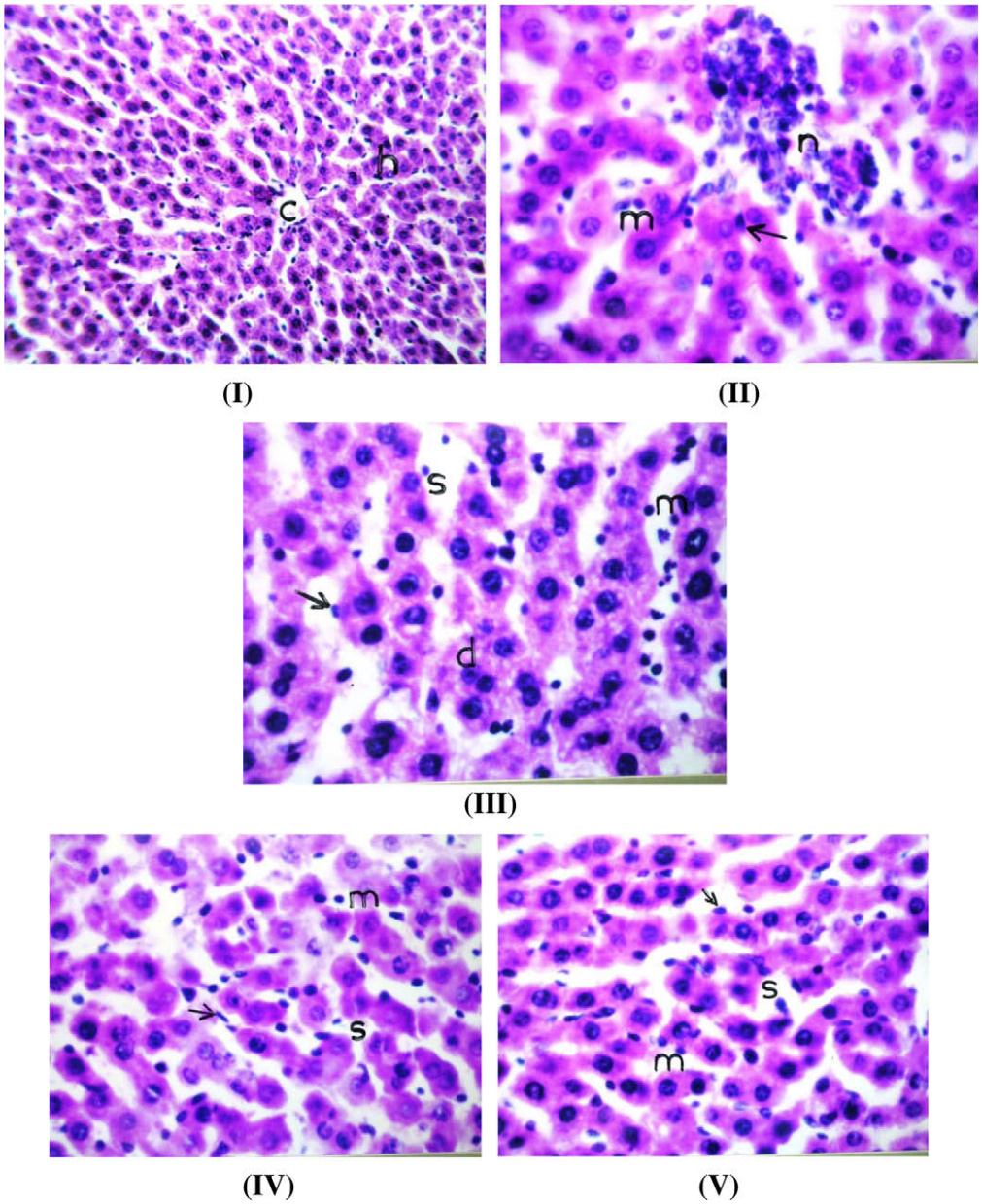

Fig. 1. liver histological structure of rats in control group (I), injected CCl_4 group (II), groups administered with hepaticum drug (III), ethyl acetate seed extract (IV) and ethyl seed extract (V) (H + E × 160). [C; central vein – h; surrounding hepatocytes – m; inflammatory cells infiltration – n; focal necrosis – s; dilated hypatic sinusoids – d; degenerated hepatocytes; arrow; diffuse kupffer cells proliferation].

documented in patients with viral or alcoholic liver disease (Loguercio and Federico, 2003). The degree of liver damage, the response of antiviral therapies may lead to new designs of new therapeutic strategies. One of the suggested mechanisms is the cleavage of CCl_4 which lead to the formation of highly unstable free radicals as CCl_3 or CCl_3O_2 and peroxides initiation.

The MDA, the end product of lipid peroxidation, showed the role of remarkable free radical in liver damage which increase significantly for positive control. In the same time, data declared significance decreasing in reduced glutathione for positive control and other protective groups. As well, the importance of plant extracts has been shown significant decrease for MDA levels and significant increase in GSH level as antioxidant content. Their remarkable significant increasing for plant extract exceeds than that for hepaticum the standard drug for liver damage Table 3. Significance increasing for reduced glutathione has been proved especially for ethyl acetate plant extract. Hepaticum drug on the other hand, is showing remarkable decreasing in MDA levels. These data are agreed with that shown by Toklu et al. (2008). Silymarin administration abolished the increase in MDA levels in lung and brain tissues, and reverses the decrease in GSH levels. As well, Raja et al. (2007) showed CCl_4 treatment caused significant decrease in GSH in liver homogenate tissue, while silymarin increased significantly the level. They also found increasing in lipid peroxidation level in CCl_4 treated rats, and significant decreasing for plant extract and silymarin.

Serum fucopyranosidase AFU the tumor marker, is considered a useful marker of hepatocellular carcinoma HCC. Increase AFU activity in serum is an early indication of HCC. Recent studies clearly demonstrated that measurement of AFU can significantly increase the detection specificity and sensitivity for HCC. AFU is reported to be a more sensitive marker especially for detecting a small tumor size of HCC.

Non-significance increasing for rats injected with carbon tetrachloride might be a sign for suspended relationship of liver damage with tumor formation Table 4. But the clear role for the standard liver damage relieve has been focused in group treated orally with hepaticum. In recent study, Kaur and Agarwal (2007) declared the cancer chemopreventive efficacy of silymarin and its active constituent silibin in both *in vitro* and *in vivo* animal models of epithelial cancers especially liver cancer.

TG, CHL, LDL levels and LDL:HDL ratio have been significantly increased for positive control comparing to control group Table 5a. Ethyl acetate seed extract dramatically decreased cholesterol and LDL level. In agreement, silymarin reduced cholesterol absorption in rats fed on high cholesterol diet, and caused significant decreases in vLDL, cholesterol and TG in the liver (Sobolova et al., 2006). Level of HDL cholesterol was significantly increased after silymarin. Inhibition of cholesterol absorption caused by silymarine and plant extracts could be a mechanism contributing to the positive changes in plasma cholesterol lipoprotein profile and in lipid content Table 5b in liver.

Histopathological examination for rat livers observes no alteration for rat control, while focal necrosis and diffuse kupffer cells proliferation in between the hepatocytes for positive control (Fig. 1 I and II). Some improvements especially for plant extracts have been shown in the protective groups as dilatation in the hepatic sinusoids associated with inflammatory cells infiltration and diffuse kupffer cells proliferation in between the degenerated hepatocytes (Fig. 1III–V). In another view, Bonis et al. (2001) mentioned that fibrosis and necrosis defined as a passive and irreversible chronic damage. Generally saying, reactive oxygen species generated in hepatocytes by Kupffer and inflammatory cells may deplete GSH level as in positive control Table 3, Fig. 1 II. Accordance to our data, Padhy et al. (2007) observed leukocytic infiltration, centrilobular necrosis and vacuolation in CCl_4 treated rats.

It's worth to say that the scientific attention is now paid to a possible redox gene therapy. Enhancing the antioxidant ability of hepatocytes could counteract oxidative/nitrosative stress and contribute to blocking the progression of liver disease. Increasing demands for natural plant products could modify the biological harmful molecules by the antioxidant potential. Additional studies should be accomplished to find many herbal medicines for both treat and prevent diseases.

Conflict of interest

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