PROTECTIVE EFFECTS OF AQUEOUS AND ETHANOLIC EXTRACTS OF SAFFRON STIGMA AND PETAL ON LIVER TOXICITY INDUCED BY CARBON TETRACHLORIDE IN MICE.

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Summary

Saffron (*Crocus sativa* L.) is one of the most valuable Iranian local plants and has been traditionally used as a medicine. The preventive effects of aqueous and ethanolic extracts of saffron stigma and petal were evaluated against CCl_4 induced hepatotoxicity in rats. CCl_4 induced fatty degeneration and vacuole formation and significantly increased the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma. Treatment with aqueous and ethanolic extracts of *C. sativus* L., stigmas and petals significantly decreased the levels of AST and ALT in plasma. Also histopathological studies showed that aqueous and ethanolic extracts of saffron (stigma and petal) reduce the incidence of liver lesions induced by CCl_4. The hepatoprotective effects of aqueous and ethanolic extracts of saffron may be due to: 1- antioxidant effects and radical scavenging 2-reduction of CCl_4 metabolic activation by cytochrome P450 inhibition 3- fixation of hepatic cell membrane.This results suggested that aqueous and ethanolic extracts of saffron exhibit hepatoprotective effects against liver damages induced by CCl_4 in mice.

Key words: saffron stigma, saffron petal, carbon tetra chloride (CCl4) and hepatotoxicity.

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Introduction

Crocus sativus L., commonly known as saffron, is a perennial stemless herb of the *Iridaceae* family and widely cultivated in Iran and other countries such as spain and Greece. Commercial saffron comprises the dried red stigma with a small portion of yellowish style attached. The important usages of saffron is in cooking and baking and it is also employed in alcoholic and nonalcoholic beverages (1). In folk medicine saffron is used as an antispasmodic, eupeptic, gingival sedative anticatarrhal, nerve sedative and antiedematogenic remedy. Furthermore, modern pharmacological studies have demonstrated that saffron extract has an antitumor effect, radical scavenger property and hypolipidemic effect (2),(3).

Carbon tetrachloride consistently causes liver toxicity, resulting in fatty degeneration, cellular necrosis, fibrosis and cirrhosis and induces hepatocellular carcinomas by many roots of administration (oral, inhalation, and parenteral exposure). There is a lot of evidence for toxic effects of carbon tetrachloride in animals. Mechanistic studies suggest following events in the carcinogenicity of carbon tetrachloride: 1) formation of trichloromethyl radical and trichloromethyl peroxy radical by CYP_2E_1, 2) lipid peroxidation by the trichloromethyl peroxy radical, 3) loss of calcium homeostasis leading to activation of degradative enzymes and cytotoxicity and 4) regenerative and proliferative changes in the liver in response to hepatotoxicity (4). So, the initial phase involves the metabolism of CCl4 by cytochrome P450 to the trichloromethyl radicals, which lead to membrane lipid peroxidation and finally to cell necrosis. The second phase of CCl4-induced hepatotoxicity involves the activation of Kupffer cells,which is accompanied by the production of proinflammatory mediators (5).
Antioxidants and radical scavengers protect liver cells from CCl4-induced damage by breaking the chain reaction of lipid peroxidation (6). Many plants and natural compounds showed protective effects against CCl4 induced liver toxicity (7-9) and in many cases the protection effects were due to antioxidant properties (10-15). Also saffron (especially active compounds crocin and safranal) showed anticancer (16-17), and antioxidant (18) properties. Based upon the antioxidant effects of *Crocus sativa* L., we investigated the hepatoprotective activities of saffron against CCl4 induced liver toxicity.

Materials and methods

Plant material

Crocus sativus L., stigmas and petals were collected from Kashmar (Khorasan province, east of iran) in november 2009, and was deposited at the Herbarium of the college of pharmacy, Mashhad University of Medical Science, Khorasan; Iran. Samples were protected from humidity and light and were air dried at room temperature (25°C).

Animals and preparation of blood and liver samples

Male white Razi mice (27-32 g, 9-11 weeks age) were purchased from Mashhad Razi institute (mashhad, iran) and were fed with standard chow diet and tap water ad libitum. All of them were kept in the same room under a constant temperature (24±1°C).

Preparation of *C. sativus* L., aqueous and ethanolic extract

Dried and pulverized petals and stigmas of *C. sativus* L, (5g) were extracted with distilated water for 24h and centrifuged. Then the aqueous extract was evaporated on the 40°C, dried and kept at 4°C.
Dried and pulverized petals and stigmas of *C. sativus* L, (5g) were extracted with 80% ethanol for 24h and centrifuged. Then the ethanolic extract was evaporated on the 40°C, dried and kept at 4°C.

Determination of serum biochemical parameters

All mice were anesthetized with ether and blood was obtain by cardiac puncture for serum biochemical testing (approximately 1ml from every mice has been obtained). After coagulation, samples were centrifuged at 4000 rpm for 5 minutes and the sera were kept at -20°C temperature for further assay.
The sera were analyzed with authoanalyzer for ALT and AST (RA-1000 from Thechnicon®).

Preparation of liver samples for histopathological studies

Samples of removed livers were fixed rapidly with 10% (v/v) neutralized formalin (pH= 7.4).
Hepatic samples were stained with routine Mayer's Hematoxylin and Eosin (H & E) staining method.
Samples sandwiched between a glass microscope slide and coverslip and mounted on the stage of a light microscope for analyzing.

Acute toxicity

Different doses of the extract were injected intraperitonealy into groups of six mice. The number of deaths was counted at 24 h after treatment.

The ethanolic extract doses for petal and stigma were 1, 1.1, 1.2 g/Kg and 0.8, 0.9, 1 g/Kg, respectively.

The aqueous extract doses for petal and stigma were 2, 2.3, 2.6 g/Kg and 1.7, 2, 2.3 g/Kg, respectively.

Determination of normal ALT and AST range and the liver histology

The blood and liver samples were collected from two groups of healthy animals and stored at -20°C temperature for further assay.

Determination of CCl4 toxic dose

For determination of CCl4 toxic dose 31.25, 62.5, 125, 250, 500, 1000 µl/kg of CCl4 in liquid paraffin (as a vehicle) prepared and injected to the groups of animals. Samples collected after 24 hours.

Evaluation of toxic effects of normal saline (as a vehicle of extracts) and liquid paraffin (as a vehicle of CCl4)

10 ml/kg of each vehicle injected intraperitonealy into group of six animals. Samples collected after 24 hours.

Evaluation of protective effects of aqueous and ethanolic extracts

0.56 and 0.8 g/kg doses of aqueous extract of stigma were injected intraperitonealy at 1 and 6 hours after injection of CCl4 into groups of six animals.

1.4 and 2 g/kg doses of ethanolic extract of stigma were injected intraperitonealy at 1 and 6 hours after injection of CCl4 into groups of six animals.

1 g/kg dose of aqueous extract of petal was injected intraperitonealy at 1 and 6 hours after injection of CCl4 and 0.7 g/kg dose of aqueous extract of petal was injected only one hour after injection of CCl4 into groups of six animals.

2.3 g/kg dose of ethanolic extract of petal was injected intraperitonealy at 1 and 6 hours after injection of CCl4 into groups of six animals.

Statistical analysis

Data were analyzed by one-way analysis of variance. Sequential differences among means were calculated at level of $p < 0.05$, using Tukey contrast analysis.

Results

The maximum tolerated dose (MTD) for the aqueous extract of petal and stigma were 1 g/kg and 0.8 g/kg, respectively. For ethanolic extract MTD were 2.3 g/kg and 2 g/kg, respectively.

The normal ALT and AST were 57±5 u/L and 179±18 u/L, respectively.

The effect of different doses of CCl4 on ALT and AST plasma levels showed in table 1 (we can see significant reduction in plasma levels of ALT and AST by reduction in CCl4 doses).

Groups that have been treated with Liquid paraffin and normal saline showed normal values of ALT and AST.

Table 1. Effects of different doses of CCl4 on ALT and AST plasma levels

CCl4 dose (µl/Kg)	AST (IU/L)	ALT (IU/L)
1000	12639±514***	20634±1102***
500	11956±1100***	16735±911***
250	12680±462***	14360±580***
125	11800±500***	13180±1150***
62.5	4312±519***	6900±608***
31.25	2436±270*	5518±500**
control	179±18	57±5

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to control, values represent the mean±SD, n=6

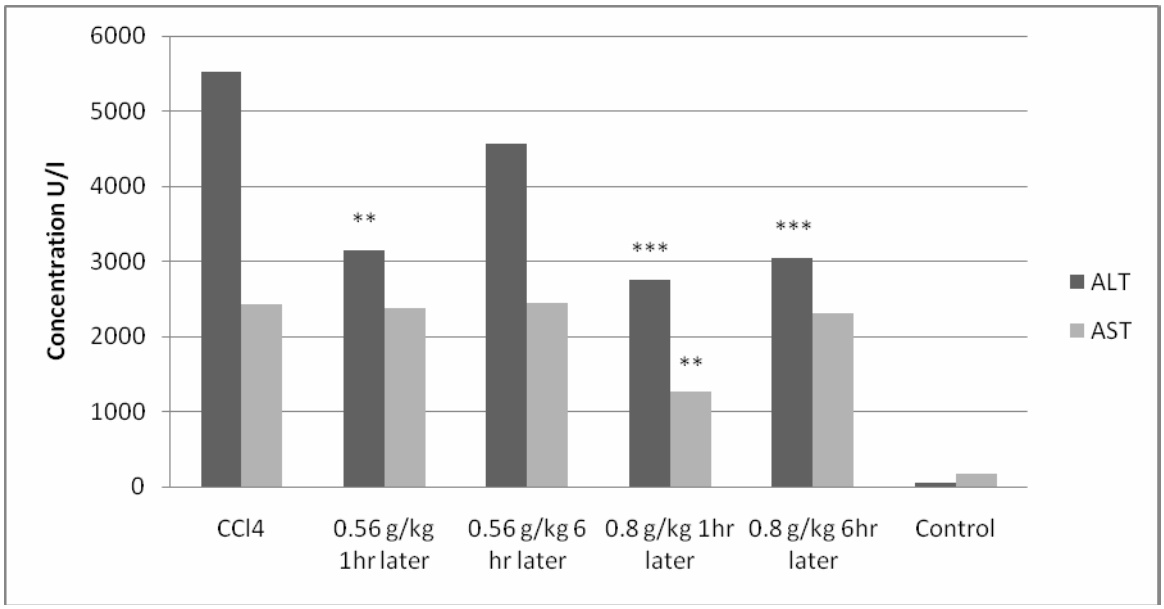

Fig 1. Effect of aqueous stigma extract of saffron on serum hepatic enzyme (ALT and AST) levels in mice after interaperitoneal injection. Data are presented as mean ±SD of six mice per group. ** $p < 0.01$ compared to control ; *** $p < 0.001$,compared to control in Tukey-Kramer Test.

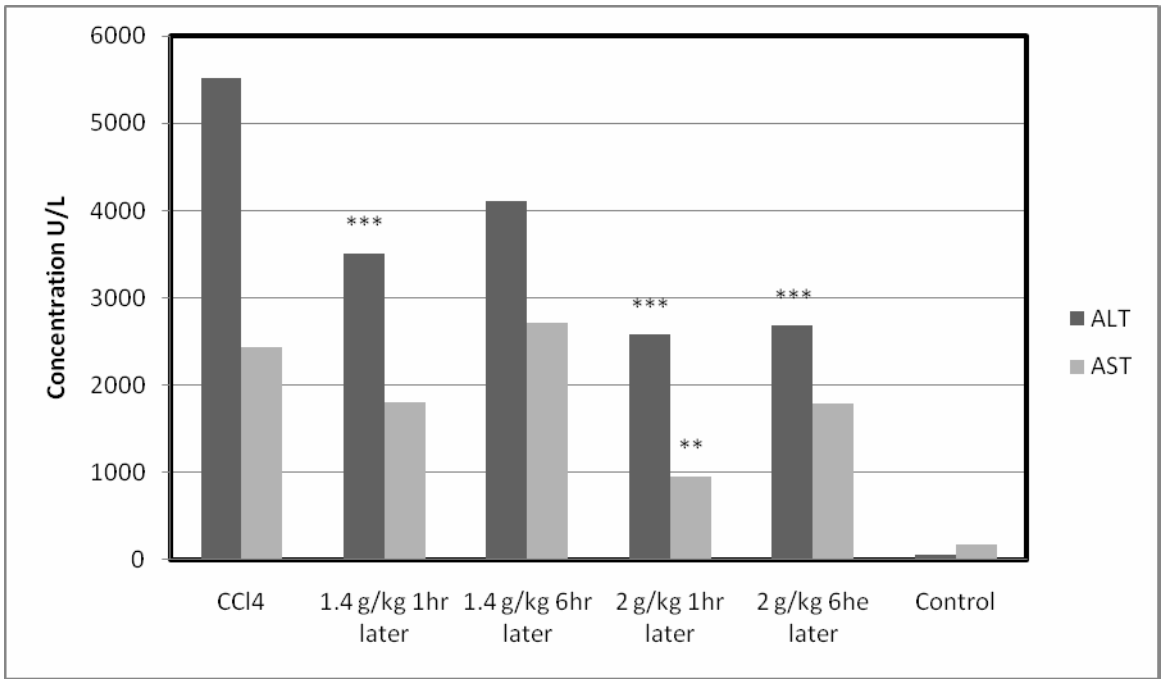

Fig 2. Effect of ethanolic stigma extract of saffron on serum hepatic enzyme (ALT and AST) levels in mice after interaperitoneal injection. Data are presented as mean ±SD of six mice per group. ** $p < 0.01$; *** $p < 0.001$,compared to control in Tukey-Kramer Test.

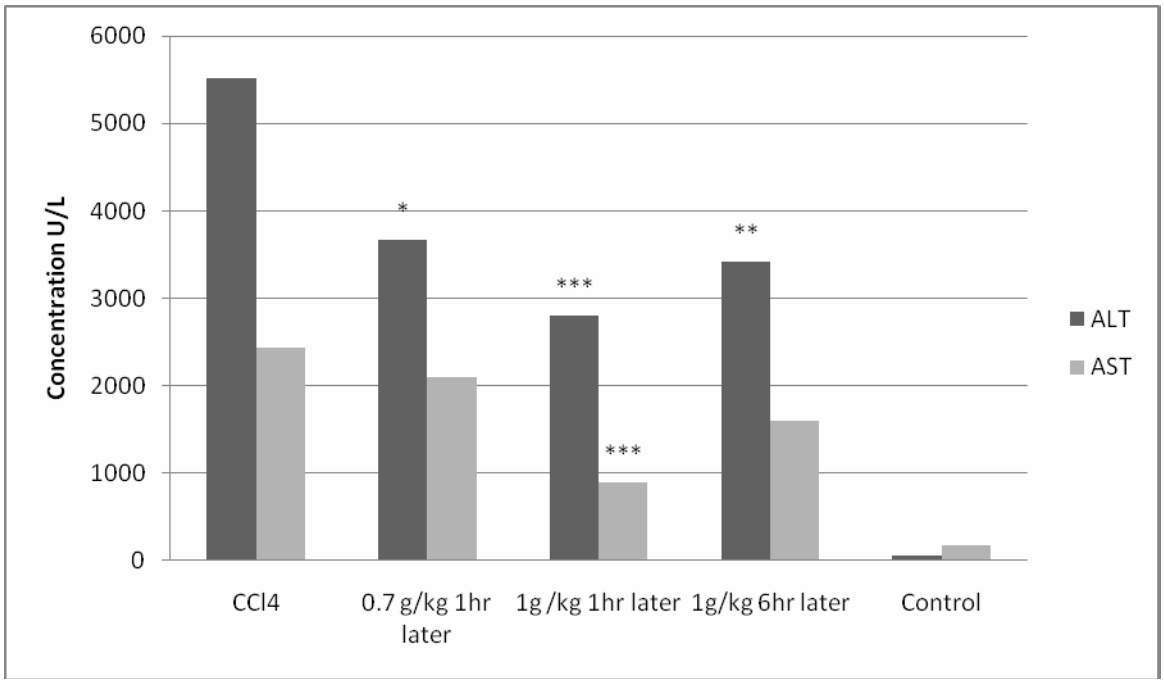

Fig 3. Effect of aqueous petal extract of saffron on serum hepatic enzyme (ALT and AST) levels in mice after interaperitoneal injection. Data are presented as mean ±SD of six mice per group. . * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control in Tukey-Kramer Test.

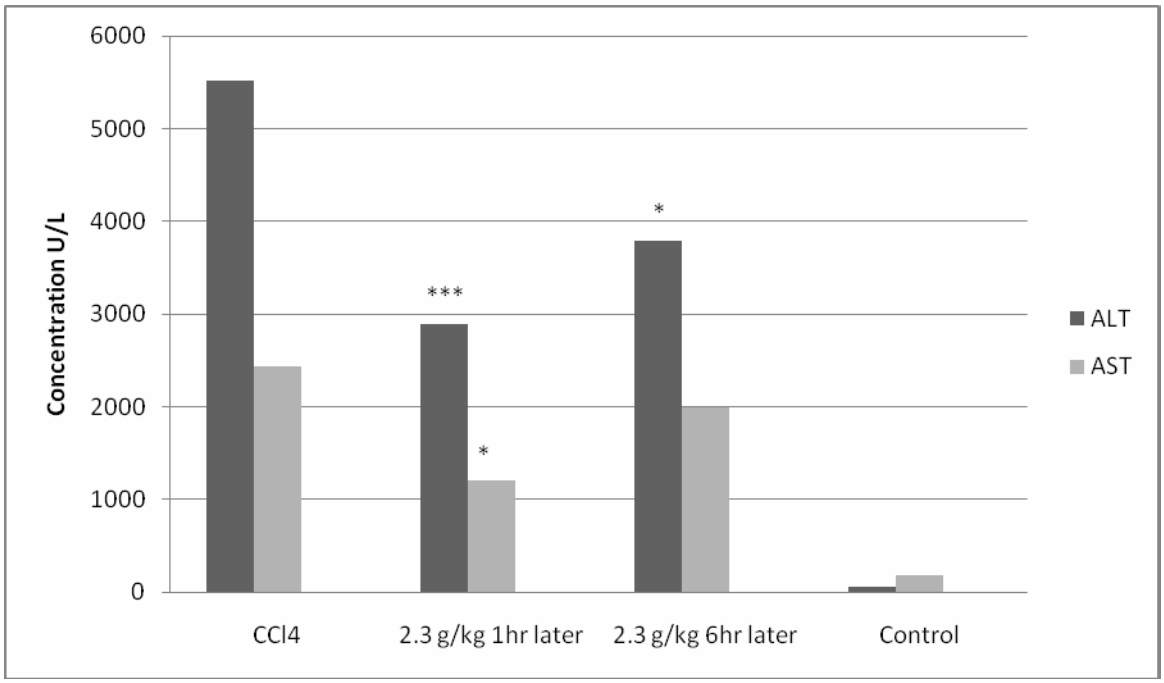

Fig 4. Effect of ethanolic petal extract of saffron on serum hepatic enzyme (ALT and AST) levels in mice after interaperitoneal injection. Data are presented as mean ±SD of six mice per group. * $p < 0.05$, *** $p < 0.001$,compared to control in Tukey-Kramer Test.

Results for histopathological studies

The histological assay of all groups had been performed with an expert pathologist in single blind pathway and mean percent of necrosis in the various slices had reported in table 4 - 6.

We used a scoring method which has been showed below:

Groups	Percent of necrosis	degree of necrosis
No necrosis	0 - 3%	1
Mild necrosis	x < 10%	2
Moderate necrosis	10% < x < 50%	3
Severe necrosis	50% < x	4

8.2% was the mean necrosis of selected toxin dose and other data compared with it. We observed no necrosis in histological results of normal group and toxin career and extract career injection.

The selective toxin dose of 31.25 µl/kg showed mild necrosis.

125, 250, 500 and 1000 µl/kg toxin doses showed 3 to 4 degrees of necrosis and sorted in moderate to severe necrosis groups.

All doses of aqueous stigma extracts reduced the necrosis in range of 4.8% to 75.6%.

All doses of ethanolic stigma extracts reduced the necrosis in range of 29.2% to 93.9% except.

All doses of aqueous petal extracts reduced the necrosis in range of 90% to 97% except 0.7 g/kg (1hr later) that increased necrosis.

In addition, the dose 2.3 g/kg of ethanolic petal extract (1hr and 6hr later) reduced necrosis in amount of 81.7% compared to mean toxin.

Table 2. Effects of all groups (Toxin, ethanolic and aqueous extract of saffron petal and stigma) on hepatic histopathology scores of liver damage in rat treated with CCl4.

Groups	type of necrosis	percent of necrosis	fatty change
31.25 µl/kg	C*	8.2%	m*
62.50 µl/kg	C	22.5%	m
125 µl/kg	C	72%	m
250 µl/kg	C	46%	m
500 µl/kg	C	34%	m
1000 µl/kg	C	64%	-

C* = Coagulative m* = Mild

Table 3. Effects of aqueous and ethanolic extract of saffron stigma on hepatic histopathology scores of liver damage in rat treated with CCl4.

Groups	type of necrosis	percent of necrosis	fatty change
Aqueous stigma exract			
1hr later			
0.56 g/kg	C*	2%	m*
0.8 g/kg	C	7.8%	m
6hr later			
0.56 g/kg	C	8.7%	m
0.8 g/kg	C	25.5%	m
Ethanolic stigma extract			
1hr later			
1.4 g/kg	C	4%	M*
2 g/kg	C	5.8%	M
6hr later			
1.4 g/kg	C	33%	m
2g/kg	C	0.5%	M

C* = Coagulative m* = Mild M* = Moderate

Table 4. Effects of aqueous and ethanolic extract of saffron petal on hepatic histopathology scores of liver damage in rat treated with CCl4.

Groups	type of necrosis	percent of necrosis	fatty change
Aqueous petal extract			
1hr later			
0.7 g/kg	C*	11.2%	M*
1 g/kg	-	0.75%	m*
6hr later			
1 g/kg	-	0.25%	m
Ethanolic petal extract			
1hr later			
2.3 g/kg	C	1.5%	M
6 hr later			
2.3 g/kg	C	8.8%	M

C* = Coagulative m* = Mild M* = Moderate

Discussion

Liver injury induced by CCl_4 is a common model for screening the hepatoprotective activity of compounds because this chemical is a potent hepatotoxin and a single exposure can rapidly lead to severe hepatic necrosis (19); (4).

The mechanism of CCl_4 toxicity is production of very unstable and toxic metabolites named trichloromethyl free radicals (CCl3). This radicals bind to hydrogen atom of cell membrane unsatturated fatty acids and initiates lipid peroxidation of the endoplasmic reticulum membrane and causes a chain reaction. In addition, injury to liver tissues alters their transport function and membrane permeability, leading to leakage of enzymes from the cells. Therefore, the marked release of AST and ALT into the circulation indicates severe damage to hepatic tissue membranes due to CCl_4 intoxication (20). Many types of oxygen radicals are existing such as superoxide, hydroxyl radicals and hydrogen peroxide that produce in intracellular actions and can bind to DNA biologic molecules, proteins and phospholipids and cause destruction of cell membrane structure and organs (21)**.**

There is some experimental models for blocking the CCl_4 hepatotoxicity by antitoxic agents that inhibit the liver injury:

1. Supporting the cell defense mechanisms such as: Antioxidant effect, free radical scavenging (eg Vitamin E) or supplying glutathione and its precursor.
2. Inhibition of CCl_4 metabolic activation by blocking the CYP450 enzymatic system.
3. Cell membrane or cell component fixation (eg Silymarin).
4. Interaction with receptor binding of CCl_4 free radicals (21).

The aqueous and ethanolic extract of saffron (petals and stigma) showed a significant effect on hepatic injury induced by CCl_4 and it caused reduction in ALT and AST hepatic enzymes. It also prevented from CCl_4 cell injury in hepatic cells.

Results obtained in this study showed that maximum doses 0.8 g/kg aqueous extract of stigma, 2 g/kg ethanolic extract of stigma, 1 g/kg aqueous extract of petals and 2.3 g/kg ethanolic extract of petals (1 hr later) are effective in decreasing CCl_4 induced hepatotoxicity. The hepatocellular protective effect of saffron is dose and time dependent. Therefore, the maximum doses of 1 hr later had both cellular and mytochondrial protection and reduced ALT and AST enzymes and necrosis. But doses of 6 hr later just had cell protection effects. Also decreasing in dose (1 hr later) showed cellular protection and just ALT reduction.

A brief review on the hepatic histology results showed reduction of necrosis in some groups following extract administration. Therefore, with decreasing in cell injury in most percent of samples the lower percents of necrosis observed.

In conclusion, this results suggests that maximum dose of each 4 extracts (1 hr later) possess the best effects on CCl_4 induced hepatotoxicity inhibition both in decreasing ALT and AST enzymes level and approvable decreasing in necrosis.

Protective mechanisms of saffron extract in CCl_4 induced liver injury:

The saffron petals have so many flavonoide and anthocyanin compounds. The anthocyanin is also a kind of flavonoids. Flavonoids can inhibit the fatty acids enzymatic peroxidation and also have free radical scavenging property. Therefore, it possesses potency to act as antioxidant agent. So we assume that most hepatoprotective effect of saffron petals is due to such compounds.

According to many researches (18, 22), crocin has antioxidant properties and a large amount of it exists in saffron stigma. Crocin and crocetin are carotenoid glycosids and soluble in ethanol and water. There has been proposed that the effectiveness of saffron stigmas in healing liver damages induced by CCl4 is due to this glycosides.

In addition, the present in vivo study showed that the saffron possess hepatoprotective effects. It is well-known that the hepatoprotective effect has a significant correlation with the antioxidant activities of saffron. This property is probably due to anthocyanins and flavonoids compounds of petals and stigma.

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