Hepatoprotective and antioxidant effects of *Zizyphus spina-christi* fruits on carbon tetrachloride induced hepatotoxicity in rats.

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Abstract: In the present study the *Zizyphus spina-christi* fruits (ZSCF) (Nabk) as an antioxidant to protect against CCl_4-induced oxidative stress and hepatotoxicity in Albino Wistar rats was investigated. Intraperitoneal injection of CCl4, administered twice a week, produced a marked elevation in the serum levels of aspartate transaminase, alanine transaminase, alkaline phosphatase and bilirubin. Daily dietary containing powder of (ZSCF) at 2.5%, 5%, 10%, and 15% of basal diet for 6 weeks produced a reduction in the serum levels of liver enzymes. Treatment with (ZSCF) normalized various biochemical parameters of oxidative stress. Furthermore, (ZSCF) has also restored normal levels of malondialdehyde and retained control activities of endogenous antioxidants such as SOD, and GSH. Therefore, the results of this study show that (ZSCF) can be protect the liver against CCl_4-induce oxidative damage in rats, and the hepatoprotective effect can be correlated with its antioxidant and free radical scavenger effects.

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1. Introduction:

The liver is one of the most important organs, owing to its biological functions such as drug metabolism, amino acid metabolism, lipid metabolism and glycolysis. Acute and chronic liver diseases constitute a global concern, and medical treatments for these diseases are often difficult to handle, and have limited efficiency ***(Lee et al., 2007)***.

Therefore, there has been considerable interest in role of complementary and alternative medicines for the treatment of liver disease. Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically **(Shen *et al.*, 2009).**

Carbon tetrachloride (CCl4) is a classical hepatotoxicant that causes rapid liver damage progressing from steatosis to centrilobular necrosis. Long-term administration of CCl4 causes chronic liver injury, and is a widely accepted model to produce hepatic fibrosis **(Pierce et al., 1987 and Hernandez-Munoz et al., 1990)**. Carbon tetrachloride (CCl4), an industrial solvent, is a well-established hepatotoxic. Extensive evidence demonstrates that as a result of the metabolic activation of CCl4, CCl4 and Cl, are formed during initiate lipid peroxidation process **(Ha and Lee, 2003)**. The fact, a major component of the antioxidant system in mammalian cells consists of three enzymes, namely, SOD, CAT and glutathione peroxidase. These enzymes work in concert to detoxify superoxide anion and H2O2 in cells. Therefore, reducing oxidative stress may be an effective therapeutic strategy for preventing and treating hepatic fibrosis.

Zizyphus spina-christi Willd belongs to the family Rhamnaceae and grows wild throughout upper Egypt and Sinai . Zizyphus has a common name ‘‘Nabka”, Arabs used it to maintain a healthy lifestyle and used for soothing properties **(Adzu et al., 2002).**

The genus Ziziphus belongs to the Rhamnaceae family which consists of about 100 species of deciduous or evergreen trees and shrubs distributed throughout the tropical and subtropical regions of the world **(Johnston 1963)**, from which twelve species are cultivated **(Hammer 2001)**. The genus Ziziphus is known for its medicinal properties as hypoglycemic, hypotensive anti-inflammatory, antimicrobial, antioxidant, antitumour, and liver protective agent and as an immune system stimulant **(Said et al. 2006).**

Zizyphus species (Rhamnaceae family) are commonly used in folklore medicine for the treatment of various diseases such as digestive disorders, weakness, liver complaints, obesity, urinary troubles,

diabetes, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anemia, diarrhea, and insomnia **(Han & Park, 1986; Kirtikar & Basu, 1984).**

They widespread in the Mediterranean region, Africa, Australia, and tropical America. Previous phytochemical studies on the different species of the genus Zizyphus led to the isolation and characterization of cyclopeptide alkaloids, flavonoids, sterols, tannins, and triterpenoid saponins **(Ikram, Ogihara, & Yamasaki, 1981; Nawwar et al, 1984)**. Phytochemical studies have revealed that from the different species of the genus Zizyphus, peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, betulinic acid and triterpenoidal saponin glycosides have been isolated and chemically identified**.** ZSC has many content of peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, butulinic acid and triterpenoidal saponin glycosides **(Ikram et al., 1981; Higuchi et al., 1984; Nawwar et al., 1984; Han et al., 1990; Barboni et al., 1994; Abu-Zarga et al., 1995; Cheng et al., 2000; Shahat et al., 2001; Tripathi et al., 2001).**

Phytochemical studies have revealed that Ziziphus jujube contains various constituents, including vitamins, flavonoids, amino acids, organic acids, microelements, and polysaccharides **(Lee et al., 2007) .**

The aim of this work was to evaluate the role of fruits of *Zizyphus spina-christi* to reduce the hepatotoxicant in rats that induced by ccl4.

2. Material and Methods:

2.1. Plant material

The fruits of *Zizyphus spina-christi* were collected from Sinai, Egypt in January 2010, and the plants were identified by the corresponding author. The fruits were cleaned; washed under tab water; seeds were separated; finally dried at 40 ◦C in under-vacuum oven and then crushed to a fine powder.

2.3. Animals

Thirty six adult male albino rats, weighing (150-170) g each from Medical Insects Research Institute, Doki, Cairo, Egypt were used in this study. Rats were housed in wire cages under the normal laboratory condition and were fed on standard diet for a week as an adaptation period. Diet was introduced to rats in special food cups to avoid scattering of food, Also water was provided to the rats by glass tubes projecting through the wire cage from an inverted bottle supported to one side of the cage. Food and water provided ad-labium and checked daily. Standard diet prepared from fine ingredients per 100 g according to ***AIN, (1993)***.

2.4. Experimental groups:

Male Wister rats were randomly divided into two main groups, the first, negative control group (n=6), fed on basal diet and the second hepatotoxic groups (n=24), this groups were subjected to subcutaneous administration of a single dose of 0.3 ml/kg CC14 mixed with equal volume of corn oil on the 7th day, 2 h after the last treatment. ***Saraswat et al***, (***1993***).

Hepatotoxic groups (n=24) divided into subgroups: group 1 positive control (n=6) fed on basal diet, group 2 (2.5% of ZSCF powder for basal diet), group 3 (5% of ZSCF powder for basal diet), group 4 (10% of ZSCF powder for basal diet) and group 5 (15% of ZSCF powder for basal diet). Food intake was calculated daily and rats were weighed weekly. Feeding and growth performance were carried out by determination of food intake and body weight gain .

2.5. Blood and tissue collection:

At the end of the experiment all period 8 weeks rats were starved for 12h then sacrificed under ether for anesthesia. Blood samples were collected into clean dry centrifuge tubes stored at room temperature for 15 minutes, put into a refrigerator for hour; centrifuged for 10 minutes at 3000 rpm to separate serum. Serum was carefully aspirated and transferred into dry clean Wassermann tubes using a Pasteur pipette and then kept frozen at - 20 C˚ till analysis.

2.6. Biochemical assays:

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) , gamma glutamine transferase (GGT), alkaline phosphates(ALP) and total bilirubin (T.B) are determined using commercially available kits (Alkan Medical, Cairo, Egypt) according to the manufacturer's instructions.

The most prominent product of lipid peroxidation (LP) is malondialdehyde (MDA) which is used as an indirect index of LP in biological system **(Sorg, 2004).** The method of **Uchiyama and Mihara (1978)** was used to determine MDA, it is based on its reaction with thiobarbituric acid to form a pink complex with absorption maximum at 535 nm, The superoxide dismutase (SOD) enzyme activity was determined according to the method described by **Sun and Zigman (1978)** .This method is based on the ability of SOD to inhibit the auto-oxidation of epinephrine at alkaline pH to adrenochrome and other derivatives, which are easily monitored in the near-UV region of the absorption spectrum .

The glutation peroxidase (GSH-Px) activity was assayed by the method of **Rotruck et al., (1973).**

Serum total cholesterol, triglyceride (TG) and high density lipoprotein (HDL-c) were determined by using

methods of **Allain et al., (1974), Fossati and Prencip (1982)and Lopez-virella (1977)** respectively. The determination of low density lipoprotein cholesterol (LDLc) and very low density lipoprotein cholesterol (VLDLc) were carried out according to the methods of **Lee and Nieman (1996)** as follows:

$$\mathbf{VLDL_c = \frac{TG}{5}} \quad \mathbf{And\ LDL_c = Total\ cholesterol - (HDL_c + VLDL_c).}$$

2.7. Statistical analysis

SPSS (version 10) (SPSS Inc., Chicago, IL, USA) was used to carry out a one-way analysis of variance (ANOVA) on our data. When significant differences were detected by ANOVA, analyses of differences between the means of the treated and control groups were performed using Dennett's t-test.

Results and Discussion

The proximate chemical composition of dry Zizyphus spina-christi fruits were presented in table (1), the results indicated that the carbohydrate (83.18%) was high in dry ZSC fruits while protein (4.7%), moisture (5.4%) and fat (0.94%) were low. These results are agreement with **Abdelmuti, 1991 and Berry-Koch *et al*.,1990** who showed the dry ZSC fruits was high content in carbohydrate (80.11/100 g dry matter) but low in protein (4.69/100g dry matter) and fat (0.9 g / 100 dry matter). Also, **Saied; *et al* (2007)** who observed that the flesh of ZSC fruits is rich in carbohydrates (80.6% in dry matter) notably starch (21.8%), sucrose (21.8%), glucose (9.6%) and fructose (16%) and in iron (3 mg 100 g-1 dried fruit; **(Nour *et al* ., 1987; Abdelmuti 1991**). One hundred gram dried fruit pulp contains 314 calories, 4.8 g protein, 0.9 g fat.

In the same table it was high content in total phenolic compounds (7.55) , ZSC has many content of peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, butulinic acid and triterpenoidal saponin glycosides **(Ikram et al., 1981; Higuchi et al., 1984; Nawwaret al., 1984; Han et al., 1990; Barboni et al., 1994; Abu-Zarga et al.,1995; Cheng et al., 2000; Shahat et al., 2001; Tripathi et al., 2001).**

Table1. Proximate chemical composition of dry ZSC fruit

Protein (%)	Moisture (%)	Fat (%)	Carbohydrate (%)	Ash (%)	T. phenolic compounds
4.7±0.8	7.4±0.2	0.94±0.3	83.18±029	3.79±0.4	7.55±0.33

Data in table (2) illustrate the mean value of liver functions for normal and hepatotoxic rats. Levels of GOT and GPT in serum were used as biochemical markers to evaluate the hepatic injury The serum GOT, GPT,GGT and ALP levels and T.B content were significantly increased ($P<0.05$) in hepatotoxic groups compared to that in normal group, but the values of GOT and GPT for 10% and 15% ZSC fruits groups were highly significant decreased as compared with other concentration, GGT and ALP values was significantly decrease ($P<0.05$) for 10% ZSC fruits group followed by 15% ZSC fruits group as compared with the hepatotoxic groups. While serum T.B content level in 15% ZSC fruits group was lower significant ($P<0.05$) compared to other hepatotoxic groups. CCl4 induced a severe hepatic damage, which represented markedly elevating activities of GOT and GPT in serum **(shen et al ., 2009)**. In similar study, **Amin and Ghoneim (2009)** found that serum AST and ALT levels were significantly increased in the fibrosis group compared to that in the normal group, but were significantly decreased in the ZSC treated groups . Compared to that of normal rat, serum GGT activity increases 6.90 times after 8 weeks of CCl4 induction; serum Alb content decreases 66% after 8 weeks of CCl4 induction. ZSC restores normal levels of both Alb and GGT in serum reduced the CCl4-induced levels of ALT and AST. **Shen et al ., (2009)** who concluded that CCl4 induced a severe hepatic damage, which represented markedly elevating activities of ALT and AST in serum.

Results in **table (3)** represent the mean values of lipid profile in normal and hepatotoxic rats. It was clear that no significant different observed with serum cholesterol of both normal group and hepatotoxic rats group which consumed 15% of ZSC fruits, while the serum level of triglyceride, LDL and VLDL were highly significant ($P<0.05$) in hepatotoxic groups compared to that in normal group (control group), but the hepatotoxic group which was feed 15% of ZSC fruits group was lower significant in serum triglyceride , LDL and VLDL levels than the other hepatotoxic groups .

In the same table, it could be noticed that, mean value of serum HDL in hepatotoxic groups was significantly increase (P<0.05) than the normal group, but the values of HDL for 2.5% ZSC fruits groups were highly significant increase as compared with other concentration of ZSC fruits, followed by 5%, 10% and 15% ZSC fruits groups respectively . **(Said et al ., 2006)** reported that the genus ZSC is known for its medicinal properties as a hypoglycemic, hypertensive, anti-inflammatory, antimicrobial, antioxidant, antitumor, and liver protective agent and as an immune system stimulant. ZSC has many content of peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, butulinic acid and triterpenoidal saponin glycosides have been isolated and chemically identified **(Ikram et al., 1981; Higuchi et al., 1984; Nawwar et al., 1984; Han et al., 1990; Barboni et al., 1994; Abu-Zarga et al., 1995; Cheng et al., 2000; Shahat et al., 2001; Tripathi et al., 2001) .**

Table (2) Effect of adding different portions of ZSC fruits to hepatotoxic rats diet on liver functions (u / l) .

	Control(-)	Control (+)	*ZSC* fruits groups				LSD
			2.5%	5%	10	15	
GOT	91.1^{d} ± 4.03	170.75^{a} ±1.7	125.47^{b}±2.98	123.69^{b}±2.36	106.56^{c}± 0.35	103.58^{c}±2.3	3.8
GPT	44.22^{d}±4.03	67.84^{a}±1.70	59.75^{b}±2.98	57.74^{b}±2.36	48.8^{c}±.35	47.76^{c}±2.3	3
GGT	17.36^{e}±0.75	65.13^{a}±2.76	36.66^{b}±3.63	33.72^{b}±1.21	27.62^{c}±1.08	24.05^{d}±0.65	3.001
ALP	34.5^{d}±2.5	112^{a}±7.5	56.4^{b}±6.2	50.0^{bc}±3.3	48.15^{c}±2.5	37.79^{d}±1.8	6.68
T.B	0.37^{d}±0.02	0.625^{a}±.03	0.615^{ab}±0.02	0.557^{bc}±0.05	0.56^{bc}±0.03	0.525^{c}±0.01	0.048

Values in the table were expressed as means ± SD
Different letters (a, b, c, d) in the same column are significantly different (P≤0.05)

Table (3). Effect of adding different portions of ZSC fruits to hepatotoxic rats diet on lipid profile

	Control (-)	Control (+)	*ZSC* fruits groups				LSD
			2.5%	5%	10%	15%	
Cholesterol (mg/dl)	96.15^{d}±3.4	143.65^{a}±7.7	115.4^{b}±4.7	108.35^{c}±3.3	101.8^{cd}±2.4	96.4^{d}±4.77	6.9
Triglyceride (mg/dl)	50.13^{d}±4.2	111.1^{a}±6.2	70.23^{b}±3.1	63.4^{bc}±7.2	62.4^{bc}±5.7	56.8^{cd}±3.5	7.8
HDLc (mg/dl)	52.1^{a}±3.7	27.5^{f}±2.3	33.33^{e}±1.6	38.4^{d}±2.2	42.65^{c}±1.7	48.3^{b}±1.8	3.5
LDLc (mg/dl)	34.1^{e}±1.7	93.94^{a}±6.8	68^{b}±5.9	57.3^{c}±4.7	46.7^{d}±0.95	$36.62s^{e}$+6.6	7.4
VLDLc (mg/dl)	10^{d}±0.82	22.21^{a}±1.24	14^{b}±0.78	12.7^{bc}±1.4	12.5^{bc}±1.3	11.37^{cd}±0.71	1.56

Values in the table were expressed as means ± SD
Different letters (a, b, c, d) in the same row are significantly different (P≤0.05)

Data in Table (4) indicated the Effect of adding different portions of ZSC fruits to hepatotoxic rats diet on antioxidant defense system.

MDA is commonly used as marker of free radical mediated lipid peroxidation injury **(Amin and Ghoneim, 2009)**. MDA level in serum of hepatotoxic groups were highly significant (P<0.05) compared to that in normal group, 15% of ZSC fruits group was significant (P<0.05) reduction of MDA levels compared to the other concentration of *ZSC* fruits groups, followed by 10%, 5% and 2.5% *ZSC* fruits groups respectively (**Amin and Ghoneim, 2009)** showed that MDA level of CCl4-induced animals showed a significant (P > 0.001) 2-fold increase compared to control level .

SOD is the first line of define to oxidative stress, and GSH is an intracellular reluctant that play major roles in catalysis, metabolism and transport **(Amin and Ghoneim, 2009)**. the mean values of SOD and GSH activity of liver in hepatotoxic group was significantly lower than that of control group , while the 15% of ZSC fruits group was significant (P<0.05) recovery compared to the other concentration of *ZSC* fruits groups, followed 10%, 5% and 2.5% *ZSC* fruits groups respectively. **(Amin and Ghoneim, 2009)** reported that pretreatment of ZSC increased the activity of tested antioxidant enzymes SOD and CAT. The antioxidant properties of flavonoids from various plant extracts reveal their stimulatory action on antioxidative enzymes **(Nagata *et al.*, 1999; Sreelatha *et al.*, 2009) .**

Amin and Ghoneim (2009) showed that ZSC exerts a therapeutic effect on CCl4-induced liver fibrosis in rats, possibly through its antioxidant action . **Shen et al ., 2009** observed that CCl4-induced generation of peroxy radicals and O2 – results in inactivation of CAT and SOD. And they showed that CCl4 challenge significantly decreased the activities of SOD and CAT in liver. **Szymonok-Lesiuk et al. (2003)** have found that CCl4 intoxication can lead to alteration in gene expression and depletion of SOD and CAT activities in kidney and heart. Oxidative stress causes depletion of intracellular GSH, leading to serious consequences **(FernàndezCheca et al., 1993).**

Ziziphus jujube administration inhibited lipid peroxidation at higher level after CCl4 treatment. Interestingly, FZJ 200mg/kg was able to increase the activities of endogenous antioxidant enzymes (SOD, CAT, and GSH-Px) and levels of GSH in hepatic tissue. FZJ pretreatment demonstrated to inhibit MDA of the reactive oxygen radical production.**(Shen et al., 2009)** .

Table (4). Effect of adding different portions of Zizyphus spina-christi fruits to hepatotoxic rats diet on antioxidant defense system .

`	Control(-)	Control(+)	*ZSC* fruits groups				LSD
			2.5%	5%	10%	15%	
MDA(nmol/ml)	$7.9^{e}\pm0.75$	$34^{a}\pm2.2$	$24.75^{b}\pm1.7$	$23.5^{b}\pm0.56$	$13.85^{c}\pm10$	$10.375^{d}\pm0.27$	1.88
SOD(unit/prot)	$7.3^{a}\pm0.28$	$2.71^{f}\pm0.35$	$3.63^{e}\pm0.75$	$4.4^{d}\pm0.45$	$5.3^{c}\pm0.3$	$6.45^{b}\pm0.39$	0.68
GSH(mg/dl)	$31.55^{a}\pm2.9$	$16.93^{f}\pm2.9$	$20.2^{e}\pm1.8$	$22.9^{d}\pm2.5$	$25.1^{c}\pm3.8$	$27.95^{b}\pm2.3$	4.12

Values in the table were expressed as means ± SD
Different letters (a, b, c, d) in the same column are significantly different (P≤0.05)
MDA (molondialdehyde), SOD(Superoxide dismutase enzymes), GSH(glutathione peroxides)

Data in **Table (5)** show the effect of adding different portions of ZSC fruits to hepatotoxic rats diet on body weight in rats. Before inducing hepatotoxicity, there was no significant difference in the body weight between groups. After the period of treatment it is clear that weight of normal group were highly significant (P<0.05) compared to the other group, followed by the weight of 15, 10, 5 and 2.5% of ZSC fruits group respectively. the relative body weight gains were increased by 33.97, 30.53, 22.32, 21.57% for 15, 10, 5 and 2.5% of ZSC fruits group respectively as compared to positive control which increased by 15.42% .

Table (5) . Effect of adding different portions of ZSC fruits to hepatotoxic rats diet on body weight in rat.

	Control (-)	Control (+)	*ZSC* fruits groups				LSD
			2.5%	5%	10%	15%	
Initial weight(g)	$162.25^{a}\pm2.22$	$162.25^{a}\pm3.86$	$157.75^{a}\pm4.19$	$160.25^{a}\pm2.87$	$159.75^{a}\pm3.20$	$160.5^{a}\pm3.69$	5.06
Final weight (g)	$233.25^{a}\pm4.27$	$187.25^{e}\pm3.30$	$191.75^{de}\pm4.78$	$196^{d}\pm3.37$	$308.5^{c}\pm2.65$	$215^{b}\pm4.08$	5.66
Relative gain (%)	$43.75^{a}\pm1.69$	$15.42^{e}\pm1.23$	$21.57^{d}\pm2.34$	$22.32^{d}\pm1.78$	$30.53^{c}\pm1.50$	$33.97^{b}\pm0.84$	2.43

Values in the table were expressed as means ± SD
Different letters (a, b, c, d) in the same row are significantly different (P≤0.05)

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