Research Article

The Protective Effect of Date Seeds on Nephrotoxicity Induced by Carbon Tetrachloride in Rats

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ABSTRACT

Carbon tetrachloride (CCl_4) causes generation of reactive oxygen species (ROS) in many tissues other than the liver including the kidney These Free radicals lead to a number of pathological changes in renal injury. Objective of the study to evaluate the potential effect of date seeds against nephrotoxicity induced by CCl4 in rats. Twenty-one Rats were divided into three groups, seven each. Group I: negative control. Group II and III (CCl_4 groups): treated with 0.5 ml of 10% CCl_4 in olive oil / rat twice a week. In addition, group III treated with daily aqueous date seeds suspension at a dose of 1gm/ kg orally. All groups treated for four weeks. At the end of the experiment, blood samples were collected and used for determination of kidney functions; urea, creatinine and creatinine/albumin ratio. The kidney tissues were subjected to histopathological examination. Malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (NO), superoxide dismutase (SOD) and glutathione-S-transferase (GST) were evaluated in kidney homogenate. Animals treated with CCl_4 exhibited significant elevations in kidney function tests, MDA, GSH, NO and exhibited significant decrease in the activities of SOD and GST. The treatment with date seeds has preserved the kidney histology, kidney function close to control values. It significantly restored the activities of SOD and GST and decreased kidney MDA, GSH and NO levels. Dry date seeds confers an appealing nephroprotective effect which might be explained partially via diminishing the generation of MDA and NO and induction of antioxidant systems.

Keywords: Date seeds, Nephrotoxicity, Carbon tetrachloride, Rats.

INTRODUCTION

CCl_4 is a potent lipid-soluble hepatotoxin bound to lipid and protein, enhances the peroxidative process.[1] The toxicity of CCl_4 depends on formation of the trichloromethyl radical (CCl_3•), which interacts with oxygen to form the more toxic trichloromethyl peroxyl radical (CCl_3O_2•).[2] Studies have demonstrated that CCl_4 can cause generation of reactive oxygen species (ROS) in many tissues other than the liver including the kidney, heart, lung, testis, brain, and blood.[3] Free radicals that induce lipid peroxidation cause cell membrane damage leading to a number of pathological changes in acute and chronic renal injuries.[4, 5] The key enzyme involved in CCl_4-induced nephrotoxicity is cytochrome P450, which is localized in the cortical tubule cells, and the increased lipid peroxidation is evident in the renal brush border. CCl_4 also affects renal mitochondrial function including calcium flux across mitochondrial membranes. [6]

Reports documented that several herbal extracts and plant-derived pure molecules could protect organs against CCl_4 induced oxidative stress by altering the levels of increased lipid peroxidation and enhancing the decreased activities of antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) as well as enhanced the decreased level of the reduced glutathione (GSH).[7] Endogenous antioxidants in medicinal herbs may play an important role as a defense against oxidative damage and protecting the biological functions of cells.[8] In the modern medicine, plants occupy a significant berth as raw materials for some important drug preparations.[9]

Date seeds (*Phoenix dactylifera*) have been reported to have relatively high amounts of antioxidants. [10, 11] Many studies have revealed that the oxidative stability of date seed oil is superior compared to other oils. This oxidative stability and high antioxidant capacity are attributed to richness in polyphenols and tocopherols compounds. [12]

Studies has demonstrated that date seed oil has in vitro chemoprotective effect against the oxidative damage induced by hydrogen peroxide in skin cell lines, [13] and exhibits antiwrinkle effect when used topically on skin of human volunteers.[14] Furthermore, a recent study by Habib and Ibrahim has demonstrated that diet containing date seeds reduces the basal level of lipid peroxidation in liver of normal rats while does not affect the antioxidant enzyme capacity of normal tissue.[15]

Although, several studies have reported that the date seeds have significant amount of antioxidants ranging from 580,000 to 929,000 µmol kg^{-1}. Considerable amount of the polyphenols can be isolated from the date seeds ranging from 31020-44300 mg gallic acid equivalent kg^{-1} depending on the variety [16] yet; date seeds are still used on a very limited scale in making a caffeine-free beverage with a coffee-like flavour.[17]

In this particular study, protective role of date seeds was evaluated against kidney damages mediated by oxidative stress produced by CCl_4 *in vivo* situations.

MATERIALS AND METHODS

Chemicals

All chemicals required for all biochemical assays were analytical grade and were obtained from Sigma-Aldrich Chemicals Co., St. Louis, USA.

Preparation of date seeds suspension:

Seeds of Hayani date (*Phoenix dactylifera. L*) were obtained from El-Sharkia date factory (El-Sharkia, Egypt). A voucher specimen number Ph-d.1 was kept in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Cairo, Egypt

The seeds were sun dried then, ground to a fine powder by hammer-mil. The date seeds suspension was freshly prepared as 1gmkg.

Animals

Adult male albino rats of Wistar strain weighing 200-250 g provided by Institutional Breeding House, Egypt were used throughout this study. The animals had free access to food and water *ad libitum* and maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12 hr light and dark cycle. The protocol of the study was approved by the Animal Ethics Committee of Faculty of Pharmacy, Helwan University on 1/11/2012. The study was conducted in accordance with EC Directive 86/609/EEC for animal experiments.

Dosage and treatment

Twenty-one rats were randomly divided into three groups each containing seven rats. Group I: act as a control group and received olive oil vehicle only (0.5 ml/rat) intrapretoneal (i.p) twice a week.

Group II - Received 0.5 ml CCl_4 (10% CCl_4 in olive oil) per rat i.p twice a week in accordance to Lin *et al*. [18]

Group III - Received 0.5 ml CCl_4 per rat i.p twice a week, and 1g/kg/day orally by gavage aqueous suspension of date seeds (after vigorous shaking to ensure uniform dose for all rats).

All groups treated for four weeks, then the rats were anesthetized and blood samples were collected from each rat through retro-orbital puncture, serum was separated and stored at -80 for biochemical analysis. Then rats were sacrificed, and kidney tissue was rapidly removed and washed in ice-cold saline solution. Kidney tissue was cut into two parts. One part of the kidney tissue was fixed in formalin for histopathological examination.

The other part was homogenized in phosphate buffer saline (0.1 M PBS, pH 7.4). The homogenates were centrifuged at 10000 rpm for 30 min at 4 °C, and supernatants were stored at −70 °C until biochemical assays could be performed.

Biochemical estimations

Kidney function tests

Serum urea and serum creatinine levels were assayed in the samples by a colorimetric method[19, 20] using commercial diagnostics kits (Diamond Diagnostics, Egypt). The levels of serum urea and creatinine were expressed as mg/dl. Serum albumin level was assessed calorimetrically using commercial diagnostic kits obtained from Diamond Diagnostics, Egypt. Creatinine /albumin ratio (C/A) was calculated from the results obtained. All serum parameters measured using Spectrophotometer 1200 UNICO Instruments.inc.USA.

Determination of lipid peroxide level

lipid peroxidation level in the kidney homogenate were determined as thiobarbituric acid reactive substances (TBARS) by measuring malondialdehyde (MDA) level spectrophotometrically in kidney homogenates according to Mihara & Uchiyama.[21] Briefly, 0.5 ml supernatant of kidney homogenate was mixed with 0.6% thiobarbituric acid (TBA) solution in water and 1% aqueous orthophosphoric acid solution, and heated in a boiling water bath for 45 min. The pink-colored chromogen formed by the reaction of TBA with MDA was extracted by n-butanol and measured at 535 nm. The results were expressed as MDA nmol/g tissue.

Nitric oxide (NO) level in kidney homogenate

Nitric oxide level in kidney tissue homogenates supernatant was determined by reacting with Greiss reagent. The assay is based on the diazotization of sulfanilic acid with nitric oxide at acidic pH (2-3) and subsequent coupling with N-(10-naphthyl)-ethylenediamine to yield an intensely pink colored product that is measured spectrophotometrically at 543 nm. Nitrite level was calculated using a standard curve for sodium nitrite and its level was expressed as umol/mg protein.[22]

Glutathione (GSH) in kidney

Levels of GSH in kidney homogenates were assayed according to the method of Ellman.[23] Briefly, the deproteinzation of kidney homogenate was made by 10% trichloroacetic acid and centrifuged at 3500 rpm for 10 min. 200µl supernatant was mixed with dipotassium hydrogen phosphate buffer (PH 8) and 0.4% 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) solution. The yellow-colored substance formed by the reaction of GSH and DTNB was measured at 412 nm. The results were expressed as GSH mg/g tissue.

Antioxidant enzymes in kidney

a. Superoxide dismutase (SOD) activity

Superoxide dismutase activity in kidney homogenate supernatant was determined spectrophotometrically according to the method of Roth & Gilbert.[24] This method is based on measuring the %inhibition in the auto-

oxidation of pyrogallol in the presence of SOD enzyme. One unit of SOD represents the amount of enzymes required to inhibit the rate of pyrogallol oxidation by 50% at 25°C. The activity was expressed as units/mg protein.

b. Glutathione-S-transferase (GST) activity

GST activity in kidney homogenate supernatant was determined spectrophotometrically by the method described by Habig *et al.* [25] The conjugation of 1- chloro-2, 4- dinitrobenzene (CDNB) with reduced glutathione was measured. The conjugation is accompanied by an increase in absorbance at 340 nm where the rate of increase in the absorbance was directly proportional to the GST activity in the homogenate. The results were expressed in U/min/mg of protein.

Determination of tissue protein content

Protein content in kidney homogenate was determined according to Lowry's method using bovine serum albumin (BSA) as a standard.[26]

Histological assessment

Immediately following animals scarification, kidney tissues were surgically excised, individually weighed, and thin kidney slices (5 µm thick) were cut and fixed in 10% neutral buffer formalin and embedded in paraffin wax blocks. Stained with hematoxylin-eosin (H&E), and then examined under light microscope at 400 magnifications for determination of pathophysiological changes. The sections were analyzed blindly by a certified pathologist and at least three different sections were examined in each sample of kidney.

Statistical analysis

All obtained data were expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons using GraphPad Instat (Graph software Inc., V 3.05, Ralf Stahlman, Purdue Univ.). P<0.05 was considered statistically significant. Appropriate graphs were plotted using Microsoft Excel 2007.

RESULTS

Effect of date seeds on body and kidney weights

The effect of CCl_4 and date seeds suspensions on kidney weight and body weight gain are represented in Table 1. The present study showed that treatment of rats with CCl_4 lead to significant reduction in the body weight compared to control group (P< 0.001). Treatment with date seeds suspension markedly improve the growth, yet this improvement is still non significant from the CCl_4 group. The average kidney weights of the CCl_4 group were significantly higher than control group (P<0.05). Administration of date seeds suspension to CCl_4 treated groups lead to significant decrease in the average kidney weight compared to CCl_4 group (P< 0.01).

Effect of date seeds and CCl4 on kidney function tests

The present study showed that administration of CCl_4 to rats caused a significant increase in serum creatinine and creatinine/ albumin ratio and significant decrease in serum albumin level compared with control group (P<0.05, P<0.05 & P< 0.001 respectively). Moreover, concomitant administration of date seeds suspension to CCl_4 intoxicated rats caused a significant decrease in serum creatinine level and in creatinine/ albumin ratio (P< 0.01) and significant increase in serum albumin level (P< 0.001) compared to CCl_4 group (Table 2).

Effect of date seeds and CCl_4 on MDA and NO concentrations

CCl_4 significantly increased the renal MDA and NO levels compared with control group (P<0.05). Concomitant treatment with date seeds suspension and CCl_4 markedly decreased MDA and NO levels in kidney tissue homogenate but only decrease in MDA reach significance (P<0.01) compared with CCl_4 group.

Table 1: Effect of CCl_4 and date seeds suspension on weight gain and kidney weights in rats

Parameter	Control	CCl_4	Date seeds (DS)
Weight gain (gm)	90.7± 9.6	22.8±10.3***	55.4±11.18
Average kidney weight (gm)	0.927±0.037	1.128±0.0646*	0.889±0.0384##

DS: Date Seeds (1gm/kg suspension). Data are presented as mean± S.E., n=7. * P<0.05, *** *P*<0.001 compared with the control group. ## *P* < 0.01 compared with CCl_4-treated group.

Table 2: Effect of CCl_4 and date seeds suspension on kidney function tests

Parameter	Control	CCl_4	Date seeds (DS)
Albumin (gm/dl)	4.748±0.156	3.467±0.1803***	4.497±0.1634###
Creatinine (mg/dl)	1.202±0.1279	1.533±0.2096*	1.043±0.1969##
Creatinine/Albumin ratio	0.264±0.0087	0.361±0.0289*	0.236±0.0221##

DS: Date Seeds (1gm/kg suspension). Data are presented as mean ± S.E., n=7. * P<0.05, *** *P*<0.001 compared with the control group. ## *P* < 0.05, ### *P* < 0.001 compared with CCl_4-treated group.

Effect of date seeds and CCl_4 on the activity of GSH

The level of GSH in the CCl_4 group is increased significantly compared to the control group (P<0.001). The group treated with date seeds suspension showed significant decrease in the level of GSH compared to the CCl_4 group (P<0.05)

Effect of date seeds and CCl_4 on SOD activities

Treatment with CCl_4 decreased the activities of SOD compared with the control group (P<0.05), treatment with date seeds suspension significantly increased the activity of SOD compared with CCl_4 group (P<0.05).

Effect of date seeds and CCl_4 on GST

As shown in Table 3, the activity of GST was decreased in kidney tissue homogenates of rats treated with CCl_4 compared to control group (P<0.05). However, treatment with date seeds suspension markedly increased its activity yet it still not significant compared to CCl_4 group.

Effect of date seeds and CCl_4 on histopathological changes in the kidney

In the control rats there was no abnormal appearance or histological changes in the kidney, where there are normal proximal and distal tubules and intact glomerular toufts (Fig 1 A). CCl_4 injection caused classical damage in the rat kidney demonstrated by vacuolations of glomerular tuft, perivascular oedema, cystic dilatation of renal tubules, atrophy of glomerular tuft and vacuolization and necrobiotic changes of epithelial lining renal tubules (Fig 1 B). Rats treated with date seeds showed no histological changes in kidney tissues. Treatment with date seeds suspension markedly prevented congestion in glomeruli and vessels and other alterations (Fig 1 C).

Table 3: Effects of date seeds suspension on MDA, NO, SOD and GST

Parameter	Control	CCl_4	Date seeds (DS)
MDA (n mol/mg protein)	0.172±0.0303	0.251±0.0482*	0.158±0.0414$^{\#\#}$
GSH (mg/g tissue)	1.061±0.0962	2.731±0.308***	1.875±0.190$^{\#}$
NO(µmol/mg protein)	5.536±1.103	10.348±1.539*	6.16±0.693
SOD(U/mg protein)	84.1±20.452	25.183±1.74*	99.2±17.14$^{\#}$
GST(U/min/mg protein)	12.227±2.28	5.024±2.02*	6.21±0.357

MDA: malondialdehyde, GSH: reduced glutathione, NO: nitric oxide, SOD: superoxide dismutase, GST: glutathione-s-transferase, DS: Date Seeds (1gm/kg suspension). Data represented as mean± S.E., n=7. $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$, $^{\#\#}$ P< 0.01 as compared to CCl_4 group.

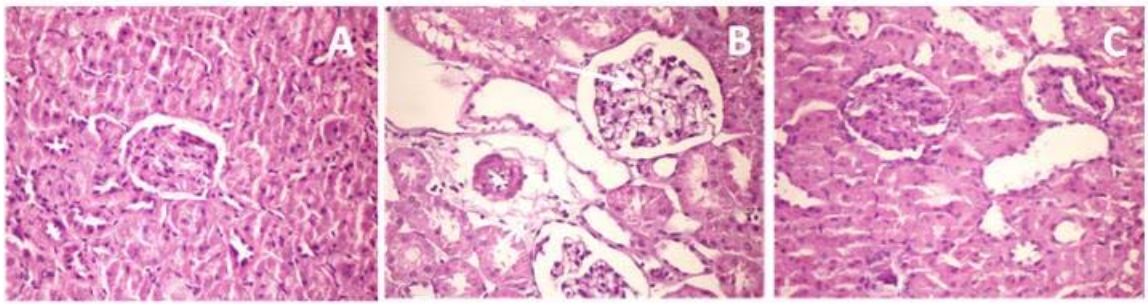

Figure 1: A: Normal control group, B: CCL_4 group, C: DS Treated group showing normal architecture of kidney

DISCUSSION

The present study revealed ameliorative effect of date seeds on CCl_4 induced renal toxicity in rats. CCl_4 when administrated is distributed and deposited to organs such as the liver, brain, kidney, lung and heart. [27] CCl_4 is converted by cytochrome P-450 into the reactive metabolites, trichloromethyl radical ($\bullet CCl_3$) and trichloromethyl peroxide radical ($CCl_3O_2\bullet$). These free radicals initiate the peroxidation of membrane poly unsaturated fatty acids, cell necrosis, GSH depletion, membrane damage and loss of antioxidant enzyme activity.

It has also been reported that systemically administered CCl_4 in rats was distributed at higher concentrations in the kidney than in the liver.[28] Since the kidney has high affinity for CCl_4 [29] and contains cytochrome P450 predominantly in the cortex, [30, 31] CCl_4 is extensively metabolized in the kidney generating more reactive metabolites. Fruits of the date palm (*Phoenix dactylifera*) are popular plants in many countries and are a vital component of arid and semiarid regions of the world. The chemical composition of date seeds has been well characterized by several reports.[18, 32, 33] Also, recent studies have shown that *Phoenix dactylifera.L* seeds possess high antioxidant activity due to abundance of phenolic compounds and flavenouids.[34] The flavonoids content in *Phoenix dactylifera* seeds is found to be Gallic acid (0.1mg/gm), Rutin (0.4 mg/gm), and Quercetin (0.9 mg/gm).[35] Habib and coworkers demonstrated that the

date seed constitutes one of the highest sources of total polyphenols, exceeding tea, grapes, flaxseed, nut seeds and even date flesh. [34] In this experimental study we investigated the protective effect of a suspension of date seeds on the nephrotoxicity occurred by CCl_4 injection in rats.

In the present study CCl_4 group showed a significant decrease in the body weight and significant increase in the kidney weight compared to control group. Administration of aqueous suspension of date seeds to CCl_4 treated group caused increase in the body weight and significant decrease in the average kidney weight compared to the CCl_4 group restoring kidney and body weights close to the control one. This results came in agreement with Ahmed & Kamal who demonstrated that CCl_4 induced a significant increases in kidney weight and relative kidney weight of rats due to a significant decrease in body weight and kidney swelling.[36]

Serum creatinine and creatinine/albumin ratio showed significant increase and serum albumin showed significant decrease in the CCl_4 group compared to control group. Treatment with date seeds suspension to CCl_4 treated rats lead to significant decrease in the serum values of creatinine and creatinine/albumin ratio and significant increase in serum albumin value compared to CCl_4 group. The results came in accordance with Ogeturk et al who reported that these pathological changes signify the potential damage to liver and kidney cells induced with CCl_4 treatment[37] and with Venkatanarayana et al who observed that rats intoxicated with CCl_4 showed significant increase in relative kidney weights and creatinine, and significant decrease in the concentration of albumin over normal control.[38] Elevation in plasma creatinine level can be attributed to the damage of nephron structural integrity.[39] In addition, decrease in the plasma albumin concentrations in CCl_4-treated rats might have resulted from remarkable leakage due to hyper cellularity of both glomeruli and tubules.[8] Histopathological examination revealed the vacuolations of glomerular tuft, perivascular oedema, and necrobiotic changes of epithelial lining renal tubules. Treatment with date seeds suspension significantly improved the concentrations of albumin in plasma, while significant recovery was noticed in the levels of creatinine and creatinine/albumin ratio. This effect may be related to the antioxidant properties of date seeds since it has been found that ROS may be involved in the impairment of glomerular filtration rate.[40, 41]

The present study showed that the value of GSH in the kidney tissue homogenate is significantly increased in the CCl_4 group compared to control group. This value decreased significantly when these rats treated with date seeds compared to CCl_4 group. The increase in the GSH activities in the CCl_4 group may be due compensatory mechanisms for the overproduction of free radicals and oxidative stress.[42, 43]

Superoxide dismutase (SOD) is one of the extremely effective antioxidant enzymes responsible for catalytic dismutation of highly reactive toxic superoxide radicals to H_2O_2 and for the catalytic decomposition of H_2O_2 to oxygen and water.[44] CCl_4 induced oxidative stress in renal tissues led to accumulation of superoxides and hydrogen peroxides. In our study it is evidenced by the decline in the activities of SOD and GST in the kidney tissue. These results came in agreement with other findings.[39] And also with accordance with Ahmed and Kamal who stated that the activities of antioxidant enzymes were markedly decreased in kidney tissue homogenates of rats treated with CCl_4.[36] Concomitant administration of date seeds suspension with CCl_4 significantly attenuated the toxic effect of CCl_4 on the activities of these enzymes.

The present study demonstrated that the level of kidney MDA and NO in CCl_4 treated group was significantly higher than the control group. The increase in MDA level suggests enhanced peroxidation leading to tissue damage and failure of the antioxidant mechanisms to prevent the production of excessive free radicals. Our result came in agreement with other reporters who demonstrated that CCl_4 significantly increased the renal MDA and NO levels compared to control group.[4, 36] Treatment with date seeds significantly improved the antioxidant enzyme activity in the kidney tissue. Our findings also in agreement with a study done on the effect of date seeds on normal rats and showed that rats fed the diets supplemented with date seeds had significantly lower levels of the serum and liver lipid peroxidation product MDA compared with the control group.[15]

The results obtained in this study suggest the protective effects of date seeds suspension against CCl4-induced oxidative stress, could be attributed mainly to the presence of high content of phenolics and flavenoids which have profound antioxidant activity.[13, 33] These compounds could scavenge the free radicals of CCl_4 generated through cytochrome P450 enzyme system thereby diminished the oxidative injuries. Histopathological examinations are in agreement with biochemical analysis.

CONCLUSION

Our study suggests that date seeds suspension may be considered as potentially combating oxidative stress and nephrotoxicity induced by CCl_4. This effect may be attributed to high antioxidant contents in date seeds.

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