Proanthocyanidin-Rich Date Seed Extract Protects Against Chemically Induced Hepatorenal Toxicity

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ABSTRACT A hydroacetone extract was prepared from seeds of *Phoenix dactylifera* L. var. Khalas, which is an industrial by-product of date processing. The proanthocyanidin nature of the extract (coded as DTX) was characterized by phytochemical and nuclear magnetic resonance (NMR) analyses. The total phenol/proanthocyanidin content and antioxidant activity of DTX were estimated by Folin–Ciocalteu, vanillin-sulfuric acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, respectively. The hepatorenal protective activity of DTX was evaluated using CCl₄-induced toxicity model in rats, in comparison with silymarin (SYL). Results of the histopathological examination and measurements of various hepatorenal serum indices and tissue biochemical markers demonstrated that DTX displayed marked protective potential against CCl₄-induced liver and kidney injury at 100 mg/kg/rat. Relative to the control CCl₄-intoxicated group, pretreatment with DTX significantly ($P < .001$) suppressed the elevated serum levels of alanine aminotransferase and aspartate aminotransferase (ALT and AST), alkaline phosphatase (ALP), γ -glutamyl transferase (GGT), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), bilirubin, creatinine, and calcium, whereas it significantly ($P < .001$) increased the diminished serum levels of high-density lipoprotein cholesterol (HDL-C) and total protein (TP). Moreover, DTX significantly decreased malondialdehyde (MDA) formation and increased TP synthesis in hepatorenal tissues compared with the intoxicated control. The improvement in biochemical parameters by DTX was observed in a dose-dependent manner and confirmed by restoration of normal histological features. The acute toxicity test of DTX in rats revealed safety of the extract. This study reveals that DTX enhances the recovery from xenobiotics-induced toxicity initiated by free radicals.

KEY WORDS: • antioxidant • hepatoprotective • nephroprotective • phenolics • *Phoenix dactylifera* • proanthocyanidins

INTRODUCTION

THE SEED OF THE DATE palm tree (*Phoenix dactylifera* L.), which constitutes 10–15% of the fruit weight, is a by-product of date processing industries.¹ With world production of dates reaching 9 million tons ~960 thousand tons of seeds are produced² and being wasted or partly used as fodder, noncaffeinated coffee, or a source of dietary fibers.³ The seeds are odorless with light to dark brown color and astringent taste, and they are rich in dietary fiber (65–69%), fat (9.9–13.5%), protein (4.8–7.5%), and phenolics (~4%).^{4,5} It is estimated that date seeds possess higher total phenolic content (about 18-fold more gallic acid equivalent GAE/100 g) and antioxidant activity (about 4- to 7-fold more Trolox equivalent/g) compared to the commonly eaten date flesh.⁶ Recently, the phenolic content of the Khalas variety

of date seeds, as analyzed by UPLC-DAD-ESI-MS, was found to contain mainly flavan-3-ol monomers to tetramers (proanthocyanidins), in addition to minor quantities of simple phenolic acids and flavonoids.⁷ Al-Farsi and Lee have optimized the extraction of total phenols using a water-acetone mixture as a solvent.⁸

Although date seeds are readily available, few studies have been undertaken to prove their antidiabetic,⁹ antioxidant,¹⁰ and antiviral¹¹ potential. In this study, we aimed to evaluate the effect of a proanthocyanidin-rich extract of Khalas date seeds in combating xenobiotic-induced hepatorenal injury via oxidative stress using *in vivo* models.

MATERIALS AND METHODS

Chemicals and analytical instruments

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antistax[®] capsules (Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany), each contains 180 mg dry aqueous extract of red vine leaves (*Vitis vinifera* L.);

Manuscript received 27 January 2014. Revision accepted 9 October 2014.

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FIG. 1. Structure of common flavan-3-ols and proanthocyanidins (a) and ^{13}C NMR spectrum (125 MHz, dimethyl sulfoxide [DMSO]- d_6) of DTX (25 mg), number of scans (NS)=3000, acquisition time in seconds (AQ)=1.091241 (b). NMR, nuclear magnetic resonance. Color images available online at www.liebertpub.com/jmf

batch No. B03820003. ^{13}C nuclear magnetic resonance (NMR) spectrum (Fig. 1 and Table 1) were recorded in dimethyl sulfoxide (DMSO)- d_5 on a Bruker Avance DRX-500 spectrometer (College of Pharmacy, KSU). Absorbance was measured on a Specord 40 UV-VIS instrument (Jena Analytik AG, Jena, Germany) in 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant, vanillin-sulfuric acid, and Folin–Ciocalteu assays; on a Reflotron® Plus Analyzer (Roche Diagnostics GmbH, Mannheim, Germany) in estimation of serum parameters; and on a Shimadzu UV mini-1240 spectrophotometer (Shimadzu Europe, Milano, Italy) in other measurements.

Preparation of DTX

Ripe Khalas dates (collected from Al-Kharj region, 16 kg) were purchased from the dates' market in Riyadh, Saudi Arabia. The date variety was identified by Dr. Mahmoud Abdel Aziz, College of Food and Agricultural Sciences, KSU. Seeds were manually separated from dates, thoroughly cleaned with distilled water, air dried in shadow (2.2 kg, 13.75% w/w yield), and then ground using a heavy-duty mill. The resulting powder was exhaustively extracted by 70% acetone in distilled water ($3\text{ L} \times 4$) for 8 days and the extracts

were combined and concentrated under vacuum at 40°C to yield a dark brown solid extract (192.68 g, 8.76% w/w yield).

Folin–Ciocalteu assay

Total phenols in DTX were determined according to the procedure of Singleton and Rossi¹² with some modifications. Briefly, aliquots (1.0 mL) of diluted extracts or standards (gallic acid and quercetin) in MeOH were mixed with 2.5 mL 0.2 N Folin–Ciocalteu reagent. After 5 min, the reaction mixture was neutralized with Na_2CO_3 (2 mL, 7.5% w/v) solution. After incubation (2 h/RT), absorbance “A” of the resulting blue color was measured at 765 nm. Calibration curves were prepared using “A” of different concentrations (6.25, 12.5, 25, 50, 100, and 200 $\mu\text{g}/\text{mL}$, $n=3$) of standards. Total phenols content was expressed as mg gallic acid or quercetin equivalent (QUE) per g extract (Table 2).

Vanillin-sulfuric acid assay

Proanthocyanidins in DTX or Antistax were estimated according to Sun *et al.*¹³ with some modifications. Samples were sonicated in MeOH followed by centrifugation and each supernatant was separated, evaporated to dryness, and then reconstituted in MeOH to make up a stock solution of 1 mg/mL. To 1 mL of (+)-catechin (CT) solutions (18.75, 37.5, 75, 150, or 300 $\mu\text{g}/\text{mL}$ MeOH) or test solution (200 $\mu\text{g}/\text{mL}$ MeOH) in a test tube, 2.5 mL of 1% vanillin solution in MeOH, and 2.5 mL of 3.6 N H_2SO_4 in MeOH were added. After incubation (20 min/ 30°C), “A” of each reaction mixture was measured at 500 nm and calculated using the equation: $A = (A_s - A_b) - (A_c - A_o)$.¹⁴ A_s , A_b , A_c , and A_o are absorbances of solutions with the test sample, without the test sample, without vanillin, and with only H_2SO_4 (total volume, 6 mL), respectively. Proanthocyanidin content was calculated from a calibration curve prepared by using A of CT dilutions and the above equation as %CT w/w (Table 2).

HPTLC densitometry for analysis of proanthocyanidins in DTX

The solvent mixture CH_3CN – H_2O – AcOH in a volume ratio of 50:50:0.5 and Merck HPTLC RP-18 F_{254} plates, 10×10 cm were used as mobile phase and stationary phase, respectively. Standard (+)-catechin and DTX methanolic solutions (1 $\mu\text{g}/1\text{ }\mu\text{L}$ to 10 $\mu\text{g}/10\text{ }\mu\text{L}$) were applied, separately, as 4 mm bands (in spray mode) using CAMAG automatic TLC sampler (ATS-4). After development in the

TABLE 1. DIAGNOSTIC ^{13}C NMR DATA (PPM) OF A- AND C-RINGS IN THE PROANTHOCYANIDINS OF DTX IN COMPARISON WITH FLAVAN-3-OL MONOMERS: (+)-CATECHIN AND (–)-EPICATECHIN, AND COMMON PROANTHOCYANIDIN OLIGOMERS³⁵

Carbon No.	DTX proanthocyanidins content	(+)-Catechin	(–)-Epicatechin	Common proanthocyanidins
C-2	76.7–82.9	82.0	79.1	76.4–82.0
C-3	67.8–73.0	67.9	66.8	66.2–73.1
C-4	28.7–29.0	28.1	28.6	28.0–37.2
C-6	96.7–104.1	96.7	96.8	96.1–108.6
C-8	96.7–104.1	95.7	96.0	95.6–108.1

TABLE 2. TOTAL PHENOL/PROANTHOCYANIDIN CONTENT AND ANTIOXIDANT ACTIVITY OF DTX

Index	Value
Phenol content ^a	
mg GAE/g ^b	282.83 ± 18.16
mg QUE/g ^c	280.47 ± 19.95
Proanthocyanidin content (%CT w/w) ^d	49.0 ± 0.52 [6.28 ± 0.08] ^e
Antioxidant activity (IC ₅₀ mg/mL) ^f	6.0 [13.4] ^e [5.2] ^g

^aIndex was measured by Folin-Ciocalteu assay.

^bThe linear regression equation was $y = 0.0212x + 0.0548$, $r^2 = 0.998$.

^cThe linear regression equation was $y = 0.0193x + 0.1714$, $r^2 = 0.993$.

^dIndex was measured by vanillin sulfuric acid assay.

^eValues obtained by a standardized proanthocyanidin extract [Antistax®].

^fIndex was measured by DPPH radical scavenging assay.

^gA value obtained by a reference antioxidant flavonoid [rutin].

GAE, gallic acid equivalent; QUE, quercetin equivalent; CT, (+) Catechin.

CAMAG automatic developing chamber (ADC-2), the developed HPTLC plates were scanned and recorded at 280 nm. The identification of proanthocyanidin bands was based on the UV/Vis spectra traced by CAMAG TLC Scanner3 at 200–700 nm before and after derivatization with the specific vanillin-HCl spray reagent. The relative content (%) of proanthocyanidins was calculated based on area under peaks densitometrically measured at 280 nm. The whole process of analysis was controlled by WinCATS software version 1.3.4. The results are presented in Table 3.

DPPH radical scavenging assay

The antioxidant activity of DTX was determined according to the method of Brand-Williams *et al.*¹⁵ at 250, 50, 10, 2, and 0.4 µg/mL concentrations. Each solution (1 mL) was mixed with freshly prepared DPPH methanolic solution (125 µL of 1 µM) and 375 µL MeOH. After incubation (25°C, 30 min), the decrease (I%) in “A” was measured at

517 nm. Inhibition percent of DPPH radical was calculated from the equation: $I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$.

Animals and monitoring

Male adult Wistar albino rats (150–170 g) were obtained from the Experimental Animal Care Center, College of Pharmacy, KSU. After a 1-week adaptation period, rats were randomly divided into groups (six rats/cage) and kept at 22°C ± 2°C, 55% humidity, and 12/12 h light–dark cycle. The animals were provided with Purina chow rat diet (UAR-Panlab, Barcelona, Spain) and drinking water *ad libitum*. All treatment protocols for this study were approved by the Ethics Committee of the Experimental Animal Care Society, KSU.

Acute toxicity test

The acute toxicity of DTX was tested in rats, which were dosed in a stepwise procedure using the fixed doses of 50–2000 mg/kg orally according to the OECD guideline No. 420.¹⁶ The animals were fasted overnight and then received DTX (suspended in 3% gum acacia in distilled water). The animals were then observed for 3 h for general behavioral, neurological, and autonomic profiles and every 30 min for the next 3 h and finally for mortality after 24 h till 14 days.

Pretreatment and CCl₄-induced hepatorenal toxicity

Five groups (I–V) of animals were used. Group I was kept as a normal control. Group II received CCl₄ and served as a CCl₄-intoxicated control. Groups III–V were assigned as treatment groups. Groups III and IV were pretreated with DTX at doses of 50 and 100 mg/kg/rat orally, respectively; whereas groups V was pretreated with silymarin (SYL) at 10 mg/kg/rat orally, for 17 days. Group I and II animals received a similar volume of vehicle once daily orally. At the 16th day, groups II–V received CCl₄ in liquid paraffin

TABLE 3. R_f VALUES, RELATIVE CONTENT, UV MAXIMA OF ISOLATED PROANTHOCYANIDINS (BEFORE AND AFTER DERIVATIZATION), AND DENSITOGAM OF DTX BY HPTLC ANALYSIS

Band	R _f value ^a	Relative content (%) ^b	Plain UV spectral data (λ _{max} nm)	UV spectral data (λ _{max} nm) after derivatization	HPTLC densitogram of DTX
1	0.51	04.96	283.1, 316.9	288.6 sh, 314.6, 502.7	
2	0.76	36.42	283.1, 316.9	288.6 sh, 314.6, 502.7	
3	0.88	11.20	283.1, 316.9	288.6, 314.6 sh, 502.7	
4	0.92	47.41	283.1, 316.9	288.6, 314.6 sh, 502.7	
CT	0.74	— ^c	283.1	250.0, 503.0	

^aAnalysis was run using HPTLC RP-18 plate and mobile phase used: CH₃CN-H₂O-AcOH (50:50:0.5).

^bBased on area under peak densitometrically measured at 280 nm.

^c(+)-Catechin (CT) peak may be overlapped with the proanthocyanidin peak at R_f 0.76.

sh, shoulder.

(1:1) at a dose of 1.25 mL/kg/rat intraperitoneally.¹⁷ After 48 h, following CCl₄ challenge, the blood was collected by cardiocentesis and serum was obtained by centrifugation at 1000 g for 20 min at 4°C. The liver and kidney were removed for biochemical and histological assessment.

Analyses of serum hepatic biochemical and lipid profile

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), γ -glutamyl transferase (GGT), and alkaline phosphatase (ALP) were determined calorimetrically by methods of Reitman and Frankel¹⁸; Fiala *et al.*¹⁹; and King and Armstrong,²⁰ respectively, whereas bilirubin was determined by Stiehl's method.²¹ Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) were measured by the methods of Demacher and Hijamaus²²; Burstein and Scholnick²³; and Foster and Dunn,²⁴ respectively, using Roche kits (Roche Diagnostics GmbH). Low-density lipoprotein cholesterol (LDL-C) and the very low-density lipoprotein cholesterol (VLDL-C) levels were calculated from the formula: LDL-C = TC – HDL-C – VLDL-C; VLDL-C = TG/5.²⁵

Analyses of serum creatinine, calcium, urea, and uric acid

Creatinine was measured by the Jaffe reaction method²⁶ using CS604 kit (Crescent Diagnostics, Jeddah, Saudi Arabia). Calcium was determined by *o*-cresolphthalein method described by Gitelman²⁷ using CE500 kit (Crescent Diagnostics). Urea and uric acid were determined by urease and uricase methods described by Munan *et al.*²⁸ and Fossati *et al.*,²⁹ respectively, using Roche kits (Roche Diagnostics GmbH).

Determination of lipid peroxidation

A modified method of Utley *et al.* was used to measure lipid peroxidation.³⁰ The liver (or kidney) was homogenized in 0.15 M KCl at 4°C; and the homogenate (10% w/v, 1 mL) was transferred into a centrifuge tube and incubated at 37°C for 3 h. Aqueous trichloroacetic acid (TCA, 10%) was then added and the mixture was centrifuged at 800 g for 10 min. The supernatant (1 mL) was removed and mixed with aqueous thiobarbituric acid (1 mL, 0.67%) and placed in a boiling water bath for 10 min. The mixture was cooled, diluted with 1 mL distilled water, and A was read at 535 nm. The lipid peroxidation was expressed as malondialdehyde (MDA) in nmol/g wet tissue using a standard curve of MDA dilutions.

Estimation of nonprotein sulfhydryl groups

Tissue nonprotein sulfhydryl (NP-SH) groups were measured according to the method of Sedlak and Lindsay after homogenization in ice-cold ethylenediaminetetraacetic acid (0.02 M).³¹ The homogenate (5 mL) was mixed with distilled water (4 mL) and TCA (50%, 1 mL), shaken for 10 min and then centrifuged. A supernatant (2 mL) was mixed with Tris buffer (4 mL, 0.4 M, pH 8.9) and 5,5'-dithiobis-(2-nitrobenzoic acid) (0.1 mL) was then added and shaken. "A" was measured within 5 min at 412 nm against a reagent blank.

Determination of albumin and total protein

Serum albumin; and serum and tissue total protein (TP) were estimated according to the method of Doumas³² by CS600 and CS610 kits (Crescent Diagnostics), respectively. The principle is based on the formation of a blue/violet complex when protein peptide bonds react with Cu(II) ions in alkaline solution (biuret reaction). KNa tartrate and KI solutions were added as stabilizers. "A" was measured at 546 nm and protein was calculated as $(A_{\text{sample}}/A_{\text{standard}}) \times \text{concentration of standard}$.

Histopathological study

The liver and kidney samples were fixed in 10% neutral buffered formalin for 24 h and processed using a VIP tissue processor. The processed tissues were then embedded in paraffin blocks and sections (5 μ m thickness) were cut by a rotary microtome (American Optical, Buffalo, NY, USA). Sections were stained with hematoxylin and eosin³³; and then microscopically examined for pathomorphological changes.

Data analysis

Values are presented as arithmetic mean $\pm$ standard error of the mean. Data were statistically analyzed by using one-way ANOVA followed by Dunnett's multiple comparison tests. The *P*-value < .05 was taken as a statistically significant difference.

RESULTS

Qualitative and quantitative determination of phenolic content and antioxidant capacity of DTX

The cherry-red color produced with vanillin-HCl reagent³⁴ indicated the presence of proanthocyanidins in DTX (Fig. 1). Furthermore, ¹³C NMR spectrum measured in DMSO-d₆ showed the diagnostic carbon signals of C-ring (C-2 to C-4) and A-ring (C-6 and C-8) of flavan-3-ol monomers and their common oligomers³⁵ (Fig. 1 and Table 1), indicating the proanthocyanidin chemical nature of the extract.

The total phenolic content in DTX was quantified with Folin-Ciocalteu reagent as 282.83 $\pm$ 18.16 mg GAE/g or 280.47 $\pm$ 19.95 mg QUE/g. Moreover, the proanthocyanidin portion of total phenols was determined with a modified vanillin assay that revealed the richness of DTX in proanthocyanidins, being eightfold more %CT relative to a standard proanthocyanidin-containing extract, Antistax (Table 2).

HPTLC densitometric analysis of 0.1% w/v DTX methanolic solution indicated the presence of four major bands of proanthocyanidins as depicted from their characteristic spectral data (Table 3) measured before and after derivatization with the specific vanillin-HCl spray reagent. Their relative percentages were found to be 4.96%, 11.20%, 36.42%, and 47.41%.

The antioxidant activity of DTX was measured by DPPH radical scavenging assay. As presented in Table 2, DTX was able to reduce the blue DPPH radical solution (125 μ L of 1 μ M) into a yellow stable compound at IC₅₀ 6.0 μ g/mL, being as

TABLE 4. SERUM CONCENTRATIONS OF LIVER ENZYMES, BILIRUBIN, ALBUMIN, TOTAL PROTEIN, LIPIDS, CREATININE, URIC ACID, UREA, AND CALCIUM FROM THE CONTROL AND THE DIFFERENT TREATED GROUPS

Variables	Normal control	CCl ₄	CCl ₄ +DTX (50 mg/kg)	CCl ₄ +DTX (100 mg/kg)	CCl ₄ +SYL (10 mg/kg)
AST (U/L)	75.78±4.08	301.66±8.33*** ^a	236.33±5.00*** ^b	209.00±6.15*** ^b	138.33±6.79*** ^b
ALT (U/L)	29.08±2.03	210.83±6.36*** ^a	161.33±4.83*** ^b	138.16±3.62*** ^b	71.66±7.09*** ^b
GGT (U/L)	3.83±0.36	17.21±0.84*** ^a	11.21±0.26*** ^b	9.31±0.24*** ^b	5.65±0.21*** ^b
ALP (U/L)	308.66±7.18	681.33±14.17*** ^a	573.16±20.98*** ^b	507.66±8.26*** ^b	418.66±7.33*** ^b
Bilirubin (mg/dL)	0.58±0.01	2.66±0.15*** ^a	1.77±0.09*** ^b	1.38±0.03*** ^b	1.05±0.06*** ^b
Albumin (U/L)	4.94±0.22	1.73±0.16*** ^a	1.82±0.12 ^b	2.20±0.16 ^b	3.49±0.28*** ^b
TP (g/dL)	9.58±0.57	4.47±0.34*** ^a	5.26±0.45 ^b	6.86±0.30*** ^b	8.02±0.38*** ^b
TC (mg/dL)	102.66±4.80	312.00±9.68*** ^a	234.66±15.82*** ^b	196.00±7.93*** ^b	169.33±10.61*** ^b
TG (mg/dL)	93.51±6.48	237.96±4.40*** ^a	210.64±20.32 ^b	189.35±16.64 ^b	159.25±6.11*** ^b
HDL-C (mg/dL)	48.08±0.75	21.05±1.11*** ^a	36.03±1.03*** ^b	38.67±1.99*** ^b	35.66±2.54*** ^b
LDL-C (mg/dL)	35.92±5.77	243.35±9.14*** ^a	156.50±17.35*** ^b	119.45±9.11*** ^b	101.68±10.42*** ^b
VLDL-C (mg/dL)	18.70±1.29	47.59±0.88*** ^a	42.12±4.06 ^b	37.87±3.32 ^b	31.85±2.34*** ^b
Creatinine (mg/dL)	1.90±0.05	7.07±0.11*** ^a	6.50±0.18 ^b	5.41±0.18*** ^b	6.38±0.14*** ^b
Uric acid (mg/dL)	1.20±0.09	6.43±0.42*** ^a	5.25±0.21 ^b	4.78±0.25*** ^b	3.18±0.21*** ^b
Urea (mg/dL)	64.78±3.77	189.66±12.09*** ^a	165.55±5.27 ^b	146.33±4.58*** ^b	134.50±0.22*** ^b
Calcium (mg/dL)	7.17±0.43	29.52±0.84*** ^a	28.00±0.57 ^b	21.90±1.46*** ^b	9.20±0.45*** ^b

All values represent mean±SEM (*n*=6). **P*<.05; ***P*<0.01; ****P*<.001; ANOVA, followed by Dunnett's multiple comparison test.

^aAs compared with normal group.

^bAs compared with CCl₄ only group.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ -glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SEM, standard error of the mean; SYL, silymarin; TC, total cholesterol; TG, triglycerides; TP, total protein; VLDL-C, very low-density lipoprotein cholesterol.

equipotent as rutin and twice as potent as Antistax. The relative high antioxidant potency of DTX was thus correlated to its proanthocyanidin content. On the basis of above findings, it was expected that DTX would exhibit a substantial protective activity against *in vivo* CCl₄-induced toxicity.

Acute toxicity test of DTX

DTX was found to be safe where no mortality or toxicity symptoms was observed in the animals that received up to a dose of 2000 mg/kg till the end of experiment (14 days).

Effect of DTX on liver-related serum and tissue markers

Pretreatment with DTX (groups III and IV) significantly (*P*<.001) reduced the CCl₄-induced elevated levels of serum

ALT, AST, ALP, GGT, and bilirubin in a dose-dependent manner (Table 4), demonstrating a capacity to restore the normal functional status of the injured hepatocytes. Moreover, the low level of serum TP induced by CCl₄ liver-injury were significantly (*P*<.001) ameliorated in rats pretreated with DTX at a dose of 100 mg/kg.

The administration of DTX to the intoxicated rats markedly ameliorated the values of TC, HDL-C, and LDL-C in a dose-dependent manner (groups III and IV), with an effect similar to that attained by the standard hepatoprotective flavonolignan SYL. However, DTX at 100 mg/kg moderately diminished the CCl₄-induced elevated levels of TG and VLDL-C relative to SYL (Table 4).

As depicted in Table 5, the level of hepatic MDA, an end product of lipid peroxidation, was significantly increased

TABLE 5. TISSUE CONCENTRATIONS OF TOTAL PROTEIN, MALONDIALDEHYDE, AND NONPROTEIN SULFHYDRYL FROM LIVER AND KIDNEY OF THE CONTROL AND THE DIFFERENT TREATED GROUPS

Variables	Normal control	CCl ₄	CCl ₄ +DTX (50 mg/kg)	CCl ₄ +DTX (100 mg/kg)	CCl ₄ +SYL (10 mg/kg)
Liver					
TP (g/L)	110.97±7.21	51.09±2.52*** ^a	61.47±3.25 ^b	79.04±3.81*** ^b	82.63±4.04*** ^b
MDA (nmol/g)	0.85±0.15	8.39±0.39*** ^a	6.22±0.47*** ^b	4.06±0.37*** ^b	2.89±0.39*** ^b
NP-SH (nmol/g)	7.24±0.69	3.36±0.46*** ^a	4.02±0.50 ^b	4.85±0.33 ^b	7.03±0.52*** ^b
Kidney					
TP (g/L)	96.20±3.68	36.32±2.42*** ^a	40.31±2.42 ^b	59.88±3.33*** ^b	71.05±3.99*** ^b
MDA (nmol/g)	0.65±0.13	9.08±0.58*** ^a	6.08±0.70*** ^b	3.57±0.35*** ^b	2.27±0.22*** ^b
NP-SH (nmol/g)	9.61±0.41	4.95±0.40*** ^a	6.35±0.29 ^b	6.41±0.41 ^b	8.63±0.37*** ^b

All values represent mean±SEM (*n*=6). **P*<.05; ***P*<.01; ****P*<.001; ANOVA, followed by Dunnett's multiple comparison test.

^aAs compared with normal group.

^bAs compared with CCl₄ only group.

TP, total protein; MDA, malondialdehyde; NP-SH, nonprotein sulfhydryl.

FIG. 2. Photomicrography of hematoxylin–eosin-stained sections of normal rat liver (A), liver of a CCl₄-intoxicated rat (B), liver of a rat pretreated with 50 mg/kg DTX (C), liver of a rat pretreated with 100 mg/kg DTX (D), and of a rat liver pretreated with 10 mg/kg silymarin (SYL) (E). Color images available online at www.liebertpub.com/jmf

($P < .001$) in CCl₄-intoxicated rat liver compared with that in normal controls. However, pretreatment with DTX significantly ameliorated the abnormal levels of MDA in a dose-dependent manner. In the same experiment, DTX increased the level of TP diminished by CCl₄-intoxication in a dose-dependent manner. Similarly, DTX dose-dependently increased NP-SH level.

Effect of DTX on kidney-related serum and tissue markers

Administration of DTX at 100 mg/kg was found to significantly ($P < .001$) inhibit the CCl₄-induced high level of creatinine comparable to SYL. The inhibitory effect of DTX at 100 mg/kg on the CCl₄-induced elevated calcium level was also significant ($P < .001$). Moreover, DTX suppression of the CCl₄-elevated levels of uric acid and urea was found to be more responsive and significant ($P < .01$) at 100 mg/kg (Table 4).

In comparison with the CCl₄-intoxicated kidney, pretreatment with DTX significantly lowered the level of renal MDA, being more pronounced at 100 mg/kg (Table 5). Furthermore, the higher dose of DTX (100 mg/kg) exerted a significant ($P < .001$) increase in TP and a moderate elevation in NP-SH of pretreated kidney relative to those of control CCl₄-intoxicated kidney.

Effect of DTX on histopathological features of liver and kidney

Results of histopathological assessment of the hepatic tissue (Fig. 2) were found to be correlated with the above-mentioned biochemical findings. In the control CCl₄-intoxicated group, the lobular architecture of liver tissue was deformed and showed evidence of extensive pericentral vein necrosis and

fatty changes with ballooning of hepatocytes and infiltration of inflammatory cells. These alterations almost disappeared and liver parenchyma returned to its normal status in rats pretreated with DTX at 100 mg/kg or with SYL at 10 mg/kg, whereas DTX at 50 mg/kg exerted minimal fatty changes and necrosis.

The kidney in the control CCl₄-intoxicated group showed necrosis, loss of tubular details, degeneration/shrinkage of glomeruli and Bowman's capsules, and infiltration of inflammatory cells. Rats pretreated with DTX exhibited dose-dependent correction of renal injury as demonstrated by improved tubular and glomerular architecture and reduced inflammatory cells. Therefore, a better correction was attained by DTX at 100 mg/kg comparable to SYL at the dose of 10 mg/kg (Fig. 3).

DISCUSSION

Many dietary modifications and chemoprevention are considered to be effective approaches against the health hazards induced by oxidative stress. Various studies have shown that several xenobiotics cause generation of free radicals, which play a major role in initiation of oxidative stress-related diseases. Extensive *in vivo* studies on rodents have demonstrated that CCl₄ causes injuries to liver,³⁶ kidney,^{37,38} and many organs *via* production of electrophilic trichloromethyl ($\bullet\text{CCl}_3$) and peroxy trichloromethyl ($\bullet\text{OCCl}_3$) radicals.^{39,40} Production of such reactive free radicals from halogenated alkanes by the metabolizing activity of cytochrome P450 2E1 (CYP2E1) was reported to induce hepatotoxicity^{40,41} and nephrotoxicity.⁴² These free radicals initiate lipid peroxidation and protein deterioration^{39,43} with a subsequent alteration of cellular membrane

FIG. 3. Photomicrography of hematoxylin–eosin-stained sections of normal rat kidney (A), kidney of a CCl₄-intoxicated rat (B), kidney of a rat pretreated with 50 mg/kg DTX (C), kidney of a rat pretreated with 100 mg/kg DTX (D), and kidney of a rat pretreated with 10 mg/kg SYL (E). Color images available online at www.liebertpub.com/jmf

permeability and function, leakage of intracellular enzymes into serum,^{44,45} and other abnormal biochemical and histopathological changes in tissues.⁴⁶ Moreover, the increase in concentration of free peroxide radical and unsaturated fatty acid peroxides can induce alterations in the cholesterol profile and lipid metabolism along with induction of oxidative DNA damage, including the formation of DNA adducts and chromosomal alterations.^{47,48} Additionally, the level of NP-SH, for example, glutathione (GSH), the non-enzymatic part of the antioxidant defense, and antioxidant enzymes (SOD, GPX, and CAT) decrease in the tissues due to their rapid consumption after combatting free radical-induced oxidative stress.⁴⁹ Lipid peroxidation and damage of hepatocyte membranes initiated by the reactive oxygen species (ROS) generated by CCl₄ was reported to be followed by the release of a myriad of growth factors, inflammatory mediators, and prostaglandins from activated hepatic macrophages, which potentiate CCl₄-induced hepatic injury by further generation of a variety of ROS.⁵⁰ Therefore, inhibition and/or scavenging of intracellular ROS would play a critical role in preventing liver and kidney diseases.

Proanthocyanidins are the most abundant polyphenols in human diets and mainly composed of dimers, oligomers, and polymers of flavan-3-ols: (+)-catechin, (–)-epicatechin, and their gallic acid esters. They possess powerful antioxidant properties, and consequently therapeutic benefit against oxidative stress-related diseases.^{51,52} The antioxidant activity of proanthocyanidins is principally based on the properties of their phenolic hydroxyl groups, which serve as electron or hydrogen donors to terminate the free radical chain reaction yielding a stable phenolic radical.^{48,53} In this study we prepared a hydroacetone extract (DTX) from date seeds, which proved to be highly rich in proanthocyanidins as identified by vanillin reaction, and by HPTLC chromatographic and NMR spectroscopic analyses (Fig. 1 and Tables 1 and 3). Thus, DTX showed a powerful *in vitro* free radical scavenging capacity (Table 2). This prompted us to evaluate the protective potentiality of DTX against CCl₄-induced free radical-mediated hepatic and renal damage on the basis of biochemical and histopathological evidence.

This study revealed that CCl₄ significantly increased the levels of serum ALT, AST, GGT, and ALP, which indicated acute hepatocyte injuries, altered membrane integrity, and consequently enzyme leakage. However, on pretreatment with DTX, the pathological levels of these enzymes were remarkably restored (Table 4), indicating the ability of DTX to protect hepatocytes against the deleterious effect of CCl₄-derived free radicals. The CCl₄-induced enhanced lipid peroxidation, which is marked by high levels of MDA, was thus significantly normalized in liver tissue on pretreatment with this proanthocyanidin-containing extract (Table 5). Furthermore, the significant decrease in the serum levels of bilirubin indicated that bilirubin was rapidly and selectively taken up into the liver as a function of healthy hepatocyte membranes maintained on pretreatment with DTX relative to that of intoxicated control (Table 4).

Administration of CCl₄ significantly increased serum TG, TC, LDL-C, and VLDL-C while decreased serum HDL-C (Table 4), an effect that was found to be in agreement with previous studies.^{47,54} However, the increased serum concentrations of TC and LDL-C, and the decreased level of HDL-C, were found to be greatly ameliorated upon pretreatment with the proanthocyanidin-rich DTX. Studies with animal models reported that proanthocyanidins significantly decrease plasma concentration of TG (in hypotriglyceridemia), TC, and LDL-C along with an increase in HDL-C.⁵⁵ Moreover, Montagut *et al.*⁵⁶ concluded that proanthocyanidins may be largely responsible for inhibiting TG and Apolipoprotein B secretion (a marker of VLDL) by hepatic cells. It also was found that proanthocyanidins repress the expression of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, HMG-CoA synthetase, and other enzymes involved in cholesterol biosynthesis.⁵⁷ Proanthocyanidins also increase the CYP7A1 activity, which has a role in the transformation of cholesterol into bile acids. Finally, the insulin-like effect of proanthocyanidins in conjunction with the activation of farnesoid X receptor (FXR) may collaborate in the repression of VLDL secretion and the hypotriglyceridemia.⁵⁸

The present results also revealed that the concentration of TP in the serum and hepatorenal tissues were markedly decreased after CCl₄ challenge, in agreement with previous studies.^{17,54} The interpretation for the depletion of TP in CCl₄-intoxicated animals may be due to the relationship between the damage occurring to DNA *via* free radicals and consequently the protein expression. Therefore, due to the free radical scavenging properties of proanthocyanidins, pretreatment with DTX significantly increased these TP levels. Better levels of liver function-related enzymes and MDA along with those of TP and lipid profile in hepatic tissue have been achieved by administration of DTX in intoxicated animals. These findings indicated a preservation of biomembrane integrity, induction of protein synthesis, and stability of cholesterol metabolism in hepatocytes against free radical-induced oxidative stress, respectively. This subsequently led to a marked parallel restoration of the histological features of the liver (Fig. 2).

In this study, we also investigated the protective effects of DTX against CCl₄-induced nephrotoxicity on relevant oxidative stress parameters, including renal MDA and NP-SH levels, renal injury serum biomarkers (creatinine, uric acid, urea, and calcium), and renal histopathology. Nephrotoxicity was evidenced by a significant alteration of these parameters in the CCl₄-intoxicated group when compared with those of controls in the same way as previously reported.^{54,59} Pretreatment with DTX markedly decreased the elevated levels of creatinine, urea, uric acid, and calcium; significantly corrected the levels of tissue MDA and TP; and improved the histopathological features in renal tissue (Tables 4 and 5 and Fig. 3).

With regard to the antioxidant defense system, CCl₄ treatment significantly reduced the total content of NP-SH (*e.g.*, GSH), which is considered as an important

nonenzymatic antioxidant defense against lipid oxidative damage in the liver and kidney eliminating the hydrogen peroxide, peroxy, and hydroxyl radicals formed. Therefore, NP-SH-dependent enzymes such as glutathione peroxidase, reductase, and S-transferase (GPx, GR, and GST, respectively), along with other antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) will also be expected to be negatively affected following. In our study, the level of NP-SH content was partially restored in the hepatorenal tissues on pretreatment with DTX at dose 100 mg/kg/rat. However, another study proved that treatment with proanthocyanidins in a higher dose (400 mg/kg/rat) significantly increased GSH along with a marked restoration in antioxidant enzymes SOD, GPX, and CAT levels in CCl₄-treated rats.⁴⁹

The overall hepatorenal protection may be explained by many characteristics of DTX. DTX contained high amount of proanthocyanidins (49.0%CT±0.52%CT w/w), which conducted high antioxidant potential through their direct free radical scavenging activity,⁵³ as revealed by the *in vitro* DPPH assay, by the *in vivo* dose-dependent decrease of the lipid peroxide marker MDA, and by the increase of the nonenzymatic antioxidant NP-SH system. Proanthocyanidins can also effectively suppress CCl₄-induced cytosolic CYP2E1 expression and thus inhibit the CYP2E1 enzyme from generating free radicals from CCl₄, thereby preventing the cascade that causes oxidative stress and liver injury.⁴⁹ Moreover, proanthocyanidins have shown a strong ability to chelate iron, which has been reported to exert some cytoprotective effects, such as decreasing iron-mediated free radical formation.⁶⁰

In conclusion, administration of hydroacetone extract of date seeds was experimentally demonstrated to be safe, and remarkably protected against chemically induced hepatorenal injury. This protection could be attributed to the total phenol/proanthocyanidin content, which can suppress the oxidative stress induced by xenobiotic-generating free radicals with the subsequent restoration of the physiological and histological features of the susceptible organs. The results suggest that date seeds can serve as an invaluable and highly available source of natural antioxidants, food additives, or dietary supplements for increasing the quality of life. The evidence-based protective activity achieved in this preclinical study provides convincing evidence to support further clinical study of standardized date seed extracts in the treatment of oxidative stress-related liver and kidney diseases.

ACKNOWLEDGMENT

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no RGP-VPP-272.

AUTHOR DISCLOSURE STATEMENT

The authors have declared that there are no conflicts of interest.

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