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***In vivo* Antioxidative and Hepatoprotective Effects of Palm Date Fruits (*Phoenix dactylifera*)**

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Abstract: Palm date fruits have been used extensively for the traditional cure of liver illnesses and malaria in the Arab Peninsula. Therefore, the aim of the present study was to evaluate for the first time the *in vivo* hepatoprotective and the antioxidative effects of different varieties of palm date fruits in relation to their total phenolic contents and total flavonoids. This study was carried out using the CCl₄-induced liver injuries in rabbits for two different periods: 4 and 20 h after CCl₄-subcutaneous injection and oral administration of palm date syrups (5 g kg⁻¹ b.wt.). The antioxidative and hepatoprotective effects of palm date syrups during the first and second experimental periods (4 and 20 h) were very clear, since results of plasma ALT and AST and TBARS (from liver homogenate) of the control were significantly higher than those obtained from animals treated with syrups. The present findings do strongly recommend to increase the consumption of palm date fruits, especially in cases of liver diseases and for the prevention of other serious diseases such as cardiovascular and cancers.

Key words: Palm date fruits, *Phoenix dactylifera*, phenolic compounds, flavonoids, polyphenolic compounds

INTRODUCTION

Free radicals, such as, active oxygen species (O₂⁻, *OH or lipid peroxy radical, LOO*), cause oxidative damage to lipids, proteins, and nucleic acids and may lead to many biological carcinogenesis, mutagenesis, aging, atherosclerosis, neuro-degenerative diseases and stress-induced depression (Bilici *et al.*, 2001; Sapolsky, 2000). On the other hand, natural antioxidants, which neutralize free radicals, have been received more attention by nutritionists and medical researchers for their potential effects in the prevention of chronic and degenerative diseases, such as cancer and cardiovascular disease as well as aging (Diaz *et al.*, 1997; Young and Woodside, 2001).

Antioxidant is any substance that present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate, including every type of molecules found *in vivo*. It was found that phenolic and polyphenolic compounds are very efficient scavengers of free radicals

(Halliwell, 1994), because of their molecular structures, which include an aromatic ring with hydroxyl groups containing mobile hydrogen. Moreover, the action of phenolic compounds can be related to their capacity to reduce and chelate ferric ion, which catalyze lipid peroxidation (Gazzani *et al.*, 1998a). Flavonoids are phenolic compounds that belong to the recently popular phytochemicals, i.e., chemicals derived from plant material with potential beneficial effects in human health. The study by Chen *et al.* (2004) showed that the polyphenols in green tea reduce the severity of liver injury in association with lower concentrations of lipid peroxidation and proinflammatory nitric oxide-generated mediators.

Palm dates (*Phoenix dactylifera* L. Areaceae) are considered to be an important fruits for most of population in the Middle-East countries. Global date's production is more than 5 million tones per year and most of this production comes from the Arab World (>80%). This fruit has great importance from nutritional (Hasan *et al.*, 2010) and economic points of view

(Mansouri *et al.*, 2005). Sugars contribute the most prevalent single component (Eltayeb *et al.*, 1999; Mrabet *et al.*, 2008), since the total sugars (at the tamr stage) on dry weight bases is about 75-80%. Date syrup (or dibs) is probably the most common derived date product and there are some efforts to broaden the field of application for date syrup as an ingredient, which can be used for beverages, in confectionery, bakery products and ice cream. It was also found that dates syrup could replace honey by up to 75%. Muslims all over the world break their fasting during Ramadan by taking palm date fruits as an ethereal rule. The study by Hong *et al.* (2006) showed that palm date fruits at their different stages of maturity contain thirteen flavonoid glycosides of luteolin, quercetin, and apigenin. In addition, procyanidin oligomers through decamers were identified (Hong *et al.*, 2006; Biglari *et al.*, 2009). In traditional medicine, this fruit is highly recommended to be consumed by people suffering from jaundice and by women before and after delivery. In addition, the *in vitro* studies have shown that this fruit possesses antioxidative and antimutagenic properties (Vayalil, 2002; Ishurd *et al.*, 2007; Biglari *et al.*, 2008).

The hepatotoxic effects of CCl₄ are related to its active metabolite trichloromethyl radical, [•]CCl₃, which binds covalently to macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides and the lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl₄ (Muriel and Escobar, 2003). This is evidenced by an elevation in the serum marker enzymes namely AST, ALT, ALP, total bilirubin and GGTP.

Palm date fruits could be one of the cheapest and safest sources of natural antioxidant compounds in Arab World and other countries in the Middle East. The aim of the present work is to evaluate *in vivo* the antioxidative property and the hepatoprotective effects of different types of palm dates in relation to their total phenolic contents and total flavonoids.

MATERIALS AND METHODS

Palm date samples: Three different varieties of palm dates (*Phoenix dactylifera* L.) were obtained from the local market. Two of them were date-tamr (100% soft-brown color) namely, Saudi (Khodairi) and Iraqi (Barhi), while the third sample was Yemeni date-rotab (50% soft brown color and 50% hard yellow color) from Tihamah. All samples were in good conditions.

Animals: Twenty five healthy white New Zealand rabbits weighing (800-1000 g) were obtained from the farm of Agricultural Faculty, Sana'a University. They were fed a balanced diet to fulfill their nutritional requirements and tap water was offered *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee.

Chemical reagents: Thiobarbituric Acid (TBA), D-catechin, quercetin and 1, 1, 3, 3-tetraethoxypropane, were obtained from Sigma, while ethanol (99.9%), CCl₄, n-butanol, KCl, phosphoric acid, sodium tungstate, sodium molybdate, lithium sulfate, hydrochloric acid (37%), sodium carbonate, AlCl₃, liquid bromine and CH₃COOK, were obtained from BDH. The Folin-Ciocalteu phenol reagent was prepared according to the method described as follows: 25 g of sodium tungstate (Na₂WO₄) and 6.25 g of sodium molybdate (Na₂MoO₄) were added to 175 mL of distilled water. Into the mixture 12.5 mL (85%) of phosphoric acid (H₃PO₄) and 15 mL of concentrated HCl were further added and refluxed on a sand-bath for 10 h. Then, 37.5 g of lithium-sulphate (Li₂SO₄), 50 mL of distilled water and 5 drops of liquid bromine were added. Excess bromine was removed by boiling the solution for 15 min, then diluted with distilled water to 1 N strength with respect to standard alkali.

Preparation of palm date syrups: All dates washed with tap water, then the seeds were removed. A portion of each sample was weighted (300-400 g) and 1200 mL of the extracting solvent (80% ethanol) was added. Then the extraction was carried out at 80°C for about 30 min and the extract was filtered through cotton wool. The residue was extracted again by 1000 mL of distilled water for about 5 min and then left for overnight in the fridge and filtered through cotton wool plug in the neck of filter funnel. The two extracts were combined, filtered on Buchner funnel and evaporated by using rotary evaporator apparatus under vacuum at 40°C. The evaporation was continued until no more water can be distilled. The obtained heavy syrups were weighed (73% for Rotab and 80% for Iraqi, and 78% for Saudi) and stored at -18°C to be used for further studies.

Measurement of total phenolic compounds in date palm syrups expressed as D-catechin equivalent (mg/100 g date palm): Five gram of each syrup was initially dissolved in some amount of distilled water and the volume was then adjusted to 25 mL. The total phenolic contents were measured according to the method described by Singleton and Rossi (1965). Briefly, 0.1 mL of the solution was added to 0.5 mL Folin-Ciocalteu

reagent, mixed for 1 min and 1 mL sodium carbonate solution (0.08 g mL^{-1}) was added. The volume was then adjusted to 2 mL with distilled water and mixed. The mixture was left for one h at room temperature in a dark place and the absorbance was measured at 760 nm using UV/VIS spectrophotometer (Shimadzu, UV-1601). Measurements were made in triplicates. The calibration curve of D-catechin was prepared by using a concentration from 50 to 400 $\mu\text{g}/100 \text{ mL}$. The concentration of each sample was calculated from the D-catechin standard curve. The total phenolic compounds were expressed as D-catechin equivalent in $\text{mg}/100 \text{ g}$ palm dates syrup.

Measurement of total flavonoids in dates palm syrups expressed as mg quercetin equivalent (mg/100 g dates palm): Aluminum chloride colorimetric method was used for flavonoids determination (Chang *et al.*, 2002). Therefore, 0.1 mL of each syrup (10 mg mL^{-1}) in methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% AlCl_3 , 0.1 mL of 1 M CH_3COOK and 2.8 mL of distilled water and kept at room temperature for 30 min. The Abs. of the reaction mixture was measured at 415 nm. A calibration curve of quercetin was prepared by using concentration from 12.5 to 100 $\mu\text{g mL}^{-1}$ in methanol and the total flavonoids were expressed as quercetin equivalent ($\text{mg QE}/100 \text{ g}$ palm dates syrup).

Treatment of animals with palm dates syrups and carbon tetrachloride induced liver injury: Twenty five healthy white New Zealand rabbits (800-1000 g) were fasted for ~18 h. They were divided to 5 groups, 5 animals per each group. The first, second and third group were given orally palm dates syrups (5 g kg^{-1} body weight) suspended in distilled water, namely Rotab (Tehamah-Yemen), Iraqi and Saudi palm dates, respectively. The fourth and fifth groups were used as positive control (injected by CCl_4 , but not given palm dates syrups) and negative control (neither injected by CCl_4 nor given palm dates syrups), respectively. One hour later, the treated groups (first, second and third group) and the positive control group, were injected subcutaneously by CCl_4 diluted in olive oil (10% v/v), while the fifth group (negative control) was not injected by CCl_4 , because they were used to know the normal range values of different measured parameters. After 4 h, the animals were anesthetized by ether. Animals were cut-opened longitudinally. Blood samples were collected from the heart for enzymes assays and the liver from each rabbit was removed immediately for the measurement of TBA-RS. The same steps were followed to carry out the second experiment, but the blood and liver samples were collected after 20 h of treatments.

Measurement of enzyme activities: Blood samples were collected from the heart by syringe containing a drop of 10% EDTA solution to prevent blood coagulation. The plasma was obtained by centrifugation (Gallenkamp centrifuge 200) at 2000 rpm for 10 min and stored frozen until required. Plasma was assayed for AST and ALT according to the method recommended by the IFCC using enzymatic kits (SPINREACT, S.A. Ctra. Santa Coloma, Spain). The rate of NADH disappearance was determined spectrophotometrically, since it is directly proportional to the activity of AST or ALT in the sample.

Preparation of liver homogenate: A portion of each liver was weighed and homogenized in 10 folds weight of ice-cold 0.15 M KCl solution. The liver homogenate was used to measure the formation of TBA-RS as an indication to the extent of lipid peroxidation in the liver.

Measurement of thiobarbituric acid reactive substances (TBARS) formed in rabbit liver: The TBARS values were obtained as described by Yoshikawa *et al.* (2002) with slight modifications. Briefly, 3 mL of 0.1 M phosphoric acid and 1 mL of 0.04 M 2-thiobarbituric acid aqueous solutions were added to 0.5 mL of the liver homogenate in 10 mL centrifuging tube. The mixture was heated for 60 min. In a boiling water bath. After cooling, 4 mL of n-butanol was added and mixed vigorously. Then the n-butanol phase was separated by centrifugation (3000 rpm, 10 min., 4°C) and absorbance was measured at 535 nm and 520 nm, the difference between the absorbance at 535 nm and that at 520 nm was used as the TBARS value. The thiobarbituric acid reactive substances value was expressed in terms of malondialdehyde ($\mu\text{mol g}^{-1}$ of wet tissue), using 1, 1, 3, 3, tetraethoxypropane as an external standard.

Statistical analysis: Differences in the enzymes activities and TBA-RS between groups were tested by one-way analysis of variance followed by the Tukey's test to find differences between groups using SPSS 13.0 Software. Significant level was taken at $p < 0.05$ unless otherwise indicated.

RESULTS AND DISCUSSION

To our knowledge this is the first *in vivo* study about the hepatoprotective and antioxidative effects of palm dates against CCl_4 -induced liver injury in rabbits. Therefore, the results in Table 1 and 2 show the activities of liver enzymes ALT and AST as an indication to the extent of hepatotoxicity, and the formation of TBARS as a marker of lipid peroxidation in the liver and how these

Table 1: Preventive effects of date palm syrups in relation to their total phenolic contents and total flavonoids on CCl₄-induced liver injury in rabbits (4 h after CCl₄ administration)

Treatment	ALT (U L ⁻¹)	AST (U L ⁻¹)	TBARS (μmol g ⁻¹ LW)	T.Ph ------(mg/100 g syrup)-----	T.Fl	T.Fl (%)
Rotab	45.92±2.88 ^f	152.48±2.59 ^f	0.990±0.014 ^f	769.6±7.2 ^a	554.0±8.7 ^a	71.9
Iraqi	62.76±1.36 ^g	202.85±9.31 ^b	1.189±0.027 ^b	434.3±1.8 ^c	310.5±2.8 ^c	71.6
Saudi	52.67±2.89 ^e	183.48±8.36 ^b	0.997±0.011 ^e	600.3±4.0 ^b	372.7±1.7 ^b	62.1
Control (+)	235.65±4.77 ^a	261.04±7.09 ^a	1.570±0.025 ^a	0.0	0.0	
Control (-)	48.30±0.77 ^e	35.62±0.92 ^d	0.965±0.015 ^e	0.0	0.0	

LW: Liver weight, BW: Body weight, T.Fl: Total flavonoids, T.Ph: Total phenols, Control (+): The animals were subjected to CCl₄-subcutaneous injection, but they were not given date palm syrups. Control (-): The animals neither subjected to CCl₄-subcutaneous injection nor given date palm syrups. Each value represents the Mean±SD, Values in the same column carrying different letters are significantly different (p<0.05)

factors are affected by the palm date syrups after 4 and 20 h of CCl₄ and syrups administration. Therefore, the outcome of the present work, allowed us to extract some important information about the hepatoprotective and antioxidative effects of palm date fruits. Results in Table 1, revealed the extent of hepatoprotective and antioxidative effects of palm date syrups within the period of 4 h after CCl₄ subcutaneous injection and palm dates syrups administration. The enzyme activities of ALT and AST (U L⁻¹) and the formed amount of TBARS (μmol g⁻¹ LW) from animals of all treatments were significantly lower than those obtained from the animals of the positive control (Table 1). In other words, the hepatoprotective effects of palm dates syrups can be easily predicted from the enzymes activities (ALT and AST). Because the enzymes activities obtained from animals of all treatments and negative control were almost similar, but they were significantly lower than those obtained from animals of the positive control (Table 1). In addition, the activity of ALT resulted from animals of the positive control was approximately 4-5 folds higher than those results obtained from treated and negative control animals. The extent of the hepatic damage is assessed by the level of increased cytoplasmic enzymes (ALT and AST) in circulation. Thus, it is well known that hepatocytes are damaged by CCl₄ and cytosolic enzymes in the injured hepatocytes are leaked out of the cells due to an increase in cell membrane permeability, cell damage and necrosis (Tezuka *et al.*, 1995). The results of TBARS accumulated during the period of 4 h of treatment are shown in Table 1 and they indicate to strong antioxidant activities of palm dates syrups, since all treatments suppressed the TBARS formation significantly as compared with that value obtained from the positive control. These findings suggest that the hepatoprotective and antioxidative effects of the palm date syrups could be due to their phenolic contents, because many phenolic and polyphenolic compounds have been reported to have hepatoprotective and antioxidative effects (Chen *et al.*, 2004; Yoshikawa *et al.*, 2002; Hewawasam *et al.*, 2004;

Essa and Subramanian, 2008; Al-Naqeeb *et al.*, 2009; Bitiren *et al.*, 2010) against CCl₄-induced lipid peroxidation. In addition, the study by Chen *et al.* (2004) has shown that phenolic compounds reduced the severity of liver injury (Krishna *et al.*, 2009; Elzaawely and Tawata, 2012) in association with lower concentration of lipid peroxidation. Since 'CCl₄ free radical, which might be resulted from CCl₄ by an enzymatic reaction in liver microsomal cytochrome P₄₅₀ (Miyazawa *et al.*, 1990) causes consecutive lipid peroxidation of the cell membrane and endoplasmic reticulum and the peroxidative products induce hypofunction of the membrane and finally cytosolic enzymes release to blood. Therefore, lipid peroxidation of hepatocytes has been recognized to be a major factor in the liver injury model.

From the present study, it seems feasible to explore the biological activities of all investigated palm date fruits in relation to the total phenolic contents and total flavonoids. Therefore, as Iraqi palm date fruits contain the lowest total phenolic contents and total flavonoids, so this might be the reason for its less suppression of hepatotoxicity and antioxidant activity as noticed from the highest values of ALT, AST and TBARS obtained from animals treated by this type of palm date syrup in comparison with those results obtained from animals treated by Rotab and Saudi palm date syrups (Table 1). The present values of TBARS as an indication to the antioxidative effect are in agreement with those findings obtained by Yoshikawa *et al.* (2002) and Tezuka *et al.* (1995) when they studied the preventive effects of natural phenolic constituents on CCl₄-induced liver injury in mice. The flavonoids-health properties are due to their peculiar chemical structures, as they are very reactive towards Reactive Oxygen Species (ROS), because of their electron deficiency (Clifford, 2000; Espin *et al.*, 2000).

Results in Table 2, were obtained from the study that was carried out during the longer period (20 h) of treatments. The enzyme activities of ALT and AST and the TBARS from all treatments and the positive control were increased dramatically and highly significant in comparison with the normal value. However, all palm date

Table 2: Preventive effects of date palm syrups in relation to their total phenolic contents and total flavonoids on CCl₄-induced liver injury in rabbits (20 h after CCl₄ administration)

Treatment	ALT (UL ⁻¹)	AST (UL ⁻¹)	TBARS (μmol g ⁻¹ LW)	T.Ph	T.Fl	T.Fl (%)
				------(mg/100 g syrup)-----		
Rotab	482.7±10.0 ^b	1917.4±32.3 ^c	1.606±0.041 ^c	769.6±7.2 ^a	554.0±8.7 ^a	71.9
Iraqi	550.6±22.3 ^b	2310.3±16.3 ^b	2.370±0.024 ^b	434.3±1.8 ^c	310.5±2.8 ^c	71.6
Saudi	485.7±9.4 ^b	2237.4±60.9 ^b	1.619±0.007 ^c	600.3±4.0 ^b	372.7±1.7 ^b	62.1
Control (+)	627.7±21.7 ^a	2509.1±32.7 ^a	4.683±0.203 ^a	0.0	0.0	
Control (-)	048.30±0.77 ^e	0035.62±0.92 ^d	0.965±0.015 ^c	0.0	0.0	

LW: Liver weight, BW: Body weight, T.Fl: Total flavonoids, T.Ph: Total phenols, Control (+): The animals were subjected to CCl₄-subcutaneous injection, but they were not given date palm syrups. Control (-): The animals were not subjected to CCl₄-subcutaneous injection and they were not given date palm syrups, Each value represents the Mean±SD. Values in the same column carrying different letters are significantly different (p<0.05)

syrups showed to have longer hepatoprotective effect against hepatotoxicity and lipid peroxidation as noticed from ALT, AST and TBARS values obtained after 20 h of treatments (Table 2) with the emphasis that Rotab revealed to possess the strongest hepatoprotective effect and antioxidant activity in comparison with other palm dates treatments. On the bases of new findings, palm date fruits can be arranged according to their total phenolic contents, total flavonoids, hepatoprotective effect, and antioxidative property as follows: Rotab>Saudi>Iraqi palm date fruits.

As a whole, the very high enzyme activities of ALT and AST obtained from the positive control indicate severe liver injuries in rabbits due to the subcutaneous administration of CCl₄, but the lower values of the enzyme activities when the palm dates syrups were administered orally, might be resulted due to the hepatoprotective effects of these syrups since they contain phenolic and flavonoid compounds (Annegowda *et al.*, 2010) (Table 2), which are known to be strong antioxidants (Gazzami *et al.*, 1998a, b; Mansouri *et al.*, 2005; Biglari *et al.*, 2008; Biglari *et al.*, 2009; Al-Mamary *et al.*, 2010). Generally, the hepatoprotective effect of palm date fruits, may be mediated through combination of multiple mechanisms, such as reported antioxidant, free radical scavenging, anti-inflammatory, calcium channel blocking and/or microsomal enzymes inhibitory action. In addition, the study by Vayalil (2002) has shown that the aqueous extract of palm dates had antioxidant activity, even though this study did not measure the phenolic compounds, but the antioxidant activity was related to the presence of compounds with potent free radical scavenging activity. Another study by Mansouri *et al.* (2005) has shown that different varieties of Algerian ripe palm date fruits were found to contain mainly p-coumaric, ferulic and sinapic acids and some cinnamic acid derivatives. Three different isomers of 5-O-caffeoylshikmic acids and different types of flavonoids, mainly flavones, flavanones and flavonolglycosides were identified (Mansouri *et al.*, 2005). These compounds were also identified in different natural sources and have shown to have strong antioxidant activities (Li *et al.*,

2009; Ozyurek *et al.*, 2008). The results of TBARS (Table 2) obtained after 20 h of treatment showed that animals from the positive control were extensively subjected to lipid peroxidation, since the TBARS value was about 4.5 folds higher than that obtained from animals of the negative control and 1.9-2.8 folds higher than those values obtained from animals treated with palm date syrups.

Generally, it seems that the hepatoprotective roles of palm dates syrups were very effective in the short period (4 h) of treatments as observed from the ALT and AST activities and the TBARS formation. These results are in agreement with those obtained by other researchers, who indicated that natural antioxidants present in dietary plants, particularly flavonoids and polyphenols towards tissue injury mediated by ROS (Duthie *et al.*, 2000; Mann, 2002; Nijveldt *et al.*, 2001). Thus, the results in Table 1 are suggestive to the hepatoprotective effect of palm date fruits, since the plasma ALT activities from animals treated by palm date syrups almost were not affected during the short period (4 h), unlike that of plasma AST activities, which show moderate increases in the same period (Table 1). Therefore, these moderate increase in AST as compared with that of ALT could be explained on the bases that, in addition to the liver, the AST exists in high concentrations in a large number of tissues, such as the heart, kidneys, skeletal muscle and pancreas, whereas the ALT is primarily limited to the cytosol of hepatocytes and the latter is considered to be a highly sensitive marker of hepatocellular damage and within limits can provide a quantitative assessment of the degree of damage sustained by the liver. It may also be of value with acute liver damage, especially when the damage is severe enough.

The present *in vivo* findings showed the hepatoprotective and antioxidative effects of palm date syrups, thus it is strongly recommended to increase consumption of palm dates, which may have positive potential health effects. Further work is required to isolate and purify the active principle compounds and determine which one is responsible for these physiological activities.

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