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Acute and Sub-Chronic Toxicity Evaluation of Aqueous Extract of *Phoenix Dactylifera* Seeds *in* Wistar Rats

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A B S T R A C T

Date, *Phoenix dactylifera,* is a commonly used food in arid and semiarid regions of the world including Iran. Date seeds, are low-cost agriculture by-products and used for several medicinal uses and animal food, however, there are no records on its likely toxic effects. To investigate the safety of *P. dactylifera*, we investigated its acute and sub-chronic toxicity in Wistar rats in both sexes. Animals were treated with four different single doses (5g/kg) of *P. dactylifera* extract oral gavage (per oral, p.o.), and checked for symptoms of toxicity two weeks after dispensation. In the sub-chronic toxicity study, *P. dactylifera* seeds were administered in three different doses (500, 1000, 1500 mg/kg) through 45 days. Fatality, clinical symptoms, body weight alteration, gross findings, organ weights, hematological and biochemical criteria, and histological specifications were screened all along the study. We found no fatality or morbidity in clinical manifestations, corpse weight, or necropsy evidence in none of the rats in the acute study. Regarding the outcome of the sub-chronic assay, a meaningful decline in urea concentration in male animals and no significant differences observed in other hematological criteria in both sexes. No significant histopathological difference in lung and heart tissue of different groups were observed but there are kidney cortex congestion and hepatocyte focal degeneration in female and male rats taking elevated doses (1500 mg/kg) of palm kernel extract, respectively. Analyses of the obtained results with the information of signs, behaviour, and health monitoring could lead to the conclusion that the no observed adverse effect level of *P. dactylifera* was defined to be 1000 mg/kg for male and female rats.

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Introduction

Phoenix dactylifera, date palm, is a monocotyledon plant from Arecaceae family [1]. Since ancient times, both fruits and seeds of dates have been used in the multiple folk medicines [2, 3]. Dates are used traditionally to treat hypertension and diabetes [4]. In ancient Egypt, the date was an important ingredient in different aphrodisiac and tonic agents. There was a belief about the indication of date palm pollen and the male flowers in aphrodisiac and enhancing fertility [2, 5]. Dried dates are also used in Ayurveda, the traditional Indian system of medicine, as expectorant, laxative, emollient and diuretic [2, 5].

It is believed that consumption of dates is highly tonic, sexual enhancer, preventive of premature graying of hair, wrinkle formation and to give the skin a glowing healthy look. The date kernels have also been reported to show anti-aging properties [6]. For pregnant and lactating mothers the boiled date pulp in milk or with almonds, quince seed, pistachio nuts, spices, and sugar is used as a body booster [7,8]. The increment of gums hardening in infants with teething complication is another folk indication of date fruits [9].

The consumption of date boiled with black pepper and cardamom is supposed to alleviate headaches, dry coughs, lethargy, mild fever and loss of appetite [9].

Several pharmacological effects are reported from the fruits such as antifungal [1, 10-12], antihyperlipidemic [13], anti-inflammatory [14], anti-cancer [15], nephroprotective [16], gastrointestinal, hepatoprotective [17-19], anti-oxidant [20, 21], anti-mutagenic [20], antibacterial [22] and antiviral [23].

Animal studies also proved anti-inflammatory [24, 25], gastrointestinal protective [26, 27], antihyperlipidemic [28], hepatoprotective [29], nephroprotective [30], anticancer [31], immunostimulatory [8] and gonadotropic [32] activities.

Date seed mostly consists of fatty acids such as capric, lauric and myristic acids [33-34]. Date fruit itself consist of moisture, fat, ash, protein, amino acids, alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, carbohydrates, fructose, glucose, sucrose, fiber, minerals like magnesium (Mg), sodium (Na), calcium (Ca), phosphorus (P), potassium (K), manganese (Mn), ferrous (Fe), zinc (Zn), selenium (Se), Vitamin A, B group, C, carotenoids, xanthins, phenolics and anthocyanins [34-35].

In recent years, many medicinal plants are used but probable toxicity and side effects have not been considered sufficient in uses. Although side effects of phytotherapeutic agents are less than that of synthetic drugs, herbal medicine is not completely safe [36-37]. There is not enough research about toxicity and side effects of palm seed, so we decided to evaluate the acute and sub-chronic toxicological evaluation of *P. dactylifera* seed total extract in Wistar rats.

Materials and methods

Plant material

Date seeds were collected from Bandar Abbas in Hormozgan province in April 2015 at an altitude of 9 meters from sea level.

Extraction of date seeds

750 g of ground seeds was extracted with deionized water for 45 min (5 L × 1). The extract was concentrated to 1/5 of the first solution to get a dark brown liquid, which was kept in -20 °C until use.

Experimental Animals

Male and female Wistar rats were obtained from the Central Animal Facilities of Kermanshah Medical Sciences University, separated according to gender and kept three per plastic cage. They were about eight weeks old at the beginning of the study. All animals were visually checked at the time of delivery and during the acclimatization; those found inappropriate were excluded.

All the animals were kept in definitive circumambient (23 ± 2°C, 12:12 h dark/light cycle, constant air flow) and access to tap water and

food *ad libido*. All procedures used in this study followed the "Principles of Laboratory Animal Care" from NIH Publication No. 85-23 and were approved by the Animal Ethics Committee of Kermanshah University of Medical Sciences.

Acute oral toxicity study

Rats of both sexes (12 males, 12 females), aged 6–8 weeks, old, weighing between 120-150 g, were used. Rats were divided into two groups of six animals in each sex and adjusted for one week before starting the practice. They were treated as follow: the control group (of both sexes) was treated with water, while another group received seed dates solution at the doses of 5 g/kg, via gavage. Animals were observed for general behavioral change, body weight change, and mortality immediately after the administration and then for a period of 14 days post-treatment. At the end of the experiment, the rats were anesthetized anesthetized with di-ethyl ether in the laboratory. The organs were excised, weighed, and examined macroscopically. The relative organ gross was (weight of organs as a fraction of the overall body weight of each rat) as then calculated.

Sub-chronic oral toxicity in rats

Rats of both sexes (20 male, 20 female), aged 4–6 weeks old, weighing between 130-150 g, were used. They were randomized into 4 groups of 5 animals and acclimatized for one week before starting the experiment. They were treated as follow, the first group was the control group and received deionized water while the other groups each were administered 500, 1000, 1500 mg/kg of aqueous extract of *P. dactylifera* seeds via gavage each day for 45 days. The date seed extract was freshly prepared with deionized water on daily basis. The behavior (salivation, fur, lethargy, and sleep) of the animals was observed daily, and they were weighed twice weekly.

Laboratory tests

At the end of the experiment, animals were fasted overnight, but with free access to water. After anesthesia with ether, blood was collected by a retro-orbital puncture [38] using capillary tubes, with and without ethylene diamine tetraacetate as an anticoagulant, for hematological and biochemical studies, correspondingly. The following hematological factors were determined with the Sysmex K-1000 fully automated hematology analyzer: erythrocyte (RBC), total and differential leukocyte (WBC), hematocrit (Hct), hemoglobin (Hb), platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC). Blood samples for biochemical analyses were centrifuged at 3,000 rpm for 5 min, and the plasma was collected and analyzed for fast blood sugar,, creatinine, urea, alkaline phosphatase (ALP), triglycerides (TG), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and lactate dehydrogenase (LDH) using a COBAS Mira S chemistry analyzer (Roche Diagnostic Systems, West Sussex, England).

Histopathological examination

The animals were killed with an overdose of ether [39] following sacrificing the treated and control rats, the heart, kidneys, liver, spleen, and lungs were removed and weighed. Isolated organs were preserved in 10% neutral-buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically for gross and microscopic pathological abnormalities.

Statistical analysis

The standard error of mean (SEM) was calculated for body weights, organ/body weight ratios, hematological and biochemical factors. The difference between dose groups and control groups was separately evaluated for males and females with a one-way analysis of variance

(ANOVA) followed by Tukey's test. P-values of 0.05 or less were considered to be significant.

Results

Acute study

All of the rats receiving total extracts of *P. dactylifera* seeds were alive for all 14 days of observation. Normal body weight gains were observed either in males and females rats receiving different doses (Figure 1 and 2). No abnormal gross findings were observed in any of the animals at doses up to 5 g/kg.

Subchronic study

Evolution of animal weight and organ weights

No death was observed in any of the groups throughout the experimental period, and no abnormality was found in any of the animals. Moreover, there was no significant difference in body weights between the control and treatment groups (Figures 3 and 4). Organ weights measured at necropsy, similarly, no significant changes were observed in any of the organs evaluated (Table 1).

Hematological parameters

The results of the hematological study including the number of lymphocyte, monocyte, neutrophil, eosinophil, basophil and etc. are shown in Table 2. No treatment-related changes in hematological parameters were observed during the study period. Although some alterations in blood parameters among our treated groups were detected, but these difference were not statistically significant. For example, the male and female groups that received 1000 mg/kg of the extract had higher WBC level than the control group.

Biochemical parameters

Biochemical parameter evaluation showed a slight but significant decrease in urea levels in male rats (Table 3). No significant differences were observed between the vehicle control and *P. dactylifera* treatment groups in the other biochemical parameters, such as blood glucose, ALP, TG, LDH, SGOT, SGPT, and creatinine.

Organ weight

Relative organ weights of 45-day treated rats are shown in Table 1. The relative isolated organ weight of rats treated with *P. dactylifera* seed extract did not show a significant difference compared to the control group in subchronic toxicity study.

Histopathological examinations

As shown in the figure 4, histopathological examinations showed mild pneumonia with mononuclear cells infiltration in the lung. No changes were observed in any of the other parameters evaluated, including alveolar collapse, septal thickness, intra-alveolar hemorrhage, alveolar edema and exudates in the lumen of the bronchus, congestion, alveolar collapse, intra-alveolar neutrophil counts and fatty changes in the lung (Figure 4).

Moreover, kidney cortex congestion (Figure 5) and hepatocyte focal degeneration were shown in the female and male rats, respectively, receiving high doses (1500 mg/kg) of *P. dactylifera* seed extract. (Figure 6). No histological findings in the spleen or heart could be attributed to the *P. dactylifera* seed (Figure 4).

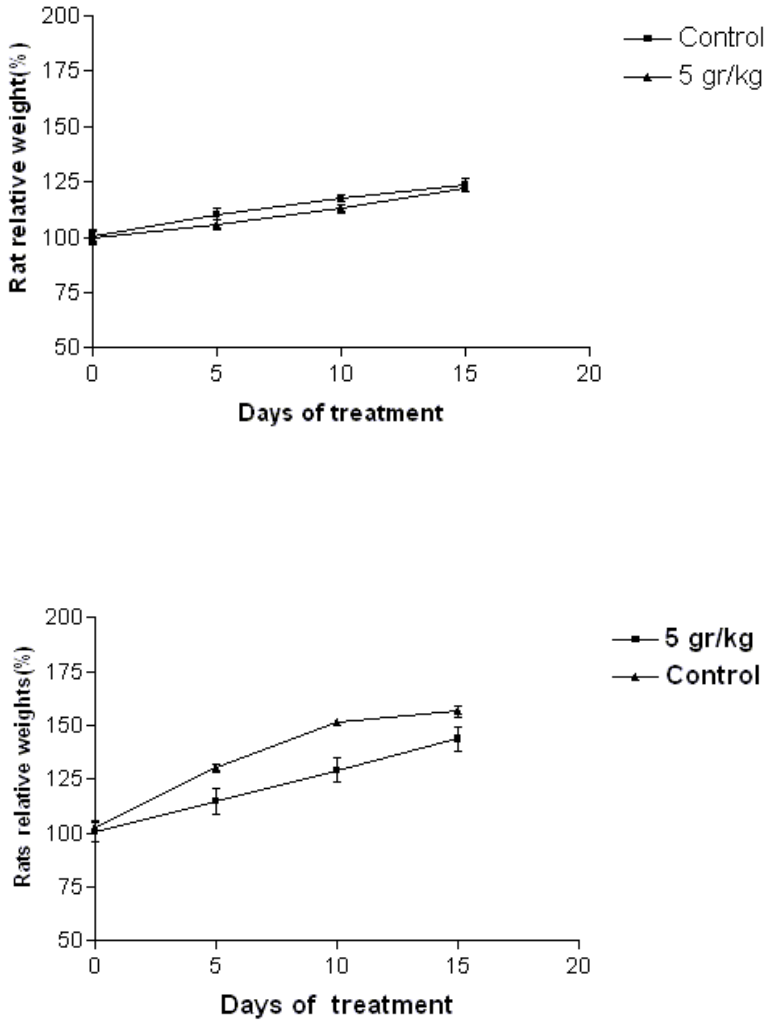

Fig. 1. Changes in A) Female and B) Male rats body weight with duration of acute treatment. Each point represents mean±SEM. N=5.

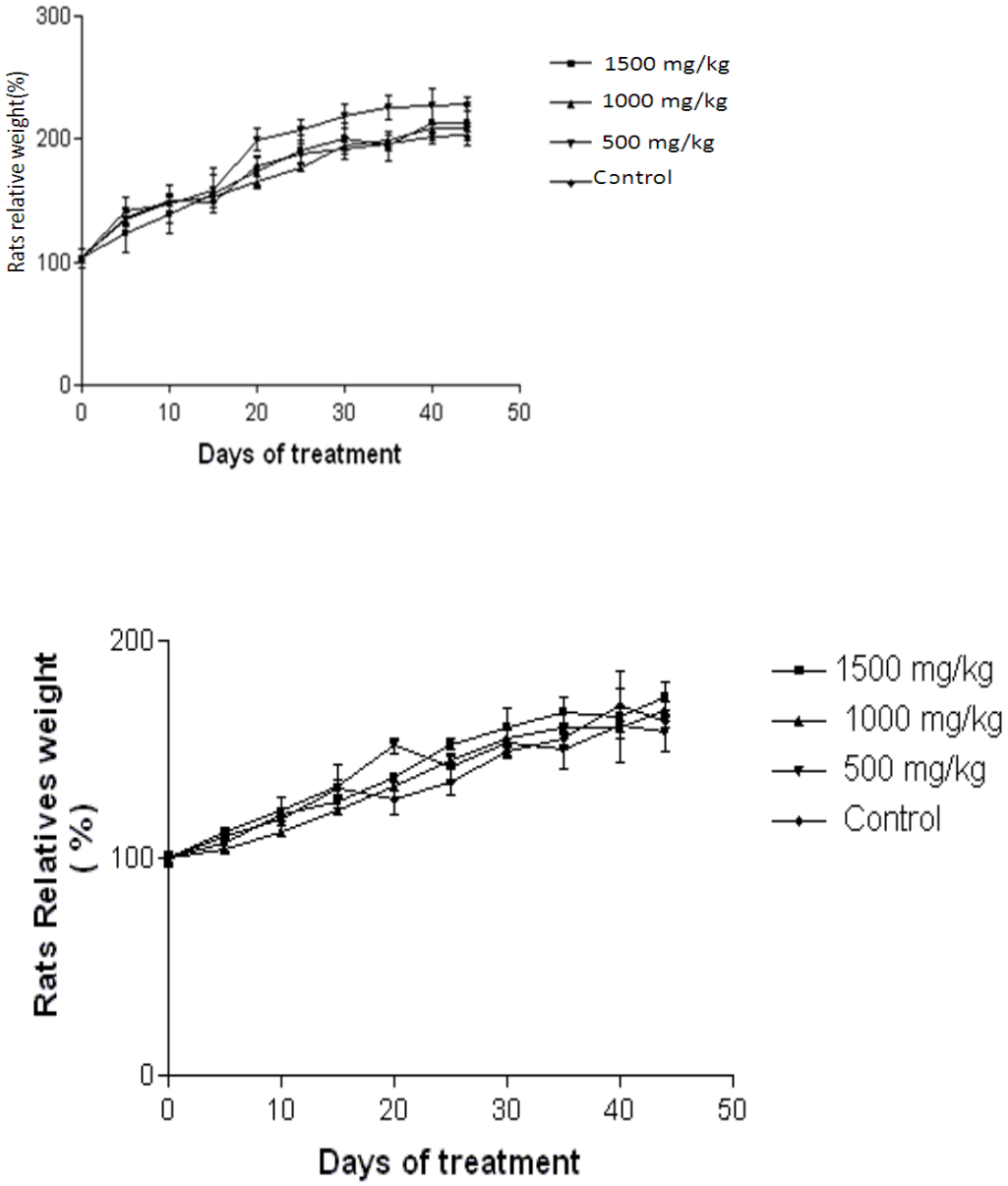

Fig. 2. Changes in A) male and B) female rat body weights with duration of sub-chronic treatment. Each point represents mean±SEM. N=5.

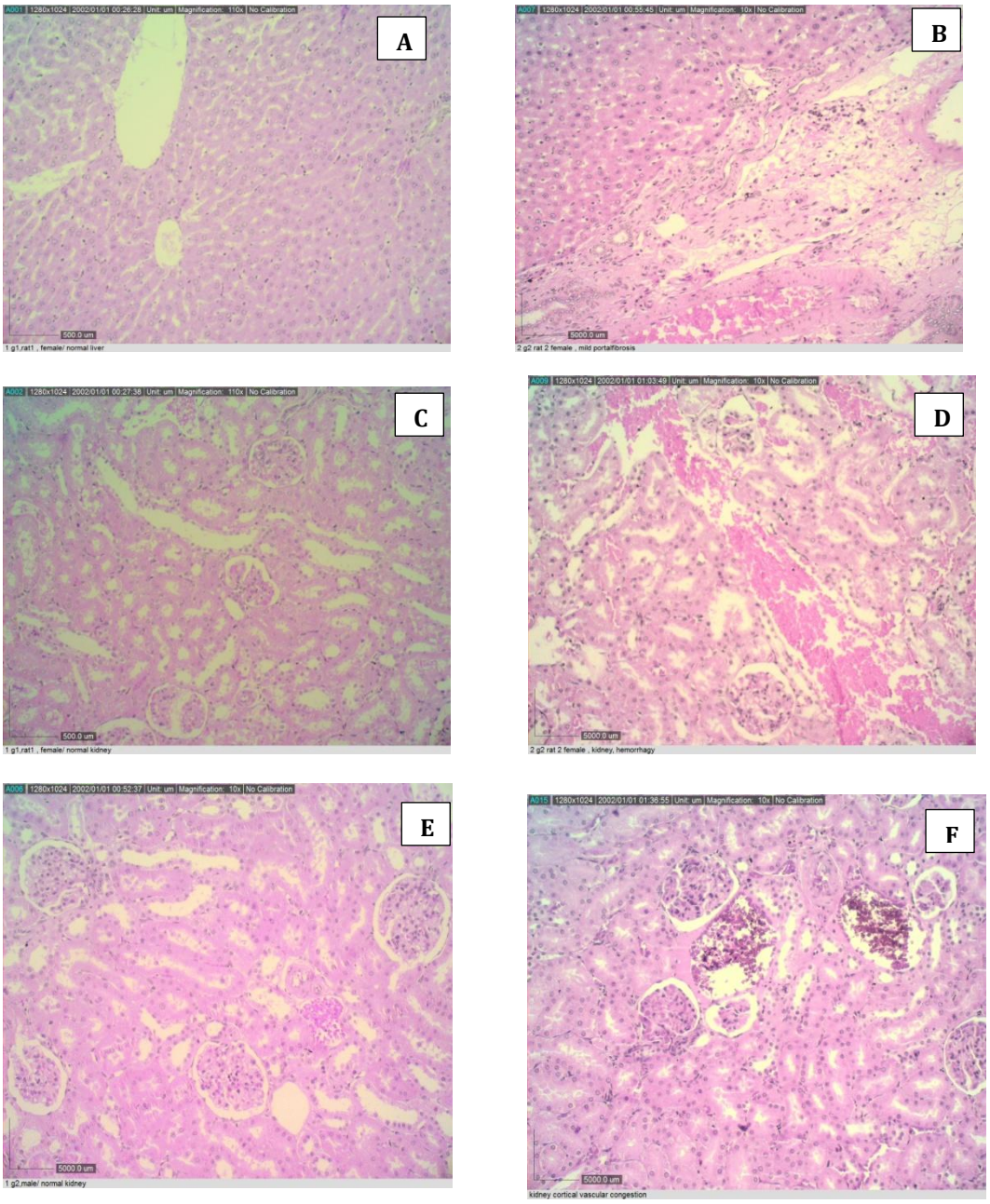

Fig. 3. Selected microphotographs of kidney and liver hematoxylin and eosin of male and female rats that received the highest dose of date seed extract. A; Negative control of female liver, B; Showing female liver mild portal necrosis, C; Negative control of female kidney, D; Showing hemorrhage in female kidney , E; Negative control of male kidney, F; Showing vascular congestion in male kidney.

Table 1. Relative organ weight at termination of treatment (g % body weight)

Sex	Dose mg/kg	liver%	kidney%	Heart%	Lung%	Spleen%
Male	Control	3.68 ± 0.02	0.34 ± 0.02	0.42 ± 0.02	0.68 ± 0.06	0.22 ±0.02
	500	4.03 ± 0.07	0.31 ± 0.05	0.39 ± 0.01	0.74 ± 0.04	0.29 ± 0.04
	1000	3.65 ± 0.25	0.40 ± 0.013	0.37 ± 0.006	0.69 ± 0.03	0.27 ± 0.007
	1500	3.80 ± 0.10	0.39± 0.01	0.38 ± 0.01	0.66 ± 0.02	0.24 ± 0.01
Female	Control	3.55 ± 0.17	0.32 ± 0.002	0.38 ± 0.034	0.80± 0.07	0.23 ± 0.04
	500	3.89 ± 0.02	0.35 ± 0.02	0.34 ± 0.009	0.74 ± 0.11	0.25 ± 0.03
	1000	3.96 ± 0.23	0.36 ± 0.03	0.33 ± 0.01	0.73 ± 0.03	0.21 ± 0.06
	1500	3.60 ± 0.13	0.31 ± 0.004	0.39 ± 0.05	0.78 ± 0.05	0.24 ± 0.02

Table 2. Hematological parameters of Wistar rats after 45 days treatment.

Sex	Dose mg/kg	MCV (fi)	MCH (pg)	MCHC (g/dl)
Male	Control	52.54 ± 2.82	18.4 ± 0.50	35.08 ± 1.15
	500	51.32 ± 3.09	18.62 ± 1.50	36.2 ± 0.90
	1000	52.02 ± 1.69	18.72 ± 0.94	35.94 ± 1.36
	1500	49.82 ± 2.03	18.1 ± 0.93	36.36 ± 1.41
Female	Control	52.3 ± 2.69	19.1 ± 0.93	36.52 ± 0.46
	500	52.04 ± 1.46	19.44 ± 0.38	37.40 ± 0.49
	1000	52.8 ± 1.93	19.94 ± 0.38	37.78 ± 0.85
	1500	50.72 ± 1.79	19.25 ± 0.71	37.90 ± 0.23

Sex	Dose mg/kg	WBC (1000/µl)	RBC (106/µl)	Hb (g/dl)	HCT (%)
Male	Control	7.16 ± 2.95	8.44 ± 0.44	15.54 ± 0.96	45.56 ± 5.95
	500	7.25 ± 0.74	8.37 ± 0.98	15.05 ± 0.97	42.82 ± 3.38
	1000	12.64 ± 6.59	8.33 ± 0.17	15.56 ± 0.52	43.38 ± 1.30
	1500	7.1 ± 1.01	7.76 ± 0.47	14.18 ± 0.86	39.02 ± 2.17
Female	Control	5.38 ± 1.67	7.49 ± 0.13	14.32 ± 0.65	39.2 ± 1.94
	500	8.9 ± 2.39	7.45 ± 0.29	14.5 ± 0.65	38.76 ± 1.82
	1000	8.08 ± 2.90	7.11 ± 0.11	14.18 ± 0.20	37.56 ± 1.05
	1500	6.07 ± 2.78	5.53 ± 3.69	14.45 ± 0.64	38.1 ± 1.67

- **Data presented as Mean±S.E.M. for N= 5.**

Table 3. Biochemical parameters of Wistar rats after 45 days treatment.

Sex	Dose mg/kg	First Blood Sugar)mg/dl(	Urea)mg/dl(	Creatinine (mg/dl(	Alkaline phosphatase)mg/dl(	Triglycerides)mg/dl(
Male	Control	157.4± 55.23	44.6 ± 3.57	0.75 ± 0.04	384.4 ± 86.78	77 ± 25.25
	500	94.5 ± 11.24	35.5 ± 2.31*	0.67 ± 0.03	260.75 ± 89.64	81.25 ± 16.25
	1000	103 ± 15.79	51.4 ± 4.15	0.72 ± 0.05	339.8 ± 39.69	58.8 ± 18.47
	1500	146.8 ± 93.32	45.8 ± 1.64	0.74 ± 0.05	348.6 ± 51.34	83.6 ± 24.08
Female	Control	107.6 ± 15.58	55.8 ± 13.48	0.77 ± 0.01	276.6 ± 81.82	82.2 ± 27.40
	500	101.2 ± 23.01	51.2 ± 7.53	0.74 ± 0.04	290 ± 121.79	101 ± 18.34
	1000	120.8 ± 23.26	45.8 ± 5.4	0.75 ± 0.04	257.6 ± 57.57	64.4 ± 25.65

Sex	Dose mg/kg	LDH)mg/dl(	S.G.O.T)mg/dl(	S.G.P.T (mg/dl)
Male	Control	1183.2± 966.76	203.8 ± 101.65	76.8 ± 24.13
	500	1041.5 ± 523.36	159.5 ± 27.40	61.75 ± 9.91
	1000	944.2 ± 330.55	145.8 ± 10.52	84.8 ± 10.78
	1500	1216.4 ± 404.33	188 ± 51.25	90 ± 36.58
Female	Control	819.4 ± 279	123.8 ± 22.44	62.8 ± 9.44
	500	1093 ± 310.32	137.4 ± 13.27	0.74 ± 0.04
	1000	1012.8 ± 648.07	139.4 ± 35.56	0.75 ± 0.04
	1500	906.25 ± 261.33	121 ± 7.16	0.72 ± 0.02

Data presented as Mean±S.E.M. for N= 5.

Discussion

This study evaluated the probable acute and subchronic toxicity of *P. dactylifera* seed extract in the rats. In the acute toxicity test (LD_{50}) no lethality was observed up to a dose of 5 g/kg. Consequently, the median lethal dose value (LD_{50}) of *P. dactylifera* is above 5 g/body weight. According to the chemical labeling and classification of the acute systemic toxicity recommended by OECD, any substance with LD_{50} higher than 2.0 g/kg when administrated orally is considered practically non-toxic [38]. Therefore, the extract of *P. dactylifera* seed is placed in the fifth category (LD_{50} > 5000 mg/kg), which was the lowest toxicity class [40].

Next, the subchronic study designed for further identifying and characterizing the specific organs affected by the *P. dactylifera* extract after repeated administration. Sub-chronic toxicity study showed minor changes in the histopathological parameters in the liver and kidney of male and female rats that received the highest concentration of the extract, respectively. Based on these results, we can conclude that no observed adverse effect level (NOAEL) of the extract of *P. dactylifera* was considered to be 1000 mg/kg/day for both sexes.

Some previous reports assessed pharmacological properties of the *P. dactylifera*. It has been showed that hydroethanolic extract of *P. dactylifera* is able to decrease rat blood lipids and sugar with no report of death among animals. Probably the hypolipidemic effect of *P. dactylifera* is due to the special compounds like linoleic acid, oleic acid. Besides, presence of Mg and Zn can cause hypoglycemia, and Mg and Zn stimulate synthesis and secretion of insulin and Mn acts like insulin. Increasing the rate of *P. dactylifera* kernel in food

can lower the rate of cholesterol and TG in blood but this decrement was not significant, statistically. Generally using palm kernel in Broiler food up to 10% can be effective [41].

The hypoglycemic effect of date seed extract combined with insulin reduces the blood glucose level meaningfully to normal when compared to the effect of insulin administered as a single drug for treatment of diabetes. Diabetic untreated rats showed significant hyperglycemia and hyperlipidemia, deterioration in kidney functions and morphology with enhanced oxidative stress in kidney tissues [42].

Methanolic and aqueous extracts of fruit and seed of palm date protected kidneys from diabetic nephropathy in rats, which might cause by their antioxidant properties [43].

Date seed extract-insulin-treated diabetic group demonstrated a statistically significant reducing in the mean blood glucose level compared to the insulin-treated diabetic group. The mean serum C-peptide level was significantly higher in date seed extract-insulin-treated diabetic group compared to insulin-treated diabetic group [44].

A diet containing *P. dactylifera* seeds reduces the basal level of lipid peroxidation in liver of normal rats while does not affect the antioxidant enzyme capacity of the normal tissues [45]. Also, it has been presented that the aqueous and ethanolic extracts of *P. dactylifera* seeds are in effect in improving gastric ulceration in rats [26]. A study [46] has shown the hepatoprotective effect of *P. dactylifera* seeds on carbon tetrachloride (CCl_4) treated rats. Actually, *P. dactylifera* seeds pointedly improved the liver function parameters like AST, ALT, ALP and albumin, oxidative stress, nitric oxide and oxidative DNA damage. It also restored the activities of hepatic antioxidant enzymes (superoxide dismutase and glutathione S-transferase) and attenuated the incidence of liver lesions including vacuolization and fibroblast proliferation, in rats with CCl_4-induced alterations [17]. Hepatoprotective [46], reducing blood glucose [47, 48] and increasing c-peptide [44], cerebroprotective [49] are another indication for date seed.

Conclusion

In the acute toxicity evaluation of the aqueous extract of *P. dactylifera* seed is placed in the fifth category of toxicity which was the lowest toxicity class and in the sub-chronic toxicity evaluation, no observed adverse effect level was considered to be 1000 mg/kg/day for both sexes. Furthermore, additional evaluation of clinical toxicology evaluations needs to be done to define a safe dose and protect the population from possible toxic effects of the *P. dactylifera* aqueous extract.

Conflict of interests

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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