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Original Article

Assessment of acute and subacute toxic effects of the Saudi folk herb *Retama raetam* in rats

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Abstract

Background: *Retama raetam* (RR) fruit is used in traditional Saudi folk medicine as a hypoglycemic herb. However, the potential toxicity of RR has not been fully investigated. The current study aimed to explore the potential acute and subacute toxicities of the methanolic extract of RR fruit in male and female rats.

Methods: The extent of acute toxicity of RR was tested 14 days after a single oral dose was administered (250 mg/kg, 500 mg/kg, or 750 mg/kg). Additionally, subacute toxicity was tested 28 days after an oral dose of 250 mg/kg/d, 500 mg/kg/d, or 750 mg/kg/d was administered for 4 weeks.

Results: Subsequent to variable dosage testing, oral LD_{50} of RR was found to be 1995 mg/kg in rats. Oral doses of 500 mg/kg and 750 mg/kg significantly decreased body weight gain. Subacute administration (750 mg/kg) was associated with significant manifestations of toxicity. Additionally, subacute administration of the extract at doses of 500 mg/kg or 750 mg/kg significantly elevated alanine transaminase and aspartate transaminase activities. Hepatotoxicity of RR was confirmed with histopathological findings. Subacute administration of RR (500 mg/kg) showed histopathological changes in the liver as indicated by degenerated hepatocytes and early fibrosis, while a dosage of 750 mg/kg showed congested central vein and vascular degeneration. Moreover, subacute administration of the extract at doses of 250 mg/kg, 500 mg/kg, or 750 mg/kg showed histopathological alterations in rat kidney that ranged from mild interstitial congestion to tubular degeneration. The extract showed positive result in the Ames test.

Conclusion: Repeated administration of methanolic extract of RR (250 mg/kg) has a low nephrotoxic subacute toxicity potential, while it might have hepatotoxic, nephrotoxic, and mutagenic effects at higher doses.

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Keywords: acute toxicity; hepatotoxicity; nephrotoxicity; *Retama raetam*; subacute toxicity

1. Introduction

Retama raetam (RR), of the family Fabaceae, is endogenous to the north and east Mediterranean regions and Sinai.[1,2] RR contains flavones,[3] quinolizidine alkaloids, dipiperidine alkaloids,[4] lupin alkaloids,[5,6] polysaccharides,[7] and essential oils.[8] Traditionally, RR has been used in folk remedies for diabetes[9] and hypertension.[10] It has been used as an abortifacient, anthelmintic, emetic, purgative, sedative, antiseptic, and an antipruritic.[11] Furthermore, RR is an effective grazing plant for sheep and camels.[12] Experimental evidence indicates that RR possesses antihypertensive,[13] diuretic,[9] lipid and body weight-lowering,[10] antioxidant,[14,15] antibacterial,[16] antihyperglycemic,[17] antibacterial, antifungal, and cytotoxic effects.[18]

There are reports indicating the potential toxicity of RR. Respiratory failure has been reported in a neonate after folk treatment with RR extract.[19] Camels that were overgrazed on RR suffered shivering and other signs of toxicity.[12] RR aqueous extract showed potent insecticide activity against

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

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whitefly adults.[20] In spite of the wide folk remedy related uses of RR, there is a scarcity of information regarding its toxicity profile. Therefore, the current study was designed to explore the acute and subacute toxicity of the methanolic extract of the fruit RR in rats.

2. Methods

2.1. Chemicals

Cyclophosphamide, ethidium bromide, histidine, and methyl alcohol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). *Salmonella typhimurium* was obtained from American Type Culture Collection (ATCC; strain number AT-2638 and AT-100; Rockville, MD, USA). Kits for blood chemistry were obtained from Dimension RxL Max Integrated Chemistry System (Siemens Healthcare Diagnostics Inc., Eschborn, Germany). Nicotinamide adenine dinucleotide phosphate, sterile top agar, benzo(a)pyrene, rat liver extract S9, and dimethyl sulfoxide were obtained from Xenometrix AG (Allschwil, Switzerland).

2.2. Collection and extraction of RR

Retama raetam (Forssk.) subsp. *gussonei* (RR) fruit were collected in April 2012 from Al-Hoferah, Saudi Arabia. The plant was identified and confirmed by the taxonomists of the King Abdulaziz University Herbarium, Jeddah, Saudi Arabia. RR fruit was left to rest at room temperature, and powdered fruit was extracted with methanol using IKA ULTRA-TURRAX T25 digital (Janke and Kunkel, IKA Labortechnik, Stauten, Germany). Subsequently, the methanol was distilled off at 40°C under reduced pressure and a dark yellow residue was obtained.

2.3. Standardization of the methanolic extract

RR extract was standardized based on its alkaloidal content using gas chromatography−mass spectrometry (GC−MS) analysis (Shimadzu GC-MS quadrupole system; model 2010, Nakagyo-ku, Kyoto, Japan). Helium was chosen as a carrier gas with a flow rate of 2.0 mL/min and an injector temperature of 240°C. The oven temperature was programmed to rise from 80−240°C at a rate of 10°C/min. MS were taken at 70 eV, with an ion source temp of 200°C and an interface temp of 250°C. Alkaloids were identified referring to retention time of authentic samples and comparing fragmentation patterns of sample compounds with those of NIST 147 library data of the GC-MS system (Mass Spectral Library, HP, Palo Alto, CA, USA). Fifteen alkaloids were detected as stated in Table 1.

2.4. Animals

Male and female Sprague-Dawley rats (150−200 g) were obtained from the animal facility of King Fahd Medical Research Center, King Abdulaziz University. The animals were provided with a standard pellet diet and water *ad libitum*. They were kept at 25 ± 2°C room temperature, 45−55% relative humidity, and had a 12 hour dark/light cycle. Procedures involving animals and their care were approved by the Bioethical Committee, King Abdulaziz University.

2.5. Experimental protocol

Oral LD_{50} of the methanolic extract of RR was determined in rats at the beginning of the study. Acute toxicity of the methanolic extract was tested in male and female rats 14 days after a single oral dose (at three dose levels; 250 mg/kg, 500 mg/kg, or 750 mg/kg). The subacute toxicity was tested in male and female rats 28 days after repeated daily oral doses for 4 weeks (at three dose levels; 250 mg/kg/d, 500 mg/kg/d, or 750 mg/kg/d). The effect of the methanolic extract of RR on the following parameters was assessed: mortality and gross symptoms of toxicity, whole body weight, hematological and blood chemistry values of rats, organ weights, and histopathological examination of liver and kidney sections. The potential mutagenic and genotoxic effects were also assessed using Ames and Comet tests, respectively.

2.6. Determination of LD_{50}

LD_{50} was estimated based on the use of the Spearman-Karber method.[21]

2.7. Acute toxicity study

This study was performed according to the Organization of Economic Cooperation and Development.[22] Sprague-Dawley rats of both sexes (150−200 g) were divided into four groups (I−IV) of 16 animals (8 males and 8 females) matched for weight. All animals had free access to water and food throughout the experiment. Groups II−IV were administered the methanolic extract of RR at single oral doses of 250 mg/kg, 500 mg/kg, or 750 mg/kg, and Group I served as the control, receiving distilled water at the same volume. The general behavior of rats was continuously monitored during the first 24 hours and then daily thereafter, for a total period of 14 days. Changes in the normal activity of rats were monitored and the time at which signs of toxicity or death appeared was documented. Changes in the whole body weights of the rats were monitored.

At the end of the observation period, all surviving animals were fasted overnight. Blood samples (2−3 mL) were collected from the common carotid vein into heparinized and dry nonheparinized centrifuge tubes. The heparinized blood was used for hematological studies (hematocrit, hemoglobin, white blood cells, and platelets), using a fully automated analyzer (Beckman Coulter ACT 10 Hematology Blood Analyzer, EKG Machines, New York, NY, USA). The serum was separated from the nonheparinized blood and was assayed for biochemical parameters, using Dimension RxL Max Integrated Chemistry System (Siemens Healthcare Diagnostics Inc.). After blood collection, animals were sacrificed with cervical dislocation. Various tissues (kidneys, liver, heart,

Table 1
Gas chromatography-mass spectrometry analysis of the alkaloidal content of *Retama raetam* fruits.

Alkaloid	t_R	Area %	Formula	[M^+]	m/z (rel. int)	Similarity %
N-methylcytisine	11.15	6.13	$C_{12}H_{16}N_2O$	204 (18)	160 (3), 146(5), 58 (100)	89
Cytisine	11.80	18.51	$C_{11}H_{14}N_2O$	190 (54)	160 (27), 146 (100), 134 (27), 117 (12), 109 (20), 93 (10), 82 (20), 68 (10), 44 (52)	95
5,6-Didehydrolupanine	13.49	18.09	$C_{15}H_{22}N_2O$	246 (30)	147 (15), 134 (18), 98 (100), 97 (54), 67 (10)	98
Argentamin	21.08	9.14	$C_{15}H_{20}N_2O_2$	260 (30)	160 (10), 146 (21), 133 (5), 114 (100), 106 (3), 96 (25), 70 (24), 41 (20)	85

m/z (rel. int) = mass number/charge number (relative intensity); t_R = retention time.

lung, and spleen) were dissected and weighed. Wet sections from the liver and kidney were removed and fixed in a 10% solution of buffered formalin. Then, they were dehydrated in ascending grades of ethyl alcohol, cleared in xylol, and mounted in molten paraffin at 58−62ºC. Tissue slices of 0.5 μm thickness were cut and stained with hematoxylin and eosin and evaluated for any structural changes under a light microscope (Olympus BX-50 Olympus Corporation, Tokyo, Japan). All sections were evaluated without prior knowledge of the animal treatment groups.

2.8. Subacute toxicity study

Four groups of 16 rats (8 males and 8 females) were used. Groups II−IV received the methanolic extract of RR at daily doses of 250 mg/kg, 500 mg/kg, or 750 mg/kg, and Group I served as the control receiving distilled water at the same volume, for a period of 28 days. During the period of administration, the animals were weighed and observed daily to detect any signs of toxicity. After 28 days, all surviving animals were investigated in the same way that was used in the acute toxicity study. Heparinized blood was used for hematological studies while the serum separated from the non-heparinized blood was assayed for blood chemistry. Additionally, various tissues (kidneys, liver, heart, lung, and spleen) were dissected and weighed, and wet sections from the liver and kidneys were examined histopathologically.

2.9. Ames test

Ames test was performed on *Salmonella typhimurium* (ATCC strain number AT-2638 and TA-100) as previously described by Ames et al.[23] Briefly, *Salmonella typhimurium* was plated on histidine-deficient media containing the methanolic extract of RR (0.1 μg/mL and 1 μg/mL) for 4 days. The extent of colony growth indicates potential mutagenicity compared to ethidium bromide-containing media (positive control).

2.10. Comet test

Comet assay was performed on rat mature sperm as previously described.[24] Briefly, six male adult Sprague-Dawley rats were treated with the methanolic extract of RR (250 mg/kg, 500 mg/kg, or 750 mg/kg) for 24 hours. At the conclusion of the experiment, the rats were euthanized and sperm was immediately collected from the cauda epididymis in a phosphate-buffered solution for comet assay. Cyclophosphamide (100 mg/kg) was used as a positive control genotoxic agent.

2.11. Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance followed by application of Tukey-Kramer multiple comparisons tests. A p value = 0.05 was used as the criterion for significance. All statistical analyses were performed using GraphPad InStat Software Version 4, and graphs were sketched using GraphPad Prism Software Version 4 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Determination of oral LD_{50} of the methanolic extract of RR in rats

The oral LD_{50} in rats was calculated mathematically according to the Spearman-Karber method[21] to be 1995 mg/kg. Furthermore, the 95% confidence limits of LD_{50} were 1218−3265 mg/kg.

Table 2
Mortality and gross signs of acute and subacute toxicity of the methanolic extract of *Retama raetam* in male and female rats.

Group	D/T	Acute signs of toxicity	D/T	Subacute signs of toxicity
Control	Male 0/8	None	Male 0/8	None
	Female 0/8	None	Female 0/8	None
250 mg/kg RR	Male 0/8	None	Male 0/8	None
	Female 0/8	None	Female 1/8	None
500 mg/kg RR	Male 1/8	None	Male 1/8	Hypoactivity, mild hyperventilation
	Female 1/8	Hypoactivity	Female 2/8	Hypoactivity, hyperventilation
750 mg/kg RR	Male 2/8	Hypoactivity, loss of appetite	Male 2/8	Convulsion, dizziness, hyperventilation, syncope
	Female 2/8	Hypoactivity, loss of appetite	Female 3/8	Convulsions, dizziness, hyperventilation, syncope

D/T = dead/total; RR = *Retama raetam.*

3.2. Effect on mortality and gross symptoms of toxicity

In the acute toxicity study, oral administration of RR-methanolic extract caused no noticeable changes in the general behavior of male and female rats at 250 mg/kg, compared with the control group (Table 2). Some female rats manifested minor symptoms of toxicity including hypoactivity (at 500 mg/kg and 750 mg/kg) and loss of appetite at 750 mg/kg of the extract. A few male rats receiving the highest dose (750 mg/kg) of the extract manifested hypoactivity and loss of appetite. No deaths occurred in both sexes at the dose 250 mg/kg. However, there was a dose-related increase in mortality rate at higher doses; one male and one female rat died after the administration of 500 mg/kg. In addition, two male and two female rats died after the administration of 750 mg/kg (Table 2).

Subacute administration of RR-methanolic extract caused a dose-related mortality with the highest mortality in the 750 mg/kg group. There was no noticeable change in the general behavior of male and female rats at 250 mg/kg, compared with the control group (Table 2). The administration of a subacute dose of 500 mg/kg caused only hypoactivity and mild hyperventilation in male rats, while female rats manifested more severe hyperventilation. Subacute administration of the highest dose (750 mg/kg) caused marked changes in the general behavior of rats of both sexes in the form of convulsions, hyperventilation, dizziness, and syncope. The mortality rate was slightly higher than that in the acute toxicity study; two male and three female rats died after the administration of 500 mg/kg. In addition, three rats of each sex died after the administration of 750 mg/kg (Table 2).

3.3. Effect on changes in body weight

No significant changes occurred in body weight, food intake, or utilization of food in male and female rats treated acutely with single oral doses of the methanolic extract (250 mg/kg, 500 mg/kg, or 750 mg/kg). Both the control and treated rats appeared healthy at the end and throughout the 14-day period of the study (Table 3). However, there were significant decreases in body weight gains of male rats treated subacutely with repeated oral doses of 500 mg/kg and 750 mg/kg, being 24.3% and 13.2%, respectively, as compared with the control group figure of 50.8% (Table 4). Similarly, body weight gains of female rats decreased significantly when treated subacutely with repeated oral doses of 500 mg/kg and 750 mg/kg, being 21.9% and 14.3%, respectively, as compared with the control group's 49.3% (Table 4).

3.4. Effect on hematological values

The hematological parameters of hemoglobin concentration, hematocrit value, white blood cells, and platelets in the treated rats did not differ significantly from those of the control group. All the values were within control limits after the experimental periods of both the acute and subacute studies (Table 5).

3.5. Effect on blood chemistry

As shown in Tables 6 and 7, single oral doses of RR-methanolic extract had no noticeable effect on blood chemistry values in both sexes of rats. No significant treatment-related changes were detected in the levels of glucose, creatinine, proteins, bilirubin, lipids, and liver enzyme activities. However, in the subacute toxicity study, the oral dose of 750 mg/kg significantly decreased blood glucose level by 19.4% in male rats and 20.1% in female rats, compared with their respective controls (Tables 8 and 9). Additionally, administration of the extract to male and female rats at repeated oral doses of 500 mg/kg and 750 mg/kg caused significant elevation of blood urea nitrogen (BUN) and creatinine (markers of renal function), alanine transaminase (ALT), and aspartate transaminase (AST) activities (markers of hepatic function; Tables 8 and 9).

3.6. Effect on organ weights

The weights of organs such as liver, kidney, lung, heart, and spleen in the treated rats of both sexes did not differ significantly ($p < 0.05$) from that of the control groups. All the values were within the control limits after the experimental period of both the acute and subacute studies (Table 10).

Table 3
Body weights of male and female rats in acute toxicity of a single oral dose of the methanolic extract of *Retama raetam*.

Group	Sex	Body weight (g)		Weight gain (%)
		D 0	D 14	
Control	Male	193 ± 13.8	226 ± 18.2	17.2
	Female	181 ± 7.15	210 ± 11.7	16.0
250 mg/kg RR	Male	191 ± 6.40	231 ± 5.70	21.0
	Female	186 ± 8.19	220 ± 8.47	18.3
500 mg/kg RR	Male	193 ± 5.44	233 ± 8.02	19.6
	Female	184 ± 7.64	217 ± 7.45	17.9
750 mg/kg RR	Male	192 ± 11.0	228 ± 11.8	19.2
	Female	183 ± 8.04	213 ± 9.47	16.9

Data are presented as mean ± standard deviation.
RR = *Retama raetam.*

Table 4
Body weights of male and female rats in subacute toxicity of the methanolic extract of *Retama raetam*.

Group	Sex	Body weight (g)		Weight gain (%)
		D 0	D 28	
Control	Male	193 ± 15.5	291 ± 25.5	50.8
	Female	199 ± 19.3	297 ± 35.5	49.3
250 mg/kg RR	Male	193 ± 27.7	300 ± 13.2	55.1
	Female	190 ± 17.7	285 ± 13.3	50.2
500 mg/kg RR	Male	196 ± 18.4	243 ± 13.6*	24.3*
	Female	192 ± 18.4	234 ± 23.7*	21.9*
750 mg/kg RR	Male	191 ± 15.0	216 ± 11.8*	13.2*
	Female	196 ± 27.3	224 ± 11.1*	14.3*

Data are presented as mean ± standard deviation.
*Significantly different from corresponding control value at $p < 0.05$.
RR = *Retama raetam.*

Table 5
Hematological values of male and female rats in acute and subacute toxicity of the methanolic extract of *Retama raetam*.

Group	Sex	HGB (g%)		HCT (%)		WBC (10^3/mm^3)		Platelet (10^5/mm^3)	
		Acute	Subacute	Acute	Subacute	Acute	Subacute	Acute	Subacute
Control	Male	15.0 ± 0.17	15.3 ± 0.22	39.9 ± 0.61	42.2 ± 1.08	4.76 ± 0.47	4.31 ± 0.41	9.13 ± 0.41	9.47 ± 0.37
	Female	14.9 ± 0.32	14.2 ± 0.32	40.3 ± 0.71	41.9 ± 1.04	4.35 ± 0.32	4.15 ± 0.38	8.83 ± 0.25	9.16 ± 0.18
250 mg/kg RR	Male	16.2 ± 0.42	14.4 ± 0.84	41.6 ± 0.71	43.3 ± 0.96	4.19 ± 0.38	4.35 ± 0.55	9.61 ± 0.12	9.38 ± 0.42
	Female	14.5 ± 0.22	14.2 ± 0.32	42.5 ± 0.76	41.3 ± 0.76	4.12 ± 0.38	4.15 ± 0.45	9.16 ± 0.18	9.66 ± 0.25
500 mg/kg RR	Male	15.3 ± 0.43	14.9 ± 0.52	40.4 ± 0.63	42.7 ± 0.94	4.25 ± 0.43	4.11 ± 0.48	9.44 ± 0.24	8.94 ± 0.34
	Female	15.3 ± 0.39	15.2 ± 0.33	41.3 ± 0.66	43.3 ± 0.67	4.25 ± 0.34	4.35 ± 0.39	9.56 ± 0.17	8.41 ± 0.28
750 mg/kg RR	Male	15.0 ± 0.33	14.3 ± 0.74	42.1 ± 0.56	42.1 ± 0.71	4.47 ± 0.37	5.19 ± 0.87	9.11 ± 0.39	9.21 ± 0.42
	Female	14.5 ± 0.33	14.2 ± 0.34	42.4 ± 0.78	42.3 ± 0.71	4.17 ± 0.41	5.15 ± 0.55	9.26 ± 0.28	9.15 ± 0.30

Data are presented as mean ± standard deviation.
HCT = hematocrit; HGB = hemoglobin; RR = *Retama raetam*; WBC = white blood corpuscles.

Table 6
Blood chemistry values of male rats in acute toxicity of the methanolic extract of *Retama raetam*.

Parameter	Control	250 mg/kg RR	500 mg/kg RR	750 mg/kg RR
Glucose (mg/dL)	136 ± 12.3	137 ± 5.50	140 ± 16.0	131 ± 32.8
BUN (mg/dL)	14.5 ± 7.82	12.7 ± 5.20	13.5 ± 2.66	12.0 ± 1.60
Creatinine (mg/dL)	0.38 ± 0.07	0.38 ± 0.07	0.42 ± 0.04	0.33 ± 0.04
Total protein (g/dL)	6.10 ± 0.52	6.40 ± 0.52	6.58 ± 0.46	6.50 ± 0.45
Albumin (g/dL)	1.33 ± 0.12	1.38 ± 0.12	1.42 ± 0.15	1.38 ± 0.10
Globulin (g/dL)	4.76 ± 0.44	4.98 ± 0.42	4.18 ± 0.38	5.10 ± 0.38
Total bilirubin (mg/dL)	0.12 ± 0.01	0.13 ± 0.03	0.12 ± 0.02	0.12 ± 0.05
Direct bilirubin (mg/dL)	0.017 ± 0.008	0.019 ± 0.001	0.020 ± 0.003	0.020 ± 0.002
LDH (IU/L)	525 ± 204	416 ± 151	546 ± 222	511 ± 159
Amylase (IU/L)	687 ± 94.3	688 ± 106	683 ± 54.8	725 ± 88.9
Cholesterol (mg/dL)	71.7 ± 21.7	67.3 ± 20.0	75.0 ± 15.0	75.7 ± 11.1
Triglycerides (mg/dL)	59.5 ± 24.3	57.1 ± 18.0	56.1 ± 10.0	51.0 ± 11.7
AST (U/L)	92.0 ± 7.80	85.2 ± 15.7	85.8 ± 11.5	101 ± 13.5
ALT (U/L)	37.7 ± 8.40	35.8 ± 08.35	34.4 ± 2.64	40.6 ± 10.0
ALP (U/L)	1.33 ± 0.10	1.38 ± 0.11	1.41 ± 0.14	1.37 ± 0.10

Data are presented as mean ± standard deviation.
ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate transaminase; BUN = blood urea nitrogen; LDH = lactate dehydrogenase; RR = *Retama raetam*.

Table 7
Blood chemistry values of female rats in acute toxicity of a single oral dose of the methanolic extract of *Retama raetam*.

Parameter	Control	250 mg/kg RR	500 mg/kg RR	750 mg/kg RR
Glucose (mg/dL)	141 ± 20.1	139 ± 7.70	143 ± 15.2	137 ± 55.5
BUN (mg/dL)	13.2 ± 7.82	11.9 ± 4.30	13.8 ± 3.14	12.7 ± 2.10
Creatinine (mg/dL)	0.35 ± 0.08	0.36 ± 0.03	0.41 ± 0.07	0.34 ± 0.08
Total protein (g/dL)	6.98 ± 0.61	7.20 ± 0.66	7.30 ± 0.74	7.30 ± 0.37
Albumin (g/dL)	1.42 ± 0.21	1.25 ± 0.17	1.39 ± 0.21	1.29 ± 0.30
Globulin (g/dL)	4.51 ± 0.46	4.24 ± 0.51	5.14 ± 0.46	5.11 ± 0.74
Total bilirubin (mg/dL)	0.16 ± 0.04	0.15 ± 0.01	0.17 ± 0.02	0.13 ± 0.08
Direct bilirubin (mg/dL)	0.019 ± 0.004	0.018 ± 0.001	0.022 ± 0.001	0.021 ± 0.007
LDH (IU/L)	497 ± 114	502 ± 95.4	512 ± 122	510 ± 117
Amylase (IU/L)	656 ± 87.5	633 ± 120	677 ± 54.8	738 ± 79.6
Cholesterol(mg/dL)	76.0 ± 11.8	72.5 ± 10.0	72.0 ± 19.0	69.9 ± 14.8
Triglycerides (mg/dL)	48.6 ± 11.4	51.1 ± 13.0	46.3 ± 9.00	45.8 ± 7.90
AST (U/L)	97.0 ± 7.10	93.2 ± 15.7	90.2 ± 12.7	98.5 ± 21.8
ALT (U/L)	35.8 ± 6.20	39.2 ± 15.1	32.5 ± 3.19	41.8 ± 11.2
ALP (U/L)	1.38 ± 0.20	1.35 ± 0.30	1.34 ± 0.12	1.34 ± 0.20

Data are presented as mean ± standard deviation.
ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate transaminase; BUN = blood urea nitrogen; LDH = lactate dehydrogenase; RR = *Retama raetam*.

Table 8
Blood chemistry values of male rats in subacute toxicity of repeated oral doses of the methanolic extract of *Retama raetam*.

Parameter	Control	250 mg/kg RR	500 mg/kg RR	750 mg/kg RR
Glucose (mg/dL)	144 ± 27.1	140 ± 9.70	130 ± 18.7	116 ± 44.1*
BUN (mg/dL)	14.3 ± 2.21	21.6 ± 2.84	23.1 ± 2.16*	27.6 ± 2.16*
Creatinine (mg/dL)	0.40 ± 0.05	0.41 ± 0.03	0.57 ± 0.05*	0.64 ± 0.07*
Total protein (g/dL)	6.47 ± 0.26	6.60 ± 0.24	6.83 ± 0.32	6.80 ± 0.29
Albumin (g/dL)	1.45 ± 0.07	1.44 ± 0.08	1.50 ± 0.11	1.56 ± 0.08
Globulin (g/dL)	5.02 ± 0.22	5.16 ± 0.20	5.32 ± 0.25	5.25 ± 0.24
Total bilirubin (mg/dL)	0.18 ± 0.05	0.17 ± 0.01	0.20 ± 0.06	0.21 ± 0.03
Direct bilirubin (mg/dL)	0.02 ± 0.006	0.02 ± 0.005	0.02 ± 0.008	0.02 ± 0.007
LDH (IU/L)	587 ± 140	520 ± 117	426 ± 155	528 ± 152
Amylase (IU/L)	858 ± 54.4	913 ± 66.3	920 ± 93.5	882 ± 60.7
Cholesterol(mg/dL)	66.1 ± 7.20	64.1 ± 6.99	62.0 ± 8.80	69.0 ± 8.20
Triglycerides (mg/dL)	74.8 ± 19.0	70.3 ± 10.9	84.6 ± 28.6	90.0 ± 30.9
AST (U/L)	104 ± 11.2	108 ± 16.9	190* ± 13.1	166* ± 38.3
ALT (U/L)	44.1 ± 6.50	58.7 ± 9.10	69.3* ± 7.90	71.5* ± 15.4
ALP (U/L)	1.45 ± 0.07	1.44 ± 0.08	1.50 ± 0.11	1.56 ± 0.08

Data are presented as mean ± standard deviation.
* Significantly different from corresponding control value at $p < 0.05$.
ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate transaminase; BUN = blood urea nitrogen; LDH = lactate dehydrogenase; RR = *Retama raetam*.

Table 9
Blood chemistry values of female rats in subacute toxicity of repeated oral doses of the methanolic extract of *Retama raetam*.

Parameter	Control	250 mg/kg RR	500 mg/kg RR	750 mg/kg RR
Glucose (mg/dL)	150 ± 26.1	143 ± 15.6	138 ± 10.6	119* ± 20.8
BUN (mg/dL)	16.5 ± 2.42	19.8 ± 3.54	20.5* ± 3.19	27.6* ± 3.78
Creatinine (mg/dL)	0.40 ± 0.12	0.49 ± 0.08	0.71* ± 0.08	0.75* ± 0.03
Total protein (g/dL)	7.12 ± 0.31	6.90 ± 0.42	7.21 ± 0.49	7.11 ± 0.31
Albumin (g/dL)	1.51 ± 0.09	1.48 ± 0.06	1.52 ± 0.20	1.49 ± 0.05
Globulin (g/dL)	5.12 ± 0.45	5.09 ± 0.30	5.29 ± 0.27	5.22 ± 0.19
Total bilirubin (mg/dL)	0.18 ± 0.08	0.17 ± 0.02	0.19 ± 0.03	0.22 ± 0.04
Direct bilirubin (mg/dL)	0.02 ± 0.005	0.02 ± 0.012	0.02 ± 0.009	0.02 ± 0.005
LDH (IU/L)	540 ± 119	534 ± 154	522 ± 111	578 ± 142
Amylase (IU/L)	921 ± 44.6	989 ± 47.5	890 ± 95.7	897 ± 32.8
Cholesterol(mg/dL)	78.2 ± 5.40	72.8 ± 6.97	69.7 ± 8.10	70.4 ± 6.90
Triglycerides (mg/dL)	79.2 ± 13.0	72.8 ± 13.9	81.2 ± 18.9	95.7 ± 20.9
AST (U/L)	101 ± 13.2	105 ± 16.9	138 ± 13.1*	171 ± 14.8*
ALT (U/L)	46.1 ± 9.50	57.7 ± 11.1	61.3 ± 17.1*	77.5 ± 14.1*
ALP (U/L)	1.39 ± 0.04	1.47 ± 0.05	1.51 ± 0.13	1.52 ± 0.04

Data are presented as mean ± standard deviation.
*Significantly different from corresponding control value at $p < 0.05$.
ALT = alanine transaminase; ALP = alkaline phosphatase; AST = aspartate transaminase; BUN = blood urea nitrogen; LDH = lactate dehydrogenase; RR = *Retama raetam*.

Table 10
Organ weights of male and female rats in acute and subacute toxicity of the methanolic extract of *Retama raetam*.

Group	Sex	Organ weight (g/100 g body weight)									
		Liver		Kidney		Lung		Heart		Spleen	
		Acute	Subacute	Acute	Subacute	Acute	Subacute	Acute	Subacute	Acute	Subacute
Control	Male	3.18 ± 0.16	3.21 ± 0.13	0.43 ± 0.03	0.46 ± 0.07	0.49 ± 0.02	0.44 ± 0.01	0.41 ± 0.01	0.39 ± 0.01	0.25 ± 0.01	0.26 ± 0.04
	Female	3.33 ± 0.31	2.97 ± 0.17	0.47 ± 0.08	0.44 ± 0.09	0.52 ± 0.05	0.41 ± 0.02	0.44 ± 0.04	0.41 ± 0.05	0.23 ± 0.02	0.22 ± 0.03
250 mg/kg RR	Male	2.97 ± 0.02	3.38 ± 0.01	0.45 ± 0.02	0.47 ± 0.02	0.51 ± 0.03	0.42 ± 0.01	0.43 ± 0.01	0.38 ± 0.04	0.26 ± 0.01	0.28 ± 0.01
	Female	3.15 ± 0.15	3.24 ± 0.19	0.41 ± 0.05	0.42 ± 0.07	0.49 ± 0.01	0.47 ± 0.05	0.47 ± 0.03	0.37 ± 0.07	0.29 ± 0.01	0.25 ± 0.05
500 mg/kg RR	Male	3.21 ± 0.01	3.41 ± 0.01	0.41 ± 0.02	0.42 ± 0.01	0.46 ± 0.02	0.36 ± 0.01	0.38 ± 0.01	0.37 ± 0.01	0.28 ± 0.01	0.24 ± 0.02
	Female	3.09 ± 0.07	3.37 ± 0.08	0.44 ± 0.07	0.47 ± 0.02	0.49 ± 0.05	0.39 ± 0.04	0.41 ± 0.03	0.42 ± 0.03	0.26 ± 0.09	0.21 ± 0.04
750 mg/kg RR	Male	3.01 ± 0.20	3.56 ± 0.20	0.42 ± 0.01	0.45 ± 0.02	0.47 ± 0.03	0.45 ± 0.02	0.39 ± 0.02	0.39 ± 0.03	0.25 ± 0.01	0.27 ± 0.01
	Female	3.58 ± 0.17	3.45 ± 0.23	0.43 ± 0.03	0.43 ± 0.07	0.45 ± 0.04	0.38 ± 0.05	0.43 ± 0.04	0.41 ± 0.01	0.27 ± 0.04	0.28 ± 0.03

Data are presented as mean ± standard deviation.
RR = *Retama raetam*.

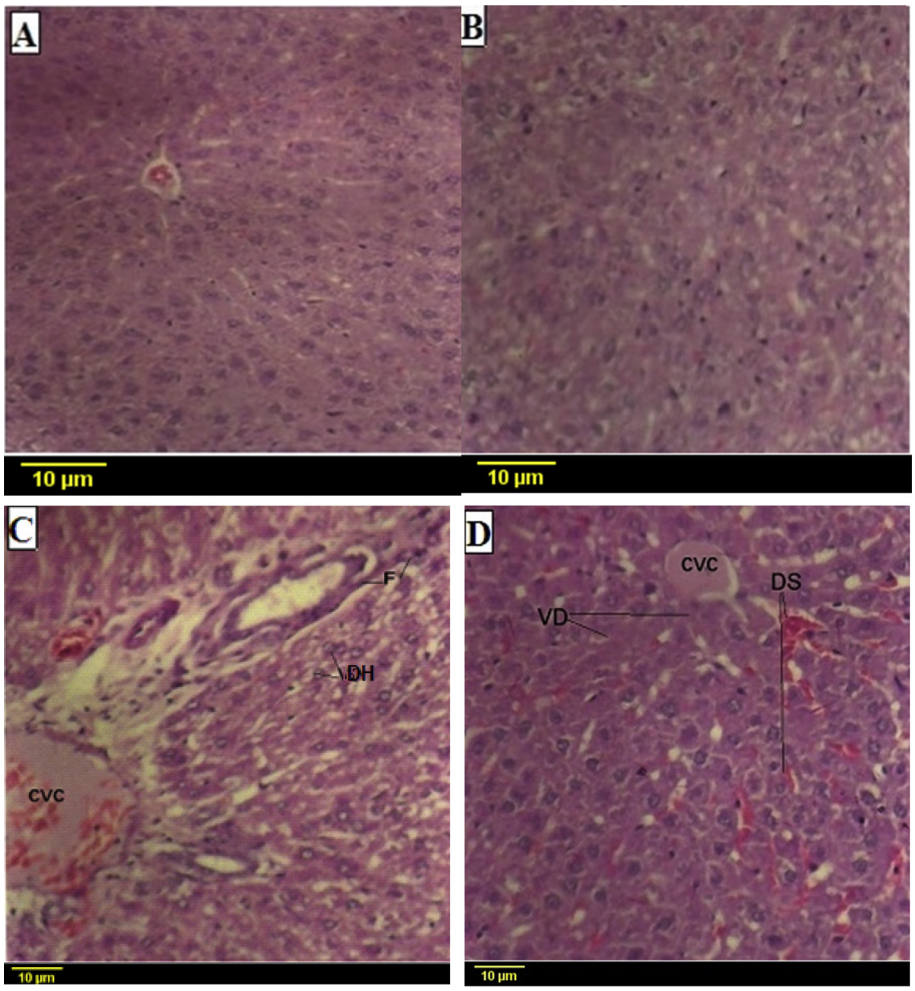

Fig. 1. Effect of subacute toxicity of *Retama raetam* (RR) methanolic extract on histopathology of rat liver. Histopathological examination of liver sections of: (A) control rats [hematoxylin and eosin stain (H&E)]; (B) rats treated with the methanolic extract of RR (250 mg/kg/d for 4 weeks) showing normal parenchymal cells, portal system, and blood sinusoids (H&E); (C) rats treated with the methanolic extract of RR (500 mg/kg/d for 4 weeks) showing congested dilated central vein with degenerated hepatocytes and early fibrosis (proliferated fibroblasts; H&E); and (D) rats treated with the methanolic extract of RR (750 mg/kg/d for 4 weeks) showing congested central vein and dilated congested blood sinusoids in addition to vascular degeneration (H&E).

3.7. Histopathological examination of liver and kidney

In the acute toxicity study, histopathological examinations of liver sections were normal for all doses (250 mg/kg, 500 mg/kg, and 750 mg/kg). Similarly, liver sections from rats treated with subacute dose of 250 mg/kg did not show any histopathological changes (Fig. 1B). However, liver sections from rats treated subacutely at 500 mg/kg of the extract showed moderate changes such as congested dilated central vein and blood sinusoids with degenerated hepatocytes and early fibrosis (Fig. 1C). In addition, liver sections from rats treated with a subacute dose (750 mg/kg) of the extract showed severe changes such as vascular degeneration (Fig. 1D).

In the acute toxicity study, histopathological examination of kidney sections were normal at all tested doses (250 mg/kg, 500 mg/kg, and 750 mg/kg). In the subacute toxicity study, however, examination of kidney sections showed dose-related histopathological changes. Kidney sections from rats treated subacutely with 250 mg/kg of the extract showed mild interstitial congestion (Fig. 2B). Sections from rats treated subacutely with 500 mg/kg showed moderate changes in the form of early tubular degeneration in addition to interstitial congestion (Fig. 2C). However, histopathological examination of kidney sections from rats treated subacutely with the highest dose of the extract (750 mg/kg) showed severe changes such as congested glomeruli, wide Bowman's capsule, red cell casts, and tubular degeneration (Fig. 2D).

3.8. Ames test

The methanolic extract of RR showed a potential mutagenic effect on *Salmonella typhimurium* (both strains AT-2638 and AT-100) manifested as significant growth of colonies plated on 1 µg/mL extract-containing medium and mild growth at 0.1 µg/mL extract-containing medium (Fig. 3).

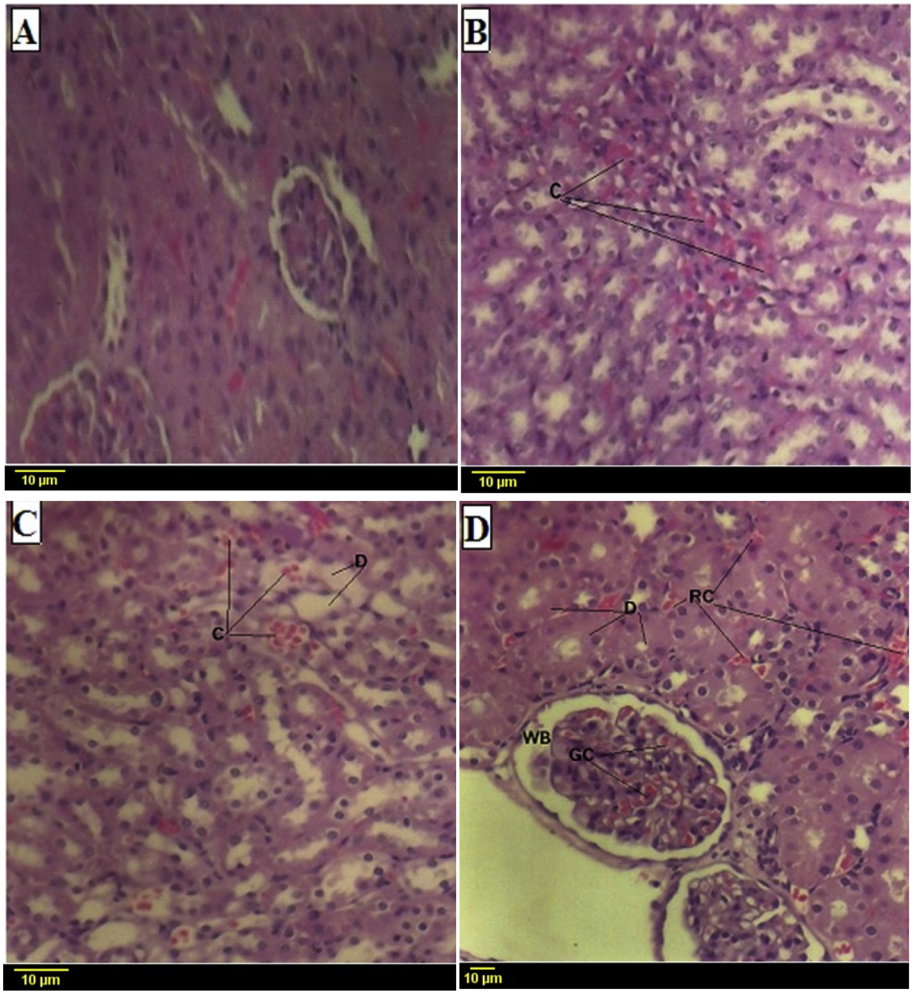

Fig. 2. Effect of subacute toxicity of *Retama raetam* (RR) methanolic extract on histopathology of rat kidneys. Histopathological examination of kidney sections of: (A) control rats [hematoxylin and eosin stain (H&E)]; (B) rats treated with the methanolic extract of RR (250 mg/kg/d for 4 weeks), showing mild interstitial congestion (H&E); (C) rats treated with the methanolic extract of RR (500 mg/kg/d for 4 weeks), showing early tubular degeneration and interstitial congestion (H&E); and (D) rats treated with the methanolic extract of RR (750 mg/kg/d for 4 weeks), showing congested engorged glomeruli, wide bowman capsule, some red cell casts, and tubular degeneration (H&E).

3.9. Comet test

No obvious DNA aberration was detected in rat sperm nuclei after treatment with the methanolic extract of RR up to doses of 750 mg/kg (Fig. 4).

4. Discussion

In Saudi folk medicine, fruit of RR is used in remedies for diabetes.[12] A World Health Organization survey indicated that ~70−80% of the global population depends on alternative medicine, predominantly herbal in nature, in their primary health care. This is especially the case in developing countries where the cost of consulting a Western-style physician and the cost of medicines are not affordable for the large majority of the population.[25,26] Although medicinal plants may produce several biological activities in humans, generally very little is known about their toxicity and the same applies for RR. Therefore, the aim of the present study was to evaluate the acute and subacute toxicity of the methanolic extract of RR in male and female rats.

In an attempt to evaluate the safety margin of methanolic extract of RR in healthy rats, oral LD_{50} was determined according to the Spearman-Karber method.[21] Oral LD_{50} was calculated to be 1995 mg/kg, at 95% confidence limits of 1218−3265 mg/kg. This dose was approximately from four to eight times the tested concentrations for antidiabetic activity,[17] indicating a relatively limited methanolic extract of RR safety margin. In addition, the classification of Loomis and Hayes[27] indicated that substances with LD_{50} between 500 mg/kg bodyweight and 5000 mg/kg bodyweight and between 5000 mg/kg bodyweight and 15,000 mg/kg bodyweight are regarded as being slightly toxic and practically nontoxic, respectively. Therefore, the present results suggest that the safety level of RR-methanolic extract falls within the first category, being slightly toxic.

In order to investigate the effect of the methanolic extract of RR fruit on mortality and gross symptoms of toxicity,

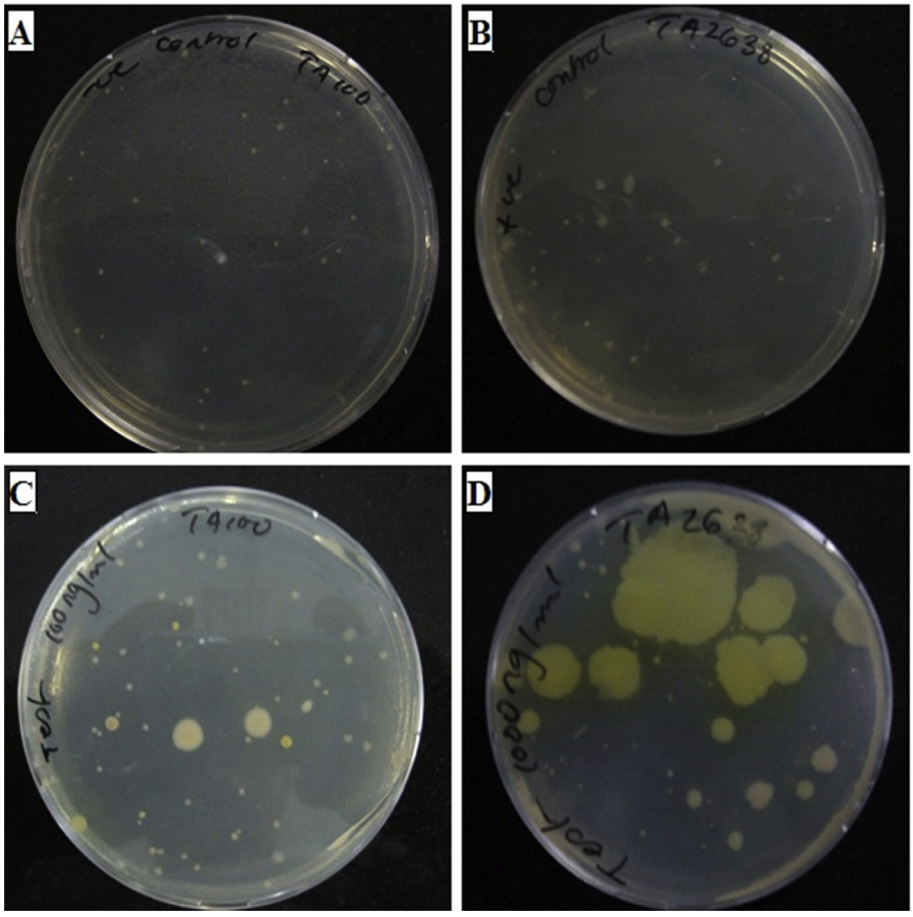

Fig. 3. Ames test for *Retama raetam* (RR) methanolic extract. Ames test for: (A) negative control; (B) positive control (ethidium bromide), showing growth of *Salmonella typhimurium* on histidine-deficient medium in the presence of ethidium bromide; (C) methanolic extract of RR (0.1 μg/mL), showing mild growth of *Salmonella typhimurium* on histidine-deficient medium; and (D) methanolic extract of RR (1.0 μg/mL), showing significant growth of *Salmonella typhimurium* on histidine-deficient medium.

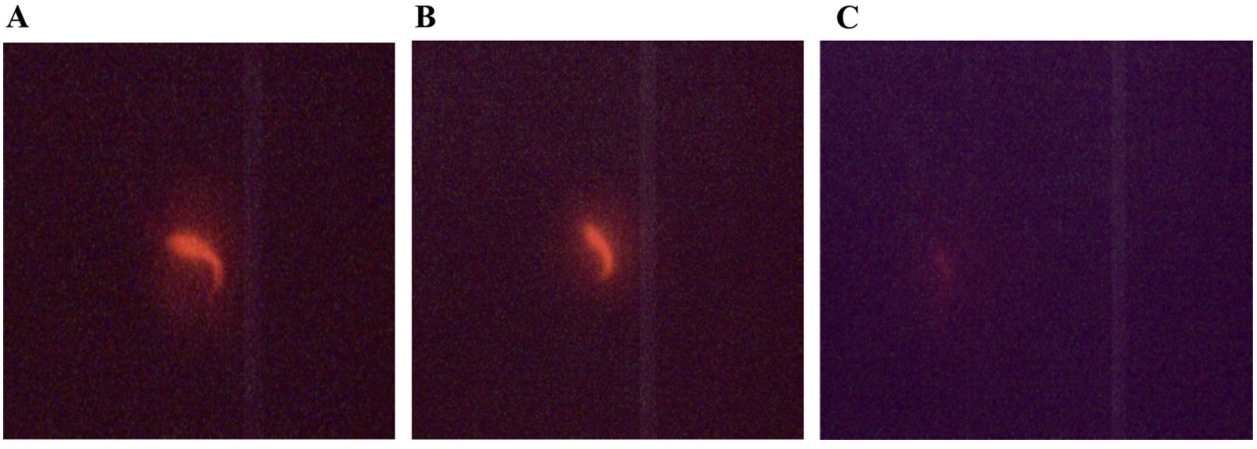

Fig. 4. Comet test for negative control (A) the methanolic extract of *Retama raetam*; (B) 750 mg/kg showing no DNA aberration; and (C) positive control (cyclophosphamide), showing significant DNA aberration.

different doses of the methanolic extract (250 mg/kg, 500 mg/kg, and 750 mg/kg) were tested in male and female rats in both an acute and subacute manner. In both studies, oral administration of RR methanolic extract caused neither noticeable change in the general behavior nor death in both sexes at the smallest dose. However, there was a dose-related increase in symptoms of toxicity and mortality rate at the higher doses. These findings could be explained by the relatively low safety margin of this methanolic extract, as classified by Loomis and Hayes.[27]

In the acute toxicity study, RR-methanolic extract, regardless of dose used, neither appeared to affect the body weight of the rats of both sexes, nor did it cause significant changes in their food intake or utilization of food. However, there were significant decreases in body weight gains of male and female rats when treated subacutely with repeated oral doses of 500 mg/kg and 750 mg/kg. This suggests that, at these oral doses, the extract could retard the growth of rats of both sexes. In both acute and subacute studies, there were no treatment-related changes in the hematological parameters in rats of both sexes (i.e., hematocrit value, hemoglobin concentration, platelets, and white blood cells). This indicates that the RR-methanolic extract did not interfere with the production of platelets. Hematopoiesis and leucopoiesis also were not affected even though the hematopoietic system is one of the most sensitive targets for toxic compounds,[28] as well as an important marker of physiological and pathological status in man and animals.[29] In addition, many of the biochemical parameters (i.e., serum proteins, lipids, bilirubin, amylase, and lactate dehydrogenase) were also unchanged in both sexes of rats by the ingested RR-methanolic extract, regardless of the dose given either in acute or subacute studies.

Concerning blood glucose level, it was significantly decreased in rats of both sexes at the subacute dose of 750 mg/kg. This might reflect the pharmacological hypoglycemic effect of RR-methanolic extract[9,30] that was ascribed, at least partly, to stimulating pancreatic insulin release and reducing intestinal glucose absorption.[17]

There were slight but significant elevations in the levels of ALT and AST at subacute doses of 500 mg/kg and 750 mg/kg in both male and female rats. These findings suggest that subacute ingestion of RR-methanolic extract at relatively high doses is hepatotoxic in rats. The transaminases (AST and ALT) are biomarker enzymes used to predict possible toxicity.[31] Generally, damage to the parenchymal liver cells results in elevation of both transaminases.[32] Interestingly, the elevation of AST level was about twice the increase in ALT. The isolated elevation of AST levels, in the presence of normal levels of other cholestatic markers, could be justified apart from liver injury. AST is present in different tissues (including heart, skeletal muscle, kidney, brain, and liver) while ALT is localized primarily in the liver. Moreover, AST can exist as a complex with an immunoglobulin, and this macromolecule can cause an elevation in serum AST activity, which may be detected in blood chemistry analysis and mistakenly be considered to highlight the occurrence of liver dysfunction.[33] Thus, RR might influence previous mechanisms that cause more elevations of the AST than ALT levels, rather than acting on the liver.

BUN and creatinine levels were significantly elevated at subacute doses of 500 mg/kg and 750 mg/kg in both male and female rats. These findings suggest that subacute ingestion of RR-methanolic extract at relatively high doses is nephrotoxic in rats. This is in contrast with previous studies that suggested the ability of the methanolic extract of the powder of the dried seeds of RR to protect against gentamicin-induced nephrotoxicity[34] and cadmium chloride-induced hepato- and nephrotoxicities[15] in rats. The apparent differences can be explained on the basis of the differences in method of extraction, the dosing regimen, as well as the differences in the part used, and environment of RR which might affect the presence and/or concentration of the active constituents. The hepatoxic and nephrotoxic effects of RR-methanolic extract require further investigation.

The weights of organs (liver, kidney, lung, heart, and spleen) in the treated male and female rats did not differ significantly from those of the control group, in both the acute and subacute studies. Therefore, it was necessary to examine liver and kidney tissues histopathologically, in order to detect any microscopic changes that might be present. Examination of liver and kidney sections taken from rats exposed to single oral doses of RR methanolic extract failed to show any histopathological changes, which comply with the normal pattern of biochemical parameters, as previously observed in the acute study. However, histopathological examination of liver sections from rats treated with subacute doses (500 mg/kg and 750 mg/kg) of the extract showed significant dose-related changes. These findings could explain the previously mentioned elevation in the transaminases (ALT and AST) levels. In a similar pattern, histopathological examination of kidney sections from rats treated subacutely with 250 mg/kg showed only minimal changes, which gradually became more evident at subacute doses of 500 mg/kg and 750 mg/kg of the extract. Thus, the significant elevation of BUN and creatinine—as observed previously—is confirmed by these histopathological findings.

In order to investigate the potential mutagenicity and genotoxicity of the methanolic extract of RR, both Ames and Comet tests were conducted. In the Ames test, the extract was shown to have a potential mutagenic effect, manifested by evident growth of colonies plated on 1.0 μg/mL extract-containing medium and minimal growth at 0.1 μg/mL extract-containing medium. These data are contradictory to the findings of Alkofahi and Al-Khalil[35] who indicated that aerial parts of RR did not show any observable mutagenic activity. This discrepancy may be due to differences in the part used as we examined RR fruit. However, use of the Comet test failed to detect any DNA aberrations in rat sperm nuclei after treatment with the methanolic extract of RR up to doses of 750 mg/kg.

In conclusion, the current study provided valuable data on the acute and subacute toxicity of the methanolic extract of RR. The extract appeared to be slightly toxic (with a relatively low LD_{50}), but caused no apparent organ damage. Under our experimental conditions, RR methanolic extract can exhibit hepatotoxic and nephrotoxic effects at moderate to high subacute doses. In addition, it could be potentially mutagenic, based on experimental studies. Further toxicity studies—to investigate its potential subchronic, chronic, developmental, reproductive, and carcinogenic effects—are needed to complete the safety profile of RR. Collectively, the findings of the present study suggest a low subacute toxicity potential for RR, and in high doses might have hepatotoxic, nephrotoxic, and mutagenic effects.

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