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Pharmacological Evaluation of the Libyan Folk Herb Retama Raetam Seeds in Mice

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Abstract: *Retama raetam (RR) is a traditional medicinal plant belongs to fabaceae family which grows in North Africa and East Mediterranean region. Locally, RR is used in several diseases including diabetes mellitus and hypertension. Thus, this study aims to investigate certain behavioral and central effects of methanolic extract of RR seeds in experimental animals (male Albino adult mice of 20 – 35 gm). Three exploratory behavioral models are used in this study, open field, elevated plus maze and light-dark box models, in addition, picrotoxin induced seizure model in mice. In elevated plus-maze test of anxiety, a dose of 25 mg/kg of RR induced a significant increase in number of open arms entries and time spent on open arms of the maze compared to the control. In light-dark model, time spent on light area and number of light-dark transitions are significantly increased after treatment with 25 mg/kg RR extract of seeds. Open field model also revealed a significant increase in general motor activity of the mouse after 25 mg/kg of RR. On the other hand, a dose of 50 mg/kg of RR significantly induced a profound central and peripheral analgesic responses. In summary, this study concludes that RR seeds have a profound anxiolytic-like effect and analgesic response with delay in latency time of seizure induced by picrotoxin in mice.*

Keywords: Retama Raetam, Anxiolytic-like effect, General motor activity, Analgesia, Mouse.

1. INTRODUCTION

Retama raetam (forsk, RR) webb and Berthel is a spontaneous desert shrub belongs to the tribe genistae of fabaceae family which mostly distributed in East Mediterranean regions and North Africa [1]. Traditionally, RR is used in diseases such as diabetes mellitus, hypertension, hyperlipidemia, sinusitis, and cancer as well as antidote for snake bite [2, 3]. In addition, RR retains an abortifacient, anthelmintic, emetic, purgative actions, sedative and also utilizes to relieve skin irritation [4]. Phytochemically, flavonoids, essential oils and quinolizidine alkaloids have been demonstrated in RR seeds [4, 5]. Further phytochemical investigations of RR seeds indicated presence of phenolic compounds which probably explain the anti-ulcerogenic and anti-oxidant effects of the plant extracts [6]. Several studies have previously been conducted on the plant extract and proved that RR has analgesic, anti-microbial, anti-oxidant, anti-leukemic and a protective effect against osteoporosis as well as nephroprotective and hepatoprotective [7 - 9]. However, it has experimentally been demonstrated that methanol extract of RR has the ability to restore kidney and liver toxicity induced by formalin in rats [10]. Toxicity liabilities of repeated administration of 250 mg/kg of RR for 14 days have earlier been reported including respiratory failure and reduction of fertility while hepatotoxic, nephrotoxic and mutagenic effects at higher doses of RR [4, 11, 12]. Therefore, there are limited and uncertain data on pharmacological effects of RR seeds despite its local human use in Libya. Further studies are required to explore its therapeutic effects as well as its potential toxicity.

2. MATERIALS AND METHODS

Plant materials

Seeds of Retama reatam (forssk.,) webb and Berthel were collected from Sobratah area, west region of Libya, in April, 2016. The plant was identified at Herbarium of Department of Botany, Faculty of Science, University of Tripoli, Libya by two taxonomists. A voucher specimen for this collection was recorded (D-688691).

Chemicals

Diazepam (Roche, Italy), morphine (Martindale pharmaceuticals, UK), acetic acid (Park Scientific limited Northampton, UK), diclofenac sodium (Novartis, Switzerland), phenobarbitone sodium (BDH Limited Poole, UK) and picrotoxin (Koch-Light Laboratory Ltd, UK) were obtained with pharmaceutical grade and used throughout this study.

Animals

Male adult Albino mice of body weight of 20 - 35 gm were used throughout this study. Mice were obtained from local central animal house (University of Tripoli, Tripoli, Libya). Mice were kept in a standard cage (n = 6 for each cage), with artificial food and tap water freely access. Mice were kept at a temperature – controlled room (24 ^{0}C ± 3) in a reversed dark-light cycle (12 L/12 D). Most of the experiments were carried out during spring time 2017 on a calm area. All animal experiments were carried out according to the animal ethics (approval was obtained by ethic university committee, No. 12524/2017). Doses and time were chosen according to our pilot studies on 3 - 4 animals for each experiment

(unpublished data). The plant extract and other agents have all been prepared freshly and used on the same day of experiments by intraperitoneal route of administration (i.p.).

Plant extract

Shade-dried and powdered seeds of RR (500 gm) were extracted by Soxhlet apparatus at 60 ^{0}C with n-hexane for 12 hours and methanol for 14 hours. The plant extract was concentrated using a rotary evaporator at 40 ^{0}C under reduced pressure using a vacuum pump, then air-dried and kept in dark colored container at - 20 ^{0}C until used [13].

Body weight: two groups of mice (n = 6, each group) were used to examine the effect of intake RR on the body weight. Each mouse was individually weighed every day for 28 days. Test group administered RR in a dose of 50 mg/kg per day and control group received vehicle, all mice kept in the same conditions. Mice were allowed to have a free access of food and water for 28 days.

Hypnosis test: Four groups of mice (n = 6, each group) were used in this method. Mice were fasted for 18 hours before testing. Group I served as a negative control, group II as a positive control (diazepam 20 mg/kg), group III was given RR extract (50 mg/kg) and group IV was given plant extract and diazepam in combination. Sleep onset time and sleep duration time (loss and regain of righting reflex, respectively) were recorded [14, 15].

Open- field model: A fiberglass box designed to form square area of 60 x 60 cm with side wall of 20 cm height. This was used as a model to record spontaneous motor activity of the mouse. The floor subdivided into 16 equal squares. General motor activity of the mouse measured by horizontal and vertical movements were counted for a period of 4 min. Mice were divided into two groups: control group received vehicle and test group received RR extract (25 mg/kg). All parameters were recorded 30 min after plant administration. Each mouse placed separately in the center of the field and allowed freely to explore the area (15, 16).

Elevated plus-maze model: This apparatus was well validated to measure anxiety level in animals. It was made of fiberglass and designed to have two open arms and two closed arms (30 x 5 cm each arm) with 15 cm side wall for closed arms. The Four arms were connected together to make a plus sign shape with a central stand (5 x 5 cm). The model rises from the floor by 50 cm to induce fear [17]. 4 groups (n = 6) were used: group I as a control, group II received (diazepam 1 mg/kg), group III given RR extract (25 mg/kg) and group IV given diazepam (1 mg/kg) and RR extract (25 mg/kg). 30 min later, each mouse was placed individually in the center of the maze facing one of the open arms and left freely to explore for 4 min. All parameters were recorded [18].

Light dark box model: This model consists of square fiber glass box (60 x 60 cm) with side height (20 cm) and was divided into two compartments. Third compartment consists of a small closed dark field (15 x 60 x 20 cm) and the rest was large lighted field, linked together by a small open hole (5 x 5 cm) in the middle [19). Group I: served as control, group II treated with (diazepam 1 mg/kg) and group III given 25 mg/kg of the extract. After 30 min, each mouse was placed individually in the center of the light arena facing the hole of dark compartment and number of transmission in between light and dark areas and time spent on squares for 6 min were recorded [20].

Hot plate method: Effect of central analgesic of the extract was measured by Eddy's method [21, 22]. Mice divided randomly into 3 groups, (n = 7). Group I acts as a control, group II was given morphine in a dose of 10 mg/kg (s.c.) and group III treated with R.R. extract (50 mg/kg). All mice received treatment prior 30 min of hot plate testing and post-treatment latency time was monitored at 30, 60, 90 and 120 min.

Writhing test: A writhe is induced by injecting acetic acid in a dose of 3% (i.p.). The animals were divided randomly into 3 groups. Group I was served as a control, group II received diclofenac sodium 25 mg/kg and group III was treated with RR extract (50 mg/kg). After 30 min, acetic acid was given to each mouse and number of abdominal contractions (writhing response) was counted for 20 min [23, 24].

Anti-convulsant activity: A dose of 3 mg/kg of picrotoxin was used to group I which served as a positive control. Groups II and III received 50 mg/kg and 100 mg/kg of the plant extract, respectively. Thereafter, 30 min, picrotoxin 3 mg/kg was given for each mouse of groups II & III. The latency (onset of seizure induction), number of convulsions and death were observed.

Statistical analysis: Statistical calculation was made with SPSS 18 for windows software package. Data was expressed as the mean ± S.E.M. of 6 dependent experiments. One-way ANOVA was used for statistical analyses and $P < 0.05$ was considered to be statistically significant.

3. RESULTS

In table 1, the body weight of mice treated with 50 mg/kg/day of RR extract and the control mice for 28 days is given. Thus, analysis of data revealed a significant decrease in the body weight of the treated mice compared with that of the controls by $p < 0.01$.

Table 1: Effect of daily intake of Retama raetam (25 mg/kg) extract on mouse body weight

Groups	Body weight in grams at			
	0-week	1-week	2-week	4-weeks
Control	22.71 ± 1.05	26.51 ± 1.33	27.43 ± 1.51	28.25 ± 1.68

RR extract	29.23 ± 0.39	29.7 ± 0.79	30.18 ± 1.00	30.4 ± 0.85

Data expressed as mean ± SEM of 6 mice. Percentage of increase in control group is 30% and in test group is 5%.

Table 2 shows that administration of 25 mg/kg of the plant extract to mice induces significant increased (60% of the control value, $p < 0.01$) in total line crossings in comparison with that of controls. This increase was equally profound in the central and peripheral line crossings of mice treated with RR extract. However, no significant changes in total rearings (central and peripheral of movements) of mice were observed (table 2).

Table 2: effect of Retama Raetam extract seeds on mouse behavior in open field model of general motor activity

Groups / parameters	Control group	R.R. Extract 25 mg/kg
Total line crossings	56.83 ± 1.84	89.50 ± 5.38**
Central line crossings	14.0 ± 1.86	24.6 ± 2.71*
Peripheral line crossings	42.83 ± 1.45	65.88 ± 6.38*
Total rearings	26.33 ± 2.68	31.00 ± 2.14

Data expressed as mean ± SEM of 6 mice. *P < 0.05 and **P < 0.01

Table 3 shows that mice treated with RR extract in a dose of 25 mg/kg induces a significant increase in number of entry into open arms ($p < 0.05$) and total arm entries of the maze (open and closed arms). In addition, combined diazepam (1 mg/kg) with RR extract (25 mg/kg) produced highly significant increase in closed and open arms compared to the controls ($p < 0.01$). With regard to the time of permanence on open arms, mice received 25 mg/kg showed more significant increase ($p < 0.01$) while the combined treated group diazepam (1 mg/kg) and RR extract (25 mg/kg) produced highly significant increase ($p < 0.01$) when compared with the controls. With regard to total arm entries (open and closed), a significant increase in animal movements compared to control group (table 3).

Table 3: effect of Retama Raetam on mouse behavior in Elevated plus maze model

Groups	Dose (mg/kg)	Time spent on open arms (sec.)	Entries number into open arms	Total arm entries
Control	-	2.83 ± 2.29	0.33 ± 0.21	2.50 ± 0.60
RR extract	25	15.46 ± 2.20**	1.83 ± 0.39*	4.75 ± 0.86*
Diazepam	1	53.34 ± 16.80*	7.5 ± 2.17*	10.25 ± 1.93*
RR + diazepam	25 + 1	80.16 ± 11.22**	9.33 ± 0.65**	9.66 ± 0.84**

Data are expressed as mean ± SEM of 6 mice. *p < 0.05 and **p < 0.01

Table 4 shows that administration of 25 mg/kg of the RR to mice induces a significant increase by ten folds ($p < 0.01$) in time spent on light area of the light-dark box and significant increase in number of line crossings compared to that of the control group. However, the plant extract has shown a clear effect than diazepam intake in this model ($p < 0.01$).

Table 4: effect of Retama Raetam on mouse behavior in light/dark box model

Groups	Dose (mg/kg)	Transition number	Time spent on light area
Controls	-	1.5 ± 0.22	17.66 ± 4.04
RR extract	25	6.33 ± 0.98**	177.83 ± 11.11**
Diazepam	1	4.0 ± 0.36**	110.2 ± 2.95**

Data are expressed as mean ± SEM of 6 mice. *p < 0.05 and **p < 0.01.

In table 5, data of hot plate method of analgesia for different periods up to 120 min is given. Thus, analysis of data revealed that the latency time is significantly increased by 200% ($p < 0.01$) when the plant extract (50 mg/kg) was given with morphine (10 mg/kg) with more potency at 60 min till 120 min. This is also true for mice with only RR, whereas, morphine alone induces only an inhibition response by 100% at 60 min.

Table (5) effect of Retama Raetam extract on mice in hot-plate analgesia method

Groups / Parameters	Controls	Morphine (10 mg/kg)	R.R. (50 mg/kg)	R.R. + morphine
00 min	6.64 ± 0.71	6.16 ± 0.54	6.57 ± 0.41	6.64 ± 0.71
30 min	6.71 ± 0.64	21.66 ± 1.61**	6.41 ± 0.99	25.5 ± 1.75**

60 min	6.57 ± 0.56	13.02 ± 0.56**	4.15 ± 0.85*	22.11 ± 1.75**
90 min	5.68 ± 0.59	5.32 ± 0.38	2.77 ± 0.79*	18.5 ± 1.26**
120 min	5.48 ± 0.35	2.66 ± 0.48**	2.20 ± 0.49**	10.78 ± 1.40**

Data are expressed as mean ±SEM of 6 mice.*p < 0.05 and **p < 0.01.

The effect of extract on acetic- acid induced writhes in mice is shown in table 6. Administration the plant extract to mice significantly inhibited writhing response by p < 0.05 in comparison with the control group. Combined RR extract with diclofenac showed highly significant reduction by in writhing counts (70%, p < 0.01).

Table 6: effect of Retama Raetam (50 mg/kg) on mice in writhing test

Treatment	Dose	Writhes number	% of inhibition
Control	5 ml/kg	28.71 ± 2.53	-
Diclofenac Na	25 mg/kg	17.21 ± 1.71**	40.1
RR extract	50 mg/kg	18.71 ± 2.78*	34.5
combination	R.R. + Diclofenac	9.5 ± 1.62**††	66.9

Data are expressed as mean ± SEM. *p < 0.05 and **p < 0.01 compared to controls and †P < 0.05 and ††P < 0.01 compared to diclofenac group.

In table 7, the effect of the plant extract seeds on picrotoxin induced convulsion in mice is given. Thus, 50 mg/kg and 100 mg/kg of RR extract were tested on mice after 3 mg/kg of picrotoxin intake. An overall analysis of the data by Kruskall Wallis test has revealed a significant change (p < 0.05) in latency between 50 mg/kg and 100 mg/kg of the extract groups and control group. Further analysis between groups by Mann-Whitney *U* test indicated a significant difference (p < 0.01) in latency between 50 mg/kg and 100 mg/kg of the extract and the control group with no change in other parameters (table 7).

Table 3: Effect of Retama raetam on mice in picrotoxin induced seizures

Treatment	Latency (min)	Number of convulsions	Mortality rate (24 hrs)
Control	16.50 ± 1.94	0.00 ± 0.00	00.00%
RR (50 mg/kg)	24.50 ± 6.96**	0.00 ± 0.00	00.00%
RR (100 mg/kg)	18.50 ± 2.48*	0.00 ± 0.00	00.00%

Data expressed as median ± SD. *p < 0.05 and **p < 0.01.

4. DISCUSSION

The main finding of this study is that Retama Raetam seeds extract has an anxiolytic-like effect in the three mouse models of exploratory behavior (elevated plus-maze, open field and light-dark box models). This finding is mainly represented by an increase in the percentage of arm entries into and time spent on open arms of the elevated plus maze model of anxiety as well as increased movements in central area of the open field test. This is in line with the previous study of anxiolytic activity of methanol extract of aerial part of RR [10]. To validate this finding, light-dark box model showed a significant increase in transitions by four folds (index of exploration) and increasing in time spent on light chamber by ten folds. Thus, the plant proves high level of anxiolytic action which indicates that it can be used in some forms of agoraphobia. Phytochemical studies of plant seeds demonstrate some biologically active metabolites as alkaloids and terpenoids. Earlier experiments had been done on cytisine to assess its exploratory and general motor activity when using both models of anxiety had inconsistent findings. Thus, this insignificant increase in time spent on open arm could be explained by a difference in genetic composition using of CD1 mice [25] or a variation in animal species [24]. However, with respect to the previous findings of light-dark compartment, cytisine treated animals are more likely to stay in the dark area (more anoxious). This could attributed to species of the animals [27] and using C57BL/6J mice knock down α2 adrenergic receptor (α2AR) in amygdale significantly spent more time on light chamber [28]. In the present study, data presented in analgesic models suggests that the plant seeds extract possesses profound centrally and peripherally mediated analgesic activities. This finding disagrees with prior one on methanol extract of aerial part of RR [29]. Existence of terpens (limonene and myrcene) and alkaloids as cytisine qualified for inducing anti-nociception. Others reported cytisine alkaloid has stronger central analgesic activity than nicotine [30], while terpens may contribute centrally and peripherally anti-nociceptive effects [31, 32]. Long term use of the plant extract does not markedly change the body weight but a previous study indicated that RR aqueous extract exhibited body weight lowering activities in severe hyperglycemic rats [33]. Body weight and food intake are directly associated with the cytisine according to Mineure et al. [34]. It is suggested that further experimental studies are needed to confirm the present findings as there are species variations and difference in experimental methods. On the other hand, the low dose of extract may decrease the latency time of

picrotoxin induced seizure but alternative previous study asserts that the plant extract has convulsant activity by containing cytisine and anagyrine which are characterized by their affinity to nAchRs [33]. The difference in both findings can be attributed to variation in the part of plant used, constituents, doses and species variation.

In conclusion, this study strongly suggests that Retama Raetam seeds extract has a profound anxiolytic-like effect and analgesic activities as well as it delayed onset of seizures produced by picrotoxin in experimental animals. However, further clinical studies are required to assess its therapeutic use and safety profile of the plant in human.

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