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Evaluation of Acute and Repeated Dose Oral Toxicity of *Phoenix dactylifera* L. Pollen Methanolic Extract in Rats

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Abstract

Pollen of the date palm (*Phoenix dactylifera* L.) has been used for thousands of years as a traditional herbal medicine for improving fertility. However, safety of plant-derived materials is an important concern to human health, and herbal remedies should be taken with adequate knowledge about their toxicity, adverse effects, purity, appropriate dosage and dose limits. The current work was undertaken to evaluate the safety of date palm pollen (DPP) and to classify it according to its oral toxicity in rats. The experiments were done according to the reported guidelines of the Organization for Economic Co-operation and Development (OECD) to assess the acute and repeated dose oral toxicity in rats. The obtained results showed neither death nor sign of toxicity or abnormality in any of the animals throughout the experimental period regarding acute and repeated dose oral toxicity. Also the results of the hematological and clinical biochemistry determinations showed no significant differences between the control and DPP-treated rat groups. It could be concluded that, the tested plant material is a relatively safe substance and its approximate LD_{50} defined by 2000mg/kg < LD_{50} < 5000mg/kg according to the OECD tests guidelines.

Keywords: Acute toxicity, date palm, pollen, repeated toxicity

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1 Introduction

Date Palm (*Phoenix dactylifera* L.) is called Nakhla and the tree of life by the Arabs [1]. The tree grows in extremely hot and dry climates, and to some extent tolerate salty and alkaline soils [2]. It is native to North Africa and Persian Gulf regions with the top producer including Iraq, Egypt, Saudi Arabia, Tunisia, Algeria, UAE, Oman, Libya, Pakistan, Sudan, and USA [3]. Palms in general possess many economic uses, the fruits of some species can be considered as an important crop used as nutrient, production of sugar, starch, fiber, wax, timber and oil which can be used in many food products [4]. The different parts of *Phoenix dactylifera* are used widely in traditional medicine for treatment of various disorders which include memory disturbance, fever, inflammation, loss of consciousness and nervous disorders, while suspension of date palm pollen (DPP) is herbal mixture that widely used for curing male infertility [5]. Currently, there are no scientific reports that address the accurate doses or dose range of DPP that have effects as traditional medicine, and the most popular forms used traditionally in our community is the pollen powder (about 2-5 grams) mixed with milk, bee honey or other herbal extracts daily (at least 2 hours before breakfast) for 1 to 2 months.

In recent years interest has refocused on traditional medicine in the management of several disorders due to the high cost of some modern drugs, time and expenditure that is necessary to bring a drug to market after appropriate clinical trials, serious side-effects of some modern drugs, and drug-resistance developed by many microorganisms [6], [7]. Also the plant kingdom serves as a valuable source of new medicinal agents, and it has been reported that approximately 25 % of modern medications have been derived from plant materials [7], [8]. However, many toxicological studies reported that toxic effects due to the use of herbal medicine are associated with hepatotoxicity, toxic effects of the kidney, nervous system, blood and cardiovascular system, as well as mutagenicity and carcinogenicity [9].

As safety and efficacy of traditional medicine is an important concern for both health authorities and the general public, the current work was undertaken to evaluate the safety of DPP as it commonly used in our community as herbal traditional medicine, and to classify it according to its oral toxicity in rats.

2 Methods

2.1 Plant material and extraction

Mature flower cluster of the male plant of date palm containing pollen were collected from El-Shamalia State, Sudan. The plant material was identified at the Herbarium of Medicinal and Aromatic Plant and Traditional Medicine Research Institute, National Center for Research, Khartoum, Sudan.

Pollen (white color dust) was obtained by shaking the split spathe that contains pollen sacs. The powdered plant material was extracted by maceration using methanol (70 %) for 72 hours, with intermittent shaking, and then filtered under vacuum using Buchner funnel. The filtrate was then allowed to evaporate at room temperature, collected, freeze dried, and stored in an amber glass container (in refrigerator) until use.

2.2 Experimental animals

Healthy mature Wister albino rats of males and females (8 - 12 weeks old) were obtained from the animal house of the Faculty of Pharmacy, University of Gezira, Sudan, and were housed in polyacrylic cages and maintained under standard laboratory conditions (temperature 25 ± 2 °C, with dark and light cycle 12/12 hours). They received standard diet and water ad libitum. Animals were acclimatized to laboratory condition for 10 days before commencement of the experiments.

2.3 Acute oral toxicity test

The Organization for Economic Co-operation and Development (OECD) guideline No. 425 was adapted for acute oral toxicity study [10]. Five female rats were used, and fasted overnight prior to the administration of the plant extract. The test consists of a single ordered dose progression in which animals were dosed, one at a time. The first animal received the first dose (175 mg/kg) using the default progression in the doses from the sequence that stated by the guideline, observed every 30 minutes during the first 4 hours, and daily thereafter, for a total of 14 days. The time interval for dosing the next animal is usually 48 hours (could be delayed by the onset, duration, and severity of toxic signs and adjusted as appropriate). İf the animal survives, the dose for the next animal will be increased to the next higher dose level (550 mg/kg); if it dies, the dose for the next animal will be decreased to the next lower dose level (55 mg/kg). The study duration (14 days) may be increased if an animal unexpectedly dies late in the study to observe the survivors at that dose and above/below or to start the study again beginning at least two steps below the lowest dose with deaths. All observations were recorded for each animal taking into consideration the principles and criteria summarized in the OECD guidance document No. 19, specially, body weights before and after treatment, changes in skin, eyes and mucous membrane, also signs of tremors, convulsion, salivation, diarrhea, sleep, coma, bleeding and respiratory problems were observed [11].

As described by the guideline [10], the testing stopped when one of the following stopping criteria first is met:

1. Three consecutive animals survive at the upper bound (2000 mg/kg).
2. Five reversals (a situation where nonresponse is observed at some dose, and a response is observed at the next dose tested, or vice versa) occur in any 6 consecutive animals tested.
3. At least 4 animals have followed the first reversal.
4. When 15 animals are dosed.

The LD_{50} was calculated using self-contained software (AOT425StatPgm) that provided with the test guideline, which allow animal data entry grids and incorporates the necessary formulas for LD_{50} estimation and confidence interval computation. The plant material was ranked and classified according to the OECD series on testing and assessment No. 33 [12].

2.4 Repeated dose oral toxicity test

This study is intended to investigate the possible health hazards likely to arise from repeated exposure over a relatively limited period of time. The method was carried out based on that reported by the OECD guideline No. 407 [13].

Based upon data from the acute oral toxicity study, the dose was selected according to the highest dose level with the aim of inducing toxic effects but not death or obvious suffering. According to the test guidelines, and because the upper bound limit dose (2000 mg/kg) in the acute oral toxicity study produces no observable toxic effects, a limit test using 1000 mg/kg body weight/day of DPP extract was used.

A total number of 30 animals (15 females and 15 males) were used, from them 10 animals (five females and five males) were administered with the DPP extract, and another satellite group of 10 animals (5/sex) at the same dose of the extract (for observation of reversibility, persistence, or delayed occurrence of toxic effects) were kept for 14 days post treatment, while a number of 10 animals (5/sex) were considered as a control groups (received distilled water only).

The animals in the test groups were dosed with the plant extract daily for a period of 28 days, and observed daily during the study period taking into consideration the principles and criteria summarized in the OECD guidance document No. 19, specially, body weights before and after treatment, changes in skin and eyes and mucous membrane, also signs of tremors, convulsion, salivation, diarrhea, sleep, coma, bleeding and respiratory problems [11].

All animals were killed after the last day of the treatment, while those of the satellite group were kept for 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects.

Blood collection from all animals was done at the end of the treatment for the following

examinations: haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and for clinical biochemistry determinations to investigate the major toxic effects in kidneys and liver such as alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total bilirubin, total proteins, blood urea and serum creatinine.

2.5 Data analysis

The obtained hematological and biochemical results were statistically analyzed using a paired t-test and expressed as the mean±standard error. For data comparisons between the control and test groups, differences were considered significant if the *P-value* was <0.05.

3 Results and Discussion

During the study period (14 days) of the acute oral toxicity test, observations of the animals that administered with DPP extract based on that described by the OECD guideline No. 425 [10], OECD guidance document No. 19 [11], and OECD series on testing and assessment No. 33 [12], revealed that, there were no signs of toxicity, severe pain, distress and/or animals found in a moribund condition even at the dose level of 2000 mg/kg. Also individual weight calculation of each animal showed no decrease in body weights. The plant material was considered to be non-toxic and was allocated, according to the OECD series on testing and assessment No. 33, to category 5 (very low acute oral toxicity), the approximate LD_{50} defined as more than 2000 mg/kg and less than 5000 mg/kg [12]. Figure 1 represents the test report generated by the software (AOT425StatPgm) provided with the OECD Guideline No. 425.

Regarding repeated dose oral toxicity test, and based on the data obtained from the acute oral toxicity study, a limit test using 1000 mg/kg body weight/day of DPP extract for 28 days was used according to that stated by OECD Guideline No. 407 [13]. During the study period (28 days for the test group and 42 days for the satellite group) no death was observed in any of the groups throughout the experimental period, and no abnormality was found in any of the animals. Among treated groups (males and females) the weights of animals were increased compared to the control groups of both sexes. Moreover, organs observed at necropsy shows no significant changes. The results of the hematological parameters including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count showed no treatment-related changes. The clinical biochemistry determinations showed no significant differences between the control and treatment groups in the biochemical parameters such as ALP, SGOT, SGPT, total bilirubin, total proteins, blood urea and serum creatinine. Data were presented in Tables 1, 2 and 3.

AOT425statpgm (Version1.0) Test Results and Recommendations
Acute Oral Toxicity (OECD Test Guideline 425) Statistical Program

Date/Time:Sunday, October 28, 2018, 9:45:37 AM
Data file name:DPP Acute Toxicity Test
Last modified:10/28/2018 9:45:04 AM

Test/Substance:DPP methanolic extract
Test type:Main Test
Limit dose (mg/kg):2000
Assumed LD50(mg/kg):Default
Assumed sigma (mg/kg):0.5

Recommended dose progression: 2000, 550, 175, 55, 17.5, 5.5, 1.75

DATA:

Test Seq.	Animal ID	Dose (mg/kg)	Short-term Result	Long-term Result
1	1	175	O	O
2	2	550	O	O
3	3	2000	O	O
4	4	2000	O	O
5	5	2000	O	O

(X = Died, O = Survived)

Dose Recommendation:The main test is complete.

Stopping criteria met:3 at Limit Dose.

SUMMARY OF LONGTERM RESULTS:

Dose	O	X	Total
175	1	0	1
550	1	0	1
2000	3	0	3
All Doses	5	0	5

Statistical Estimate based on long term outcomes:
The LD50 is greater than 2000 mg/kg.

Figure 1 DPP acute oral toxicity test report

Clinical data observed during the study period for both acute and repeated dose oral toxicity showed no changes in skin, eyes and mucous membrane. There was also no signs of tremors, convulsion, salivation, diarrhea, sleep, coma, bleeding and respiratory problems. Assessment of animal sensory reactivity to stimuli of different types (auditory, visual and proprioceptive stimuli) showed normal behaviours and no treatment-related changes.

Table 1 Mean weights (grams) of animals during the study of repeated dose oral toxicity test

Group (N*=5)	Day 1	Day 7	Day 21	Day 28	Weight gained during the study period (%)
Control Males	184	192	201	205	11.4
Test Males	176	182	199	203	15.3
Control Females	175	182	189	194	10.9
Test Females	172	181	188	196	14

*N: number of animals.

Table 2 Hematological results of animals after 28 days of repeated dose oral toxicity test

Group (N*=5)	Erythrocyte count (100/µl)	Differential leucocyte count (1000/µl)	Haemoglobin concentration (g/dl)	Haematocrit (%)
Control Males	7.52 ± 0.67	9.16 ± 1.91	15.64 ± 0.86	42.66 ± 3.96
Test Males	7.17 ± 0.44	8.64 ± 2.05	16.38 ± 0.52	39.82 ± 5.38
Control Females	7.86 ± 0.39	6.38 ± 1.78	14.56 ± 0.65	38.42 ± 2.94
Test Females	8.11 ± 0.78	7.08 ± 2.13	15.45 ± 0.96	36.76 ± 5.67

*N: number of animals.
No significant differences between the control and test groups (*P-value* >0.05).

Table 3 Biochemical results of animals after 28 days of repeated dose oral toxicity test

Group (N*=5)	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	Total Bilirubin (mg/dL)	Total proteins (g/dL)	Serum creatinine (mg/dL)	Blood urea (mg/dl)
Control Males	94 ± 13	189 ± 65	258 ± 12	0.13 ± 0.03	5.82 ± 0.12	0.82 ± 0.04	46.4 ± 5.4
Test Males	89 ± 15	194 ± 34	276 ± 21	0.12 ± 0.03	6.13 ± 0.14	0.77 ± 0.05	54.1 ± 5.1
Control Females	85 ± 17	241 ± 88	288 ± 13	0.12 ± 0.02	5.74 ± 0.12	0.77 ± 0.03	53.8 ± 5.2
Test Females	77 ± 28	218 ± 56	301 ± 18	0.11 ± 0.02	5.59 ± 0.12	0.74 ± 0.02	48.5 ± 4.5

*N: number of animals.
No significant differences between the control and test groups (*P-value* >0.05).

Based on the published literature, for animal experimental designs it is critical to use control groups as an integral part of the study for comparison of data in the animals given the treatment to non-treated ones, this aims to minimize the impact of plethora variables, validates the experiment, provide the basis for data analysis and comparisons, and can also discriminate outcomes caused by the treatment or intervention from those caused by other factors [14], [15], [16]. Therefore, the data obtained from the control groups (non-treated animals) were used as reference values for comparisons.

Most of the toxicological studies reported that, toxic effects due to the use of herbal medicine could be associated with hepatotoxicity, kidney toxicity, nervous system, circulatory system, as well as carcinogenicity [9]. Regarding the current study, organs observed at necropsy along with biochemistry determinations showed no treatment-related changes. Furthermore, individual weight calculation of each animal showed no decrease in body weights among treated groups (the weights of animals were increased compared to the control groups of both sexes), and these findings could support that the plant extract is a safe substance based on the OECD guidance document No. 19 [11], which stated that, body weight decrease by more than 25% over a period of 7 days or more usually considered as a sign of toxicity and usually accompanied by reduced or absence of food intake.

From the published data, it has been noted that the plant parts exhibited significant antioxidant, anticancer, hepatoprotective, nephroprotective, neuroprotective, haemopoitic and anti-inflammatory activities [17], [18].

The obtained data were in close agreement with other previous reported findings [19], [20], although the parts used in the mentioned studies (seeds and fruits respectively) are different from the part used in the current study (pollen), but all these parts could be considered as complementary to each other and the product of evolution of the same cells. Thus, these findings indicated that the plant materials are relatively safe substance. However, alertness should be exercised concerning its use because the plant contains phytates, tannins, and calcium oxalates, and these constituents could cause some serious health effects and

complications such as stomach irritation, mineral deficiency and kidney stone [17].

4 Conclusions

It could be concluded that, the acute oral toxicity test findings indicated that the tested plant material is of very low acute oral toxicity and its approximate LD_{50} defined by 2000 mg/kg < LD_{50} < 5000 mg/kg, while the repeated dose oral toxicity test showed neither death nor sign of toxicity or significant differences regarding biochemistry determinations in any of the tested animals throughout the experimental period, which indicated that, the plant material is relatively safe substance. However, efficacy, safety, and possible role of DPP in treatment of some disorders should be further evaluated by well-designed controlled clinical studies.

5 Declarations

5.1 Author Contributions

All authors contributed equally in concept, design, resources, experiments, data interpretation and critical review of the study.

5.2 Funding Statement

There is no funding body, and the study was carried out by self-financing.

5.3 Conflicts of Interest

The authors declared that, there are no any conflict of interest regarding the current study.

5.4 Ethic

The current study was ethically approved by the Faculty of Pharmacy - University of Gezira ethical committee, and the experimental protocols and procedures were carried out in accordance with the guide for the care and use of laboratory animals of The National Academies [21].

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