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Research Article

A Reproductive Toxicological Evaluation of 90 Days Repeated Exposure of *Pterospermum acerifolium* (L) Willd in Rodents

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Abstract

The present study aimed to evaluate the effects of repeated administration of leaves of *Pterospermum acerifolium* (L) Willd (MEPA) on the reproductive system of male and female Wistar albino rats and its developmental effects on subsequent generation. MEPA was administered to Wistar albino rats by gavage for 90 days repeatedly at doses of 250, 500 and 1000 mg/kg body weight, in accordance to OECD guidelines No. 408, 414 and 416. Male fertility was estimated after 24 hours after 90 days MEPA by sperm count and estimation of glycogen content, superoxide dismutase activity and ascorbic acid. Body weight and food intake behavior was also monitored regularly. After treatment the male and female animals were allowed to mate. Parenteral females were regularly observed for signs of parturition and allowed to give birth to the F1 generation. The pups were clinically observed and the following parameters were recoded – litter size, number of still born pups, number of live born pups, pup body weight and sex. At the dose levels tested MEPA does not significantly alter body weight or food consumption behavior. Sperm count was found to be normal in treatment groups. The glycogen content was however found to be slightly raised in the highest dose levels tested, but not significant ($p>0.001$). Female reproductive system tolerates MEPA well as indicated by normal gestational period and normal birth rates of F1 pups, compared to the control group. The no observed effect level (NOEL) for maternal and development toxicity was 1000mg/kg/BW/d, the highest dose evaluated.

Keywords: *Pterospermum acerifolium;* repeated; toxicity; reproductive; developmental.

Introduction

Medicinal herbs are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize eco-friendly and bio-friendly plant-based products for the prevention and cure of different human diseases [1]. Inspite of increased demand of herbs in market for its use in different medicinal products still there are several issues related with their safety. Very less (< 10 %) marketed herbal products are standardized and strictly followed quality control measures [2]. Very little information is available regarding the toxicity of these products. Some plants produce toxic constituents for defense purposes. These are *Aconitum columbianum, Blighia sapida, Trifolium hybridum, Digitalis purpurea, Gymnocladius dioica, Hyoscyamus niger, Solanum nigrum, Sanguinaria Canadensis, Atropa belladonna, Physostigma venenosum, Pteridium aquilinum and Podophyllum peltatum,* these toxic substances are not distinguished from therapeutically active ingredients. Most commonly used herbal formulae have no documented evidence on quality, safety and efficacy. The plant *Pterospermum acerifolium* Linn Willd (Family Sterculiaceae), common name Muchkunda is used in traditional medicines for its haemostatic and wound healing properties. Preliminary pharmacological screening also shows the presence of anti-inflammatory, analgesic, antioxidant, antiulcer, wound healing and antipyretic properties in the leaves of the plants [3-4]. Evaluation of work of earlier researchers revealed that no significant documented evidence is there regarding the safety profile of the leaves [5-6]. Recently there has been growing concern over the effects of either natural or synthetic products on the reproductive health owing to the growing problem of infertility and impotence. The toxicological implications may be both on male fertility or female reproductive health. Nowadays, little is known about the possible toxic effects of leaves of *Pterospermum acerifolium Linn* Willd on the fertility and reproductive system of male and female rats [7-9]. Work carried out by earlier researchers have shown that on repeated administration leaves of *Pterospermum acerifolium* Linn Willd can cause cumulative toxicity, leading to altered liver, kidney function and hematological anomalies [9-11]. Thus the study was undertaken to delineate whether methanolic extract of leaves of *Pterospermum acerifolium* Linn Willd exerts any effects on reproductive and developmental systems of rodents (male and female Wistar albino rats) after 90 days repeated oral administration.

Materials and Methods

Plant Material

The leaves of *Pterospermum acerifolium* (L) Willd were collected from Asansol, West Bengal, India in September 2015. A herbarium sheet was prepared and authenticated by the Botanical Survey of India, Howrah, West Bengal, India after macroscopic and microscopic evaluation. The leaves were dried in shade and coarse powdered by using a grinder.

Preparation of the leaf extract

The air dried crushed leaves (500g) were soaked for 12 h in Methanol (2L) at room temperature. The residue was extracted with hot Methanol under reflux 3 times (each 1000 ml) after vacuum filtration. The extract was concentrated in Rotary evaporator and lyophilized, to yield approximately (10% w/w) of the residue, which was stored at 20 ^{o}C until use in a dessicator. The concentrate of methanolic extract of *Pterospermum acerifolium* (MEPA) was suspended in 5% w/v Tween 80 for oral administration in Wistar albino rats.

Preliminary Phytochemical studies

Preliminary qualitative phytochemical studies were carried out to detect various phytoconstituents present in MEPA [12-13].

Study Method

The studies were conducted in accordance with the Organization for

Economic Co-operation and Development (OECD) guidelines No. 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents), No. 414 (developmental study) and 416 (reproductive study) and were in compliance with ethical standards set by CPCSEA and Institutional Animal Ethics Committee .

Animals

Wistar albino rats (5weeks old, weighing from 100 to 125 g) were selected after physical and behavioral veterinary examination from Institutional Animal House of Gupta College of Technological Sciences (Reg no. 955/RO/A/2006/CPCSEA). All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-2˚C and humidity 60-65% with 12:12 light: dark cycles). Food was provided in the form of dry pellets and water *ad libitum.* The animals were allowed to get acclimatized to the laboratory conditions for 7 days before the commencement of the experiment. All experiments involving animals complies with the ethical standards of animal handling and approved by Institutional Animal Ethics Committee.

Test formulations and dosing

The test and control formulations were administered by suspending in 5% Tween 80 by gavage once daily for 90 days throughout the study period. The dose levels based on the study were 250, 500 and 1000 mg/kg body weight (BW)/day. Earlier studies have reported to be safe upto 2g/kg BW/day. The highest dose selected did not exceed the maximum tolerated repeated dose exposure for MEPA for the current study designs [9-11].

In-life examinations

All animals were observed at least two times a day throughout study period for signs of abnormal toxicity. Morbidity, mortality, availability of food and water and any overt evidence of toxicity was observed. Animals showing signs of severe debility or toxicity were euthanized, and post mortem analysis done for evaluation of the toxicity.

Examination of male fertility

Fertility was estimated in adult male Wistar rats according to the method of Manson and Kang (1989) [14]. 24 h after 90 days MEPA, each male was placed in a separate cage with two virgin untreated female of the same strain. They are left together for 7 days, and during this period, one estrous cycle should have elapsed [15]. Following positive identification of mating, female were placed to individual cages. Positive mating was confirmed by the presence of a copulatory plug or the presence of sperm in the vaginal smears. Each mating set was examined daily and when evidence of mating was identified that day was determined day 0 of pregnancy (GD0). The dams were sacrificed on GD20 for confirmation of pregnancy. The fertility index was analyzed, i.e., (no. of males that became sire/no. of male placed with female) x100.

The left epididymis of each animal (three per group) was frozen immediately after euthanasia. They were frozen until evaluation. After thawing at room temperature, the cauda epididymis were homogenized, for 1 min, in 10 ml normal saline (0.9% NaCl) containing 0.05% Triton X- 100. Sperm count was determined at 400x magnification using Neubauer Chamber [16].

Male toxicity

Male toxicity was evaluated by monitoring the body weight changes following each period of exposure at mating. Hematological evaluation was done of all the animals at the end of study period to assess response of hematological system towards repeated MEPA administration at various dose levels. Clinical and behavioral observations were also recorded throughout the study. The testes were used for determination of the toxicological biochemical parameters such as glycogen content, superoxide dismutase and ascorbic acid determination [17-19].

Female toxicity

Individual body weights were measured on GD 0 and every 3 days during the gestational phase of the developmental toxicological study. In the developmental toxicological study, food consumption was measured over GDs 0-20, after every 3 days.

P-generation parturition and F1 litter observations in the multigenerational study

Parenteral female animals were regularly observed for signs of parturition and allowed to give birth to the F1 generation. The day of gestation was observed, and the day the pups were delivered was designated ad lactational day 0 (LD 0). The pups were clinically observed and the following parameters were recoded – litter size, number of still born pups, number of live born pups, pup body weight and sex. Any intact dead pup was subjected to post-mortem analysis.

Statistical evaluation

All the data were presented as Mean ± SEM. The differences between group were evaluated by the one-way analysis of variance (ANOVA) followed by Dunnette's multiple comparison test's ($p<0.01$) was considered to be significant.

Results and Discussion

Preliminary phytochemical screening

Preliminary phytochemical studies showed the presence of flavonoids, carbohydrates and alkaloids.

Toxicity Studies

Herbal preparations have a great role to play in the modern system of medicine. However they need to be evaluated for toxicological implications upon long term use [2, 20].

Effect of methanolic extract of leaves of *Pterospermum acerifolium* (MEPA) on the body weight of male and female rats is depicted in Figure 1 and 2 respectively.

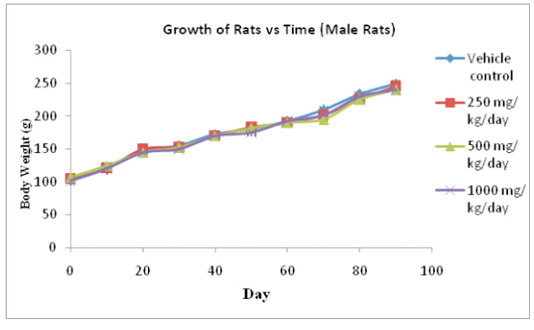

Figure 1: Mean body weights of male rats administered daily (0, 250, 500 and 1000 mg/kg b.w.) doses of *Pterospermum acerifolium* methanolic leaf extract by gastro-gavage for 90 days(n=10)

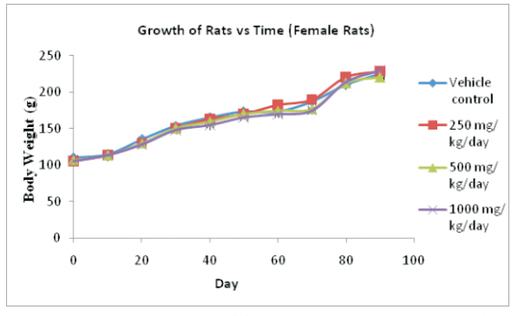

Figure 2: Mean body weights of female rats administered daily (0, 250, 500 and 1000 mg/kg b.w.) doses of *Pterospermum acerifolium* methanolic leaf extract by gastro-gavage for 90 days (n=10)

Normal body weight gains were observed in males and females of all dose groups, compared to control group. No abnormal gross findings were observed in any animals. During the 90-day treatment period, mortality was observed in only one animal in the highest MEPA treatment group. Rest of the animals was normal. Daily recording of external morphologic characteristics revealed no drastic changes in most of the treated animals. Only two animals in 500 mg/kg group showed temporary loss of fur and three animals in 1000 mg/kg group showed skin peeling in the tail after 30 days of treatment that continued till the 90 day treatment period. In all other animals, the fur, skin, eyes, animal behavior, gait and posture, reactivity to handling and grip strength were recorded normal. However two animals showed hypoactivity in the 1000mg /kg treatment group. Rests of the animals were active throughout and did not show any unusual behavior such as self mutilation, walking backward and so forth. No tremors, convulsions, mucus discharge, salivation and diarrhea were observed during the entire 90-days treatment period. Food and water intake showed daily fluctuations within the control limit. Anorexia was noticed only in the 500mg/kg MEPA treatment group.

Change in body weight is frequently used as an indicator of adverse effects of drugs and chemicals on various organ system [21]. Significant changes were observed in the general behavior, body weight and food intake of rats in the treated groups as compared to the control group after 90 day period of daily treatment, thus it may be concluded that at the sub-chronic oral doses administered, leaves of *Pterospermum acerifolium* had no effect on the normal growth of rats. Though one some of the rats depicted anorexia and hypoactivity, that may be attributed to biological and intra species variation, frequently encountered in animal studies. Work done by earlier researchers had already shown that MEPA does not cause any significant alteration of body weight or on hematological parameters after 28 days repeated administration at dose levels of 250, 500 and 1000mg/kg b.w. (p.o.) [22-24].

No significant change was observed on reproductive performance. Mating and fertility indexes were similar in MEPA treated and the control groups (Table 1).

Table 1: Effect of MEPA exposure on fertility and reproductive performance of male Wistar rats

Parameters	MEPA (mg/kg b.w.)			
	Control[a]	250	500	1000
No. evaluated	10	10	10	10
No. died or sacrificed moribund	0	1	0	1
No. with evidence of mating	9	9	8	9
No. not siring 1 litter	0	0	1	1
No. siring at least 1 litter	7	7	7	7
No. siring more than 1 litter	2	2	0	1
No. of fertile males	10	10	8	8
Mating index (%)	90	90	80	90
Fertility index (%)	90	90	70	80

[a]Control animals received 5% v/v Tween 80 (1ml/100g b.w.)

According to this, sperm counts were similar in all groups evaluated (Table 2).

In the female animals body weight gain (Figure 2) and food consumption behavior was found to be normal in the treatment period, and the gestational phase. Also normal reproductive behavior and pregnancy was observed (Table 1).

Table 2: Effect of MEPA exposure on sperm counts of Wistar rats[a]

	Sperm Count
Control (5% v/v Tween 80)	98.45 ± 08.85
MEPA (250mg/kg b.w.)	93.38 ± 05.44
MEPA (500mg/kg b.w.)	95.65 ± 08.33
MEPA (1000mg/kg b.w.)	93.36 ± 10.34

[a]Data are present as x106 cells/ml. Data are expressed as mean ± SEM (n=10)When compared with control *p<0.01 (One way ANOVA followed by Dunnett's multiple comparison test).

From the results of the sub chronic study on the reproductive and developmental effects, it may be clearly stated that the methanolic extract of leaves of *Pterospermum acerifolium* (MEPA) does not cause any alteration of reproductive functions or organs upto the highest dose tested (1000 mg/kg b.w.)(p.o.). The rats show normal sexual behavior or reproductive performance. The parenteral NOEL (No observed adverse effect level) was ⩾1000 mg/kg BW/day.

Regarding to other parameters studied, there was a decrease in testes glycogen content on male rats treated with 1000 mg/kg b.w. MEPA for 90 days (Figure 3).

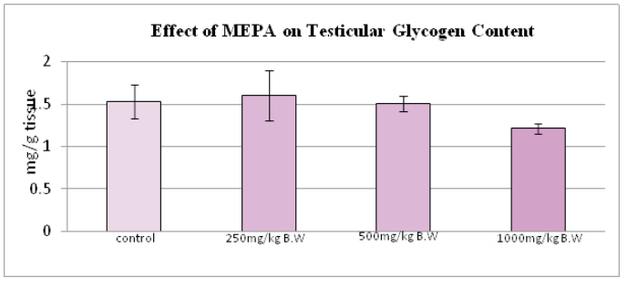

Figure 3: Effect of MEPA exposure of male rats administered daily (0, 250, 500 and 1000 mg/kg b.w.) doses of *Pterospermum acerifolium* methanolic leaf extract by gastro-gavage for 90 days on testicular glycogen content. Data are expressed as mean ± SEM (n=5).

However the ascorbic acid levels were found to be elevated in the group. There was slight decrease in glycogen content in the 500mg/kg b.w. treated group, but not significant. No alterations were found in the level of SOD (data not shown) activities in groups that received MEPA 250mg/kg and 500 mg/kg b.w.

A decrease in the glycogen content was observed in male animals treated sub chronically with MEPA 1000 mg/kg b.w. MEPA for 90 days The changes in the glycogen level may be due to interference in glucose metabolism either increased catabolism of the carbohydrates to meet the enhanced energy demand of animals or their reduced synthesis due to impaired tissue function. Besides, the absence of carbohydrates also suppresses Leydig cell function [21, 25]. Increase in ascorbic acid levels may be attributed to reflex increase in body antioxidant levels, owing to repeated administration of MEPA [26]. Leydig cells produce the testosterone hormone, essential to growth and division of the germinative cells. In spite of the slightly alteration in the glycogen level, MEPA exposure seems does not impair the reproductive performance of the animals, or any other biochemical parameter including superoxide dismutase activity. Elevation in ascorbic acid levels indicate oxidative stress on repeated MEPA administration, leading to reflex stimulation of anti-

oxidant levels in the body [21-23]. The results further substantiate the oxidative injury inflicted by MEPA, as observed with elevation in WBC and neutrophil levels.

Normal mating behavior and normal offspring is often taken as index of normal male and female reproductive architecture. The standard method for determining adverse effects of chemical compounds on fertility in animal experiments is based on mating trials to determine whether a given compound affects the reproduction [27]. Our data clearly demonstrated that male rats exposed to MEPA presented: normal sexual behavioral, reproductive performance and epididymal sperm counts unaltered, suggesting the lack of the male reproductive toxicity induced by these compounds. In this study, histopathology evaluation revealed that no modification was found in the testicular morphological architecture on MEPA treated groups. The connective tissue, seminiferous tubules, Sertoli and Leydig cells that support spermatogenesis and provide nutrition for sperm cells were not adversely affected in all groups exposed to repeated 90 days of MEPA. The seminiferous tubules showed successive stages of transformation of spermatogonia into spermatozoa and lumen filled with spermatozoa [27-28].

Histological evaluation showed no significant alteration of the testicular tissue in the MEPA treated animals. The testis of rats exposed (Figure 4) to 90 days MEPA exhibited normal morphological architecture.

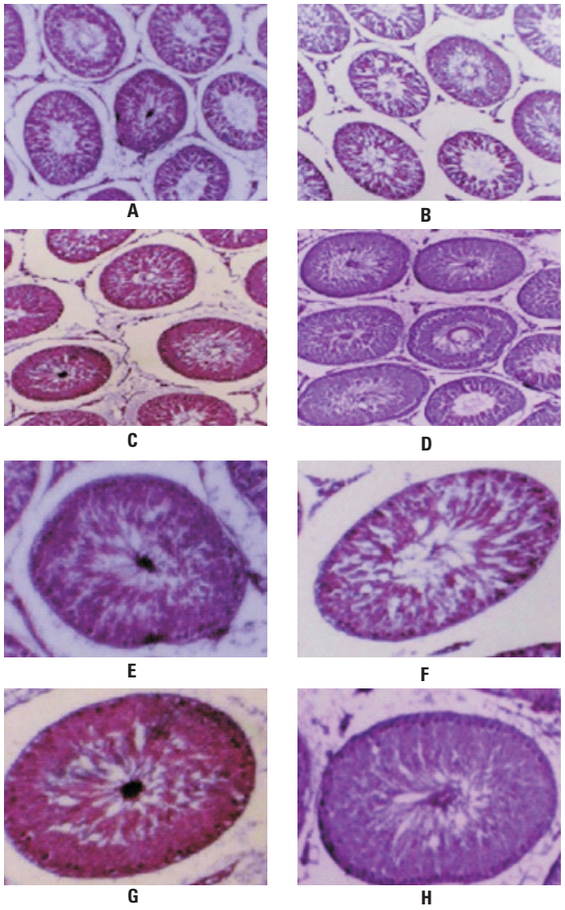

Figure 4: Testicular sections of the control rat (A and E), MEPA (250mg/kg b.w.) (B and F), MEPA (500 mg/kg b.w.) (C and G) and MEPA (1000 mg/kg b.w.) (D and H) sub-chronically (for 90 days) treated rats. Hematoxylin and eosin. (A), (B), (C) and (D): 40x; (E), (F), (G) and (H): 400x.

The seminiferous tubule showed successive stages of transformation of spermatogonia into spermatozoa and lumen filled with spermatozoa.

Compared with the controls, the 250, 500 and 1000 mg/kg p.o. MEPA did not significantly alter any of the reproductive parameters investigated, gestation length and neonatal development of pups born. Further, no obvious external gross morphological deformities were detected in pups treated both with extract and control. This may be explained by the fact that MEPA does cause significant abnormality or reproductive functions in female animals at the dose levels tested [7], [29].

Conclusion

In the present study it may be concluded that methanolic extract of leaves of *Pterospermum acerifolium* does cause significant alteration of male and female reproductive functions in Wistar albino rats upon 90 days repeated administration.

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Conflicts of interest

The authors declare no competing interest.

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