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A REPRODUCTIVE AND DEVELOPMENTAL STUDY FOLLOWING 28 DAYS REPEATED ORAL EXPOSURE OF LEAVES OF *PTEROSPERMUM ACERIFOLIUM* (L.) WILLD IN WISTAR ALBINO RATS

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Keywords:

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ABSTRACT: The present study was aimed to evaluate the effects of repeated administration of methanolic leaf extract of *Pterospermum acerifolium* Linn. 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 28 days repeatedly at doses of 250, 500 and 1000 mg/kg body weight, in accordance to OECD guidelines 414 (developmental study) and 416 (reproductive study). Male fertility was estimated by sperm count. Biochemical evaluations included estimation of glycogen content, superoxide dismutase activity and ascorbic acid. Body weight and food intake behavior was also monitored regularly. MEPA treated male and female rats were allowed to mate. After mating females were observed for signs of parturition and allowed to give birth to the F1 generation. The pups were clinically observed. At the dose levels tested MEPA does not significantly ($p<0.01$) alter sperm count of male rats. Female reproductive system tolerates MEPA well. Normal gestational period was observed. Normal birth rates of F1 pups showed absence of developmental toxicity of MEPA. The no observed effect level (NOEL) for maternal and development toxicity was 1000 mg/kg/bw/d, the highest dose evaluated ($p<0.01$). MEPA does not cause male or female toxicity in Wistar albino rats upon 28 day repeated oral administration upon repeated MEPA administration upto 1000 mg/kg/bw/d.

INTRODUCTION: An herb is a plant or plant part used for its scent, flavor, or therapeutic properties. Herbal medicines are one type of dietary supplement. They are sold as tablets, capsules, powders, teas, extracts, and fresh or dried plants.

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People use herbal medicines to try to maintain or improve their health [1]. Many people believe that products labeled "natural" are always safe and good for them. This is not necessarily true. Herbal medicines do not have to go through the testing that drugs do. Some herbs, such as comfrey and ephedra, can cause serious harm. Kumari *et al.*, has already shown that some herbs can interact with prescription or over-the-counter medicines [2].

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 by Saboo *et al*., 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 such as Basu *et al*., revealed that no significant documented evidence is there regarding the safety profile of the leaves [5, 6]. Male and female reproductive toxicology has recently become a rapidly expanding area of research and testing.

In the last decades 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. 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, 8].

Work and review carried out by Nandy *et al*., Chatterjee *et al*., and Datta *et al*., have shown that repeated administration leaves of *Pterospermum acerifolium* Linn. Willd. can cause cumulative toxicity, leading to altered liver, kidney function and hematological anomalies [9, 10, 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 28 days repeated sub-acute oral administration.

MATERIALS AND METHODS:

Chemicals and Reagents: Methanol (S.D. Fine-Chem Ltd., Mumbai), tween 80 [E. Merk (India) Ltd.], Iodine-Iodide Reagent [E. Merk (India) Ltd.]. Other chemicals and kits were purchased from Hi Media Laboratories Pvt., Ltd., (Mumbai, India) and Sigma Aldrich, India.

Plant Material: The leaves of *Pterospermum acerifolium* (L.) Willd. were collected from Asansol, West Bengal, India in September 2013 and 2016. A herbarium sheet was prepared and authenticated by the Botanical Survey of India, Howrah, West Bengal, India after macroscopic and microscopic evaluation. Reference number of the authentication letter is CNH/I- I (144)/2013/Tech. II. The leaves were shade dried and coarse powdered by using a grinder.

Preparation of the Leaf Extract: The air dried crushed leaves (500 g) were soaked for 12 h in methanol (2 L) 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 (12% w/w) of the residue, which was stored at 20 ºC until use in a dessicator. The concentrate of MEPA was suspended in 5% w/v tween 80 for oral administration in rodents.

Preliminary Phytochemical Studies: Preliminary qualitative phytochemical studies were carried out to detect various phytoconstituents present in MEPA in accordance with Benariba *et al.,* and Kaur *et al.* [12, 13]

Study Method: The studies were conducted in accordance with the Organization for Economic Co-operation and Development (OECD) guidelines no. 414 (developmental study) and 416 (reproductive study).

Animals: Wistar albino rats (6 weeks old, weighing from 125 to 150 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 ºC 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 per day for 28 days throughout the study. Based data available from research done by previous workers, dose levels selected for these studies were 250, 500 and 1000 mg/kg body weight (bw)/day.

The animals were grouped into 4 groups. Each group containing 20 rats (10 male and 10 females). Group – I served as vehicle control. Group – II, II and IV received MEPA at a dose of 250, 500 and 1000mg/kg BW MEPA by gavage. The highest dose selected did not exceed the maximum tolerated repeated dose exposure for MEPA for the current study designs as shown by Nandy *et al.,* Chatterjee *et al.,* and Datta *et al.* [9, 10, 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 Pandey *et al.* [14] 24 h after 28 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 as described by Low *et al.* [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) × 100.

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 was homogenized, for 1 min, in 10 ml normal saline (0.9% sodium chloride) containing 0.05% triton X-100. Sperm count was determined at 400x magnification using neubauer chamber as described by Ikpeme *et al.* [16]

Male Toxicity: Body weight changes were monitored to evaluate male toxicity. Following each period of exposure at mating, boy weight was evaluated. Clinical and behavioral observations were also recorded throughout the study. Glycogen content was evaluated as described by Tawab *et al*., Superoxide dismutase activity was evaluated as described by Kalender *et al.*, and ascorbic acid as described by Reddy *et al.* [17, 18, 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 F_1 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 observed. Various parameters were recorded. Litter size, number of still born pups, number of live born pups, pup body weight and sex were noted. 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:

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

Toxicity Studies: Effect of MEPA on the body weight of male and female rats is depicted in **Fig. 1** and **2** respectively. 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 28 day treatment period, mortality was not observed in any of the MEPA treatment groups.

Daily recording of external morphologic characteristics revealed no drastic changes in most of the treated animals. One animal in 500 mg/kg group showed temporary loss of fur and two animals in 1000 mg/kg group showed skin peeling

in the tail after 4 weeks of treatment that continued till the 28 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. No tremors, convulsions, mucus discharge, salivation and diarrhea were observed during the entire 28 day treatment period. The animals were active throughout and did not show any unusual behavior such as self mutilation, walking backward and so forth. Food and water intake showed daily fluctuations within the control limit.

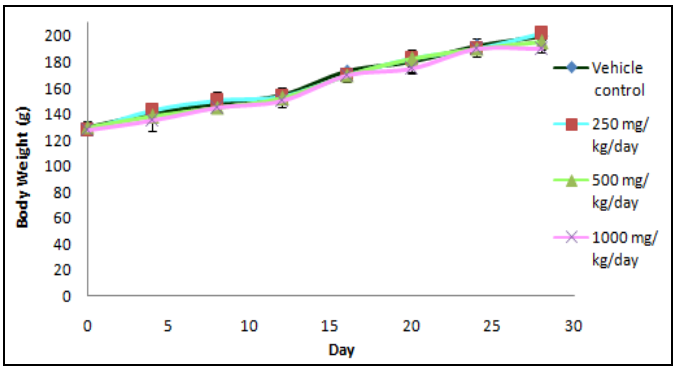

FIG. 1: MEAN BODY WEIGHTS OF MALE RATS

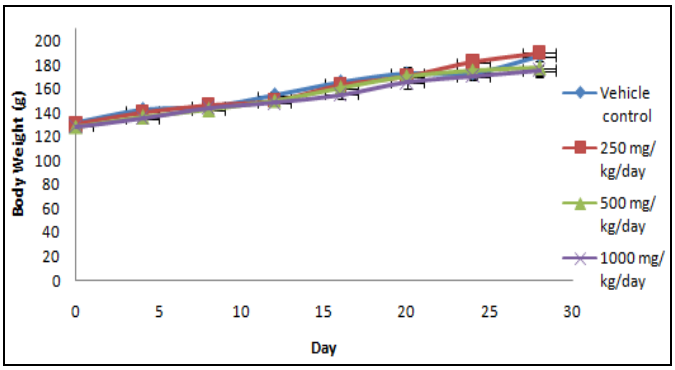

FIG. 2: MEAN BODY WEIGHTS OF FEMALE RATS

No significant change was observed on reproductive performance. Mating and fertility indexes were similar in MEPA treated and the control groups **Table 1**. According to this, sperm counts were similar in all groups evaluated **Table 2**.

TABLE 1: EFFECT ON FERTILITY AND REPRODUCTIVE PERFORMANCE OF MALE WISTAR RATS

Parameters	Control[a]	MEPA (mg/kg b.w.)		
		250	500	1000
No. evaluated	10	10	10	10
No. died or sacrificed moribund	0	0	0	0
No. with evidence of mating	10	10	8	9
No. not siring 1 litter	0	0	0	1
No. siring at least 1 litter	6	7	8	7
No. siring more than 1 litter	4	3	0	1
No. of fertile males	10	10	8	8
Mating index (%)	100	100	80	90
Fertility index (%)	100	100	80	80

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

TABLE 2: EFFECT OF ON SPERM COUNTS OF MALE WISTAR RATS

Group	Sperm Count
Control (5% v/v Tween 80)	99.53 ± 13.79
MEPA (250 mg/kg b.w.)	91.33 ± 02.34
MEPA (500 mg/kg b.w.)	96.60 ± 10.23
MEPA (1000 mg/kg b.w.)	93.33 ± 02.34

Data are present as $x10^6$ 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).

Regarding to other parameters studied, except for a decrease in testes glycogen content ($p < 0.01$) as compared to the respective control group) on male rats treated with 1000 mg/kg b.w. MEPA for 28 days **Fig. 3**, no alterations were found in the level of ascorbic acid concentration and SOD (data not shown) activities in groups that received MEPA.

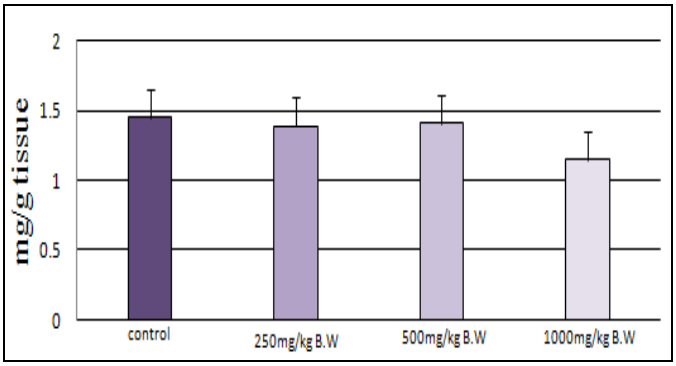

FIG. 3: EFFECT ON TESTICULAR GLYCOGEN CONTENT

Histological evaluation showed no significant alteration of the testicular tissue in the MEPA treated animals. The testis of rats exposed sub-chronically **Fig. 4** to MEPA exhibited normal morphological architecture.

The seminiferous tubule showed successive stages of transformation of spermatogonia into spermatozoa and lumen filled with spermatozoa. In the female animals body weight gain **Fig. 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**.

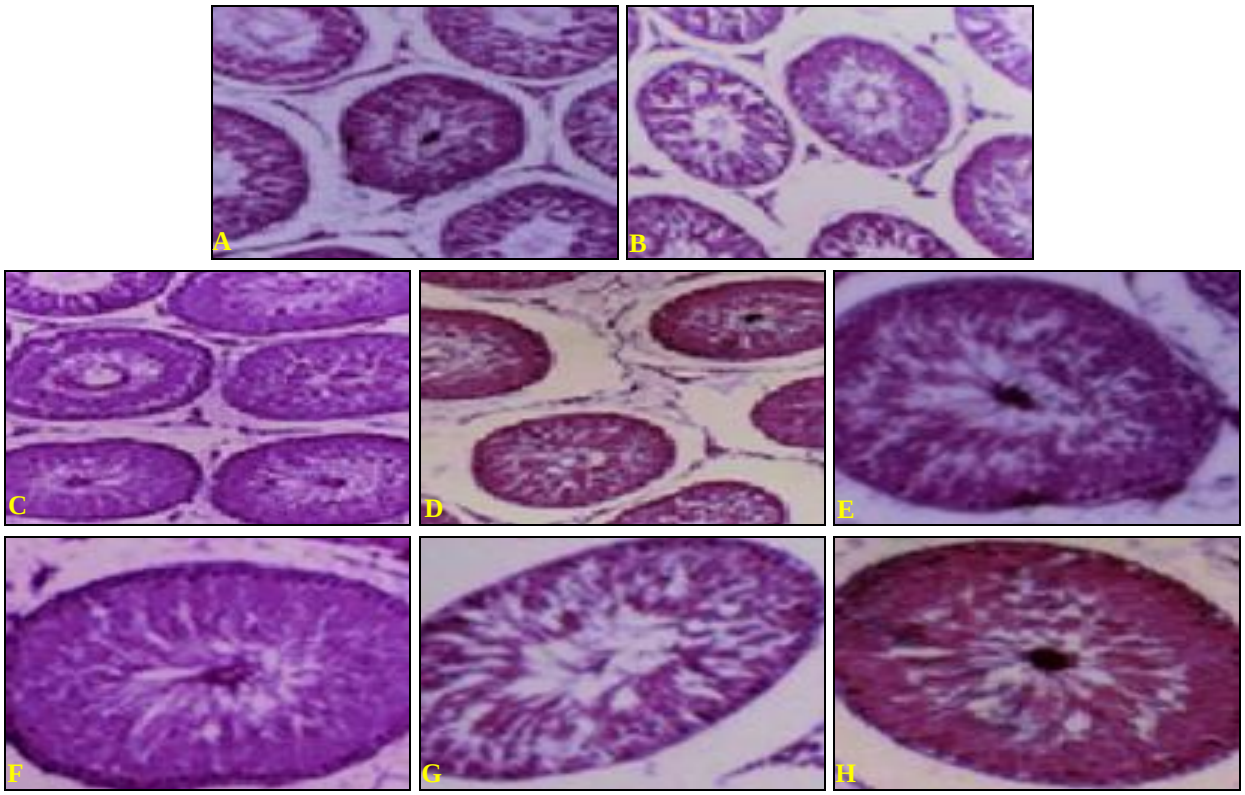

FIG. 4: TESTICULAR SECTIONS OF THE CONTROL RAT (A AND E), MEPA (250 mg/kg b.w.) (B AND F), MEPA (500 mg/kg b.w.) (C AND G) AND MEPA (1000 mg/kg b.w.) (D AND H) SUB-ACUTELY (FOR 4 WEEKS) TREATED RATS. HEMATOXYLIN AND EOSIN. (A), (B), (C) AND (D): 40X; (E), (F), (G) AND (H): 400X

DISCUSSION: Herbal preparations have a great role to play in the modern system of medicine. However Izzo *et al.*, has clearly shown that they need to be evaluated for toxicological implications upon long term use [20]. From the results of the sub-acute 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 no observed adverse effect level (NOEL) was ≥ 1000 mg/kg bw/day.

Changes in body weight have been used as an indicator of adverse effects of drugs and chemicals by researchers such as Lonare *et al.* [21] Since, no significant changes ($p<0.01$) were observed in the general behavior, body weight and food intake of rats in the treated groups as compared to the control group after 28 day period of daily treatment, it suggested that at the sub-acute oral doses administered, leaves of *Pterospermum acerifolium* had no effect on the normal growth of rats.

A decrease in the glycogen content was observed in male animals treated sub acutely with MEPA 1000 mg/kg b.w. MEPA for 28 days. The changes in the glycogen level may be due to interference in glucose metabolism. Besides, the absence of carbohydrates also suppresses Leydig cell function as explained by Lonare *et al.* and Owagboriaye *et al.* [21, 22, 23] 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 indicating that the dose tested of this compound was not deleterious. Mating trials give a good indication about possible toxic effects of a compound. The method is outlined by Da Silva *et al.* [24]

Our data clearly demonstrated that male rats exposed to MEPA presented normal sexual behavioral. Also epididymal sperm counts were unaltered. 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 sub acutely to MEPA.

As explained by Ding *et al.,* and Meena *et al.*, the seminiferous tubules showing successive stages of transformation of spermatogonia into spermatozoa and lumen filled with spermatozoa indicate health male sexual architecture [7, 25].

Compared with the controls, the 250, 500 and 1000 mg/kg p.o. MEPA did not significantly ($p<0.01$) 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. Forster *et al.,* has clearly shown that this may be explained by the fact that the drug (in this case MEPA) does not cause abnormality or alteration of reproductive functions in female animals at the dose levels tested [26].

CONCLUSION: In the present study it may be concluded that methanolic extract of leaves of *Pterospermum acerifolium* does not cause alteration of male and female reproductive functions in Wistar albino rats upon sub-acute repeated dosing of 28 days.

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CONFLICT OF INTEREST: The authors have no conflicts of interest that are directly relevant to the content of this manuscript.

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