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Dominant Lethal Assay Following Administration of Methanol Sub-fraction of the Seeds of *Carica papaya*

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Abstract: Background: The seed extracts of *Carica papaya* have been proven to possess male antifertility properties. In the present investigation dominant lethal test following oral administration of Methanol Sub-Fraction (MSF) of benzene chromatographic fraction of the chloroform extract of the seeds of *Carica papaya* in rats and rabbits was evaluated. The animals were divided into two groups. Group I was served as vehicle treated control. Group II was treated with MSF @ 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days and mated with untreated female in 1:1 ratio at 5, 30, 45 and 60 days of treatment. Pregnant females were necropsied in the second half of pregnancy to determine dominant lethal effect, based on comparison of the live implants per female in the treated group and vehicle treated control group. **Results:** Both pregnant rats and rabbits did not show any signs of distress or toxic symptoms. No mortality or morbidity was observed. The fetuses were normal and the status of ovary, uterus and implantation, fetal body weight, soft tissues and skeletal structures were recorded normal and the data were comparable to those of vehicle treated control. **Conclusions:** In conclusion, the test substance had no dominant lethal effect when administered at 20x CD for 5 consecutive days in albino rats and rabbits.

Key words: *Carica papaya*, MSF, contraceptive dose, dominant lethal test, gravid uterus

INTRODUCTION

To curtain the population at a level consistent with the requirements of sustainable economic growth, social development and environmental protection, there is an urgent unmet need in developing world for contraception. Several chemical and hormonal methods for control of male fertility have been identified, none of them so far reached the stage of drug formulations as yet owing to their relative systemic side effects (Bajaj, 1999; Griffin, 1999; Handelsman, 2000). The main advantages in developing an antifertility agent from a plant source are that, it could be cost effective, likely to have a long folklore history as a contraceptive and that it would have low toxicity potential (Chaudhury, 1985).

The Indian Council of Medical Research, New Delhi has published a special report on plants with antifertility activities emphasizing the need to explore the antifertility potential of herbal resources (Chaudhury, 1993). Some of the possible outcome from these were from *Hibiscus rosa-sinensis* (Tewari, 1974), *Vicoa indica* (Chaudhury, 1985) and *Embelia ribes* (Chaudhury, 1993, 2001) for the females and *Carica papaya* (Lohiya *et al*., 2001) for the male.

Carica papaya, a small tree with succulent stem, is native to West Indies and Central America and widely grown throughout India for its edible fruit. It is an important medicinal plant having properties like abortifacient, emmenagogue, digestive, anti-implantation and antibacterial etc. Preliminary investigations carried out with different extracts and fractions of the papaya seeds revealed that benzene chromatographic fraction of chloroform extract of the seeds of *Carica papaya* found to be more effective in inducing total inhibition of sperm motility in rats after 60 days of treatment (Pathak *et al*., 2000; Manivannan *et al*., 2004). Preclinical investigations in langur monkeys revealed that the chloroform extract at 50 mg kg^{-1} (body wt. day^{-1}) for 360 days resulted in to azoospermia after 90 days of treatment that continued until the 360 days study period (Lohiya *et al*., 2002). Benzene chromatographic fraction showed contraceptive efficacy without adverse effects, mediated through inhibition of sperm motility (Lohiya *et al*., 2008). The Methanol Sub-Fraction (MSF) of benzene chromatographic fraction of chloroform extract of the seeds of *Carica papaya* showed motility inhibition properties in rats following 30 days of treatment and azoospermia in rabbits within 15 days of treatment

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(Lohiya *et al*., 2001). Preclinical toxicological investigations viz., acute, sub-chronic, chronic toxicity and carcinogenicity in Wistar albino rats carried out so far with methanol sub-fraction of the benzene chromatographic fraction of the chloroform extract of the seeds of *Carica papaya* have shown that there were no associated health hazards due to treatment of MSF (Lohiya *et al*., 2006; Goyal *et al*., 2009, 2010). Owing to proven contraceptive efficacy, in order to initiate further progress of research towards clinical trials, the objective of the present study was to investigate the induction of a dominant lethal event, if any, after exposure to MSF in albino rats and rabbits (OPPTS 870.5450, 1998).

MATERIALS AND METHODS

Animals: Adult Wistar male albino rats (*Rattus norvegicus*), 3-4 months old, weighing between 150 and 180 g and adult New Zealand white rabbits (*Oryctologus cuniculus*), of 6 months old, weighing 1.5 to 2.0 kg, of both sexes were used in the present investigation and were maintained in the Departmental Animal House Facility, with 12:12 h light and dark schedule. Rats were housed in polypropylene cages (size 15×9×6) and rabbits were maintained in individual metallic cages (size 16× 18×15), fed with pellet diet (M/s Ashirwad Industries Limited, Chandigarh, India) and free access to safe drinking water. The animals were given extensive care and kept under veterinary supervision and the Guidelines for Care and Use of Animals in Scientific Research of the Indian National Science Academy, New Delhi (INSA, 2000) were strictly followed. The experimental protocol has the approval of the Institutional Ethical Committee.

Test material: The seeds of *Carica papaya* L (family: Caricaceae, Voucher No. RUBL 16590) of pure honey dew variety were obtained commercially, shade dried and powdered. The powdered material was refluxed in chloroform for 12×3 h at 58-60°C. The resultant chloroform extract was subjected to silica gel column chromatography (60-120 mesh) eluted with benzene. The benzene chromatographic fraction of the chloroform extract was concentrated under reduced pressure and sub-fractionated with methanol and termed as Methanol Sub-Fraction (MSF).

Characterization of the *Carica papaya* seed products through ^{1}H-NMR, IR, Mass Spectra (E1/C1/FAB) and gas chromatography revealed several sub-components and appeared to be a mixture of compounds. Spectral data showed evidences of long chain fatty alcohols, long chain ester, glycerides of unsaturated fatty acids and long chain unsaturated acids. Based on the available evidences, the most probable fatty acids could be saturated myristic acid with molecular formula ($CH_3(CH_2)_{12}COOH$), palmitic acid with molecular formula ($CH_3(CH_2)_{14}COOH$) and stearic acid with molecular formula ($CH_3(CH_2)_{16}COOH$). Further purification through analytical HPLC using the solvent system acetonitrile: tetrahydrofuran: methanol: water in 45:5:35:15 ratio resulted into an inhomogeneous mixture of several compounds (Bhande, 2004).

At each extraction level, the extract/fraction/sub-fraction of the seeds of *Carica papaya* have been tested and proven for antifertility activity and the Methanol Sub-Fraction (MSF) of the benzene chromatographic fraction of the chloroform extract of the seeds of *Carica papaya* which possessed the desired antifertility activity in all animal trials, was used as an Investigational New Drug (IND), for preclinical reproductive toxicity testing, in the present investigation.

Experimental design: The initial assessment of dominant lethality for the MSF was carried out in two groups, each consisting of 5 male rats and rabbits.

Group I: Animals served as vehicle treated control
Group II: Animals were treated orally with 1000 mg kg^{-1} body wt. for a period of 5 days

After 5, 30, 45 and 60 days of treatment, animals were mated with untreated virgin females in a 1:1 ratio. Successes of mating were confirmed through vaginal smear/plug. The females were sacrificed in the second half of pregnancy and the uterine contents were examined to determine the total numbers of implants and the number of live and dead embryos. The calculation of the dominant lethal effect was based on comparison of the live implants per female in the treated group with the live implants per female in the vehicle treated control group. The increase of dead implants per female in the treated group over the dead implants per female in the vehicle treated control group reflected the post-implantation loss. The post-implantation loss was calculated by determining the ratio of dead and total implants from the treated group compared with the ratio of dead and total implants from the vehicle treated control group. Pre-implantation loss was estimated on the basis of corpora lutea counts or by comparing the total implants per female in treated and vehicle treated control groups. The dominant lethal index was calculated as under (Ehling and Malling, 1968):

$$\text{Dominant Lethal Index (DLI)} = 1 - \frac{\text{Live implants/Pregnant female (experimental group)}}{\text{Live implants/Pregnant female (vehicle treated control group)}} \times 100$$

Parameters

Food and water intake: Individual consumption of food and water was recorded daily. Rats and rabbits were fed individually with 150 g of rat pellets and 200 mL of drinking water per day. On the next day the remaining food and water were measured and the consumed food and water was recorded.

Body weight: The body weight was recorded before treatment and following 5, 30, 45 and 60 days of post treatment periods.

Terminal scarification: On day 14 of gestation in rats and on day 21 in rabbits all the pregnant animals were sacrificed under an over dose of ether anesthesia and intravenous injection of sodium thiopentone (NEON Laboratories Ltd., Mumbai; THIOSOL* Sodium), respectively. Uterine horns and ovaries were removed, freed from fat and adherent tissues. Non-gravid uteri were subjected to ammonium sulphide staining for confirmation of non-pragnant status (Kopf *et al*., 1964). Number of corpora lutea, number of implantations, early resorptions/embryonic deaths and late resorptions/fetal deaths were observed.

Fetal observations: The fetuses were removed by opening uterus and were placed in sequential manner in 0.9% saline solution. All dead and live fetuses were counted and following observations were made:

- **Sexing of fetuses:** The fetuses were sexed by observing the anogenital distance. The numbers of male and female fetuses were counted and sex ratio was noted
- **Fetal body weight and length:** Individual fetuses were weighed to the nearest milligram on an electronic balance and fetal length (crown to rump) was measured
- **External examination:** The fetuses were sacrificed using diethyl ether vapour. All the fetuses were examined for external malformations in an orderly manner starting from head, face, nostrils, eyes, external ears (pinna), trunk to tail and limbs
- **Visceral examination:** Half number of male and half number of female fetuses from each group were fixed overnight in 70% ethanol and examined by the modified Wilson's technique/fetal necropsy (Monie *et al*., 1965). Visceral malformation present, if any, was recorded under a stereozoom microscope (Model: Stereoscopic Zoom Microscope, SMZ1000, Nikon, Japan)
- **Skeletal examination:** Remaining half number of the fetuses from each of control and treated groups were skinned, fixed in 70% ethanol, macerated in 1% KOH and stained with alcian blue and Alizarin red S according to the method of Taylor (1986). The stained skeletal systems were cleared in grades of glycerol and evaluated for skeletal malformations. The skeletal malformations, viz., skull, vertebral column, sternebrae, forelimbs and hind limbs, fore and hind paw, etc., if any, were observed using magnifying glass

Statistical analysis: Values are represented as Mean±Standard Deviation (SD). One-way analysis of variance (ANOVA) was employed for statistical comparison. The difference between means was analyzed by Holm-Sidak multiple comparison test to detect the inter-group difference by using the statistical software SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). The p value less than 0.05 was considered as significant.

RESULTS

Clinical signs and pre-terminal deaths: In both vehicle treated control (group I) as well as in MSF treated (group II) at dose regimen of 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days pre-terminal deaths were not present. The neurological, respiratory, gastrointestinal and urogenital systems appeared normal. All male rats and rabbits during the entire observation schedule of treatment in group II appeared normal and no signs of overt toxicity has been detected, when compared with vehicle treated control (group I) animals.

Visible toxicological symptoms: Skin, fur, eyes and nose showed normal characteristics throughout the treatment and observation period. All the animals were active throughout the study period. Neurological symptoms like tremors, convulsions and salivation, diarrhoea and bizarre behaviour such as self mutilation, walking backward, etc. were not found. Normal animal behaviour, feeding pattern, motor activity, gait and posture, reactivity to handling or sensory stimuli and grip strength were present, when compared with vehicle treated control (group I) animals:

Food and water intake: Food and water intake was fluctuated daily in vehicle treated control (group I) and MSF treated rats and rabbits at the dose of 1000 (mg/kg b. wt. /day) for 5 consecutive day (group II)

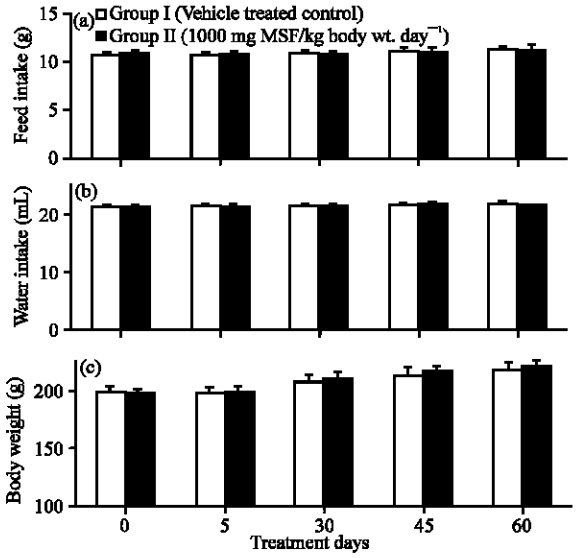

Fig. 1: Feed intake, water intake and body weight gain in group I (vehicle treated control) and group II (MSF @ 1000 mg kg^{-1} body wt. day^{-1}) rats (Each column and the vertical bar denotes Mean±SD). (a) The feed intake in group II was comparable to the group I (b) The water intake in group II was comparable to the group I and (c) The body weight gain in group II was comparable to the group I

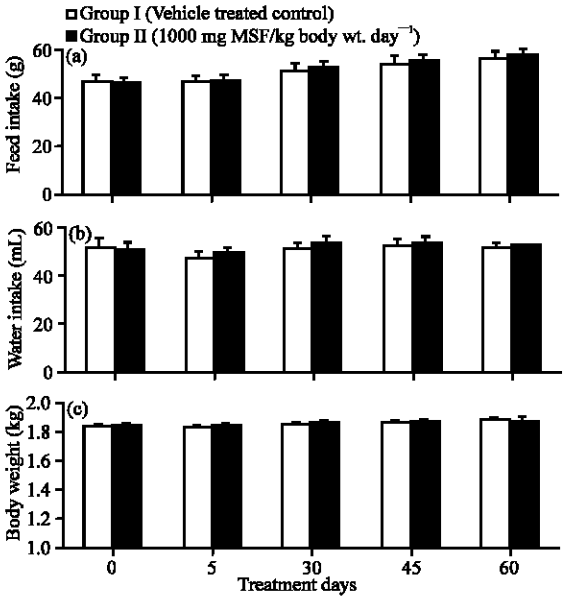

Fig. 2: Feed intake, water intake and body weight gain in group I (vehicle treated control) and group II (MSF @ 1000 mg kg^{-1} body wt. day^{-1}) rabbits (Each column and the vertical bar denotes Mean±SD). (a) The feed intake in group II was comparable to the group I (b) The water intake in group II was comparable to the group I and (c) The body weight gain in group II was comparable to the group I

throughout the study period. However, the initial and final values of food and water intake, in group II were comparable to those of group I (vehicle treated control) (Fig. 1a, b and 2a, b).

Body weight: The initial and final body weights in the vehicle treated control male rats were 195±5.00 and 214±5.47 g, respectively. Likewise, the initial and final body weights of the MSF treated male rats were 194±4.18 and 217±4.47 g, respectively (Fig. 1c). The initial and final body weights in the vehicle treated control (group I) male rabbits were 1.83±0.01 and 1.88±0.01 kg, respectively. Likewise, the initial and final body weights in the group II were 1.84±0.008 and 1.87±0.03 kg (Fig. 2c). Oral administration of MSF at 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days showed no change in the initial and final body weights in males, till 60 days of study period, comparable to those of vehicle treated control.

Pregnancy record: All females mated with vehicle treated control males (group I) and MSF at the dose of 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days treated male rats and rabbits (group II) were become pregnant following mating at 5, 30, 45 and 60 days after the treatment. The pregnancy record, in group II mated females, viz., weights of gravid uterus, ovary and placenta and the fetal sex ratio was unaltered when compared with vehicle treated control mated females (group I) (Table 1).

Implantation status: The vehicle treated control rats (group I) showed a total of 34 (6.80±0.44) *Corpora lutea*, 34 (6.80±0.44) implantations, 0 resorption and 34 (6.80±0.44) viable fetuses. The vehicle treated control rabbits (group I) showed a total of 20 (4.00±0.70) *Corpora lutea*, 20 (4.00±0.70) implantations, 0 resorption and 20 (4.00±0.70) viable fetuses. The implantation data in the females mated with males of group II following 5, 30, 45 and 60 days of post-treatment periods was not significantly different when compared with the females mated with vehicle treated control (group I) (Table 2).

Offsprings record: The body weight and body length of rats fetuses in group I (vehicle treated control),

Table 1: Pregnancy record of the normal female rats and rabbits mated with vehicle treated control and MSF treated (1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days) male rats and rabbits for assessment of dominant lethality

	Weight of gravid uterus (g)		Weight of ovary (g)		Weight of placenta (g)		Fetal sex ratio (M/F)	
Groups	Rats	Rabbits	Rats	Rabbits	Rats	Rabbits	Rats	Rabbits
Group I								
(Vehicle treated control)	23.56±1.78	65.62±2.07	0.08±0.01	0.32±0.01	0.70±0.01	1.78±0.03	1.03±0.29	1.30±0.44
Group II (MSF treated)								
5 days of treatment	23.52±1.69	69.32±1.37	0.08±0.007	0.32±0.02	0.69±0.01	1.74±0.02	1.09±0.39	1.30±0.97
30 days of treatment	23.92±0.88	68.25±0.87	0.08±0.01	0.37±0.03	0.67±0.01	1.71±0.02	1.19±0.29	1.26±0.68
45 days of treatment	24.94±1.29	66.57±2.39	0.08±0.015	0.36±0.01	0.69±0.01	1.75±0.02	0.94±0.26	1.03±0.58
60 days of treatment	24.15±1.55	67.03±1.87	0.08±0.007	0.36±0.02	0.69±0.008	1.73±0.03	1.14±0.26	1.13±0.50

Values are represented as Mean±SD

Table 2: Assessment of dominant lethality of the male rats and rabbits treated with MSF of 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days and mated with normal females for implantation status

	Total No. of *Corpora lutea*		Total No. of implantation sites		Resorption		Total No. of viable fetuses	
Group	Rats	Rabbits	Rats	Rabbits	Rats	Rabbits	Rats	Rabbits
Group I								
(Vehicle treated control)	6.8±0.44	4.0±0.70	6.8±0.44	4.0±0.70	0	0	6.8±0.44	4.0±0.70
Group II (MSF treated)								
5 days of treatment	7.4±0.54	3.8±0.44	7.4±0.54	3.8±0.44	0	0	7.4±0.54	3.8±0.44
30 days of treatment	7.0±1.00	4.0±1.00	7.0±1.00	4.0±1.00	0	0	7.0±1.00	4.0±1.00
45 days of treatment	6.6±1.14	4.0±1.00	6.6±1.14	4.0±1.00	0	0	6.6±1.14	4.0±1.00
60 days of treatment	7.2±0.44	4.0±1.00	7.2±0.44	4.0±1.00	0	0	7.2±0.44	4.0±1.00

Values are represented as Mean±SD

Table 3: Assessment of dominant lethality of the male rats and rabbits treated with MSF of 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days and mated with normal females for offsprings record

	Mean body weight of fetuses (g)		Mean body length of fetuses (cm)		Pre-implantation loss		Post-implantation loss loss		Dominant lethal mutations (%)	
Group	Rats	Rabbits	Rats	Rabbits	Rats	Rabbits	Rats	Rabbits	Rats	Rabbits
Group I										
(Vehicle treated control)	4.89±0.04	26.40±0.42	4.68±0.19	6.87±0.05	0	0	0	0	0	0
Group II (MSF treated)										
5 days of treatment	4.81±0.07	25.90±0.51	4.70±0.15	6.83±0.12	0	0	0	0	0	0
30 days of treatment	4.83±0.09	26.08±0.52	4.78±0.13	6.76±0.07	0	0	0	0	0	0
45 days of treatment	4.85±0.05	26.09±0.61	4.8±0.10	6.66±0.22	0	0	0	0	0	0
60 days of treatment	4.91±0.05	26.76±0.72	4.84±0.05	6.74±0.25	0	0	0	0	0	0

Values are represented as Mean±SD

respectively, were 4.89±0.04 g and 4.68±0.19 cm. The body weight and body length of rabbits fetuses in group I (vehicle treated control), respectively, were 26.40±0.42 g and 6.87±0.05 cm. No significant difference was noticed in offsprings data of group II (1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days), following 5, 30, 45 and 60 days of mating, when compared with group I (vehicle treated control) (Table 3).

Pre-implantation loss: In both group I (vehicle treated control) and group II (MSF at the dose of 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days) following 5, 30, 45 and 60 days of post-treatment intervals the number of corpora lutea were remained equal to the number of implantations, therefore, the calculated pre-implantation loss was found to be zero percent in rats and rabbits (Table 3).

Post-implantation loss: In both group I (vehicle treated control) and group II (MSF at the dose of 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days) following 5, 30, 45 and 60 days of post-treatment period, the number of implantations were similar to the number of viable fetuses. Resorptions were also found to be zero, hence, the calculated post-implantation loss was zero percent in rats and rabbits (Table 3).

Dominant lethal index: As the calculated pre-and post-implantation losses were zero percent, in both group I (vehicle treated control) and group II (MSF at the dose of 1000 mg kg^{-1} body wt. day^{-1} for 5 consecutive days), the dominant lethal index in rats and rabbits was zero percent (Table 3).

DISCUSSION AND CONCLUSION

Herbal drugs have been used since ancient times as medicines for the treatment of a wide range of diseases. Toxicological studies constitute an essential part of the effort in developing a herbal medicine into a drug product.

One of the major challenge in the drug development is the accurate assessment of human drug toxicity. In the history of male contraceptive research, three plant products viz., gossypol, *Tripterygium wilfordii* and *Ecballium elaterium*, claimed to have better contraceptive efficacy in clinical trails, however, failed to reach the level of commercial launch, due to the side effects at unacceptable level. Further research on herbal male contraceptive warrants special attention in terms of toxicity considering the lessons learnt from the previous herbal contraceptive products.

Dominant lethal mutation is one occurring in a germ cell which does not cause dysfunction of the gamete but which is lethal to the fertilized egg or developing embryo. Dominant lethal effects cause embryonic or fetal death. Induction of a dominant lethal event after exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. Dominant lethals are generally accepted to be the result of chromosomal damage (structural and numerical anomalies).

An aqueous solution of khat (*Catha edulis*) extract administered in albino mice produced a dose-dependent reduction in the rate of fertility in the first week after mating which was irreversible in the second week at the highest dose (200 mg kg^{-1}). Khat extract also induced post-implantation loss during the first week following treatment. A comparison of the results of the first and second weeks showed a reversible pattern of dominant lethality (Tariq *et al*., 1990). Whole Tobacco Smoke (TS) induced significant dominant lethal mutations in BALB/c, BDF1 and H mice. In BALB/c and BDF1 mice TS induced dominant lethal mutations mainly in spermatocytes, spermatogonia and gonial stem cells, while in H mice only spermatids and spermatocytes were affected (Stoichev *et al*., 1993). Sasser *et al*. (1993) observed significant male dominant lethal effects in male rats orally administered sulfur mustard (HD) and mated with untreated females at 2 and 3 weeks' post-exposure. These effects included increases of early fetal resorptions and preimplantation losses and decrease in total live embryo implants. A significant increase in the percentage of abnormal sperm was detected in males. Dominant lethal mutation of crotonaldehyde was evaluated in Swiss albino mice. Treatment of mice resulted in statistically significant decrease in the fertility indices and total number of implants per female and appreciably higher frequencies of dominant lethal mutation during 8-14, 15-21 and 22-28 days post-treatment mating periods. The overall result suggests a positive dose-response relationship between treatment and induction of dominant lethal mutation in the germ cells (Jha *et al*., 2007). Dominant lethal effects of Trypsin inhibitor (ATI) isolated from *Ascaris suum*, were analyzed in three groups of adult BALB/c males. In the females bred to ATI-treated males, significant increase in pre-implantation loss was observed at post-injection week 1 and 3 for higher doses of the inhibitor ($p<0.05$ or $p<0.01$). During mating days 15-21 a statistically significant increase in post-implantation loss and dominant lethal effects were observed, preliminary findings shows that ATI may be one of the factors causing disturbances in spermatogenesis (Blaszkowska, 2010).

However, Generoso *et al*. (1984) reported that corn oil, sesame oil, peanut oil or olive oil, injected intraperitoneally to female mice prior to insemination, did not induce dominant-lethal mutations. Likewise, Oral treatment of male mice with extracts of irradiated potatoes did not increase the frequency of dominant lethal mutations in male mice (Zajcev *et al*., 1975). Kojic acid, used as a food additive for preventing enzymatic browning and in cosmetic preparations has been proven negative in *in vivo* mammalian dominant lethal assay (Burdock *et al*., 2001). Similarly, in present study of dominant lethal assay, in both group I (vehicle treated control) and group II (MSF at the dose of 1000 mg kg^{-1} (body wt. day^{-1}) for 5 consecutive days) following 5, 30, 45 and 60 days of post-treatment intervals of mating, the dominant lethal index in rats and rabbits was found zero percent, suggests that MSF is not genotoxic and it does not induce dominant lethal mutations following administration of MSF at 20 times the therapeutic dose (1000 mg kg^{-1} body wt.) for 5 days, when compared with vehicle treated control.

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