Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Effect of Aqueous/Methanolic Extract of *Ocimum sanctum* (OciBest) on the Male and Female Reproductive Performance of Wistar Rats

Raina P[1], B. Bharathi[3], A. Senthilkumar[3], S. Sagar[2], C. V. Chandrasekaran[3], D. Prabhu[2], M. Deepak[3], A. Amit[3], Kaul-Ghanekar R[1]

[1]Cancer Research Lab, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth (Deemed to be University), Pune, [2]SA-FORD (Sanctuary for Research and Development), Maharashtra, [3]R& D Centre, Natural Remedies Private Limited, Bengaluru, Karnataka, India

Submitted: 30-11-2017 Revised: 25-01-2018 Published: 28-06-2018

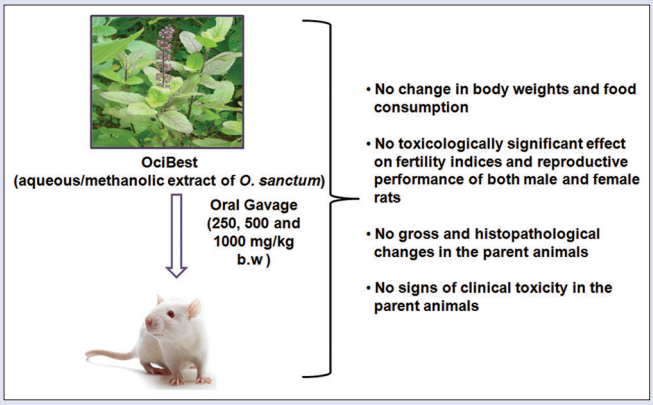
ABSTRACT

Background: *Ocimum sanctum* (OSE) has been used in the Indian system of traditional medicine (Ayurveda) for the treatment and prevention of various diseases. However, the contradictory reports regarding the reproductive safety of OSE prompted us to re-verify its effect on the reproductive system by following internationally accepted Organization for Economic Cooperation and Development guideline 415. **Objective:** To study the effect of aqueous/methanolic extract of OSE (OciBest) on the male and female reproductive performance of Wistar rats. **Materials and Methods:** Rats were orally gavaged with OciBest at dose levels of 0, 250, 500, and 1000 mg/kg. Males were administered with OciBest for 12 weeks before and during the mating period. On the other hand, females received OciBest for 2 weeks before mating until the end of the lactation period. **Results:** OciBest at 1000 mg/kg did not induce any adverse effects on the reproductive performance of male and female rats. All the treated parent animals survived until the end of the study period with no major signs of clinical toxicity. The body weights, food consumption, male and female fertility indices, organ weights, as well as gross pathological and histopathology observations in parent animals, did not reveal any treatment-related adverse effects. Moreover, in comparison to the control groups, OciBest did not induce any treatment-related adverse effects to the offsprings. **Conclusion:** Thus, no-observed adverse effect level was found up to 1000 mg/kg dose, suggesting the safety of OciBest for reproductive system.

Key words: Fertility, no-observed adverse effect level, *Ocimum sanctum*, Organization for Economic Cooperation and Development 415, reproductive toxicity

SUMMARY

- In the present study, we have investigated the one-generation reproductive toxicity study (Organization for Economic Cooperation and Development 415) of standardized extract of *Ocimum sanctum* (OSE). We found that OSE at 1000 mg/kg did not reveal any major adverse effects on the male and female reproductive performance. All the parent animals survived until the end of the study period with no major signs of clinical toxicity. The body weight (BW), male and female fertility indices, organ weights, and gross pathological and histopathology observations in parent animals and in their offsprings did not reveal any treatment-related adverse effects. Thus, the no-observed adverse effect level was found to be 1000 mg/kg in male and female rats.



Abbreviations used: OECD: Organization for Economic Cooperation and Development; NOAEL: No-observed adverse effect level; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; OA: Oleanolic acid; UA: Ursolic acid.

Correspondence:

Dr. Ruchika Kaul-Ghanekar,
Cancer Research Lab, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth (Deemed to be University), Pune Satara Road, Katraj, Pune - 411 043,
Maharashtra, India.
E-mail: ruchika.kaulghanekar@gmail.com
DOI: 10.4103/pm.pm_562_17

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Ocimum sanctum (OSE), commonly known as Tulsi, has been used from time immemorial in Ayurveda, for treating and preventing various ailments. The different parts of this plant have been used for treating various diseases such as cough and cold, bronchitis, asthma, digestive and gastric disorders, diarrhea, dysentery, malaria, arthritis, skin problems, eye and ear infections, undifferentiated fever, as well as snake and scorpion bites.[1,2] There has been extensive preclinical evidence that suggests immunomodulatory, antistress, adaptogenic, antidiabetic, anti-inflammatory, hypolipidemic, antimicrobial, radioprotective, neuroprotective, cardioprotective, and anticarcinogenic activities of OSE.[3,4] Moreover, *Ocimum* extract has also been tested at clinical level for the treatment of number of disorders. It has been

Cite this article as: Raina P, Bharathi B, Senthilkumar A, Sagar S, Chandrasekaran CV, Prabhu D, *et al*. Effect of aqueous/methanolic extract of *Ocimum sanctum* (OciBest) on the Male and Female reproductive performance of Wistar rats. Phcog Mag 2018;14:S256-63.

reported to exhibit significant efficacy in the management of metabolic syndrome.[5] Administration of aqueous extract of OSE at 5 ml twice daily for 3 months in patients with metabolic syndrome significantly attenuated blood glucose, blood pressure, and lipid levels compared to placebo group.[5] Another double-blind placebo-controlled study in symptomatic control of general stress revealed that administration of OSE extract for 6 weeks at 1200 mg dose per day significantly reduced the total stress symptom scores compared to placebo group.[6] Mondal *et al*., 2011 investigated immunomodulatory effects of Tulsi in healthy volunteers in a double-blinded randomized controlled cross-over trial.[7] Administration of 300 mg of ethanolic extract of Tulsi leaves or placebo after 4 weeks significantly increased the levels of interferon gamma and interleukin-4 and the percentages of T-helper and natural killer-cells in Tulsi-treated group compared to placebo group. A clinical trial showed that general anxiety disorder patients, administered with 500 mg OSE twice daily for 60 days, showed mild symptoms.[8] All these clinical studies on OSE did not report any treatment-related adverse effects.

There have been some confounding reports regarding adverse effects of OSE on the reproductive system. Studies by Vohora *et al*., 1969;[9] Kasinathan *et al*., 1972;[10] Seth *et al*., 1981;[11] Kantak and Gogate, 1992;[12] Akbarsha *et al*., 1998;[13] Sardessai *et al*., 1999;[14] Mankapure *et al*., 2013;[15] and Srinivasulu and Changamma, 2017[16] have reported antifertility effects of OSE. In contrast, Gautam and Goel, in 2014, reported that there were no toxic effects on the reproductive system, following a 28-day repeated dose administration of OSE in rats at 200, 400, and 800 mg/kg doses. However, our previous acute,[17] sub-acute,[18] and genotoxicity[17] studies have shown that OSE is safe in rats. Thus, the contrasting results on the reproductive toxicity of OSE prompted us to reevaluate the toxic effects of methanolic/aqueous preparation of OSE (OciBest) on the reproductive system of rats by following Organization for Economic Cooperation and Development (OECD) test guideline 415 in a good laboratory practice (GLP) certified laboratory. We tested the toxicity of methanolic/aqueous preparation of OSE on reproductive performance such as gonadal function, estrous cycle, mating behavior, conception, parturition, lactation, and weaning in male and female Wistar rats.

MATERIALS AND METHODS

Experimental animals and housing

Healthy male and female Wistar rats (6–8 weeks) used in the present study were bred and reared at SA-FORD, Mumbai, India, for the study (Institutional Animal Ethics Committee [IAEC] approval no.: SA-FORD_AUG_2013_13_024). The animals were housed two per cage in polycarbonate cages during the premating (each sex) and mating (1 male and 2 females) and postmating (2 males) periods. The mated females were housed individually in the polycarbonate cage during the gestation period. The lactating animals with pups were housed in the same cage. Cages were provided with bedding of clean corn cob and were fed standard laboratory rodent diet (M/s Nutrivet Life Sciences., Pune, India) and filtered (Aqua guard filter) water *ad libitum*. The animals were maintained in a temperature controlled animal room (22°C ± 3°C) with a relative humidity of 35%–68% and 12 air changes per hour and illumination cycle set to 12-h light and 12-h dark. The IAEC of SA-FORD, Mumbai, India, approved the protocols for the animal study which was conducted in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals and OECD guidelines.

Test substance

The test substance (OciBest) is an extract of whole plant of OSE Linn. (Source: India; Batch number: FOCEX/2012120002) developed by M/s Natural Remedies, Bangalore, India. OSE Linn. was procured from regions of Tamil Nadu, India. It was identified and authenticated at National Institute of Science Communication and Information Resources, New Delhi. A voucher specimen (no. 106) was deposited in the herbarium of Natural Remedies. The quality control data have been provided in Supplementary Table 1. The extract was ensured to comply with phytochemical specifications, namely, ociglycoside-I (hydroxychavicolglucoside/4-allyl-1-O-β-Dglucopyronosyl-2-hydroxybenzene; ≥0.1% w/w), rosmarinic acid (≥0.2% w/w), and triterpene acids (≥2.5% w/w). The details on the preparation and phytochemical analysis of OciBest have been described by Chandrasekaran *et al*., 2013. The composition adhered to the international quality requirements which included analysis of solvent residues, heavy metals, pesticide residues, and microbial contamination. The phytochemical analysis was performed as per the United States Pharmacopeia 32.

High-performance liquid chromatography profiling

High-performance liquid chromatography (HPLC) profiling of OciBest was done as described earlier.[17] Briefly, standards of ociglycoside, rosmarinic acid, oleanolic acid (OA), ursolic acid (UA), and OciBest were prepared in methanol (HPLC grade, Qualigens, Mumbai, India). The analytical method was validated for specificity, linearity, precision, accuracy, and range of quantification. Standards and OciBest were injected into HPLC system (Model LC 2010 A; Shimadzu, Kyoto Japan) consisting of quaternary pump with ultraviolet detector, auto-injector, and column oven with class LC software. The stationary phase used for standards 1 and 2 was Phenomenex Luna column (C18, 5 µm, 250 mm × 4.6 mm) and Phenomenex Luna column (C18, 2.5 µm, 100 mm × 3 mm) for standards 3 and 4. All the standards were procured from Sigma-Aldrich. Two different columns were used for the HPLC analysis of four standards. The reason being, ociglycoside I (≥95% pure) and rosmarinic acid (96% pure) are polar as compared to OA (97% pure) and UA (≥95% pure). Thus, in one column, system suitability parameters and separation could not be achieved. The mobile phase used for the detection of standards 1 and 2 was a gradient mixture of acetonitrile (solvent B) and 0.001 N monopotassium phosphate in HPLC grade water (solvent A). Solvents A and B were mixed in such a manner that the concentration of solvent B was increased from 10% to 30% as linear gradient in the first 18 min. From 18 min to 25 min, the concentration of solvent B was increased from 30% to 85% as a linear gradient at a flow rate of 1.5 mL/min. The detection wavelength was set at 278 nm. The mobile phase used for standards 3 and 4 was a degassed mixture of 67 volumes of acetonitrile and 33 volumes of water containing 0.25% ammonium acetate at a flow rate of 0.3 mL/min. The detection wavelength was set at 205 nm. HPLC chromatograms of OSE were recorded, and the quantification of standards was achieved by external standard method.

Experimental groups

Healthy adult male ($n = 48$) and nulliparous, nonpregnant female ($n = 96$) Wistar rats were assigned randomly to four experimental groups, each consisting of 12 male and 24 female rats. The animals were administered orally with OciBest at 0, 250, 500, and 1000 mg/kg doses, respectively. The above dose levels were selected based upon the limit dose recommended in the OECD test guideline 415.

Dose preparation and administration

OciBest was solubilized in distilled water to prepare desired concentration for each dosage, and volume of the doses was kept constant at 10 ml/kg. The control group was administered with 10 ml/kg of distilled water.

The doses were prepared daily fresh. The rats were given OciBest by oral gavage at approximately the same time each day, using a graduated syringe, and the dosage volume administered to individual rat was adjusted according to its BW. The test substance was administered to the male rats for 12 weeks before and during mating and to females from 2 weeks before mating to day 21 of lactation [Scheme 1].

Observations and procedures

The one-generation reproductive toxicity study was performed in compliance with principles of GLPs and in accordance with the OECD test guideline 415.

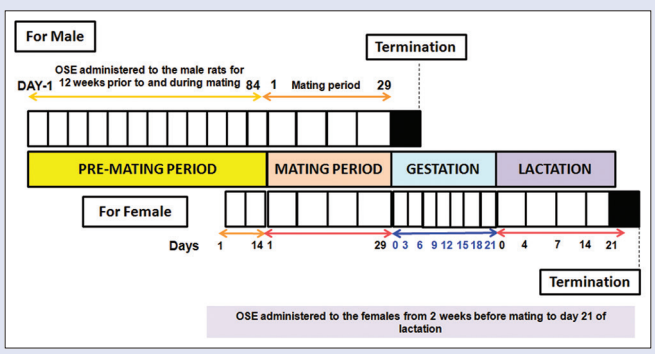
Observations of parent animals

All the animals were observed twice daily for mortality and once daily for clinical symptoms and pertinent behavioral changes in response to treatment. The time of onset, intensity, and duration of the symptoms, if any, were recorded.

BW of male rats was recorded on the day of initiation of dosing at weekly intervals during premating (84 days) and mating period (29 days), just before terminal sacrifice [Scheme 1]. Female rats were weighed on the day of initiation of dosing and at weekly intervals during premating (14 days), mating (29 days), gestation (on day 0, 3, 6, 9, 12, 15, 18, and 21), and lactation (on day 0, 4, 7, 14, 21, and 22) periods. Food consumption was recorded once in a week during the premating period in male and female rats. In the female rats, food consumption was recorded during gestation on day 0, 3, 6, 9, 12, 15, 18, and 21, as well as during lactation on day 0, 4, 7, 14, 21, and 22 [Scheme 1].

All the female rats were evaluated for the stage of estrous by vaginal cytology. Vaginal washes were collected once daily from the beginning of premating period and continued through the mating period until copulation was confirmed. The day a vaginal plug and/or sperm in the vaginal smear observed, it was designated day 0 of pregnancy. The duration of the gestation was calculated from day 0 of pregnancy, and the evidence of onset, progress, and completion of parturition were checked. Females during lactation were examined for normal behavior [Scheme 1].

All the males surviving were euthanized by carbon dioxide inhalation followed by the exsanguination at the end of the mating period, while the surviving females were sacrificed at the end of the lactation period and were subjected to a full, detailed gross necropsy [Scheme 1]. The latter included a careful examination of the external surface of the body; all orifices, cranial, thoracic, and abdominal cavities and their contents with special attention paid to the organs of the reproductive system. At necropsy, male and female reproductive organs (testes, epididymis, seminal vesicles with coagulating glands and their fluids [as one unit], prostate, ovaries, uterus with oviducts, and cervix) and pituitary were weighed. All the male and female reproductive organs along with other organs that showed macroscopic findings were subjected to histopathological examination.



Scheme 1: Treatment window of the male and female parent Wistar rats with Ocimum sanctum

Observations of F_1 animals

The day on which a litter was delivered was designated as lactation day 0. All the pups were examined immediately once they were delivered, to establish the number and sex of pups, stillbirths and live births. The number of live and dead pups and clinical observation were recorded once daily during the lactation period. Somatic markers such as time of eye-opening, ear-opening, hair growth, tooth eruption, and gross anomalies of litter were recorded along with common clinical signs. BW of the pups was measured during lactation on day 0, 4, 7, 14, 21, and 22. All surviving male and female pups were euthanized by carbon dioxide inhalation, followed by the exsanguinations, at the end of the lactation period, and were subjected to an external and internal macroscopic examination. At necropsy, organs with macroscopic findings were further subjected to histopathological examination.

Statistical analysis

The data of BW, food consumption, and absolute and relative organ weights were compared by Bartlett's test for homogeneity of variances. The data with homogeneous variances were subjected to one-way analysis of variance, and when the variances were heterogeneous, the data were subjected to Dunnett's or Dunn's test for parametric and nonparametric data, respectively. Analyses were performed using statistical software Sigma Plot 11.0. The statistical significance was set at $P < 0.05$.

RESULTS

High-performance liquid chromatography profiling of *Ocimum sanctum*

OSE was found to contain ociglycoside-I (>0.1% w/w), rosmarinic acid (>0.2% w/w), and triterpene acids (>2.5% w/w) (OA and UA) that account for approximately 3% since these are specific to OSE [Figure 1]. This standardization was done using HPLC system that ensures repeatability and reproducibility of the experimental results. Apart from these phytoactives, OSE also contains 4.34% moisture, 17.27% polyphenols (tannic acid), 3.05% total bitters, 27.47% saponins, 5.5% total polysaccharides, and 15.93% total ash content. All these phytoconstituents account for >75% of OciBest. This standardization was done using HPLC system that ensured repeatability and reproducibility of the experimental results.

Parent animals

Mortality and clinical signs

There was no mortality observed among the animals administered with OciBest at different dose levels. No test substance-related clinical signs of toxicity were observed during the study period in any dosage group [Table 1]. Male and female rats in both control and OciBest-treated groups were found to have normal-mating behavior. Females during the lactation period exhibited normal behavior at all dose levels.

Body weight and food consumption

There was no statistically significant change in the BW of the treatment groups during the premating and mating periods in males [Figure 2] and during the premating, gestation, and lactation periods in females [Figure 3]. There were no significant changes in food

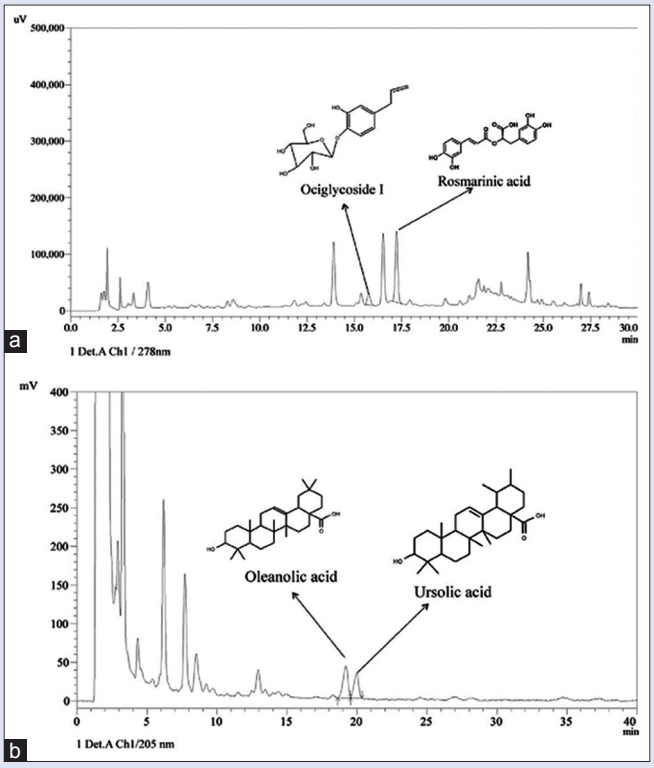


Figure 1: High-performance liquid chromatography of *Ocimum sanctum*. (a) Identification of ociglycoside I and rosmarinic acid in *Ocimum sanctum*. (b) Identification of oleanolic acid and ursolic acid in *Ocimum sanctum*

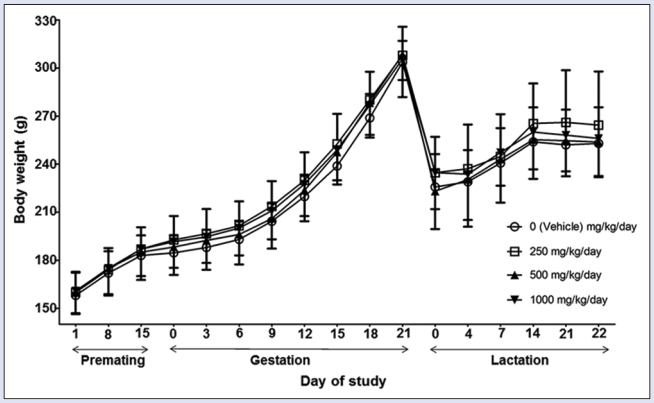


Figure 3: Mean body weights of P (parent) female rats during the premating, gestation, and lactation periods

consumption. Interestingly, males gavaged with 250 (at weeks 4 and 5); 500 (at weeks 4 and 9); and 1000 mg/kg (during weeks 5, 9, and 10) as well as females dosed with 250 (during days 4–6), 500 (during days 4–6), and 1000 mg/kg/day (during days 4–6 and 13–15) showed slightly higher food consumption [Figures 4 and 5].

Fertility data

There were no test substance-related effects on mating or fertility parameters at any of the dose levels [Table 2]. The mean estrous cycle length, male and female fertility indices, female fecundity index, mean gestation length, and gestation index were comparable across all the doses. The sex ratio, live birth index, and lactation index were also found to be similar across all the groups.

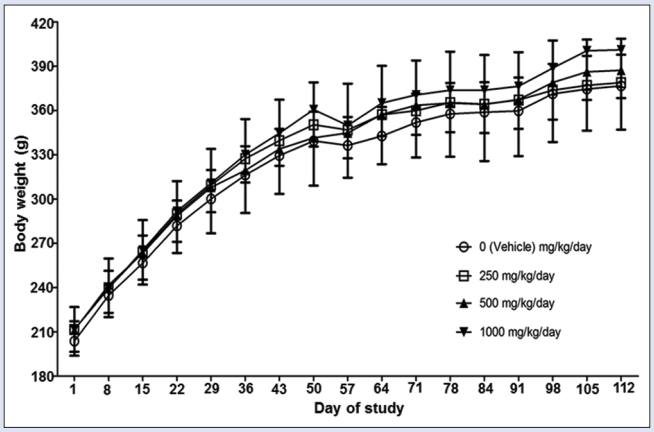


Figure 2: Mean body weights of *P* (parent) male rats during the premating and mating periods

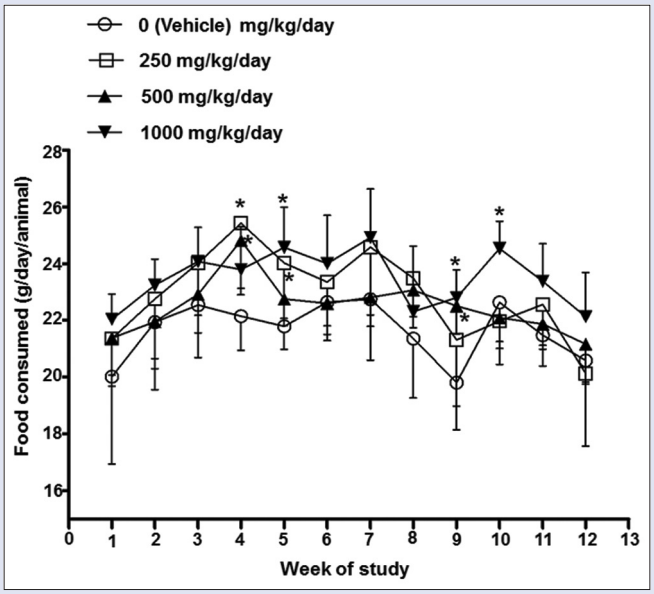


Figure 4: Mean food consumed by *P* (parent) male rats during premating periods

Organ weights, gross pathology, and histopathology

The gross pathological examination did not reveal any treatment-related alterations, except for pyometra observed in single female of the untreated control group. Further, histopathological examination revealed mild polymorphonuclear cell infiltration in the uterus. Absolute and the relative organ (testes, epididymis, seminal vesicles with coagulating glands, and their fluids [as one unit], prostate, pituitary, ovaries, uterus with oviducts, cervix) weights of male and female rats in OciBest-treated groups were comparable to the control groups [Tables 3 and 4].

F_1 generation

Mortality and clinical signs

F_1 generation mortality rate in OciBest-treated groups was comparable to the control group between day 0 and day 21. The live birth and the lactation indices were similar across all the groups, and the treatment showed no effect on the ratio of genders [Table 2]. Although cannibalism was observed, the incidence of this finding for 0, 250, 500, and 1000 mg/kg doses of OSE-treated groups was 7%, 6%, 5%, and 6%, respectively, in

male pups and 8%, 5%, 6%, and 4%, respectively, in female pups that was comparable to the control group.

Table 1: Summary of mortality and clinical observation of P (parents) from the reproductive toxicity study on *Ocimum tenuiflorum*

	Dose (mg/kg/day)			
	0	250	500	1000
Number of treated animals				
Number of treated males	12	12	12	12
Number of treated females	24	24	24	24
Mortality				
Number of males alive till the end of premating phase	12	12	12	12
Number of females alive till the end of premating phase	24	24	24	24
Number of males alive till the end of mating phase	12	12	12	12
Number of females alive till the end of mating phase	24	24	24	24
Number of females alive till the end of gestation phase	24	24	24	24
Number of females alive till the end of lactation phase	24	24	24	24
Clinical signs				
Clinical signs in male and female during premating phase	-	-	-	-
Clinical signs in male and female during mating phase	-	-	-	-
Clinical signs in female during gestation phase	-	-	-	-
Clinical signs in female during lactation phase	-	-	-	-

Premating phase: Day 1 to 84 for males and 1to15 for females; Mating phase: Day 85 to 113 for males and 16 to 44 for females; Gestation phase: Day 0 to 21; Lactation phase: Day 0 to 21

Body weight

The BWs of the male and the female pups did not show any significant difference between the control and OciBest-treated groups [Table 2]. There was no treatment-related effect on the physical development, that is, hair growth and ear-opening [Tables 5 and 6]. However, significant difference between the control and the OSE groups was observed in case of eye-opening and incisor eruption characters [Tables 5 and 6].

Gross pathology and histopathology

Gross pathological examination of all the surviving pups, assessed at the end of the lactation period, did not show any treatment-related effects. The pups that were found dead showed injuries on the neck, thoracic region, and legs; they had no milk in the stomach and showed discoloration of liver, brain, and lungs. Histopathological examination revealed that at dose levels of 250, 500, and 1000 mg/kg OciBest, the number of pups that showed histopathological changes were 7, 12, and 3, respectively. The histopathological findings such as hepatocyte degeneration, necrosis, and congestion in the brain and alveoli of OciBest-treated groups were considered incidental.

DISCUSSION

A large number of world's population relies upon medicinal herbs as an alternative and complementary therapy for basic health-care needs. Although herbal products have been often perceived as natural and relatively safe, phytopreparations could not always be nontoxic.[19]

Table 2: Summary of reproductive performance parameters of P (parents), F1 generation live birth indices, lactation indices, and body weights from the reproductive toxicity study on *Ocimum sanctum*

	Dose (mg/kg/day)			
	0	250	500	1000
Number of females placed with males	24	24	24	24
Estrous cycle length (days)[a]	3.89±0.54	3.93±0.40	4.05±0.55	3.98±0.61
Number of females mated, *n* (%)	24 (100)	24 (100)	24 (100)	24 (100)
Number of females pregnant, *n* (%)	24 (100)	24 (100)	24 (100)	24 (100)
Number of females with liveborn	24	24	24	24
Male fertility index	100	100	100	100
Female fertility index	100	100	100	100
Female fecundity index	100	100	100	100
Gestation index	100	100	100	100
Mean gestation length (days)[a]	21.67±0.56	21.17±0.82	21.58±0.72	21.33±0.76
Number of pups born[a]	10.21±2.62	9.13±3.14	10.63±3.21	9.67±3.21
Number of viable pups (total alive/total number)				
On lactation day 0	238/245	210/219	249/255	232/232
On lactation day 4	234/245	198/219	240/255	218/232
On lactation day 7	233/245	193/219	238/255	217/232
On lactation day 14	233/245	191/219	235/255	217/232
On lactation day 21	222/245	191/219	221/255	208/232
Sex ratio	1.47±1.50	1.39±1.59	1.28±1.22	1.09±0.76
Live birth index-male (%)[a]	96.53±12.02	93.06±24.04	96.36±17.06	100±0.00
Live birth index-female (%)[a]	96.25±12.79	91.88±24.61	98.96±5.10	100±0.00
Lactation index-male (%)[a]	91.15±28.19	87.06±30.12	89.35±29.37	87.36±29.21
Lactation index-female (%)[a]	90.48±28.15	84.98±30.70	87.47±28.69	85.25±30.91
Mean pup weight (g)				
On lactation day 0 (male)[a]	5.74±0.64	5.74±0.60	5.63±0.35	5.74±0.55
On lactation day 0 (female)[a]	5.45±0.61	5.55±0.49	5.43±0.29	5.5±0.45
On lactation day 4 (male)[a]	9.14±1.46	8.8±1.84	8.58±0.99	9.26±1.41
On lactation day 4 (female)[a]	8.83±1.30	8.68±1.35	8.18±1.20	8.83±1.39
On lactation day 7 (male)[a]	12.72±2.05	12.46±2.33	11.82±1.35	12.71±1.90
On lactation day 7 (female)[a]	12.22±1.92	12.16±2.18	11.41±1.67	12.1±1.75
On lactation day 14 (male)[a]	22.53±4.37	22.63±2.55	21.06±2.71	22.41±3.16
On lactation day 14 (female)[a]	21.86±4.41	22.57±2.32	20.47±3.40	22.06±3.07
On lactation day 21 (male)[a]	33.13±5.83	33.68±5.19	31.08±5.52	33.03±4.83
On lactation day 21 (female)[a]	32.06±5.63	33.33±4.49	30.12±6.00	32.33±4.99

*Significantly different from control ($P<0.05$)

Table 3: Summary of organ weights of P (parent) male rats from the reproductive toxicity study on *Ocimum tenuiflorum*

	Dose (mg/kg/day)			
	0	250	500	1000
Absolute weight (g)				
Terminal body weight	358.5±15.56	360.83±5.66	368.17±21.92	380.42±19.09
Pituitary	0.013±0.001	0.013±0.002	0.012±0.000	0.012±0.002
Prostate	2.247±0.025	2.264±0.33	2.239±0.301	2.334±1.405
Seminal vesicles with coagulating gland	1.046±0.028	1.204±0.234	1.079±0.321	1.204±0.946
Testes (right)	1.834±0.041	1.819±0.011	1.785±0.042	1.838±0.144
Epididymis (right)	0.749±0.008	0.709±0.097	0.739±0.006	0.755±0.066
Testes (left)	1.843±0.07	1.795±0.056	1.79±0.007	1.827±0.244
Epididymis (left)	0.746±0.073	0.711±0.177	0.717±0.033	0.733±0.144
Relative weight (g)				
Pituitary	0.004±0.001	0.004±0.001	0.003±0.000	0.003±0.001
Prostate	0.643±0.063	0.625±0.119	0.611±0.099	0.612±0.16
Seminal vesicles with coagulating gland	0.291±0.062	0.333±0.058	0.293±0.06	0.315±0.103
Testes (right)	0.512±0.039	0.506±0.04	0.486±0.034	0.482±0.042
Epididymis (right)	0.209±0.016	0.197±0.018	0.202±0.029	0.2±0.022
Testes (left)	0.515±0.033	0.5±0.044	0.488±0.041	0.479±0.038
Epididymis (left)	0.209±0.016	0.198±0.023	0.195±0.013	0.193±0.016

*Significantly different from control (P<0.05). Values are mean±SD, Relative weights are reported as percent terminal body weight. SD: Standard deviation

Table 4: Summary of organ weight of P (parent) female rats from the reproductive toxicity study on *Ocimum sanctum*

	Dose (mg/kg/day)			
	0	250	500	1000
Absolute weight (g)				
Body weight (g)	251.58±23.03	259±33.45	251.04±25.29	254.54±24.08
Pituitary	0.012±0.001	0.012±0.001	0.012±0.001	0.012±0.001
Uterus	0.496±0.174	0.534±0.269	0.477±0.139	0.501±0.221
Ovary (right)	0.075±0.018	0.078±0.016	0.071±0.021	0.073±0.019
Ovary (left)	0.077±0.022	0.084±0.016	0.078±0.014	0.079±0.022
Relative weight (g)				
Pituitary	0.005±0.001	0.005±0.001	0.005±0.001	0.005±0.001
Uterus	0.197±0.063	0.208±0.104	0.193±0.07	0.199±0.092
Ovary (right)	0.03±0.007	0.03±0.007	0.029±0.008	0.029±0.008
Ovary (left)	0.031±0.008	0.033±0.007	0.031±0.006	0.032±0.01

*Significantly different from control (P<0.05). Values are mean±SD. Relative weights are reported as percent terminal body weight. SD: Standard deviation

Table 5: Summary of physical signs of postnatal development in male pups from the reproductive toxicity study on *Ocimum tenuiflorum*

	Dose (mg/kg/day)			
	0	250	500	1000
Eye-opening	13.95±0.73	13.34±1.00	13.18±0.97*	13.09±0.8*
Ear-opening	15.73±0.68	15.67±0.71	15.72±0.65	15.29±0.92
Hair growth	5.94±0.78	5.92±0.94	5.53±0.90	5.48±0.97
Tooth eruption	11.82±0.97	11.71±0.87	11.91±0.58	11.74±0.72

*Significantly different from control (P<0.05). Values are mean±SD. SD: Standard deviation

Hence, phytopreparations need to be evaluated for their toxicity using scientifically validated tests to ensure safety upon consumption. Keeping this in view, we previously evaluated OciBest for its acute toxicity study in Wistar rats. The rats treated with 5000 mg/kg of OciBest did not reveal any signs of toxicity at such a high dose. Moreover, OSE was found to be nongenotoxic in bacterial reverse mutation, chromosomal aberration, and micronucleus tests.[17]

OSE is being used from many decades for the treatment of number of disorders. There are few studies on OSE that reported its untoward effects on male and female reproductive systems, but only few of them have been performed using any OECD guideline, which became the rationale of the present study. Thus, we wanted to study the safety of OSE on the reproductive toxicity of parent animals and their offsprings using internationally accepted guideline OECD 415. In the toxicity studies, where internationally accepted guidelines are not followed, the only effects recorded are considered of low or minimal toxicological significance and classification may not necessarily be the outcome. The OECD guidelines provide complete information on male and female reproductive performance along with preliminary information regarding the developmental toxic effects of the test substance. It uses relatively larger number of animals in the dose groups with longer duration of exposure of the animals to the test substance.[20-22] A limit dose of 1000 mg/kg BW was chosen for the current reproductive toxicity study.[23] Treatment with OSE did not cause any mortality, clinical or behavioral signs of toxicity, signs of pain, distress, and suffering in male and female rats up to 1000 mg/kg dose up to the end of the study period. This indicated that the test substance was safe for use.

A significant BW change has been considered to be one of the most sensitive indicators for the deteriorating condition of animals that is usually accompanied with change in the food consumption.[23] No such significant changes were observed in OciBest-treated groups at different time intervals compared to the control group. Although BW remained normal, an increase in the food consumption was observed in males and female rats at specific time periods during the study duration. Since these findings were not associated with significant BW changes, there was a lack of dose–response relationship and the observations were transient;

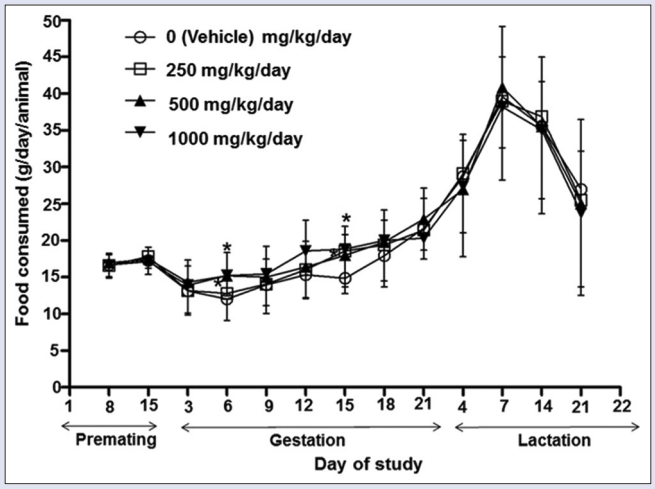


Figure 5: Mean food consumed by P (parent) female rats during premating, gestation, and lactation periods

Table 6: Summary of physical signs of postnatal development in female pups from the reproductive toxicity study on *Ocimum tenuiflorum*

	Dose (mg/kg/day)			
	0	250	500	1000
Eye-opening	14.28±0.64	13.47±0.82*	13.50±0.83*	13.32±0.63*
Ear-opening	16.15±0.60	15.94±0.64	16.09±0.58	15.92±0.47
Hair growth	6.37±0.72	6.16±1.00	5.96±1.01	5.73±0.68
Tooth eruption	12.10±0.79	11.52±0.58*	11.97±0.70	11.95±0.70

*Significantly different from control (P<0.05). Values are mean±SD. SD: Standard deviation

hence, the effects on food consumption did not indicate any toxicological relevance. The effects of OciBest on BW and food consumption were in agreement with the previous studies on OSE reported by Lagarto *et al.*, 2005[24] and Gautam and Goel, 2014.[25]

OciBest did not induce any adverse effects on reproductive performances of male or female animals. There was no effect on the estrous cycle length and gestation period. Similarly, the female fertility, fecundity, and gestational indices as well as male fertility indices were not affected by OciBest. The test substance did not induce any adverse effect on the number of the total births, sex ratio, and number of viable pups on day 0 compared to control group. No still pups were born in any of the treatment groups including the highest test dose group of 1000 mg/kg. OciBest did not affect the functional fertility in male or female rats.

Absolute and relative weights as well as histopathological observations of the reproductive tissues are relatively sensitive indicators of damage and are valuable for the assessment of reproductive toxicity.[26] Treatment with OciBest did not show any significant changes in the organ weights of male and female reproductive systems, except for polymorphonuclear cell infiltration in the uterus of one female in the control group. There were no major histopathological changes in the treated animals. Such nontoxic effects on reproductive organ weights and histopathology of male and female reproductive organs have been previously reported in rats that received OSE extract by gavage for 28 days and 90 days.[26,27]

In terms of F_1 generation, although mortality was observed between day 0 and the weaning period, the number of live pups in the treated groups was comparable to the control group. No major treatment-related clinical signs were observed, except for signs such as weakness in body condition and cannibalism in the pups that were found dead before the weaning period. However, a similar incidence of such effects was noticed in pups of all the groups and could be considered incidental. The BWs of the litter in all the treated groups were comparable with the control group.

The early developmental changes such as ear unfolding, first coat, upper and lower incisor eruption, and eye-opening were evaluated in the pups. The early eye-opening and tooth eruption observed in the treated groups were not dose dependent and did not correlate with BWs of pups. Thus, such differences were not considered to be treatment-related postnatal effects. Gross pathological examination revealed some common signs such as no milk in the stomach. Few pups showed congestion in the lungs and brain, atelectasis, collapsed alveoli, and pallor liver that could be due to exsanguination. Such common findings have been reported earlier by Endres *et al.*, 2011.[28] These changes could be considered incidental and of no toxicological relevance. Thus, there was no treatment-related adverse effect on gross pathology of litter of all the tested animals.

In contrast to our findings on the male reproductive system, previous studies by Mankapure *et al.*, 2013;[15] Srinivasulu and Changamma, 2017;,[16] and Seth *et al.*, 1981[11] reported that administration of OSE extract significantly affected the male reproductive organ weights and spermatogenesis. Similar untoward effects were reported on reproductive behavior of adult male Wistar rats by Kantak and Gogate, 1992 and on the female sexual behaviour by Sardessai *et al.*, 1999.[14] The reports attributed the change in sexual behavior and disruption of the estrous cycle to *Ocimum* administration. Mankapure *et al.*, 2013 studied the effect of OSE Linn. (400 mg/100 g BW/day) on the testis and epididymis in male albino rat. They reported that high dose of OSE decreased the weight of testis and seminiferous tubules with corresponding increase in the interstitium, arrested spermatogenesis, and caused derangements in the histoarchitecture of the testis as well as epididymis. Moreover, they found that in the control recovery group, there was a partial regaining of the normal weights of testis and epididymis. Srinivasulu and Changamma, 2017 studied the effect of OSE (Linn.) leaf extract and UA on spermatogenesis in male rats.[16] They reported that administration of OSE leaf extract decreased sperm count and motility of spermatozoa, caused androgen depletion. They did not find any adverse effect of UA on sperm count and on the levels of testosterone, follicle-stimulating hormone, and luteinizing hormone.

However, our study that was conducted according to the OECD protocol and was in compliance with GLP did not reveal any such treatment-related adverse effect on the reproductive parameters and fertility indices. Moreover, recent subacute toxicity studies published on OSE (OciBest) revealed no evidence of toxicity on male and female reproductive organs.[17,18,25] Such discrepancies in the results could be due to several reasons such as lack of proper phytochemical and analytical specifications of test substance or use of high doses of OSE that could have affected the reproductive system of the tested animals.[27] Thus, it becomes important to firmly consider experimental conditions such as nature of the test substance, type of solvent used for extraction, study methodology, and analysis of contaminants to ensure safety of the test drug.

CONCLUSION

In the present one-generation toxicity study, OSE extract did not induce any adverse toxicological effect on the reproductive performance of the male and female rats up to 1000 mg/kg BW (no-observed adverse effect level). Based on these data, OciBest could be proposed for its future clinical usefulness.

Acknowledgements

We would like to acknowledge the Department of Biotechnology, Government of India (BT/PR10467/PBD/17/561/2008), for funding the project.

Financial support and sponsorship

We would like to acknowledge the Department of Biotechnology, Government of India (BT/PR10467/PBD/17/561/2008), for funding the project.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Pattanayak P, Behera P, Das D, Panda SK. *Ocimum sanctum* Linn. A reservoir plant for therapeutic applications: An overview. Pharmacogn Rev 2010;4:95-105.
2. Prakash P, Gupta N. Therapeutic uses of *Ocimum sanctum* Linn (Tulsi) with a note on eugenol and its pharmacological actions: A short review. Indian J Physiol Pharmacol 2005;49:125-31.
3. Pandey G, Madhuri S. Pharmacological activities of *Ocimum sanctum* (Tulsi): A review. Int J Pharm Sci Rev Res 2010;5:61-6.
4. Singh V, Amdekar, S, Verma O. *Ocimum sanctum* (Tulsi): Bio-pharmacological activities. Webmedcentral Pharmacol 2010;1:1-7.
5. Devra DK, Mathur KC, Agrawal RP, Bhadu I, Goyal S, Agarwal V. Effect of Tulsi (*Ocimum sanctum* Linn) on clinical and biochemical parameters of metabolic syndrome. J Nat Remedies 2012;12:63-7.
6. Saxena RC, Singh R, Kumar P, Negi MP, Saxena VS, Geetharani P, *et al*. Efficacy of an extract of *Ocimum tenuiflorum* (OciBest) in the management of general stress: A Double-blind, placebo-controlled study. Evid Based Complement Alternat Med 2012;2012:894509.
7. Mondal S, Varma S, Bamola VD, Naik SN, Mirdha BR, Padhi MM, *et al*. Double-blinded randomized controlled trial for immunomodulatory effects of Tulsi (*Ocimum sanctum* Linn.) leaf extract on healthy volunteers. J Ethnopharmacol 2011;136:452-6.
8. Bhattacharyya D, Sur TK, Jana U, Debnath PK. Controlled programmed trial of *Ocimum sanctum* leaf on generalized anxiety disorders. Nepal Med Coll J 2008;10:176-9.
9. Vohora SB, Garg SK, Chaudhury RR. Antifertility screening of plants 3. Effect of six indigenous plants on early pregnancy in albino rats. Indian J Med Res 1969;57:893-9.
10. Kasinathan S, Ramakrishnan S, Basu SL. Antifertility effect of *Ocimum sanctum* L. Indian J Exp Biol 1972;10:23-5.
11. Seth SD, Johri N, Sundaram KR. Antispermic effect of *Ocimum Sanctum*. Indian J Exp Biol 1981;19:975-6.
12. Kantak NM, Gogate MG. Effect of short term administration of tulsi (*Ocimum sanctum* Linn.) on reproductive behaviour of adult male rats. Indian J Physiol Pharmacol 1992;36:109-11.
13. Akbarsha MA, Palanisamy M, Murugaian P, Lakshmi Latha PN. Ursolic acid generates symplasts in rat spermatogenic clones. Phytother Res 1998;12:32-6.
14. Sardessai SR, Borker AS, Abraham ME. Effects of short-term administration of Tulsi leaves on sexual behaviour in female rats. Indian J Physiol Pharmacol 1999;43:398-400.
15. Mankapure VA, Mankapure AG, Sohani PV. Male antifertility effect of *Ocimum sanctum* Linn*:* A study in albino rat. J Endocrinol Reprod 2013;17:123-32.
16. Srinivasulu K, Changamma C. A study on the effect of *Ocimum sanctum* (Linn.) Leaf extract and ursolic acid on spermatogenesis in male rats. Ind J Pharm Sci 2017;79:158-63.
17. Chandrasekaran CV, Srikanth HS, Anand MS, Allan JJ, Viji MM, Amit A, *et al*. Evaluation of the mutagenic potential and acute oral toxicity of standardized extract of *Ocimum sanctum* (OciBest™). Hum Exp Toxicol 2013;32:992-1004.
18. Raina P, Chandrasekaran CV, Deepak M, Agarwal A, Ruchika KG. Evaluation of subacute toxicity of methanolic/aqueous preparation of aerial parts of *O. sanctum* in Wistar rats: Clinical, haematological, biochemical and histopathological studies. J Ethnopharmacol 2015;175:509-17.
19. George P. Concerns regarding the safety and toxicity of medicinal plants – An overview. J Appl Pharm Sci 2011;1:40-4.
20. OECD. Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment; 2008.
21. OECD. Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation, ENV/JM/MONO; 2000.
22. OECD. OECD Guidelines for Testing of Chemicals. No. 415. One-Generation Reproduction Toxicity Study. Paris, France: Organisation for Economic Co-operation and Development; 1983.
23. Reuter U, Heinrich-Hirsch B, Hellwig J, Holzum B, Welsch F. Evaluation of OECD screening tests 421 (reproduction/developmental toxicity screening test) and 422 (combined repeated dose toxicity study with the reproduction/developmental toxicity screening test). Regul Toxicol Pharmacol 2003;38:17-26.
24. Lagarto A, Tillan J, Bueno V, Chavez I, Guerra I, Vega Y. Acute and subchronic oral toxicity in rats of a lyophilized aqueous extract of *Ocimum tenuiflorum* L. Rev Toxicol 2005;22:175-9.
25. Gautam MK, Goel RK. Toxicological study of *Ocimum sanctum* Linn leaves: Hematological, biochemical, and histopathological studies. J Toxicol 2014;2014:135654.
26. Lanning LL, Creasy DM, Chapin RE, Mann PC, Barlow NJ, Regan KS, *et al*. Recommended approaches for the evaluation of testicular and epididymal toxicity. Toxicol Pathol 2002;30:507-20.
27. Vogel R, Seidle T, Spielmann HA. Modular one-generation reproduction study as a flexible testing system for regulatory safety assessment. Reprod Toxicol 2010;29:242-5.
28. Endres JR, Qureshi I, Farber T, Hauswirth J, Hirka G, Pasics I, *et al*. One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient. Food Chem Toxicol 2011;49:1174-82.