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Anti-fertility Effects of Aqueous *Ocimum sanctum* Leaf Extract on Male Mouse

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Research Article

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ABSTRACT

Background and aim: The increasing population is a pressing issue in India. When it comes to contraception, far greater emphasis is laid on the role of a female. However, male contraception is easily developable, thanks to the far simpler hormonal makeup of the male as compared to the female. In this regard, considering the toxicity associated with chemicals, herbal contraception is the need of the hour owing to less toxicity and better adaptability to the system. Thus, the present study explored the possibility of *Ocimum sanctum* as a probable candidate for herbal male contraception. **Experimental procedure:** Male mouse were administered with three different doses (10 mg, 40 mg and 80 mg) of aqueous *Ocimum sanctum* leaf extract orally for a period of 8 weeks after which the animals were sacrificed. **Results:** There was a dose dependent decrease in sperm motility and concentration together with a decline in litter size and serum testosterone levels. The enzymatic markers for oxidative stress were found to be elevated dose dependently. In addition, testis histopathology revealed loss of germ cells and shrinkage of germinal epithelium with increasing dose and a complete lack of sperms in the 80 mg group. The mRNA expression of Catsper gene, responsible for sperm motility was also altered dose dependently. Contrastingly, the withdrawal of treatment for 8 weeks led to reversal of deleterious effects.
Conclusion: The present study revealed the deleterious effects of aqueous *Ocimum sanctum* leaf extract on male fertility. The reversible nature of damage makes *Ocimum sanctum* a potential candidate for safe herbal male contraception.

Keywords: *Ocimum sanctum*; Male fertility; Herbal contraception; Phytochemical; Testis

HIGHLIGHTS

- At a dose level of 80 mg/kg b.wt., *Ocimum sanctum* led to a significant decline in sperm concentration and motility after 8 weeks of treatment.
- The enzymatic markers for oxidative stress were found to be elevated dose dependently.
- In addition, testis histopathology revealed loss of germ cells and shrinkage of germinal epithelium with increasing dose and a complete lack of sperms in the 80 mg group.
- The mRNA expression of Catsper gene, responsible for sperm motility was also altered dose dependently.
- Contrastingly, the withdrawal of treatment for 8 weeks led to reversal of deleterious effects.

INTRODUCTION

Population growth is one of the world's most pressing issues, particularly in developing nations like India. The birth rate has traditionally been managed using a variety of contraceptive techniques [1]. However, despite being readily available, contraception is not frequently utilized due to a number of myths and religious restrictions [2]. A man and a woman are equal partners in the act of conception; however, far more emphasis has been placed on the role of the female in the development of contraceptive technology [3]. Although multicultural surveys demonstrate the willingness of the male to participate in contraception, condoms and vasectomy are the only two time-tested methods of contraception available to men at the moment [4], as hormonal methods have a slew of side effects [5]. As a result, it is necessary to look for alternative, non-hormonal, biologically active substances or fertility-regulating agents derived from plants that are cost-effective, environment friendly, and have the potential to interfere with fertility.

Herbal contraception is currently receiving more attention in modern contraceptive research [6]. The development of new fertility-regulating drugs derived from medicinal plants appears to be an appealing proposition [7] since humans have relied on plants and their products as sources of medications, and therapeutic agents from time immemorial. Fruits, vegetables, whole grains, legumes, beans, herbs, spices, nuts, and seeds contain phytochemicals, which are classified based on their chemical structures and functional properties [8]. Phytochemicals include substances like salicylates, phytosterols, saponins, glucosinolates, polyphenols, protease inhibitors, monoterpenes, phytoestrogens, sulphides, terpenes, lectins, and many others. They are believed to be useful in preventing disease due to their antioxidant capabilities [9].

It is however, interesting to note that some phytochemicals have been reported to act as pro-oxidants at higher dose levels, producing free radicals or ROS, which are extremely harmful to our bodies as they cause dysfunctional redox control and impaired cell signalling [10]. Spermatozoa produce a small amount of ROS under physiological conditions, which are required for motility, hyperactivation, capacitation, acrosomal reaction, and fertilization [11]. During the capacitation process, the levels of intracellular calcium, ROS, and tyrosine kinase rise, resulting in an increase in cyclic Adenosine Monophosphate (cAMP) [12]. This increase in cAMP promotes spermatozoa hyperactivation [13]. ROS thus participate in normal physiological processes at low concentrations. At higher concentrations, they cause adverse changes to cell components such as lipids, proteins, and DNA, resulting in oxidative stress in the testicular compartment leading to infertility [14].

In this regard, *Ocimum sanctum* (Linn), also known as Tulsi, is an important medicinal plant whose unique properties have long been recognized. The plant is used as an antibacterial and insecticidal agent, a diaphoretic in malarial fever, and an antiperiodic in the gastric and urinary systems. There are numerous reports suggestive of its role as an anticancer agent, anti-diabetic, anti-fungal [15,16], hepatoprotective, cardioprotective, analgesic and antioxidant [17]. *Ocimum sanctum* has also been reported to exert its ameliorative effects against cigarette smoke exposure [18,19].

In contrast to the well reported anti-oxidant activities of *Ocimum sanctum*; the literature bears reports suggestive of its anti-fertilty properties as well [20]. Speculating the probability of *Ocimum sanctum* as a potential candidate for male contraceptive development in future, the present study was designed with an aim to explore the effect of its aqueous extract at different doses; if any on testis (reversible/irreversible), with an aim to shed light on the contrasting reports already existing in literature, thereby paving way towards the development of a safe herbal male contraceptive in future.

MATERIALS AND METHODS

The present study followed the methods of Pavitra Ranawat et al., and Pavitra Ranawat et al.

Plant collection

Ocimum sanctum saplings were purchased from Punjab Agricultural University (PAU), Ludhiana, India and were grown thereafter in the herbarium of Department of Botany, Panjab University, Chandigarh, under strict supervison.

Animals and treatment

Male Laca mice (25-35 g) were taken from the Central Animal House, Panjab University, Chandigarh, and kept in polypropylene cages bedded with rice husk. They were given free access to clean drinking water and a standard animal pellet diet throughout the experiment. Proper ventilation and ambient room temperature of 25°C ± 2°C were maintained where the animals were kept for the experiment. The experimental protocols were approved by the Institutional Ethics Committee (IAEC), Panjab University, Chandigarh and conducted according to Indian National Science Academy Guidelines. The study is also reported in accordance with ARRIVE Guidelines. The animals were randomly divided into four groups (8

animals each) on the basis of the treatment they received.
Group I (control) was given normal drinking water and standard pellet diet ad libitum. Group II (OSE 10) received aqueous *Ocimum sanctum* leaf extract at a dose of 10 mg/kg body weight orally for eight weeks. Group III (OSE 40) received aqueous *Ocimum sanctum* leaf extract at a dose of 40 mg/kg body weight orally for a period of eight weeks. Group IV (OSE 80) received aqueous *Ocimum sanctum* leaf extract at a dose of 80 mg/kg body weight orally for a period of eight weeks. Weekly changes, if any in body weight were monitored in all groups throughout the treatment period. On completion of the treatment schedule, animals were sacrificed by cervical dislocation. Testes, epididymis, vas deferens and liver were removed immediately for further evaluation.

Preparation of aqueous leaf extract of *Ocimum sanctum*

The leaves of *O. sanctum* were dried in shade and finely powdered. The leaf powder (100 g) was then refluxed with 750 ml of double-distilled water for 1 h at 75–80℃. It was then cooled and filtered. This was repeated in three trials. The extracts were pooled and evaporated using a lyophilizer and the powder so obtained was weighed and stored in the refrigerator in an airtight container. The extract was reconstituted in DDW immediately before use.
Standard ABTS and DPPH assays were carried out with the extract to check for radical scavenging activity.

Evaluation of reproductive potential

Sperm motility: Sperms were teased out from the vas deferens into a watch glass containing 1 ml normal saline at 37℃. After thorough mixing, 100 µL of the sperm suspension was put on a glass slide and covered with a cover slip. The motile sperms were counted in several random fields under the light microscope and percentage of motile sperms was calculated.
Sperm concentration: For concentration measurements, sperms were teased out from the epididymis in 1 ml normal saline, mixed properly with a Pasteur pipette and counted on the WBC squares of the Neubauer's chamber. Sperm concentration was then expressed as millions/ml.
Fertility studies: At the end of the treatment period, male mice were allowed to mate with normal females in the ratio of either (1:3 or 1:2) for 7 days, after which the male were separated. The females were observed for 21 days for signs of pregnancy or for giving birth to pups.

Percentage fertility=No. of females giving birth/No. of females exposed to mating X 100

For each female giving birth, the litter size was recorded and averaged for each group.
Hormone analysis: Serum testosterone level in all treatment groups was measured using commercially available kit (Reckon Diagnostics Pvt. Ltd.)

Biochemical estimations

Reactive Oxygen Species (ROS): Reactive oxygen species were determined using the method of Wang et al. ROS accumulation was detected with the carboxy-H2-DCFDA (Molecular Probes) staining method. This assay is based on the principle that the nonpolar, non-ionic dichlorofluorescein diacetate dehydrate (H2-DCFDA) crosses cell membranes and is enzymatically hydrolyzed into nonfluorescent dicholorofluorescein dehydrate (H2-DCFDA) by intracellular esterases. In the presence ROS, H2-DCF is rapidly oxidised to become highly fluorescent dichlorofluorescein (DCF) which is then measured
Lipid peroxidation: The method of Trush was used for estimating lipid peroxidation in testicular homogenates. The levels of MDA produced serve as an index of the intensity of oxidative stress. Lipid peroxidation is the oxidative deterioration of lipids by free radicals generated due to various enzymatic and non-enzymatic reactions. Cycloperoxides formed in the deterioration process form Malondialdehyde (MDA). MDA forms a pink colored complex with thiobarbituric acid (MDA-TBA chromophore), which can be read at 532 nm.
Reduced glutathione: Levels of reduced glutathione were estimated by the method of Moron et al, 1979. 25% TCA was added to 10% homogenate and the precipitates so formed were removed by centrifugation at 1500 g for 10 min. Reaction buffer was then added to the supernatant thus obtained. DTNB was then added to the above solution, and the resultant yellow coloured complex was read at 412 nm. A standard GSH plot was also performed to calculate the content of GSH.
Lactate Dehydrogenase (LDH): LDH activity was measured by the method of Bergmeyer and Bernt. LDH catalyzes the reduction of pyruvate with NADH to form NAD+. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the LDH activity in the sample.
Liver marker enzymes: Liver Function Tests (LFTs or LFs) which include liver enzymes are group of clinical biochemistry

laboratory tests designed to give information about the state of an animal's liver. The activity of Alkaline Phosphatase (ALP), Alanine Transaminase (ALT) and Aspartate Transaminase (AST) was determined using commercially available kits (Reckon Diagnostics Pvt. Ltd.).

Protein estimation: Protein concentration of samples was estimated by the method of Lowry et al.

Histopathological analysis: Histopathological analysis of testis tissue was done using Hematoxylin and Eosin staining as described by Humanson et al. Testes were removed and immediately transferred to neutral formalin and allowed to fix for 12 h. Next, the tissue was dehydrated gradually in ascending series of ethanol. For embedding, the dehydrated samples were placed in benzene, then sequentially in 1:1 benzene: Paraffin wax with two changes in pure wax before finally embedding. 5 µm thick sections were obtained using a manual hand-driven microtome and transferred to glass slides. These were then dewaxed in xylene, rehydrated in descending series of ethanol, and stained with Hematoxylin and Eosin. Stained sections were mounted in DPX and viewed under a light microscope.

RNA expression studies

mRNA expression analysis by RT-PCR was performed using the INVITROGEN RT-PCR kit.

Total RNA isolation: Total RNA was isolated from mouse testis using TRI-REAGENT. 50 mg tissue from different treatment groups was homogenized after which 80 µl chloroform was added. After mixing vigorously for about 15 sec the homogenates were kept at room temperature for 10 min followed by centrifugation at 12,000 rpm for 15 min at 4℃. The upper colourless aqueous phase containing RNA was collected and an equal volume of isopropanol was added to precipitate RNA. This was then spun thereafter at 12,000 rpm for 10 min at 4℃. RNA precipitate so obtained was washed by adding 75% ice-cold ethanol and spinning at 7500 g for 5 min at 4℃. After removing ethanol, the RNA pellet was briefly air-dried (not completely) and then dissolved in DEPC treated water. Purity, integrity, and concentration of the isolated RNA were checked by taking absorbance at 260 and 280 nm and finding their ratio. The concentration of RNA was estimated by using A_{260}=1=40 µg/ml.

RT-PCR procedure: RT-PCR was done using specific primers for the respective genes (Catsper and β-actin). RT-PCR for β-actin was done along with to rule out the experimental errors. INVITROGEN one-step RT-PCR kit was used for the purpose. 2 µg of total RNA was used in RT-PCR reaction from different groups. To this, the following reagents were added as follows: 10 µl 5x Invitrogen one-step RT-PCR buffer, 2 µl dNTP mixture, 5 µl each of forward and reverse primers. (10 µM stock), 2 µl enzyme mix, and 1 µl RNase inhibitor (1 U/µl). Finally, PCR grade RNase-free water was added to make the total volume 50 µl. The components were mixed with a gentle vortex and centrifuged to collect all the components at the bottom of the tube. The PCR reaction was performed in the thermal cycler (Techne Ltd, England) using the following conditions: RT reaction was performed at 50℃ for 50 min and activation at 94℃ for 15 min. PCR was followed by 35 cycles of 94℃ (denaturation) for 45 sec, T_m (annealing) for 45 sec, 68℃ (extension) for 1 min. Finally, the products were incubated at 68℃ for 5 min to extend any incomplete single strands. Final PCR products formed were analysed on 1.5% agarose gel electrophoresis and densitometric analysis of the bands was done by Image J software (NIH, USA). The mean of four independent densitometric analyses of PCR product bands were determined for comparison of each analysis (Table 1).

Table 1. Primer sequences and their melting temperatures (Tm) for CatSper and β-actin genes.

Catsper		**Tm (◦C)**
Left primer	AGCGCTTCCAGAACATCTTTAC	58.4
Right primer	CACTTTCTCTAGGCCTTTGAGC	60.3
β-actin		**Tm (◦C)**
Left primer	ATCCGTAAAGACCTCTATGC	55.3
Right primer	AACGCAGCTCAGTAACAGTC	57.3

Statistical analysis

The data from individual groups were compared using Statistical Package for Social Sciences software (SPSS version 14.0) and the groups were presented as the mean ± S.D. Differences between groups were analyzed using a one-way Analysis of Variance (ANOVA) and a post hoc test. Significant Difference (LSD) test and minimum criterion for statistical significance was set at $p<0.05$ for all comparisons.

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RESULTS

The radical scavenging activity of *Ocimum sanctum* leaf extract was evaluated by DPPH and ABTS assay. A concentration dependent increase was observed in the activity of both ascorbic acid as well as aqueous extract of *Ocimum sanctum* against free radicals in DPPH assay along with ABTS assay respectively.

Evaluation of reproductive potential

Sperm motility (%): A dose dependent decrease in sperm motility was observed in all treatment groups with a maximum reduction observed in the 80 mg OSE group (2.41% ± 1.94, p<0.001) as compared to control (63.45% ± 5.35). Sperm motility was reduced to 9.78% ± 1.22, p<0.001 at 10 mg OSE and 4.45% ± 5.05, p<0.001 at 40 mg OSE administration.

When OSE administration was withdrawn for 2 months, sperm motility in the 80 mg OSE group was restored to 62.84% ± 4.15, p<0.001, thereby highlighting damage reversal.

Sperm concentration (millions/ml): Epididymal sperm concentration was found to be significantly decreased in all treatment groups. The decrease was found to be dose dependent with maximum reduction observed in 80 mg OSE group (3.81 ± 0.76) as compared to control (7.97 ± 1.92). Sperm concentration was also significantly reduced in 40 mg OSE group (5.37 ± 0.83, p<0.05) and 10 mg (5.29 ± 1.12, p<0.05) OSE group as compared to control (7.97 ± 1.92).

However, when OSE administration was withdrawn for 2 months in the 80 mg OSE group, the epididymal sperm concentration was restored to normal (7.85 ± 0.70) signifying the reversal of damage.

Fertility studies: The litter size decreased significantly in 10 mg OSE treated group (6 pups) and 40 mg OSE treated group (5 pups) as compared to the control (10 pups). However, no pups were produced in the 80 mg OSE treated group signifying that OSE treatment negatively affected the fertility status of the male at a high dose.

Contrastingly, when the male from the 80 mg OSE withdrawal group was mated with healthy females, 4 pups were produced thereby highlighting that the damage caused by high dose of OSE was reversible in nature. Our results are in accordance with a similar study done by MC et al., and Naik et al.

Hormonal parameters: OSE administration led to a significant dose dependent decrease in testosterone levels in all treatment groups. The levels were 72.15 ± 0.46, p<0.05 in 10 mg OSE group, 69.61 ± 0.56, p<0.001 in 40 mg OSE group, 66.47 ± 0.94, p<0.001 in 80 mg OSE group and 98.06 ± 1.4, p<0.001 in control group.

However, when OSE administration was withdrawn for 2 months in the 80 mg OSE group, the testosterone levels were restored in the near normal range 80.5 ± 1.5, p<0.001.

Biochemical estimations

ROS generation: A dose dependent increase in the relative fluorescent intensity of DCF (measure of ROS generation) in the testicular compartment was found in all OSE treated groups as compared to control. The relative fluorescent intensity was (1.69 ± 0.11) in 10 mg OSE group, (2.9 ± 0.09, p<0.001) in 40 mg OSE group and (3.29 ± 0.19, p<0.001) in 80 mg OSE group as compared to control (1.8 ± 0.06, p<0.001).

However, after withdrawal of treatment for 2 months, the relative fluorescent intensity of DCF attained near normal values (2.24 ± 0.16, p<0.01), in the 80 mg OSE group.

The relative fluorescent intensity of DCF in liver was also found to be significantly increased dose dependently in all OSE treated groups. The relative fluorescent intensity was (1.53 ± 0.47) in 10 mg OSE group, (2.29 ± 0.18) in 40 mg OSE group and (2.67 ± 0.16) in 80 mg OSE group as compared to control (1.40 ± 0.28).

However, after withdrawal of the treatment for 2 months, the relative fluorescent intensity of DCF attained near normal values (1.31 ± 0.02) in the 80 mg OSE group.

Lipid Peroxidation (LPO): The levels of MDA produced in the testis were measured as an index of lipid peroxidation. Significant increase in the levels of testis MDA was observed in all OSE treated groups as compared to control. The levels of MDA were (0.96 ± 0.03, p<0.001) in 10 mg OSE group, (2.00 ± 0.32, p<0.001) in 40 mg OSE group and (2.79 ± 0.04, p<0.001) in 80 mg OSE group as compared to control (0.89 ± 0.10). Withdrawal of the treatment for 2 months showed improvement in MDA levels in the 80 mg OSE group (0.72 ± 0.07, p<0.001).

Similarly, an increase in the levels of MDA was observed in the liver of all OSE treated groups. The levels of MDA were (1.71 ± 0.36) in 10 mg OSE group, (1.79 ± 0.41) in 40 mg OSE group and (2.32 ± 0.59) in 80 mg OSE group as compared to control (1.60 ± 0.24). After withdrawal of the treatment for 2 months, MDA levels in the 80 mg OSE group were comparable to control (1.63 ± 0.09).

Reduced Glutathione (GSH): Decline in the GSH levels were found in testicular compartments in 10 mg OSE group (1.29 ±

0.09), 40 mg OSE group (1.25 ± 0.14, p<0.05), and 80 mg OSE group (0.74 ± 0.17, p<0.001) as compared to control (1.88 ± 0.16). However, after withdrawal of treatment for 2 months in the 80 mg OSE group, the GSH levels were comparable to control (1.56 ± 0.11).

Decline in the GSH levels were also found liver homogenates in all treatment groups as compared to control. The levels were (3.29 ± 1.06) in 10 mg OSE group, (2.04 ± 0.58) in 40 mg OSE group and (1.21 ± 0.26) in 80 mg OSE group and (3.43 ± 0.57) in control group. However, GSH levels improved after withdrawal of the treatment for 2 months in 80 mg OSE group (3.49 ± 0.02).

Lactate Dehydrogenase (LDH): A dose dependent increase in the LDH activity was observed in testis in all OSE treated groups as compared to control. The levels were (0.24 ± 0.04) in 10 mg OSE group, (0.31 ± 0.11) in 40 mg OSE group, (0.43 ± 0.05, p<0.05) in 80 mg OSE group and (0.21 ± 0.02) in control group.

After withdrawal of the treatment for 2 months LDH levels returned to normal range in 80 mg OSE treated group (0.21 ± 0.001).

No significant increase in the LDH activity in liver was observed in the 80 mg dose (0.08 ± 0.02), 40 mg dose (0.07 ± 0.00) and 10 mg dose (0.06 ± 0.00) as compared to the control group (0.04 ± 0.01). After the withdrawal of the treatment for 2 months LDH levels remained in the normal range in 80 mg OSE treated group (0.04 ± 0.01).

Liver marker enzymes: Increase in the ALP activity was observed in 10 mg OSE group (100.31 ± 0.02), 40 mg OSE group (105.26 ± 6.82) and (113.04 ± 18.41) in 80 mg OSE group as compared to the control group (93.59 ± 18.89). This increase was within the normal range and after withdrawal of treatment for 2 months ALP levels in 80 mg OSE group were (106.48 ± 20.14).

Significant increase in the ALT activity was observed in the 10 mg OSE group (44.81±3.30), 40 mg OSE group (60.06 ± 16.74) and 80 mg OSE group (69.23 ± 18.13) as compared to control group (43.47 ± 12.59).

After withdrawal of treatment for 2 months, the ALT activity was brought to normal value in the 80 mg OSE treated group (48.01 ± 3.70).

Increase in the AST activity was observed in the 10 mg OSE group (62.85 ± 5.55), 40 mg OSE group (63.99 ± 17.52) and 80 mg OSE group (68.79 ± 14.09) as compared to control group (57.11 ± 12.07). The increase was within the range and after withdrawal of the treatment for 2 months AST levels returned to control range in 80 mg OSE treated group (59.63 ± 0.30).

Histopathological analysis

The control sections revealed normal testicular histomorphology. All the stages of transformation of seminiferous epithelia from spermatogonia to spermatids were seen in seminiferous tubules. The mice from 10 mg OSE treated group showed normal testicular histomorphology. 40 mg OSE treated group exhibited many distorted disorganized and shrunken Seminiferous tubules (St). Some tubules showed marked reduction in the thickness of the germinal epithelium (arrows) and decreased sperms, whereas others exhibited destruction of the basal lamina. Some seminiferous tubules showed irregular basement membranes and detachment of germinal epithelium in some of the seminiferous tubules. In 80 mg OSE group, an excessive shrinkage of seminiferous tubules was observed with destruction of its epithelia. Loss of Leydig cells was prominent and extensive vacuolization of seminiferous epithelium was observed with almost complete loss of spermatozoa from the lumen of the seminiferous tubules. Sloughing of the germinal epithelium was also evident in some of the tubules. Seminiferous tubules of testis from the 80 mg OSE withdrawal group were lined with normal germinal epithelium with normal appearance of all germ cell lineages. Clusters of Leydig cells were also seen.

m-RNA expression studies

The mRNA expression of CatSper gene significantly decreased dose dependently as compared to the control group in all treatment groups.

DISCUSSION

The qualitative analysis of aqueous extract derived from the leaves of *Ocimum sanctum* validated the presence of phytochemical constituents; namely, phenols, flavonoids, carbohydrates and alkaloids with reference to ascorbic acid taken as a standard. The results were in accordance with the study by Devendran and Balasubramanian, which have also observed the presence of the above in aqueous *Ocimum sanctum* leaf extract.

The percent radical scavenging activity of aqueous extract of *Ocimum sanctum* and ascorbic acid (reference compound) was assessed using DPPH and ABTS *in vitro* assay. The concentration for 50% inhibition (IC) was calculated from the graph, and was found to be 0.97 mg/ml and 85.05 mg/ml for ascorbic acid and test sample respectively in case of DPPH assay and 0.228 mg/ml and 58.98 mg/ml in ascorbic acid and test respectively in case of ABTS. The present results also demonstrate the radical scavenging activity in aqueous *Ocimum sanctum* leaf extract in comparison to ascorbic acid, a well-known antioxidant.

No significant change in the body weight was seen in the treated groups as compared to control group, showing that the aqueous extract of *Ocimum sanctum* does not affect the body weight even at a higher dose.

A significant decline in sperm motility and sperm concentration was observed in all the *Ocimum sanctum* treated groups presently. The presence of oxidative stress in the testicular compartment might have changed the ROS balance and would have altered the antioxidant defense system in the treatment groups. The H_2O_2 generated as a consequence of oxidative stress, diffuses across the cell membrane into the cells and inhibits the activity of some vital enzymes such as Glucose-6-Phosphate Dehydrogenase (G6PDH). G6PDH is an enzyme that controls the rate of glucose flux *via* the hexose monophosphate shunt and in turn, controls the intracellular availability of NAPH, which is then used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH Oxidase. Decreased G6PDH leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione. These changes can cause a decrease in the antioxidant defense of the spermatozoa, which ultimately lead to the peroxidation of membrane phospholipids and hence decrease in sperm motility and concentration. However, after withdrawal of the treatment (after 2 month), the motility and concentration attained their normal values, thereby implying that the damaging effects caused by *Ocimum sanctum* are reversible.

The litter size decreased significantly in 10 mg (6 pups), 40 mg (5 pups) as compared to the control (10 pups). No pups were produced in the 80 mg group signifying that the treatment with *Ocimum sanctum* caused infertility at high doses. However, after the withdrawal of the treatment in the 80 mg group, a litter of 4 pups was obtained thereby strengthening the observation regarding the reversible nature of damage by *Ocimum sanctum*.

Further, the dose dependent decrease in the testosterone levels in all treatment groups is in agreement with the previous study reported by Obianime et al. The decrease in the level of serum testosterone, as reported presently, might be due to decrease in the serum levels of LH/FSH. Leydig cells secrete testosterone by the stimulatory effect of LH. In males reduction of testosterone level may impair spermatogenesis and cause male infertility. Thus, the present observations might have resulted due to the adverse effects of *Ocimum sanctum* on leydig cells function (testosterone production). This may also be due to hinderance in oxidative cleavage of the side-chain of cholesterol due to oxidative stress caused by *Ocimum sanctum*.

There was a dose dependent increase in the ROS generation observed presently in 40 mg and 80 mg group in both testis and liver tissue. The results are in agreement with the previous study by Ranawat et al, demonstrating an increase in the generation of ROS in the testicular compartment resulting from an oxidative insult. This may further result in increased lipid peroxidation following higher doses of *Ocimum sanctum*. A decrease in ROS generation in 10 mg group might be due to antioxidant property of *Ocimum sanctum* at a lower dose, while increase in other groups might be due to its pro-oxidant activity at high dose levels. However, withdrawal of the treatment for 2 months in 80 mg group led to near normal ROS levels.

Lipid peroxidation is an important parameter to study the oxidative damage due to the presence of large number of Polyunsaturated Fatty Acids (PUFAs) in the sperm membrane. Increase in the levels of MDA was observed in all the treated groups as compared to the control, depicting oxidative damage in the testis. The results are in accordance with a study by Mahboob et al., which have also observed increase in LPO with increase in level of toxicity. These increased levels have been associated to the loss of fluidity, inactivation of membrane enzymes and increase in membrane permeability to the ions, which results in disruption of the cellular membrane. The production of lipid peroxide in the biological membrane is regulated by the availability of substrates in the form of PUFAs, pro-oxidants that promote peroxidation and antioxidant defenses such as GSH, SOD, beta-carotene. Administration of *Ocimum sanctum* showed dose dependent increase in lipid peroxidation but these modifications turned to their normal values with the withdrawal of the treatment for 2 months, suggesting the reversible effects of *Ocimum sanctum*. Similar observations were reported in liver tissue as well.

Glutathione is an important tri-peptide (glutamate, cysteine and glycine) that contains a gamma peptide linkage between carboxyl group of glutamate side chain and amine of cysteine (γ-glutamylcysteinylglycine) and is synthesized by nearly all mammalian cells. Glutathione participates in the maintenance of reduced thiol groups on intracellular enzymes/proteins/ amino acids and plays a vital role in defense against toxins and free radicals and in storing and transferring cysteine. Glutathione plays an essential role in the formation of phospholipid hydroperoxide glutathione peroxidase–an enzyme that is present in spermatids and forms the structural part in the mid piece of mature spermatozoa. Glutathione deficiency can lead to instability of the mid piece of mature spermatozoa, resulting in defective motility. A significant decrease in glutathione levels was observed in 80 mg group, owing to the pro-oxidant nature of *Ocimum sanctum* at high doses. Withdrawal of the treatment for 2 months reversed these effects suggesting the reversible nature of damage incurred by *Ocimum sanctum*.

Generation of ROS, causes membrane degradation and its permeability gets disrupted and as a result of this, LDH leaks out of the cell into extracellular matrix. Hence, it can be detected in blood. Significant dose dependent increase in the LDH activity was observed in all the treated groups which are in agreement with a study by Zaman et al. Thus, this may be due to dose dependent increase in ROS generation caused by Ocimum sanctum in the testicular compartment. However, there was a reversal of damage after withdrawal of treatment for 2 months in the 80 mg group, depicting its reversible nature. Similar observations were reported in the liver tissue as well.

To evaluate the systemic toxicity of *Ocimum sanctum* in mice, some liver function tests were performed. Estimation of liver marker enzymes *i.e.*, ALP, ALT, AST was done in serum. Our results were similar to a study performed by Uboh et al. The levels of ALP were increased but this increase was within the range. However, the levels of ALT/SGPT were increased in 40 mg and 80 mg group but that too was not that significant. The levels of AST/SGOT and ALP were also increased but the increase here too, was not that significant. All the increased values retained their normal range after the withdrawal of the treatment for two months as well in the 80 mg group.

Histopathological examination of testicular architecture of control mice revealed closely arranged seminiferous tubules with intact basement membrane followed by different stages of spermatogenic cells, clusters of Leydig cells in interstitium, and usual interactions between germ and Sertoli cells with fully mature sperms in the lumen. Many histoarchitectural changes were observed in the testis of *Ocimum sanctum* treated groups. The disruption in the histology was dose dependent. 10 mg showed the least disruption or almost no changes as compared to 40 and 80 mg group. Significant reduction in diameter of seminiferous tubules and thickness of germinal epithelium, reduced number of sperm in the lumen and sloughing off the germinal epithelium was observed in high dose groups indicative of oxidative damage as also reported earlier by Ranawat et al. Germinal epithelial damage might have led to oligo or azoospermia thereby, causing infertility in the high dose group. Hyper cellularity of leydig cells was also seen presently. However, all adverse histoarchitectural changes got reversed after the withdrawal of the treatment for 2 months, depicting the reversible nature of damage by *Ocimum sanctum*. Our results are in accordance with the results of Obianime et al.

Histopathological examination of liver tissue of control showed a vast interanastomosing network of hepatocytes arranged in single-cell thick plates separated by vascular sinusoids. Innumerable lobules were seen, each of which was a hexagonal structure consisting of a central vein (terminal hepatic venule, thin arrow) surrounded by radiating hepatocyte plates. Sinusoidal lining cells consist of specialized fenestrated endothelial cells and specialized macrophages or Kupffer cells (A). However, no histo-architectural changes were observed in the 10 mg and 40 mg group. They showed the same histology as that of the control. 80 mg group showed sinusoidal dilation (A) and infilteration of inflammatory cells (Kupffer cells) in the lobular region.

Calcium influx through the flagellar Ca^{2+} ion channel, CatSper, triggers hyperactivation and leads to changes in the flagellar envelope during capacitation. In hyperactivated spermatozoa, the transverse flagellar force is larger than the propulsive flagellar force due to the increase in mid-piece curvature (α angle), which enables a larger range of motion compared to non-hyperactivated spermatozoa. Transverse force facilitates sperm penetration through the cumulus and zona pellucida. CatSper mRNA expression was induced in OSE treated cells. Calcium channels regulate the sperm protein (CatSper). The dose dependent decrease in the expression of CatSper gene might be due to oxidative stress in the testicular compartment which may have caused hinderance in the proper functioning of this channel (due to membrane degradation), leading to decrease in motility thereby causing infertility at high dose.

CONCLUSION

Keeping in view the above observations, it is evident that aqueous *Ocimum sanctum* leaf extract was quite effective in rendering anti-fertility effects at the highest dose of 80 mg/kg b.wt. However, after a withdrawal period of 2 months, all anti-fertility effects encountered in the 80 mg treated group got reversed automatically thereby strengthening the hypothesis that *Ocimum sanctum* has a huge potential in serving as a herbal male contraceptive. However, future research in this direction would help in extrapolating this research to humans.

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CONFLICT OF INTEREST

None.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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