A Study on the Effect of *Ocimum sanctum* (Linn.) Leaf Extract and Ursolic Acid on Spermatogenesis in Male Rats

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***Ocimum sanctum* L. leaf extract has been reported to possess antifertility. This plant is a rich source of various components that include eugenol, carvacrol (3%) and eugenol-methyl ether (20%). It also contains caryophyllin, ursolic acid, rosmaric acid, thymol, methyl chavicol, citral, carvacrol and β-caryophyllene. It has also been reported that the antifertility property was due to the presence of ursolic acid. Hence, in the present study rats were treated with *O. sanctum* L. leaf extract and ursolic acid. Administration of *O. sanctum* leaf extract caused significant decrease in sperm count and motility of spermatozoa by modulating testosterone levels. The extract caused androgen depletion at the target level, particularly in the cauda epididymis thereby affecting physiological maturation of the sperm. Ursolic acid, probably due to the low dose, it does not showed much effect on sperms and also on testosterone follicle-stimulating hormone and luteinizing hormone levels.**

Key words: Cauda epididymis, sperm count, testosterone, FSH, LH

Ocimum sanctum, the queen of herbs has made important contribution to the field of science from ancient times to modern research because of its large number of medicinal properties. At the same time different scientific studies including *in vitro*, *in vivo* and human experiments revealed that the *tulsi* has unique medicinal properties. The extraction from different parts of *O. sanctum* has its own value as either a therapeutic or curative effect[1]. Effects of *O. sanctum* on reproductive systems of laboratory animals have attracted scientists globally, and extensive toxicological studies have been carried out, especially the benzene extract of *O. sanctum* leaves has been suggested to reduce spermatogenesis in male rats by retarding the sertoli cells activity without affecting the germ cells[2].

The leaves of *O. sanctum* contain a variety of constituents that may have biological activity, including saponins, flavonoids and tannins[3]. Flavonoids serve as health promoting compounds with free radical scavenging potential[4]. Saponins are important phytoconstituents present in different plants including *O. sanctum* in which these constitute an important chemical class and include pentacyclic triterpenoids. Ursolic acid, one of the active constituent of *O. sanctum* has several medicinal properties such as hepatoprotective, antiallergic and antimicrobial. Ursolic acid, one of the major constituents of the *tulsi* leaves has been suggested to possess antifertility effect in rats of both sexes and in male mice[5]. Even though a lot of studies have proved the beneficial effects of *O. sanctum*, further studies are demanding to decipher the mechanism responsible for antifertility. Hence, the present study was focused in this direction in order to find out whether ursolic acid has antifertility effect or some other compound showing this antifertility effect.

The leaf extract of *O. sanctum* was prepared following the standard reported protocol[6]. The leaves of *O. sanctum* were collected and extract was prepared by adding 500 g of dried, crushed and powdered leaves of *O. sanctum* in 1500 ml of 95% ethanol in a round bottom flask and was kept at room temperature for 3 d in dark place. The mixture was filtered through muslin cloth and Whatman filter paper. The filtrates were evaporated using a rotary evaporator; the remnants of ethanol content were removed by drying at 40-50°. The extract was subjected to further concentration by keeping in a water bath for complete dryness. The final yield of leaf extract weighed 30 g (6%), the extract obtained in this method were stored for future use at 4°.

In the present study, healthy adult (4 mon old, weight 160±10 g) male Wistar rats were obtained from an authorized vendor (Sri Venkateswara Traders, Bangalore, India). Upon arrival, rats were housed in polypropylene cages containing sterilized paddy husk as the bedding material, and provided with filtered tap water and standard rat pellet diet *ad libitum*. Animals were maintained in a well-controlled laboratory facility (temperature 26±2°; 12:12 h light:dark cycle,

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humidity 50±5%). The usage of animals was approved by the Institutional Animal Ethics Committee (IAEC) (Regd. No. 438/01/a/ CPCSEA/ dt.17/07/2001) at SV University, Tirupati, India.

All animals were grouped into three groups in which the first group served as a control group (group-I), the second and the third groups served as experimental groups. The second group (group-II) animals have received *O. sanctum* leaf extract and third group (group-III) animals ursolic acid, a bioactive compound procured from Sigma Aldrich. Group I animals were administered 1 ml distilled water/rat/d orally for 20 d and the test group II received ethanol extract of *O. sanctum* leaves of 500 mg/kg/d orally for 20 d[7], while group III received ursolic acid 5 mg/kg/d for 20 d[8]. Six animals from each group were used for test. Twenty four hours after the last dose, the control and treated animals were sacrificed by cervical dislocation and the cauda epididymis was used for sperm analysis.

The reproductive tissues like testes, epididymis, seminal vesicle and prostate gland were dissected out, trimmed off from adherent fats and weighed and recorded. Gained weight was divided into whole body weight and obtained result was multiplied to 100 and the statistical analysis was done.

The spermatozoa were counted using an improved Neubauer Chamber, as described by Belsey *et al.*[9]. The cauda epididymis was removed at autopsy from each rat and the spermatozoa were squeezed into a petri dish containing 5.0 ml of physiological saline at 37°. For the sperm analysis, 0.5 ml of the epididymal fluid was added to 1 ml of the semen diluting fluid (sodium bicarbonate 5 g, formalin 1 ml, distilled water 99.0 ml) and subsequently mixed well. One drop of diluted epididymal fluid was added to the haemocytometer in humid place for 10 min. The number of spermatozoa in the appropriate squares of the haemocytometer was counted under an Olympus microscope. The number of spermatozoa per ml epididymal plasma derived employing the following Eqn., sperm count = number of spermatozoa×dilution factor×depth factor/number of areas counted.

Sperm sample was placed on Neubauer chamber and progressive sperm motility was determined by the method reported by Belsey *et al.*[9]. The whole process was performed within 5 min following their isolation from caudal epididymis. Sperm motility was scored in 10 separate fields. First non-motile sperms were counted followed by motile sperms. Sperm motility was expressed as a percent of total sperm counted.

The ratio of live to dead spermatozoa was determined using 1% tryphan blue solution by the method of Talbot and Chacon[10]. About 0.2 ml of sperm sample was incubated with 1% tryphan blue stain for 15 min at 37°. A drop of the suspension was placed in a Neubaur haemocytometer chamber under a cover slip, allowed to settle for 1 min and observed under an Olympus microscope. The number of stained (blue) and unstained (colourless) spermatozoa were scored in 10 separate fields. The spermatozoa, which were not stained with tryphan blue were considered as viable. Sperm viability was expressed as a percent of total sperm counted. Blood was collected from the heart using a heparinized syringe from control and experimental rats. The serum was collected by centrifugation at 2000 g for 15 min. After centrifugation, serum was collected and stored at –20° until further analysis. Serum testosterone[11], follicle stimulating hormone (FSH) and luteinizing hormone (LH) were estimated using the enzyme-linked fluorescent assay (ELFA) technique. It is an aid in the diagnosis and management of conditions involving excess or deficiency of this androgen. Results have been expressed in ng/ml, mIU/ml.

The data were expressed as mean values with their standard deviation (SD). Results of the six different groups were compared using one-way analysis of variance (ANOVA) analysis with Dunnett's multiple comparison test. Statistical analysis was performed using SPSS (Version 16.5; SPSS Inc., Chicago, IL, USA). Using MSOffice Excel, the data has been analysed for the significance of the main effects (factors) and treatments along with their interaction. The results were presented as F-values and the level of significance was set at $P<0.001$.

The data in Tables 1 and 2 represent changes in body weight and organ weight. The result revealed that the administration of *O. sanctum* leaf extract and ursolic acid did not show any effect on body weight. The reduction in weights of testes, epididymis, and seminal vesicle might be due to low level of androgen, which was not enough to maintain the weight of gonads and accessories. The epididymis, an androgen-dependent target organ, manifested differential sensitivity to androgens with regard to its structure and function. The decreased serum testosterone levels noticed in the present study would have affected the internal microenvironment of epididymis and seminal vesicles. A decrease in sperm reserve might have resulted in a

TABLE 1: EFFECT OF *OCIMUM SANCTUM* LINN. LEAF EXTRACT AND URSOLIC ACID ON BODY WEIGHT

Administration	Body weight (g)	
	Initial	Final
Group-I (control)	152.15±2.12	177.35±2.87
Group-II (OS)	156.45±2.23	186.16±2.15+4.96
Group-III (UA)	158.38±3.16	191.19±2.34+7.80

Mean±SD of six individual observations, OS is *O. Sanctum* leaf extract and UA is ursolic acid

TABLE 2: EFFECT OF *OCIMUM SANCTUM* LINN. LEAF EXTRACT AND URSOLIC ACID ADMINISTRATION ON ORGAN WEIGHTS

Name of the tissue	Control (group-I)	*O. Sanctum* leaf extract administered (group-II)	% Change between group I and II	Ursolic acid administered (group-III)	% Change between group I and III
Paired testes	1.54±0.10	1.13±0.09	-26.62*	1.34±0.08	-12.98
Paired epididymis	1.16±0.06	0.78±0.05	-32.75*	0.87±0.03	-25.00*
Paired seminal vesicle	0.62±0.04	0.43±0.02	-30.64*	0.55±0.02	-11.29*
Prostate gland	0.21±0.02	0.18±0.01	-14.28	0.19±0.01	- 9.52

Mean±SD of six individual observations, *P<0.001 indicates the level of significance

reduction in the weight of epididymis. The reduction in weight of accessory sex organs might represent atrophy of glandular tissue and reduction in secretary cells, thus leading to reduced testosterone levels. However, the extract did not show any weight changes in the prostate gland. Administration of ursolic acid slightly increased the testicular weight and reduced the weights of epididymis and seminal vesicle without producing any changes in the prostate gland.

The data in Table 3 represented the results of sperm analysis that included the sperm count, sperm motility and sperm viability. Administration of *O. sanctum* leaf extract caused significant reductions in the sperm count, motility and viability to the extent of 29.58, 20.15 and 11.83%, respectively. Ursolic acid administration on the other hand caused negligible reductions in the sperm count, motility and viability, which were 0.30, 0.94 and 3.07%, respectively. These results suggest a possibility that these effects could have resulted from a general disturbance in the protein and alteration in the milieu of cauda epididymis probably resulted due to androgen deficiency caused by the antiandrogenic property of *O. sanctum* leaves.

Mammalian epididymis, being a dynamic organ, is also dependent on the testicular androgens for maintenance of its structure and secretory, resorptive, biosynthetic and other metabolic activities. Administration of *O. sanctum* leaf extract showed degenerative changes in the tubular epithelium, which became incapable of keeping the sperm viable, leading to infertility/ sub fertility[12]. It has been shown that androgens are essential for survival and motility of spermatozoa in the rat epididymis, cauda region appears to be the most favourable site. Sperm possessed two principal prerequisites for fertilization, motility and the fertilizing ability. Any negative impact on motility would seriously affect the fertilizing ability[13].

The quality and quantity of spermatozoa produced depended on normal functioning of the testicular structures and reproductive hormones[14]. The administration of *O. sanctum* leaf extract caused significant decrease in sperm count and motility of spermatozoa by modulating testosterone levels as sperm count and their motility were androgen-dependent. The extract caused androgen depletion at the target level, particularly in the cauda epididymis thereby affecting physiological maturation of the sperm[15].

Earlier reports suggest that seminal pH could be a major factor influencing motility of the spermatozoa[16]. Hence, *O. sanctum* leaf extract administration might have caused some alterations in seminal pH, which might have affected the motility of spermatozoa. The analysis of cauda epididymis fluids obtained from these treated rats revealed a concomitant decrease in the sperm concentration, which might be due to the partial arrest of spermatogenesis and also due to oxidative stress[17]. Therefore, these observations suggested that the sperm anomalies in *O. sanctum*-treated rats might have resulted from the alteration in the milieu of epididymal tissue due to androgen deficiency following antiandrogenic property of the *O. sanctum* leaf extract.

Ursolic acid administration produced only non-

TABLE 3: EFFECT OF *OCIMUM SANCTUM* LEAF EXTRACT AND URSOLIC ACID ADMINISTRATION ON SPERM COUNT, SPERM MOTILITY AND SPERM VIABILITY

Name of the parameter	Control (group-I)	*O. Sanctum* leaf extract administered (group-II)	% Change between group I and II	Ursolic acid administered (group-III)	% Change between group I and III
Sperm count (millions/ml)	66.46±1.28	46.80±1.20	-29.58*	66.26±1.92	-0.30
Sperm motility (%)	69.57±1.76	55.55±2.35	-20.15*	68.91±1.57	-0.94
Sperm viability (%)	67.62±5.02	59.62±1.97	-11.83	65.54±3.01	-3.07

Mean±SD of six individual observations, *P<0.001 indicates the level of significance

TABLE 4: EFFECT OF *OCIMUM SANCTUM* LEAF EXTRACT AND URSOLIC ACID ADMINISTRATION ON TESTOSTERONE, FSH AND LH LEVELS IN THE SERUM

Name of the parameter	Control (group-I)	*O. sanctum* leaf extract administered (group-II)	% Change between group I and II	Ursolic acid administered (group-III)	% Change between group I and III
Testosterone (ng/ml)	7.51±0.16	4.61±0.25	-38.61*	7.13±0.24	-5.05
FSH (mIU/ml)	4.33±0.10	5.55±0.19	+28.17	4.41±0.14	+1.84
LH (mIU/ml)	2.46±0.13	3.34±0.22	+35.77*	2.62±0.12	+6.50

Mean±SD of six individual observations, *P<0.001 indicates the level of significance

significant changes. Ursolic acid has been found to be the most abundant triterpene acid in the leaves of *O. sanctum*. Earlier reports suggested that ursolic acid might have the potential of inhibiting sperm motility and induce antifertility activity in rats and mice[18,19]. In the present study, probably due to the low dose, ursolic acid failed to show any significant effect on the sperms. The data in Table 4 demonstrated changes in the levels of FSH, LH and testosterone. FSH and LH levels were significantly increased (+28.17 and +35.77%, respectively) in the serum of *O. sanctum* leaf extract administered rats compared to controls. However, *O. sanctum* leaf extract caused a significant decrease (−38.61%) in testosterone levels in comparison to those in control rats. Ursolic acid administration did not show any significant change in these hormone levels (FSH+1.84%, LH+6.50% and testosterone -5.05%).

Gonadotropins and testosterone are the main regulators of germ cell development. Testosterone was reported to act on the seminiferous tubules contributing to initiation and maintenance of spermatogenesis. Testosterone production is directly dependent on the concentration of LH in the milieu secreted by the anterior pituitary gland[20]. Hormonal regulation of spermatogenesis is via the hypothalamo-pituitary-gonadal axis[21]. Thus *O. sanctum* leaf extract administration causes the elevation in FSH and LH levels leading to the suppression of testosterone synthesis and there by reduces the reproductive ability.

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Conflict of interest:

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