Research Article

Spermicidal activity and antifertility activity of ethanolic extract of *Withania somnifera* in male albino rats

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ABSTRACT

The aim of this study is to highlight the work on plant drugs and their bioactive extracts involved in male anti-fertility mechanism. The ethanol extract of *Withania somnifera* was found to be water soluble. In the present study we have evaluated the effective spermicidal concentration of this extract on male albino rat sperm, by conducting "Sander-Cramer test". The minimum effective spermicidal concentrations of *Withania somnifera* stem extract were found to be 10±0.06 mg/ million sperm. After exposure of extract, there were no morphological changes observed in the head, mid –piece & tail of sperm. In the in-vivo study, a dose dependent reduction in the epididymal sperm count and percentage motility were observed. These results showed that *Withania somnifera* extract has antifertility effect on male rat reproduction, sexual behavior and epdidymal sperm concentration. So ethanol extract of *Withania somnifera* stem is a potent spermicide which completely immobilized the one million of rat sperm within 20s.

Keywords: Spermicidal, Sperm motility, Sperm viability, *Withania somnifera*.

INTRODUCTION

The current world population is around 6.46 billion and that of India in particular is around 1.1 billion.[1] One of the critical problems of the developing countries like India is its geometrical increase in the human population. This population explosion will have negative impact on our economic policies and would be simultaneously misbalance our socioeconomic infrastructure. Thus the control of human fertility in the sense of its limitation is the most important and urgent requirement. In this search several potential approaches for induction of infertility have been investigated over a long period, including chemical, hormonal, and immunological approaches. However, no suitable method has emerged that is effective and free from side effect.[2,3] Hence, there is a need for development of new fertility regulating drug from medicinal plants because from times immemorial humans have relied on plant products as sources of drugs and therapeutic agents. In recent times due to low toxicity and long standing experience of exposure, these drugs are used in ethnic medicine system like Ayurveda. In this search *Withania somnifera* have antifertility properties which are describe in Ayurveda. It has beneficial effect in treatment of wide range of disorder as digestive ailments, nerve afflictions, heart ailments, inflammation, nervous system, and rashes. The ethanol extract of *Withania somnifera* was believed to suppress "KamVasna" (desire of sex). It was consumed by Sanyasees in shrines and the pupils studying in Gurukul for the same purpose.[4,5] In this sequence *Withania somnifera* stem extract was daily orally fed for two month to study its effect on reproductive function of male albino rats. It was observed that control albino rat showed 100% fertility rate. In *Withania somnifera* stem extract treated animals; the antifertility effect was 70%. This study was carried out to evaluate in vitro spermicidal & *in vivo* antifertility activity of this extract against male albino rats. Thus the results suggest a possible antifertility property of the ethanol extract of *Withania somnifera* in male albino rats.[6-9] Study has been approved by local clinical ethics committee.

MATERIALS AND METHODS

Animal model

Experiment was carried out by using sexually mature albino rats of proven fertility. Animal colonies were developed by breeding animals under normal husbandry conditions. The study will be carry out under the supervision of the ethical committee of the Rajasthan University, Department of Zoology, Jaipur, India and CPCSEA (ICMR, 2006) guidelines will be followed for maintenance and use of the experimental animals.[10]

Preparation of Plant Ethanol extract

Withania somnifera stem was collected from Jaipur area of Rajathan, India, and identify this plant from Department of Botany University of Rajasthan, Jaipur India (Herbarium No. RUBL19445). It is abundantly available in this part of the country. Dry stem of *Withania somnifera* (250 gm) were ground in a mixture then, shocked in 50% ethanol for overnight, boiling it for 24 hours and finally filtered with gauze. The filtrate was concentrated under the reduce pressure at 50 ± 5°C to obtained ethanolic extract (15gm) of this plant for experiment.[11]

Phytochemical studies

The methods described by Harborne were used to test for the presence of the active ingredients in the test sample.[12]

Test for steroids

A 10 ml of plant extract [methanol-leaf and root, aqueous-leaf and root extract] was evaporated to a dry mass and the mass is dissolved in 0.5 ml of chloroform. Acetic anhydride [0.5 ml] and 2 ml of concentrated sulphuric acid were added to above.[13]

Test for alkaloids

The plant extract [0.5 g] was stirred with 5 ml of 1% HCl on a steam bath. The solution obtained was filtered and 1 ml of the filtrate was treated with two drops of Mayer's reagent. The two solutions were mixed and made up to 100 ml with distilled water.[13, 14]

Test for tannins

About 1 g of plant extract powder was weighed into a beaker and 10 ml of distilled water added. The mixture was boiled for five minutes. Two drops of 5% $FeCl_3$ were then added.[15]

Test for flavonoids

A few drops of 1% NH_3 solution is added to the plant extract [0.5 g] in a tube for observation of Yellow coloration.[13]

Treatment protocol for spermicidal activity study

Sperm preparation

After cervical ostiolysis rat epididymis was puncher then, collect semen in incubated normal saline water for *in vitro* study of rat sperm. Sample has motility (≥50%) and sperm concentration (≥20 million/ml).

Spermicidal activity study

The spermicidal activity was determined by using a modified version of the original protocol (Sander and Cramer method) which measures the minimum concentration of spermicidal agent required to kill 100% sperm within 20s. Test ingredients of various concentrations (2 mg, 4 mg 6mg .... 10mg) were mixed with sperm suspension containing 1 million sperm. The mixture was observed under microscope for 20s at 10X and read for motile sperm. The concentration was recorded if any motile sperm were seen. Two - hundred fifty microliter of buffer was added to all the mixture that passed the test and incubated at 37°C for at least 60 minute. The solution was slowly vortexed and observed again for presence of any motile sperm. The concentration at which it was tested was recorded as effective if both test indicated absence of motile sperm. The end point was the lowest concentration of the *Withania somnifera* stem extract that caused complete immobilization of all the sperm within 20 s of mixing .The dose and time dependent study for spermicidal activity was done by using the above test.[16]

Sperm function test

Ability of fertilization is not only dependent on the ovum but also on other sperm functional characteristics. Therefore sperm morphology, motility and viability, reflects the sperm fertilizing capacity was now being increasingly assessed to predict a successful outcome in IVF setting.[17, 18]

Sperm viability test

Sperm were mixed with *Withania somnifera* stem extract separately for 20 s. Sperm viability was checked by using Eosin- Nigrosin technique. Unstained spermatozoa were counted as live sperm and stained spermatozoa were counted as dead sperm.[17]

Treatment protocol for antifertility activity study

Hormonal nature and antifertility effect of the ethanol extract were conducted in three experiments. Animals were equally distributed into three treatment groups, each consisting of 6 animals.

Group A: Animals of this group were given sterile distilled water alone orally for 60 days. This group was serves as control treated vehicle.

Group B: Animals of this group were fed with ethanolic extract of *Withania somnifera* at the oral dose of 25 mg/kg body wt/day, for 60 days. Doses were freshly prepared and administrated orally during the study duration.

Group C: Animals of this group were fed with ethanolic extract of *Withania somnifera* at the oral dose of 50 mg/kg body wt/day, for 60 days. A suspension of the ethanolic extract of *Withania somnifera* (50mg/ml) was daily made in distills water for administration. The required drug was administered orally with a glass syringe fitted with a feeding needle.

Sperm motility and density

For determining sperm motility and sperm density, 100 mg of cauda epididymis was minced in 1 ml of physiological saline within a scarification period of 5 minutes. One drop of evenly mixed sample was applied to a glass slide under a cover glass. The sperm motility percentage was determined by counting both motile and immotile spermatozoa per unit area. Next, cauda epididymis sperm density was determined by routine procedure and expressed as million/mm^3 of suspension.[18]

Fertility test

Successful mating (male female ratio 1:2) was carried out with all the animals, five days prior to sacrifice period. The mated females were allowed to complete the gestation period. The numbers of pups delivered, litter size and fertility percentage were recorded.[18]

Body and organ weights

The initial and final body weights of the animal were recorded. Then the testes, epididymis, seminal vesicle and ventral prostate were dissected out, freed from adherent tissue and weighed accurately up to milligram level.

Serum biochemistry

Serum was isolated and stored for detection of protein content,[19] total cholesterol,[20] phospholipids,[21] alkaline phosphatase[22] and LDH[23] by respective calculations.

Hormone assay

Blood samples were collected for estimation of serum testosterone, FSH and LH by using radioimmunoassay. Serum samples were separated by standard procedures and stored at 20°C for subsequent analysis. Serum levels of testosterone, FSH and LH were assayed in duplicate by using radioimmunoassay kit.[24]

Hematology

The blood samples were collected from the heart and analyzed for blood urea,[25] blood sugar,[26] RBC, WBC and hematocrit levels.[27]

Statistical Analysis

Data are expressed as mean ± S.E. and analyze for statistical significance by using student's "t" test. The data are considered as significant at $p \leq 0.01$ and non-significant at $p \leq 0.001$.[28]

RESULTS

Phytochemical studies

Qualitative phytochemical investigation discovered presence of steroidal compounds (Appearance of blue or green color or a mixture of the two shades); alkaloids and tannins (The turbidity or yellow precipitation shows the presence of alkaloids and greenish precipitate indicated the presence of tannins) and absence of flavonoids (Not observed yellow coloration) in all mentioned extracts of plant.

Spermicidal activity test

The Spermicidal activity of graded doses of the extract was studied in vitro by using rat semen. The results of Sander–Cramer test showed potent activity of *Withania somnifera* stem extract. The minimum effective concentration of ethanol extract required to kill 1million sperm in 20s was around 10 ± 0.066 (Table 1). For the positive control spermicidal activity test, used the normal saline, there was no motility changes observed (data not shown). The results revealed that with an increase in concentration, there is linear decrease in motility percentage. Approximately 10 mg of extract was required for 100% immobilization of one million sperm in 20 s.

Sperm morphology

The morphological; study of sperms were done by using Eosin-Nigrosin stain and no morphological changes were found in sperms head mid-piece or tail when compared with untreated sperms.

Antifertility studies

Sperm motility and density

Caudaepididymal sperm motility was significantly diminished in the dose regimens. The dose regimens also produced a significant reduction in caudaepididymal sperm density (Table 2).

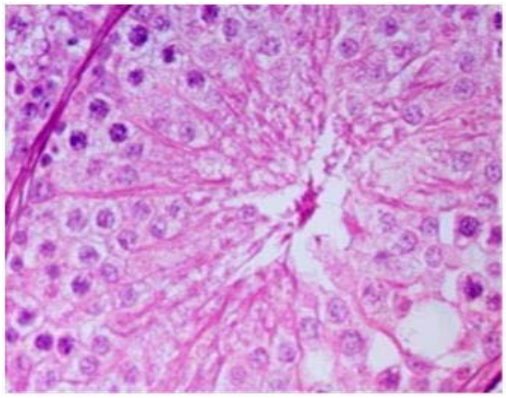

Figure 1: Photomicrograph of testes of a rat of group A (vehicle treated control) showing normal features with successive stages of transformation of somniferous epithelium to spermatozoa. H and E x400

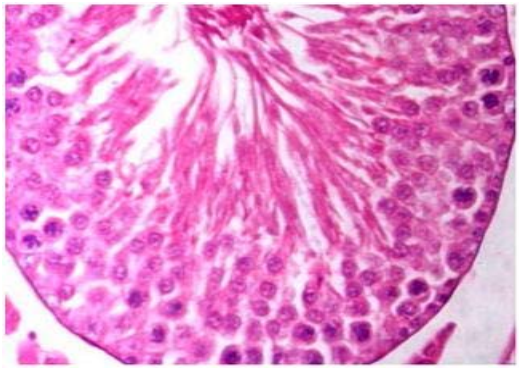

Figure 2: Photomicrograph of testes of a rat of group B (25 mg kg^{-1} b wt. *Withania somnifera* extract) after 60 days of treatment showing reduced seminiferous tubular diameter and cellular damage of tubular elements. H and E x 400

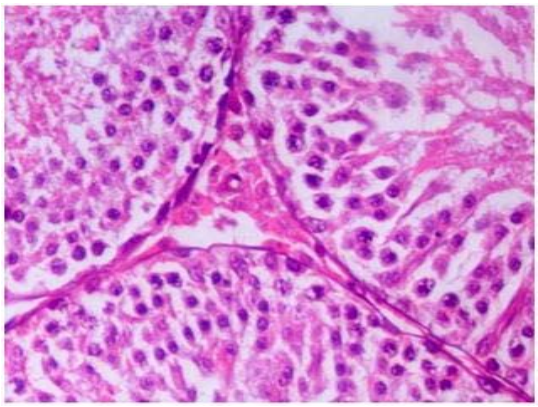

Figure 3: Photomicrograph of testes of a rat of group C (50 mg kg^{-1} b wt. *Withania somnifera* extract) after 60 days of treatment showing reduced seminiferous tubular diameter and cellular damage of tubular elements. H and E x 400

Table 1: Minimum effective concentration (MEC) of ethanolic extract of *Withania somnifera* stem required to kill 1 million sperm in 20 s.

Sample no.	semen sample sperm count (million/ ml)	Amount of semen taken containing 1 million sperm (µL)	% Motility	MEC %(mg)	Motility after MEC (mg) treatment
1	45	22	70	10	0
2	50	20	80	11	0
3	45	22	70	10	0
4	40	25	60	10	0
5	35	28	50	09	0
6	40	25	60	10	0

MEC (mg) mean ± SD 10.00 ± 0.066

Table 2: Caudaepididymal sperm motility and density levels after 60 days treatment with ethanolic extract of *Withania somnifera* stem in male rats

Treatment Group	Sperm motility%	Sperm density(million/ml)
Group A Control	77 ± 1.75	51.6 ± 1.25
Group B 25 mg /kg b.wt./day	65 ± 4.28[b]	42.5 ± 2.14 a
Group C 50 mg /kg b.wt./day	43.01 ± 4.78[c]	29.17 ± 0.98[c]

Data are expressed as Mean ± SEM of 6 animals, Groups B and C was compared with Group A, a Significant (p≤0.05), b Non-significant, c highly significant.

Table 3: Fertility of male rats in control and 25, 50 mg /kg body weight treated with Ethanol extract of *Withania somnifera* stem for 60 days when mated with females (male :female ratio 1:2)

Treatment Group	No. of females delivering	No of pups	Percent fertility
Group A Control	12	42	100
Group B 25 mg /kg b.wt./day	08	28	66.6
Group C 50 mg /kg b.wt./day	04	12	28.57

Table 4: Body and organ weight after 60 days treatment with Ethanol extract of *Withania somnifera* stem in male rats

Parameter	Group A (Control)	Group B (25 mg /kg b.wt./day)	Group C (50 mg /kg b.wt./day)
Body weight (gm.)			
Initial	176 ±10.54	174.16±1.54	175.83 ±1.53
Final	216.66±12.29	215.00±4.28	211.67±3.07
Organ weight (mg / 100 gm.)			
Testes	774± 28.70	544.42± 33.53[c]	481.51 ±11.44[c]
Epididymis	355±20.47	176.12±12.48[c]	143.61±9.12[c]
Seminal vesicle	347±21.47	226.78±4.32[c]	157.13 ±12.64[c]

Data are expressed as Mean ± SEM of 6 animals, Groups B and C was compared with Group A, a Significant (p≤0.05), b Non-significant, c highly significant.

Table 5: Levels of serum Testosterone, LH, FSH, in control and Treated group of animals, after 60 days treatment with Ethanolic extract of *Withania somnifera* stem in male rats

Treatment Group	Testosterone (ng/ml)	LH (mIU /ml)	FSH (mIU /ml)
Group A Control	1.90 ± 0.24	3.66± 0.63	3.26 ± 0.42
Group B 25 mg /kg b.wt./day	1.26 ± 0.17 [b]	3.56 ± 0.21[b]	2.06 ± 0.17 [b]
Group C 50 mg /kg b.wt./day	0.93 ± 0.03 [a]	3.45 ± 0.21[b]	2.02 ±0.04 [a]

Data are expressed as Mean ± SEM of 6 animals, Groups B and C was compared with Group A, a Significant (p≤0.05), b Non-significant, c highly significant

Fertility

A dose dependent reduction in the fertility was observed in treated group. The fertility in the 50 mg/kg body wt/day of *Withania somnifera* extract was70% control by following 60 day of treatment. There was a mark declined inpups delivered in treatment group as compare to control group. All the delivered pups were normal and healthy (Table 3).

Body and organ weight

The weight of testes, epididymis and seminal vesicle were decreased significantly. However the weight of the ventral prostrate, Heart, Liver, Kidney and Adrenal gland was non-significantly decreased in all treated animal (data was not shown) while dose regimen did not altered body weight of the animals when compared with control group animals (Table 4).

Serum biochemistry

Cholesterol, protein phospholipids, alkaline phosphates and LDH levels in serum of all treated group were non-significantly low after treatment of 25and 50 mg /kg body weight of *Withania somnifera* extract.

Hormone levels

Testosterone and Follicular stimulating hormone levels was not significantly decreases, while level of Luteinizing hormone was non-significantly in the serum of treated group animals (Table 5).

Hematology

No appreciable alterations were observed in hematological parameters in animals of treated group in comparisons to control group.

Histopathology of testes

Histological studies of control rat testes showed all successive stages of spermatogenesis, where the lumen was filled with sperm. Leydig cells were situated in between the tubules with prominent nuclei (Figure1). The tests of the treated animals revealed the arrest of spermatogenesis. The seminiferous tubules appeared reduce in size. Vacuolization was observed in the sertoli cells, spermatogonia and spermatocytes. Germ cell proliferation beyond the level of the spermatocyte was also affected. The lumen contained sloughed debris and few germ cells. Leydig cell nuclei diameter area and seminiferous tubular diameter were significantly reduced in treated rats (Figure 2 & 3).

DISCUSSION

The result of this investigation demonstrated that the ethanol extract interferes with the structure and function of major elements of male fertility as reflected by a marked decrease in the rate of fertility. On the basis of LD50 of *Withania somnifera,* selected dose in present study provide the information on the mechanism of toxic action and provide data on which user risk-benefit relationship may be assessed.[29] Phytochemical constituents such as Steroids, alkaloids, flavonoids, tannins, phenol, and several other aromatic compounds are secondary metabolites of plants that may serve as male antifertility agent. The ethanol extract of *Withania somnifera* are also rich in these secondary metabolites. The plant based contraceptive, inhibit male fertility so after administration of (25, 50 mg /kg body weight) *Withania somnifera* extract exhibited a marked reduction in counts and motility of caudaepididymal sperms in dose dependent manner. The sperm density was decreased concurrently with an increase in the percentage of non-motile and mature sperms. This could be caused by androgen deprivation.[30] There was decrease in both testicular and epididymal sperm count after chronic administration of drug and this suggested that inhibit may affect androgen binding secretion by sertoli cell *via* its action on FSH. The reduction of sperm density is also confirmed by histological and hormonal investigation of testis and serum along with fertility status of the animals. The testes of the treated animals revealed the arrest of spermatogenesis. The testicular weight loss is due to the absence of spermatogenic stages particularly in spermatid and spermatozoa, which is further due to decrease in level of testosterone.[31] The testicular damage occurred due to decrease in the seminiferous tubules diameter and in the volume of Lye-dig cell. Their atrophy is due to inhibition of hypothalamus hypophyseal axis. Since the Androgen binding protein is required for maintaining intra-tubular androgen concentration for cytological differentiation. The loss of sperm motility and structural defects exerted by steroids administration is well known by changing their membrane permeability.[32,33] These observations indicate that there is a strong interaction between the ethanol extract and plasma membrane of sperm cell. The low cauda EP, low testicular sperms counts, presence of non-motile spermatozoa and significant reduction in the organ weight imply that extract induced infertility might be consequence of an array of factors. One of these factors may be that, these extract interfere with enzymatic reaction including an oxidative phosphorylation uncoupling.[34] This oxidative phosphorylation is required for the ATP which in turns is responsible for sperm motility; slight reduction in ATPase leads to motility inhibition and thus causes infertility.[35] After treatment of *Withania somnifera* extract there was no significant changes observed in serum biochemistry and hematological parameters of treated group animals. These results suggested that *Withania somnifera* extract are free from side effect. However in the fertility test, there was reduction in fertility (number of pups) which indicating the fertilization might be due to stored epididymal sperms.

CONCLUSION

Oral administration *Withania somnifera* extract at 50 mg /kg body wt/day in male albino rats did produce antifertility effects. Further long term studies are warranted for evaluation of complete reversible sterility

mechanism of action and detailed toxicological screening with this and other extract.

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