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## Effects of Aqueous Extract of *Anethum graveolens* (L.) On Male Reproductive System of Rats

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**Abstract:** Involvement of men in control of population is lesser than women that may be due the limited contraceptive choices. For this purpose, the infertility effect of plants such as *Anethum graveolens* (L.) that used in folk remedy to decrease male fertility have been tested in animal models. Thirty adult male Wistar rats were divided into 3 groups of treatment group I, 70 mg kg<sup>-1</sup>, treatment group II, 100 mg kg<sup>-1</sup> of aqueous extract that received in a volume of 1 mL by gavage daily for 32 days and control group were treated with 1 mL of distilled water. Body weights of all animals were recorded before and after of experiment. Serum testosterone levels at the beginning and the end of the experiment were measured. The animals were sacrificed and left testis, epididymis, seminal vesicle and prostate were dissected out and weighted. Sperm count, motility and nuclear maturity were examined. After tissue preparation of testis, seminiferous tubular diameter were measured and frequency of stage of the germinal epithelium cycle of seminiferous tubules were evaluated by using staging map. The treatment did not affect body and organ weight, serum testosterone levels, sperm count, motility and nuclear maturity from that of the control group. Spermatogenic cycle in seminiferous tubules and seminiferous tubular diameter did not altered by the treatment. These results suggest anti fertility effect for *Anethum graveolens* seed aqueous extract in rats at levels of 70 and 100 mg kg<sup>-1</sup>.

**Key words:** *Anethum graveolens*, spermatogenesis, sperm nuclear maturity, seminiferous tubular diameter, male rat

### INTRODUCTION

Control of population growth is very important in populated countries. In this regard, these countries require developing family planning programs. Generally, involvement of men in these programs is lesser than women. Lack of male involvement may be due in part to the limited contraceptive choices that they have (Ringheim, 1996). So, it requires more research to develop new methods for them. For this purpose, several chemical substances and plant extracts have been tested in animal models. So, plants used in folk remedy to decrease male fertility are good sources to look for new agents.

The changes underlined Population continues to be a major problem in developing countries. Conventional drugs used as male contraceptive are often inadequate (Ringheim, 1996) therefore; any efforts to explore antifertility effect of any natural product in males carry a great clinical significance as this can help males also to participate significantly in population control programs. *Anethum graveolens*, commonly known as dill, is an annual herb that belongs to family Umbelliferae. In Iranian traditional medicine, dill seed has been indicated for a number of medical problems such as flatulence,

indigestion, stomachache, colic and over consumption of it decreases male potency (Ames and Duck, 2002). Umbelliferae family includes several plant species. Some of these species have been studied for antifertility effects like *Ferula hermonis* (Khleifat *et al.*, 2001) and *Coriandrum sativum* (Al-Said *et al.*, 1987). Although, this herb prolongs the length of diestrus phase of estrous cycle and elevate the level of plasma progesterone (Monsefi *et al.*, 2006a) and increase the number of mitochondria and dilate the smooth endoplasmic reticulum of granulosa cells of corpus luteum in female rats (Monsefi *et al.*, 2006b) and these ultrastructural changes can causes the elevation of progesterone level, thereby indicating to act as an antifertility agent in female rats. However, no pharmacological or medical studies have evaluated the effects of dill seed on male reproductive system. Therefore, the present study was planed to study antifertility effects of aqueous extract of *Anethum graveolens* seed in male rats.

### MATERIALS AND METHODS

**Plant material:** Dried seeds of dill were purchased from a commercial source in Shiraz, Iran. The identity of the

seeds was confirmed by the Department of Biology, Shiraz University, Shiraz-Iran. A voucher specimen (40110) was kept in Herbarium of Department of Biology, Shiraz University.

**Preparation of aqueous extract:** Dried dill seeds were homogenized to a fine powder. Two hundred grams of powdered dill seed were macerated in 600 mL of distilled water for 24 h. It was filtered and concentrated over the water bath. Then, the extract was dried in desiccator. The yield of the extract was 6.3%.

**Experimental design:** Thirty adult male Wistar rats, weighting  $229 \pm 20$  g, were purchased from Razi Research Institute of Shiraz. Animals were kept under standardized condition (12 h light/12 h dark) with an ambient temperature of  $24 \pm 2^\circ\text{C}$  in animal house of Biology Department of Shiraz University from years of 2003 to 2005. They were received rat chow pellets and water *ad libitum*. Principles of laboratory care established by the National Institute of Health (Anonymous, 1985) were followed. Body weights of animals were recorded. The animals were divided into treatment group I,  $70 \text{ mg kg}^{-1}$  of aqueous extract, Treatment group II,  $100 \text{ mg kg}^{-1}$  of aqueous extract that received in a volume of 1 mL by gavage once daily for 32 days. These doses determined based on its LD 50 of aqueous extract,  $3.04 \text{ g kg}^{-1}$  in ip administration, of this plant (Hosseinzadeh *et al.*, 2002). Control group were treated with 1 mL of distilled water for the same period. The total period of spermatogenesis in rats is 48-53 days and we selected approximately 2/3 of this period. On the 33rd day of the experiment, body weights of all animals were recorded, again. The animals were sacrificed under ether anesthesia. Left testis, epididymis, seminal vesicle and prostate of each animal were dissected out and weighted.

**Hormone assay:** In order to indicate plasma concentration variations of testosterone, at the beginning of the experiment, blood samples were collected from the tail vessel. Animals were anesthetized using diethyl ether and the lateral or ventral tail vein was punctured with a needle of gage 23 and heparinized canola. At the end of the experiment, blood was collected by cardiac puncture. Serum was separated by centrifugation of blood at 2000 rpm for 15 min. Then testosterone concentration of serum at the beginning and the end of experiment were measured by Eliza method in Research Center of Nemazee Hospital with Human Co. Kit, Germany.

**Sperm count and motility:** One centimeter of distal region of left vas deferens was cut and placed in a petri dish containing 5 mL of Hanks solution ( $37^\circ\text{C}$ ). Sperm was collected by diffusion method (Seed *et al.*, 1996). One drop of sperm suspension was placed on a slide. A motic

camera (model 350, Japan) that was installed on light microscope recorded film of sperm motility. Percent of motile (with fast and slow progressive and nonprogressive movement) and immotile sperms were calculated by observing the films after counting 100 sperms in each slide.

A sample of sperm suspension was taken and the number of sperm counted using a hemocytometer with improved double neubauer ruling. Counts for four chambers were averaged (Da Silveira *et al.*, 2003).

**Sperm nuclear maturity:** Acidic amiline blue staining Sperm smears were fixed in 3% glutaraldehyde in 0.2 M phosphate buffer for 30 min and stained with 5% aniline blue in 4% acetic acid (pH 3.5) for 10 min (Hoffman and Hilscher, 1991). One hundred sperms for each rat were evaluated then Sperms were categorized according to color: blue stained (immature nucleus) and unstained (mature nucleus).

**Acridine orange staining:** Sperm smears were fixed in acetic acid and methanol (3:1) for 2 h and stained with acridine orange solution (0.02% acridine orange in citrate phosphate buffer, pH 2.5) according to the procedure of Tejada *et al* (Da Silveira *et al.*, 2003). After 10 min of staining, each smear was washed with water and covered with a coverslip. Smears were examined immediately using a fluorescence microscope (Zeiss, Germany) with excitation filters. Nuclei of 100 sperms were examined and categorized according to color of fluorescence: Green (sperm with stable double-stranded DNA) or yellow (sperm with denatured DNA).

**Histological studies:** Left testis from each rat was removed and fixed in 4% buffer formalin solution for 1 week and then the paraffin blocks were prepared (Hoffman and Hilscher, 1991). The blocks were sectioned at  $6 \mu\text{m}$  thickness and were stained with hematoxylin and eosin for histological observations. Seminiferous tubular diameters (STD) of 20 circular random selected tubules were measured in each section by using ocular and stage micrometers (Ziess, Germany). Then thirty random selected sections of seminiferous tubules per animal were analyzed in order to evaluate the frequency of stages I-III, IV-VI, VII-VIII, IX-XI, XII-XIV of the germinal epithelium cycle by using staging map (Tejada, 1984).

**Statistical analysis:** The data for organ weight, sperm count, motility, nuclear maturity and seminiferous tubular diameter were analyzed using one-way ANOVA, followed by Duncan test. Data from germinal epithelium cycle of seminiferous tubules was analyzed by non-parametric Chi-square test. Statistical analyses were performed using SPSS 11.5 software.  $p < 0.05$  was considered as a significant level.

Table 1: Effect of aqueous extract of *Anethum graveolens* L. seed on body and organ weights of male rats

Treatments	Initial body weight (g)	Final body weight (g)	Standard weight of male reproductive organs (mg/100g body weight)			
			Testis	Epididymis	Seminal vesicle	Prostate
Control	227.05±6.41	258.50±10.13	0.550±0.034	0.261±0.019	0.202±0.022	0.311±0.026
Group I	231.38±5.74	254.85±6.07	0.569±0.023	0.271±0.014	0.211±0.013	0.315±0.016
Group II	230.35±7.03	274.8±8.54	0.525±0.017	0.238±0.012	0.197±0.009	0.332±0.017

Table 2: Effect of aqueous extract of *Anethum graveolens* L. seed on sperm count, motility and nuclear maturity

Treatments	Sperm count (×10 <sup>6</sup> mL)	Sperm motility (%)	Unstained sperm
Control	3.91±0.85	78.33±3.80	63.38±7.59
Group I	4.51±0.14	67.89±4.45	56.11±12.22
Group II	6.26±0.16	78.78±1.88	45.36±10.16

Table 3: Effect of aqueous extract of *Anethum graveolens* L. seed on initial serum testosterone (IST), final serum testosterone (FST) level and seminiferous tubular diameter (STD) in male rats

Treatments	IST (ng mL) <sup>-1</sup>	FST (ng mL) <sup>-1</sup>	STD (µm)
Control	4.60±1.23	3.38±1.14	0.278±0.003
Group I	3.85±1.31	3.21±1.02	0.267±0.003
Group II	2.62±0.46	2.41±0.90	0.279±0.003

Table 4: Frequency of stages of the germinal epithelium cycle in male rats of control and treatment groups

Treatments	I-III	IV-VI	VII-VIII	IX-XI	XII-XIV
Control	4	19	75	50	2
Group I	2	8	83	56	1
Group II	3	10	87	48	2

**RESULTS**

Administration of aqueous extract of *A. graveolens* seed at doses of 70 and 100 mg kg<sup>-1</sup> for 32 days did not produce any significant organ weight loss or gain compared with control rats. No significant changes were shown in standardized organ weight of testis, epididymis, seminal vesicle and ventral prostate between control and treatment groups (Table 1).

Sperm concentration did not alter in treatment groups compared with control group (Table 2). Total percentage of immotile sperm was 32.1 and 21.2% in treatment group I and II, respectively, which were not significantly different from total percentage of immotile sperm (21.7%) in control group (Table 2).

The percent of spermatozoa unstained by amiline blue with normal condensed chromatin were not significantly different between three groups. The results of amiline blue staining are detailed in Table 2.

Total sperms that stained by acridine orange in all three groups had nuclei with green fluorescence stain i.e., had stable double stranded DNA.

Serum testosterone levels did not significantly change in treatment groups, compared with control group (Table 3).

Seminiferous tubular diameter (STD) did not differ between treatment groups with control (Table 3). Germinal epithelium cycle was similar between control and treatment groups (Table 4). The sections indicated no pathologic changes in tissue of the testis, epididymis, seminal vesicle and prostate tissues at the level of light microscopy.

**DISCUSSION**

Treatment of rats with *A. graveolens* seed aqueous extract did not affect somatic growth compared to the control. This suggests that this extract at the applied doses has no general toxic effect on body growth.

Seminal vesicle and prostate gland are androgen dependent organs. From the results, there was no significant alteration in weight of testis, epididymis, seminal vesicle and prostate. Therefore, it suggests no antiandrogenic activity for this extract at these dosages. This result is also confirmed by the normal serum testosterone levels in treatment groups as compared with control group.

The treatment with *A. graveolens* did not show differences in sperm concentration. Because sperm production is depended on androgen production, therefore, this result could be due to no alterations in steroidogenesis function (Bankroft and Stevens, 1991).

Determination of sperm concentration, motility and normal morphology is a major tool in the evaluation of male fertility. However, at present, it is known that these parameters have significant limitations as fertility indicators (Hess, 1999, Bidwai and Wangoo, 1988, Collins *et al.*, 1983) Zaneveld and Jeyendran (1993). This has led to the development of new sperm assays to assess the fertilizing capacity of spermatozoa. Acidic aniline blue and acridine orange staining are such assays. The parameters of chromatin condensation and DNA stability that can be identified by amiline blue and acridine orange staining, respectively, may be a valuable index of sperm quality, reflecting the possible disorders of spermatogenesis and epididymal sperm maturation (Polansky and Lamb, 1988). The results from this study showed that treatment with dill did not affect epididymal sperm maturation including DNA stability.

Seminiferous tubular diameter and frequency of cells such as spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid and spermatozoid in germinal epithelium were similar in control and treatment groups. This result could be due to no alterations in steroidogenesis function (Bidwai and Wangoo, 1988).

In conclusion, the administration of *Anethum graveolens* seed aqueous extract following the protocol presented in this investigation did not have antifertility effects in adult male rats. With regard of our previous studies, this plant affected steroidogenesis function and ultrastructure in related cell of reproductive system of female rats without it produce any pathologic changes but in higher dose compared to that of this project (Monsefi *et al.*, 2006a, 2006b). ) Therefore, we guess administration of higher doses and increasing of experimental period may result in significant differences of mention parameters and more studies are needed to clarify the properties of this herb.

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