

## Fertility suppression by the fruit extract of *Opuntia elatior* in the male rat: Possible extragonadal action

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### Summary

Plant – derived male antifertility agents need to address the basic requirements of reversibility and fertility suppression without affecting libido. This study was undertaken to evaluate the male antifertility potential of the fruit extract of *Opuntia elatior* in the male rat and its reversibility. The methanolic extract was administered through oral route (300, 900 mg/kg bw) to male rats for 30 and 60 days, and fertility was assessed by analysing spermatogenesis, sperm count and testosterone levels. Reversibility of fertility suppression was studied by withdrawal of treatment for two weeks and mating with normally cycling virgin females. Sperm count and motility were markedly reduced in both the groups of treated rats, without commensurate decline in serum testosterone levels and testicular hydroxysteroid dehydrogenase (HSDH) activity. Fertility of the treated rats was suppressed when mated with normally cycling virgin female rats without affecting libido. Withdrawal of treatment for two weeks restored the sperm count, testicular HSDH activity, serum testosterone levels and fertility. The methanolic extract of the fruit of *O. elatior* produces reversible male antifertility effect without affecting the testosterone levels and libido.

**Keywords:** Male antifertility, *Opuntia elatior*, sperm analysis, steroidogenesis, reversibility.

### Introduction

Despite widespread use of herbal preparations in folk medicine for various ailments including fertility control, discovery of effective drugs for birth control from natural sources has remained elusive (Chopra et al., 1958; Nadkarni and Nadkarni, 1976; Satyavati et al., 1984; Qureshi et al., 2005). There is great interest in plants as potential sources of newer contraceptives. The need to develop male antifertility agents is well recognized and many plants have been investigated for their antifertility effects in the male (Akbarsha et al., 1990; Chowdhury et al., 2001; Kamal et al., 2003). Many studies on plant extracts have been reported to affect testicular function including spermatogenesis and steroidogenesis in laboratory animals (Hiremath et al., 1999, 2000; Udoh and Kehinde, 1999; Akbarsha and Murugaian., 2000). However, most of the studies on plant extracts reporting antifertility activity in the male fall short of the requirements for a viable antifertility agent. An ideal and safe male antifertility agent should be able to selectively affect sperm production and function without altering androgen levels and libido in addition to reversibility (WHO, 1998).

Species of the genus *Opuntia* (Family: Cactaceae) occur in many parts of the world and some are also cultivated for their food and medicinal value. Fruits and phylloclade of *Opuntia* are considered natural health foods and recognized as valuable source of nutraceuticals

(Feugang et al., 2006). The cactus *Opuntia dillenii* grows wild and also cultivated in India; its fruits are edible and contain a variety of nutrients including minerals, salts, amino acids and vitamins (Askar and El-Samahy, 1981; Gurrieri et al., 2000; Stintzing et al., 2001; Piga, 2004; Habibiet al., 2005; Lee et al., 2005; Tesoriere et al., 2005). Phylloclade and fruits of *O. dillenii* are used in folk medicine for various ailments such as allergies, rheumatism and ulcers, and reported to possess hypolipidemic and hepatoprotective properties (Lee et al., 2001; Galati et al., 2001, 2003). Extracts of the phylloclade of *O. dillenii* have been reported to show antispermatogenic effect and fertility suppression (Gupta et al., 2002; Gupta and Sharma, 2006). *O. elatior* is a common cactus in southern India and there is an unsubstantiated claim of its use in folk medicine for fertility control (Jain et al., 2007). The objective of this study was to investigate the male antifertility potential of the fruit pulp of *O. elatior* and its reversibility, with regard to sperm production, steroidogenesis and fertility, in order to evaluate it as a source of natural male antifertility agent.

### Materials and methods

#### Chemicals

The fine chemicals such as dehydroepiandrosterone (DHEA, 5-androsten-3 $\beta$ -ol-17one) and testosterone (17 $\beta$ -hydroxyandrost-4en-3one) were obtained from Sigma

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Chemical Co., Mo, USA; nicotinamide adenine dinucleotide sodium salt (NAD), nicotinamide adenine dinucleotide reduced (NADH), iodonitrotetrazolium chloride (INT) and phenazinemethosulphate (PMS) were purchased from Sisco Research Labs, Mumbai, India; ELISA kit for testosterone was purchased from Labor Diagnostika Nord GmbH, KG, Germany.

### Preparation of the extract

The taxonomic identity of the plant, *Opuntia elatior*, was ascertained by a taxonomy expert (Prof. G. R. Shivamurthy) and a voucher specimen is available at the department of Botany, University of Mysore, Mysore. The fruits collected from the fields of Kolar (Karnataka State, Southern India- 13.1333° N - longitude, 78.1333° E – latitude ) during the months of Jan-Mar (2011, 2012) were cut into pieces, seeds removed manually, air-dried, and powdered in a blender. The dry powder was subjected to sequential extraction for 36 hours using the solvents, petroleum ether, ethyl acetate and methanol. The methanolic extract was selected and evaporated to dryness in a flash evaporator. The dried extract was dissolved in water (100 mg/ml) for the experiments.

### Animals

Ninety day old male albino rats of Wistar strain (weighing 180 – 200 g) were housed in polypropylene cages, maintained under standard conditions (25 - 26° C, 70% relative humidity, 12:12 light/dark) in the animal house facility, and provided with laboratory chow and water *ad libitum*. Guidelines of the Institutional Animal Ethical Committee (Approval No. 841/b/04/CPCSEA) were followed.

### Experimental design

The study involved three experiments as described below:

**Experiment I:** Rats were divided into three groups of ten animals each. Group I (control) received only water, Groups II and III were administered orally with the methanolic extract at 300 mg and 900 mg/kg bw, respectively, for a period of 60 days. The doses were selected based on a preliminary study showing effectiveness of the extract (Ramyashree et al., 2013). On 61<sup>st</sup> day, autopsy was performed on half of the animals in each group and the remaining animals were maintained without treatment (withdrawal) for a period of two weeks before autopsy.

**Experiment II:** In order to assess if short term treatment affects the male reproductive system, a group of male rats were treated for a period of 30 days as above, and autopsied.

**Experiment III:** A further study to examine if sperm count is affected by two week treatment, a group of male rats were treated with the extract as above for two weeks and the animals were autopsied on 15<sup>th</sup> day for sperm analysis.

### Autopsy

At the end of the respective treatments, the animals were bled and dissected under ketamine anaesthesia. The reproductive organs (testis, epididymis, seminal vesicles, and ventral prostate) were dissected out and weighed. Blood was allowed to clot to obtain serum for testosterone assay.

### Sperm analysis

Sperms were obtained from the cauda epididymidis by mincing in physiological saline, and centrifuged at 3000 rpm. The supernatant was used for sperm count using Neubauer chamber. The sperm motility was assessed by standard protocols (WHO, 1999).

### Histology

Slices of testes, epididymides, seminal vesicles and ventral prostate were fixed in Bouin's fluid for 24 hours and processed for paraffin embedding. Paraffin sections (5µm thick) were stained with hematoxylin and eosin and observed under microscope.

### Testosterone assay

Serum testosterone levels were measured by ELISA using a commercial kit (LDN, Germany). The sensitivity of the assay was 0.022 ng/ml.

### Hydroxysteroid dehydrogenase assay

Testes were homogenised in 0.1M tris buffer (pH 7.4) and centrifuged at 5000 rpm for 15 min in a cooling centrifuge and the supernatant was used for enzyme assay. Activity of  $\Delta^5 3\beta$  and  $17\beta$ -hydroxysteroid dehydrogenases were assayed using pregnenolone and testosterone, respectively, as the substrates (Shivanandappa and Venkatesh, 1997). Protein was determined using bovine serum albumin as the standard (Lowry et al., 1951).

### Reversibility study

After 60 days, the treatment was withdrawn for a recovery period of two weeks, and the animals were sacrificed under anaesthesia. Investigations on sperm count, motility and fertility were done.

### Fertility

In experiment I (60 days), the treated and control male rats were allowed to mate with normally cycling

(untreated) virgin females in the ratio of 1:2 (WHO, 1999). The vaginal smear was checked for sperm for positive mating. The mated female rats were kept for observation for pregnancy and allowed to litter. The males were autopsied and the reproductive organs and blood were collected for sperm analysis, testosterone measurement and histology as described earlier.

### Statistical analyses

The data were analyzed by one-way analysis of variance (ANOVA) using the statistical software (SPSS, version 14). The significance was determined at  $p < 0.05$ .

## Results

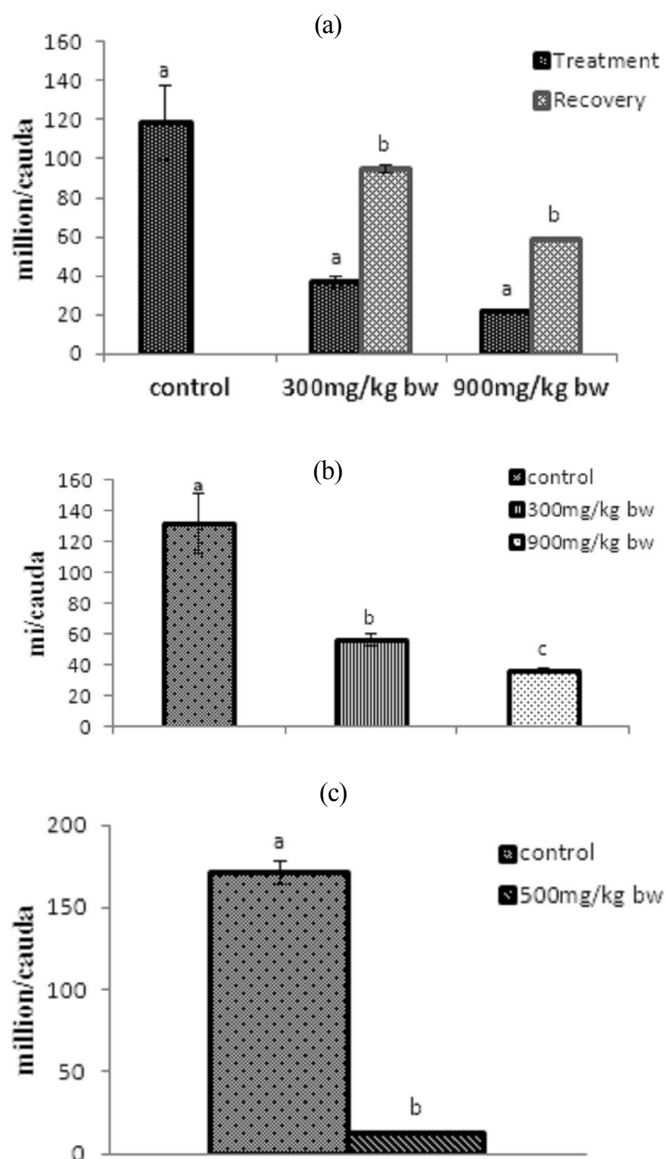
### Body weight and reproductive organ weights

Body weights of treated rats were not significantly affected in both 30 day and 60 day treatment groups and were comparable to the respective control groups.

The relative weights of testes in both 30 day and 60 day treatment groups were marginally lower but not statistically significant (Table 1). The weights of the accessory reproductive organs, epididymis, seminal vesicles and ventral prostate, of the 30 day study were also comparable to those of the control group. In the 60day study, the weights of epididymis of the treated group were marginally higher, whereas weights of ventral prostate and seminal vesicles were slightly lower, but the differences were not statistically significant (Table 1).

### Sperm analysis

Sperm count from the cauda epididymidis was markedly reduced in both 30 day and 60 day treatment groups upto 90% when compared to the respective control groups. The sperm motility was also markedly lower in the treated rats. Both sperm count and motility were recovered upon withdrawal of treatment. Interestingly, the sperm count and motility were also significantly less in rats treated with the extract for just two weeks (Fig. 1 and 2).

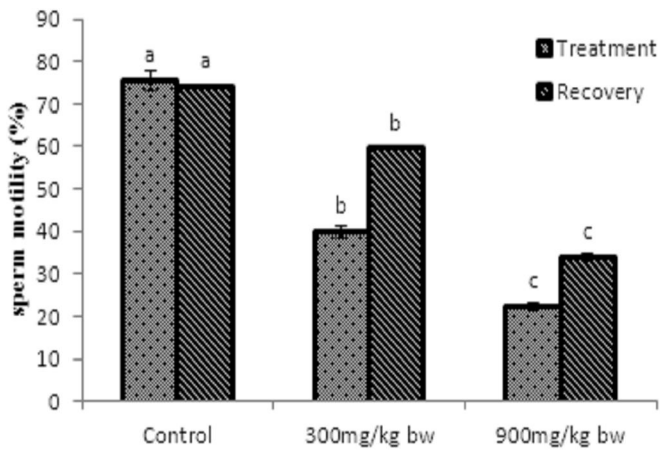


**Fig. 1:** Antifertility effect of *Opuntia elatior* fruit extract in the male rat: Sperm count (a) 60 day treatment and recovery; (b) 30 day treatment; (c) 15 day treatment. Values (Mean  $\pm$  SE) denoted by different alphabets differ significantly ( $P < 0.05$ ; DMRT)  $n=5$ .

Table 1. Antifertility effect of *O. elatior* fruit extract in the male rat: reproductive organ weights.

Values (Mean  $\pm$  SE) denoted by different alphabets differ significantly ( $P < 0.05$ , DMRT)  $n=5$ .

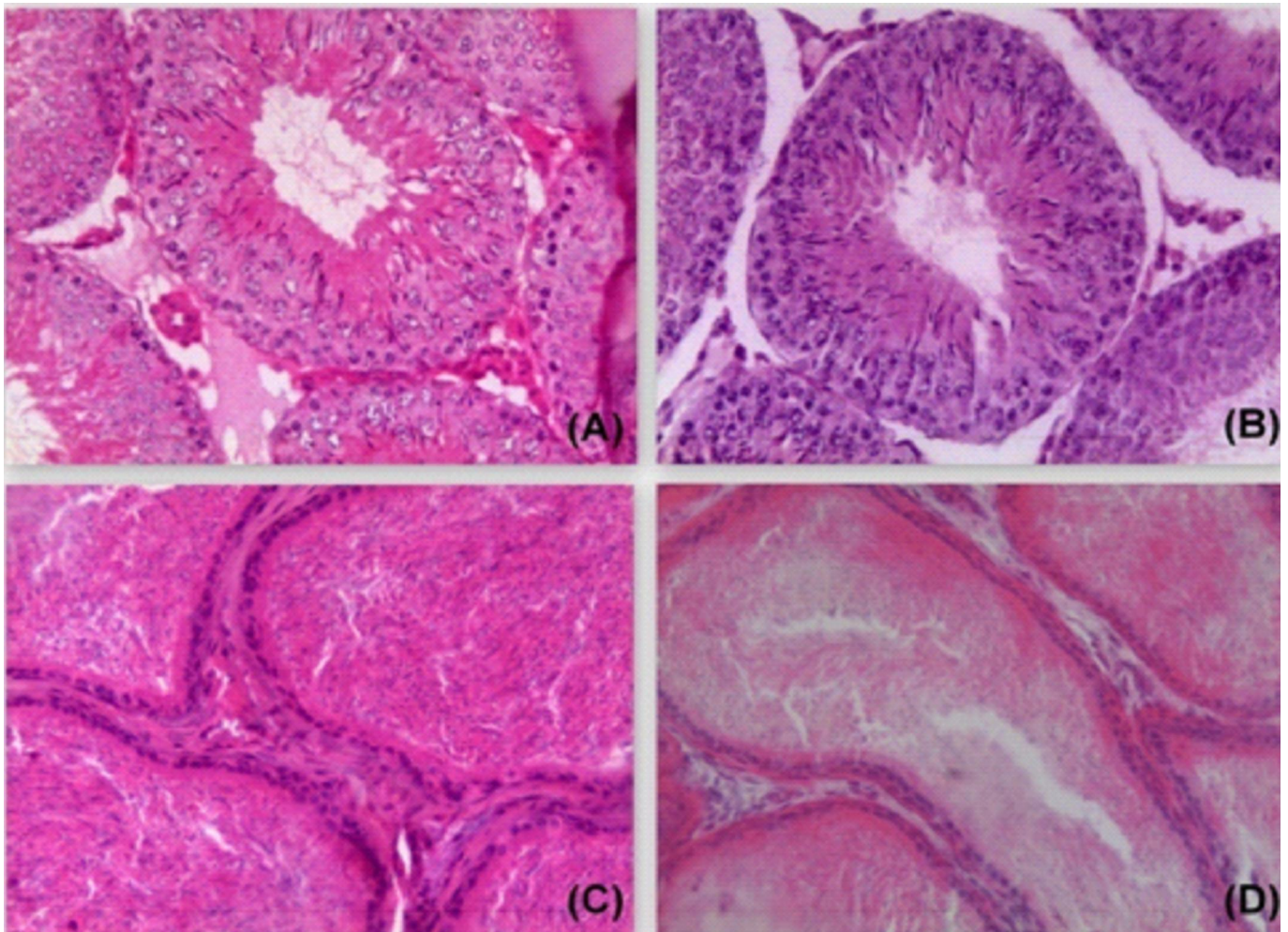
Experiment	Dose	Final body weight (g)	Relative organ weight (g/100 g bw)			
			Testis	Epididymis	Seminal vesicles	Ventral prostate
I. 60 days	Control	222.00 $\pm$ 3.54 <sup>a</sup>	1.04 $\pm$ 0.034 <sup>a</sup>	0.26 $\pm$ 0.031 <sup>a</sup>	0.21 $\pm$ 0.017 <sup>a</sup>	0.06 $\pm$ 0.045 <sup>a</sup>
	300 mg/kg bw	233.11 $\pm$ 3.06 <sup>a</sup>	0.98 $\pm$ 0.036 <sup>a</sup>	0.32 $\pm$ 0.014 <sup>ab</sup>	0.10 $\pm$ 0.006 <sup>b</sup>	0.05 $\pm$ 0.004 <sup>a</sup>
	900mg/ kg bw	216.22 $\pm$ 3.34 <sup>b</sup>	1.00 $\pm$ 0.035 <sup>a</sup>	0.32 $\pm$ 0.021 <sup>ab</sup>	0.12 $\pm$ 0.027 <sup>ab</sup>	0.05 $\pm$ 0.01 <sup>a</sup>
II. 30 days	Control	204.00 $\pm$ 4.6 <sup>a</sup>	1.16 $\pm$ 0.01 <sup>a</sup>	0.39 $\pm$ 0.01 <sup>a</sup>	0.20 $\pm$ 0.03 <sup>a</sup>	0.08 $\pm$ 0.009 <sup>a</sup>
	300 mg/kg bw	201.00 $\pm$ 5.5 <sup>a</sup>	1.15 $\pm$ 0.03 <sup>a</sup>	0.39 $\pm$ 0.01 <sup>a</sup>	0.20 $\pm$ 0.03 <sup>a</sup>	0.10 $\pm$ 0.01 <sup>a</sup>
	900 mg/kg bw	194.00 $\pm$ 6.1 <sup>b</sup>	1.14 $\pm$ 0.02 <sup>a</sup>	0.38 $\pm$ 0.04 <sup>a</sup>	0.24 $\pm$ 0.02 <sup>a</sup>	0.08 $\pm$ 0.01 <sup>a</sup>



**Fig.2:** Antifertility effect of *Opuntia elatior* fruit extract in the male rat treated for 60 days and recovery: Sperm motility. Values (Mean ± SE) denoted by different alphabets differ significantly ( $P < 0.05$ ; DMRT) n=5.

**Histology**

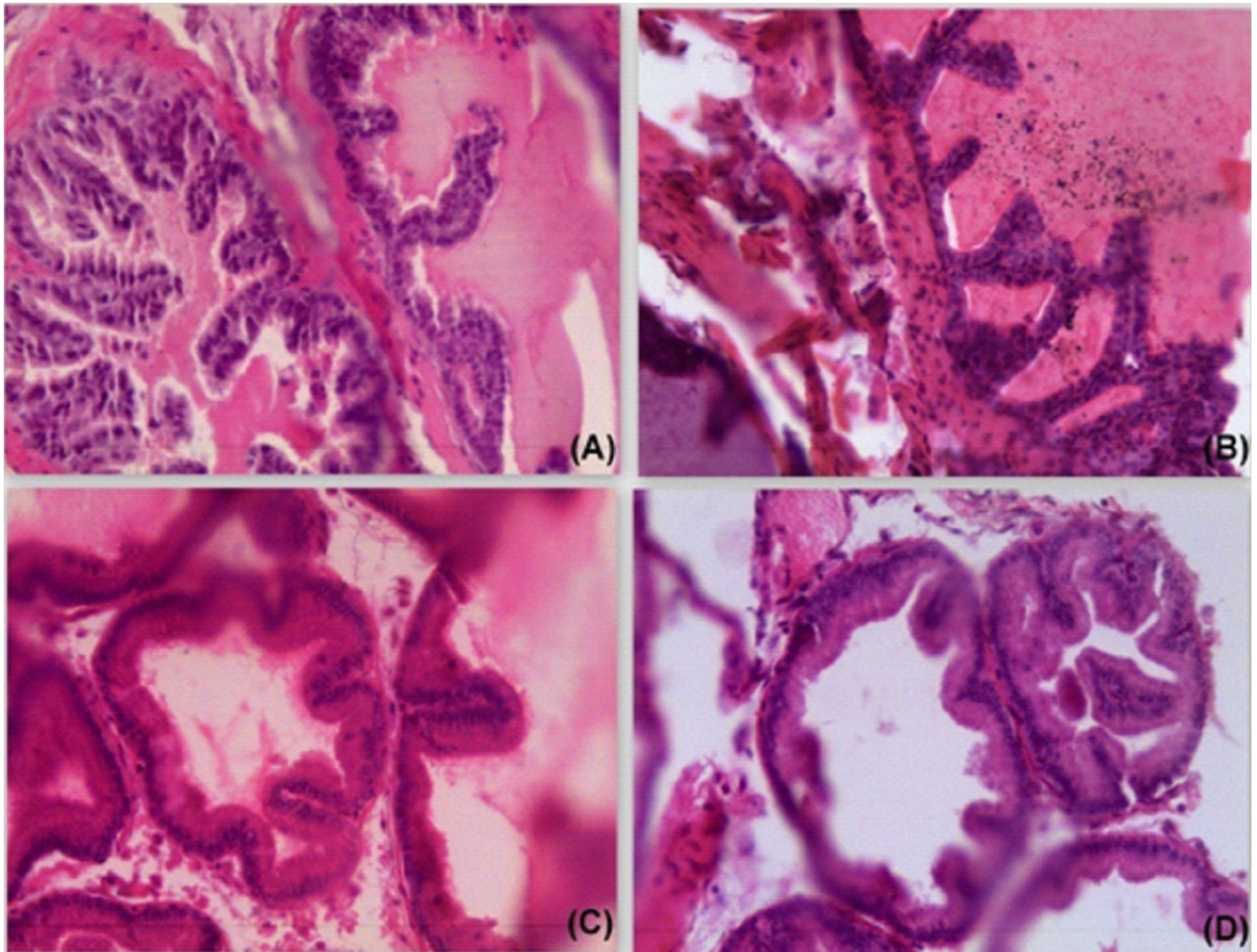
The testicular volume, the seminiferous tubule diameter and seminiferous epithelium of the treated rats of both 30 day and 60 day groups were comparable to those of the control group showing no signs of testicular degeneration or spermatogenic arrest. The Leydig cells also appeared normal in the testis of the treated rats (Fig. 3). The histological features of the epididymis of treated rats showed no visible changes in the tubule diameter and epithelial cell height which were comparable to those of control animals. However, sperm density was affected but with ample secretions in the tubules of the cauda epididymidis of treated rats (Fig. 3). Similarly, the histological profile of the seminal vesicles and ventral prostate of treated rats showed no significant changes from that of control animals (Fig. 4).



**Fig.3:** Histology of the testis (A- control, B - treated) and cauda epididymidis (C - control & D – treated) of male rats treated with *O. elatior* for 60 days (H & E staining; x200)

A discernible effect on spermatogenesis in the testis was not seen whereas epididymis showed lumen with reduced sperm density and secretions in treated animals.



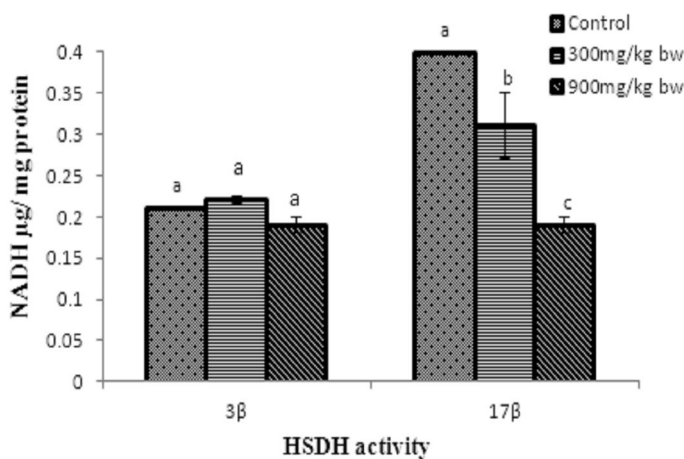


**Fig.4:** Histology of the seminal vesicles (A- control, B - treated) and ventral prostate (C - control & D – treated) of male rats treated with *O. elatior* for 60 days (H & E staining; x200). No effect on the histology of both seminal vesicles and ventral prostate was observed in treated animals.

### Hydroxysteroid dehydrogenase activity

The activity of  $\Delta^5 3\beta$  HSDH was not significantly affected in the testis of treated rats.  $17\beta$ -

HSDH activity was significantly lower in the testis of treated animals (Fig. 5).

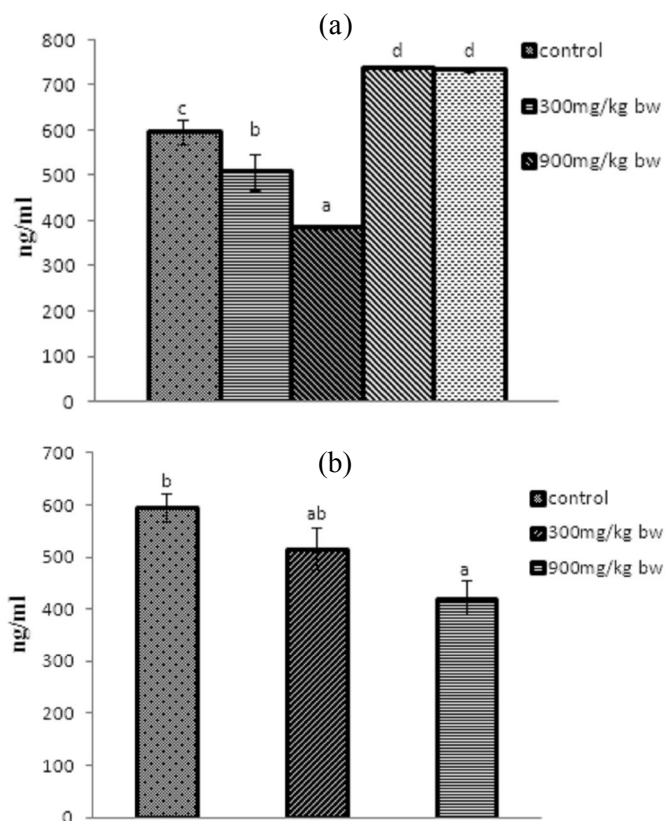


**Fig.5:** Antifertility effect of *Opuntia elatior* fruit extract in the male rat treated for 60 days: Testicular hydroxysteroid dehydrogenase activity.

Values (Mean  $\pm$  SE) denoted by different alphabets differ significantly ( $P < 0.05$ ; DMRT); n=5.

**Serum testosterone**

Serum testosterone levels were marginally lower in treated rats of 60 day study. However, serum testosterone levels were found to be slightly higher than those of control group after withdrawal of treatment (Fig. 6). In rats treated for 30 days, the serum testosterone levels were slightly reduced in the higher dose group (Fig. 6).



**Fig. 6:** Antifertility effect of *Opuntia elatior* fruit extract in the male rat: serum testosterone (a) 60days and post-treatment recovery; (b) 30 day treatment. Values (Mean±SE) denoted by different alphabets differ significantly (P < 0.05; DMRT) n=5.

**Fertility**

Fertility of male rats treated with the extract for 60 days, when mated with untreated females, was markedly affected. The vaginal smears of the mated female rats showed sperm indicating that the male rats from the treated group mated successfully and the libido was not affected. The pregnancy was 50% in the lower dose (300 mg/kg bw) group, whereas, there was no pregnancy at the higher dose (900 mg/kg bw). Fertility of the treated male rats was restored after the withdrawal of treatment for a period of two weeks. However, litter size was reduced in rats of the higher dose group (Table 2).

Table 2: Effect of *O. elatior* fruit extract on the fertility of male rats

Groups	Mating success (vaginal sperm)	Number of rats pregnant (%)	
		60 day treatment	Withdrawal of treatment
I (Control)	+	100	100
II (300 mg/kg bw)	+	0	33.3
III (900 mg/kg bw)	+	0	16.6

**Discussion**

Several medicinal plants have been shown to have varied degrees of antifertility effects in the male, mainly affecting spermatogenesis and often affecting androgen production in the testis (Akbarsha et al., 1990; Akbarsha and Murugaian, 2000; Naseem et al., 1998; Anderson and Baird, 2002; Ghoshet al., 2002; Sharma and Jacob, 2002; Parveen et al., 2003; Qureshiet al., 2005; Gupta and Sharma, 2006; Singh and Singh, 2009). The medicinal value of the cactus *Opuntia* is widely known in many parts of the world and several studies have reported the neutraceutical value, hepatoprotective properties and anti-inflammatory potential in experimental studies (Park et al., 2001; Lee et al., 2001; Feugang et al., 2006). The medicinal properties of *Opuntia* have been attributed to a variety of phytochemicals present in the cladode and fruits (Feuganget al., 2006). The methanolic extract of phylloclade of the cactus *Opuntia dillenii* has been reported to affect fertility in male rats with reduced sperm count and decreased serum testosterone (Gupta et al., 2002; Bajaj and Gupta, 2011). However, in most of the studies, the effect on fertility was only partial, including reduction in testosterone which is not a desirable feature since it could affect the libido. Male antifertility activity of the phylloclade extracts of *O. dillenii* has been investigated by Gupta et al. (2002) and Bajaj and Gupta, (2012) wherein they have reported antispermatogenic effect in rats and suppression of fertility. Their studies reported decline in testicular weight, suppression of spermatogenic activity, reduction in serum testosterone, lower sperm count and partial fertility. However, their studies did not show 100% efficacy on fertility suppression and reversibility (Bajaj and Gupta, 2012).

In our study, 60 day treatment of the methanolic extract of the fruit of *O. elatior* produced marked decline in sperm count and motility in rats which was dose-dependent. The fertility of the treated male rats was suppressed in both the dose groups, being 100% at the

higher dose. Our results show that the weights of testes and accessory organs viz., epididymis, prostate and seminal vesicles, were not significantly affected. Histological examination of testis showed no discernible effect on spermatogenesis, and the Leydig cells appeared normal. The serum testosterone levels were marginally lower but not significant enough to affect the libido. Therefore, the lack of effect of the *O. elatior* extract on accessory organs or libido could be attributed to the normal levels of serum testosterone in the treated rats. The results of serum testosterone levels are consistent with the testicular enzyme activities of  $\Delta^5$  3 $\beta$  HSDH, a key enzyme in androgen biosynthesis, which was not significantly affected in the testis of treated rats. However, 17 $\beta$  HSDH, although showed reduced activity in the testis of treated rats, was not reflected in the serum testosterone levels.

Our results of reversibility studies showed that withdrawal of treatment after 60 days for a period of two weeks was enough to restore the sperm count, testicular HSDH activity and serum testosterone levels, which are consistent with the recovery of fertility. Further, a short term experiment with 30 day treatment of the extract in male rats showed similar results without any significant effect on the testes and accessory organs and serum testosterone levels. However, sperm count and motility were drastically affected in short term treatment which indicates probable extragonadal action, possibly the epididymal targets that could be involved in sperm motility and survival. We could further show that even a shorter duration of treatment for only two weeks was effective in lowering the sperm count significantly. Therefore, our

results of short term treatment indicate the effectiveness of the fruit extract in suppressing male fertility without affecting spermatogenesis and steroidogenesis. The active chemical principles present in the methanolic extract of the fruit of *O. elatior* are being investigated, and may contain many phytochemicals which need to be identified.

Overall, our results show that the methanolic extract of the fruit pulp was quite effective in affecting male fertility without affecting libido and it was totally reversible. The mode of action of the antifertility effect appears to involve an extragonadal target since short term treatment (30 and 15 days) was enough to suppress sperm count and motility.

Over all, our study has shown that the methanolic extract of the fruit pulp of *O. elatior* could be a promising source of a natural male antifertility agent which is effective in suppressing fertility without affecting libido and was reversible after withdrawal of treatment. Since spermatogenesis and steroidogenesis in the testis were not affected, the reduction in sperm count and motility could be attributed to an extragonadal action, possibly epididymal targets, for the antifertility action. Efforts are under way to isolate the bio-active compound in the extract responsible for the antifertility effect.

## Acknowledgments

Authors acknowledge the financial support in the form of a research grant by the University Grants Commission, New Delhi, and thank the Chairman, Department of Zoology, University of Mysore, for providing facilities.

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