



## Antifertility Activity of Simple Ascidian, *Microcosmus exasperatus* Heller, 1878

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### ABSTRACT

Increase in life expectancy and decrease in death rate, as a result of recent advances in the domain of medicine, pharmaceuticals and public health has led to a rapid population explosion. Plant based natural products have been used as antifertility agents from time immemorial. But due to various reasons they are exploited and the resources are depleting. Hence there is an urgent need for the search of an effective and safe male antifertility agent from other sources. The present investigation was designed to find out the effect of ethanol extract of simple ascidian *Microcosmus exasperatus* on the fertility of male albino rats. For the study, experimental animals were divided in to four groups of five each. Group I received normal saline and served as control. The second, third and fourth group of animals were administrated with the extracts at a dose of 50, 100, 150 mg/kg body weight respectively for a period of 14 days. Significant decrease in the weight of body, testis and the accessory sex organs were noted. A dose related reduction in sperm count of testis, epididymis, motility and increase in the level of abnormal sperms were observed. The serum biochemical parameters and liver marker enzymes did not show any significant variations.

**Keywords:** *Microcosmus exasperatus*, Ascidian, Antifertility activity.

### INTRODUCTION

Rapid population growth is becoming a problem which causes severe pressure on economic, social and cultural resources. The quest for an antifertility agent that can control human population is as old as recorded history. A wide variety of synthetic contraceptive agents are available but they cannot be used continuously due to their severe side effects.<sup>1,2</sup> Several devices are available and in use for female contraception.<sup>3</sup> In contrast, except for the barrier method and vasectomy, there are no ways available for male contraception. The risk on the use of drugs has triggered the need to develop newer molecules. As concerns regarding side effects and inconvenience of these existing methods prevent their universal acceptance there is need to develop multiple male contraceptives.<sup>4</sup> Development of additional male antifertility agents can provide tremendous social and public health benefits. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources.<sup>5</sup> In the search for an effective agent to regulate male fertility or inhibit the millions of sperms produced a day towards male contraception and population control, a large number of plants have been screened. However, the attention of researchers to bring out a safe and effective male contraceptive from marine organism has not been carried out so far. *Microcosmus exasperatus* is a marine sedentary animal. A review of literature shows that studies on the nutritive value, biochemical components, toxicity, GC- MS analysis, HPTLC and pharmacological activities like antimicrobial, antidiabetic, hepatoprotective, CNS depressant activity, antitumor and immunomodulation are available.<sup>6-16</sup> But antifertility

studies on ascidians of Indian waters especially *Microcosmus exasperatus* is lacking and a preliminary attempt has been made.

### MATERIALS AND METHODS

#### Animal Material

Samples of *Microcosmus exasperatus* were collected from Thoothukudi coast, cleaned with sea water, shade dried and powdered. A voucher specimen AS 2240 has been deposited in the museum, Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin - 628002. *Microcosmus exasperatus* belongs to the Class: Ascidiacea, Order: Pleurogona, Suborder: Stolidobranchia and Family: Pyuridae. It has a hard leathery orange coloured tunic. Both siphons are clearly visible. The pharynx has 8-9 folds. There is one gonad in each side of the body, divided in to three portions. It has been found to breed throughout the year.<sup>17</sup>

#### Preparation of Extract

100 gm powder was extracted with ethanol using Soxhlet apparatus, cooled to room temperature and evaporated in a rotary evaporator to get a residue. This residue was used for further studies.

#### Experimental Animal

Mature adult male Wistar albino rats weighing about 180 - 200 gm were selected for the study. They were maintained in a well ventilated animal house with constant 12 h of darkness and 12 h light schedule, room temperature (24±2°C) and humidity (60-70%). Clean water and standard pellet diet "ad Libitum" (Hindustan Lever Ltd., India) were given to them. The animals were kept under fasting for 16 hours before the experiment.



### Acute Oral Toxicity Studies

The acute oral toxicity studies (LD<sub>50</sub> determination) were carried out as per OECD guidelines.<sup>18</sup> Albino rats were divided into five groups containing six animals each. The control group received normal saline. The second, third, fourth and fifth group received 100, 500, 1000, 2000 mg/kg body weight of the extract respectively. After the administration of the extracts, the animals were observed for gross behavioural changes like irritability, tremor, laboured breathing, staggering, convulsion and death. They were kept under continuous observation for the first two hours and then frequently during the next twenty four hours. The number of dead and surviving animals after twenty four hours was recorded.

### Experimental Protocol

Male albino rats were randomly divided into four groups consisting of 5 animals each. Group I served as control and was given normal saline. Group II, III and IV were administered different doses of extracts orally by using IGC (50, 100 and 150 mg/kg body weight respectively) for 14 days. After the experimental duration of 14 days, blood was collected by cardiac puncture and was centrifuged at 3000 rpm for 10 minutes, sera was separated and stored at -20 °C until use for assay of biochemical parameters, enzymes and hormones.

### Weight of Body and Reproductive Organs

The body weight of adult mice was recorded throughout the treatment period. Testes, epididymis (caput and cauda), vas deferens, seminal vesicle and prostate were dissected out, blotted free of blood and weighed. The organ weights were expressed in terms of mg/100 gm body weight.

### Sperm Concentration, Motility and Abnormality

The caput and cauda region of epididymis was teased separately into two petridishes and 1 mL of normal saline at temperature of 36°C was added to the semen to enhance sperm survival *in vitro* during the period of the study. The semen mixture was then sucked into a red blood pipette to the 0.5 mark, diluted with warm normal saline, sucked up to the 101 mark. The normal saline at the stem of the pipette was discarded and the content was mixed thoroughly. A drop of the semen mixture was placed on the neuber counting chamber which spread under the cover-slip by capillary action. The chamber was mounted on the stage of the microscope, viewed under the magnification of x40 and counted and expressed in million per ml.<sup>19</sup> A drop of the sperm saline mixture was taken in a separate glass slide. One slide was covered with a cover slip and examined under the microscope. Sperm motility at the caudal epididymis was then assessed by calculating the motile spermatozoa per unit area. A smear was made on another slide and total morphological abnormalities were observed.<sup>20</sup>

### Serum Biochemical Profile

Protein, albumin, globulin, urea, creatinine and liver marker enzymes like SGPT, SGOT, ALP was estimated by using standard procedures.<sup>21-24</sup>

### Serum Enzyme Assay

Catalase, Glutathione peroxidase, Glutathione-Stransferase, Superoxide Dismutase and Glutathione reductase was estimated by using standard procedures.<sup>25-29</sup>

### Fertility Test

Fertility was estimated in adult male rats treated with ethanol extract of *Microcosmus exasperatus* with the control males' counterparts. Each male was placed in an individual cage with two virgin untreated females of the same strain. They were left together for 10 days during which two estrous cycles had elapsed. One week after the removal of the exposed males, pregnant females were killed by cervical dislocation under light ether anesthesia and the number of implantation sites, fetuses and resorption sites were recorded.

### Assay of Sex Hormone and Pituitary Gonadotropins

The blood was removed from the animals by intra-cardiac puncture method, centrifuged at 2000 rpm to separate the serum for the measurement of testosterone, luteinizing hormone (LH), estrogen and follicle stimulating hormone (FSH). The quantitative determination of hormones was done by using Enzyme Immunoassay Method (EIA).

### Statistical Analysis

Values are expressed as mean  $\pm$  SEM. The statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test. P-values less than 0.05 were considered to be significant.

## RESULTS AND DISCUSSION

The animals administered with 100, 500, 1000, 2000 mg/kg body weight did not show any mortality during the twenty four hours experimental duration. A higher dose above 1000 mg/kg body weight induced irritability, tremor, laboured breathing, staggering and convulsion but not mortality indicating its safety.

In the present study a reduction in the weight of body, testis and accessory sex organs like epididymis, vas deferens, seminal vesicle and prostate was noted on treatment with the extract of *Microcosmus exasperatus* (Table 1). In rats the whole spermatogenic cycle process requires 53 days out of which spermatozoa spend the last 6 to 7 days in its final transition through epididymis.<sup>30</sup> The most common causes of male infertility include abnormal sperm production or function and impaired delivery of sperm.<sup>31</sup> The decrease in GSI (testis weight/body weight) and epididymis weight has been attributed to the increased level of damage on the tissue of experimental rats.<sup>32-34</sup> The reduction in the weight of testis and



accessory sex organs observed might be due to low level of testosterone which was not enough for the maintenance of gonads and accessory sex organs.<sup>35,36</sup> The process of spermatogenesis, growth and function of accessory reproductive organs are dependent on androgen. Hence any changes in the production of androgen would reflect the growth, maturity and functional status of the reproductive system. The same reason can be considered to support the present observation on treatment with the extract of *Microcosmus exasperatus*.

The development of normal and mature sperm is the key to optimum male fertility. Sperm density and motility in the caput and cauda epididymis was adversely affected after the treatment (Table 2). The reduction in sperm motility in cauda epididymis is of importance with regard to fertilization.<sup>37</sup> Inadequate concentration and immobile spermatozoa means they cannot penetrate the cervical mucus and thus fail to fertilize the ova.<sup>38</sup> Androgen deprivation not only suppresses spermatogenesis, leading to low sperm concentration, but alters the epididymal milieu also, which renders it hostile for physiological maturation and survival of the spermatozoa.<sup>39-41</sup> The percentage abnormality of the head and tail of the sperm in the extract treated groups were found to increase significantly. A similar observation was noticed on administration of the extract of *Azadirachta indica*.<sup>42</sup> One of the key factors for male fertility is the presence of

normal mature sperms. The anterior lobe of the pituitary release trophic factors like FSH and LH which regulates the process of spermatogenesis and secretion of testosterone by the testis. Production of sperm cells in the sertoli cells of the seminiferous tubules is initiated by FSH where as the Leydig cells are stimulated to produce testosterone by the LH.

Spermatogenesis is the process of gametogenesis leading to the formation of spermatozoa. FSH causes proliferation of the germinal epithelial cells promoting the formation of secondary spermatocytes by meiotic division. The spermatids formed undergo elaborate morphological differentiation and develop into the sperm. As the process of spermatogenesis is complex, involving many stages and hormones influencing it, any change in the normal cycle can lead to the formation of abnormal sperms. On treatment with the extract of *Microcosmus exasperatus* for 14 days, sperm abnormality was more in the head region than in the tail. The inhibitory activity on the proliferation of spermatogonia in experimental animals using various plant extracts have been demonstrated by earlier workers.<sup>43-45</sup> The presence of abnormal sperms observed on treatment may be due to the effect of the extract on normal function of sertoli and Leydig cells. It may also be because of an effect on hypothalamic release factors and anterior pituitary secretion of gonadotropins resulting in alteration of spermatogenesis.<sup>46-47</sup>

**Table 1:** Effect of ethanol extract of *Microcosmus exasperatus* on the weight of body, testis and accessory sex organ of male albino rats.

Groups/ Parameters	Body wt (gm)		Testis (gm)	Epididymis (mg)		VD (mg)	SV (mg)	Prostrate (mg)
	Before	After		Caput	Cauda			
Group I Normal	226.23±5.11	223.21±3.21	2.451±0.42	216.41±3.81	279.13±8.12	129.43±9.11	281.81±8.35	187.71±5.91
Group II 50 mg/kg	194.22±7.25	161.58±4.11*	1.634±0.13	179.35±2.17*	259.71±3.75*	97.18±6.11**	275.91±8.91	168.12±6.47
Group III 100 mg/kg	202.34±9.35	166.18±6.14*	1.341±0.82*	169.12±4.35*	243.52±5.18**	98.54±3.71**	239.84±9.51*	152.34±5.15*
Group IV 150 mg/kg	213.99±5.61	158.41±6.20*	1.232±0.59*	161.61±5.33*	237.11±5.31**	95.51±2.60**	248.18±14.34**	135.14±8.21*

Data represented as mean ±SEM, (N=5). Significance between \*Normal control and extract treated group; \*p <0.05, \*\*p <0.01.

**Table 2:** Effect on the sperm concentration, motility and abnormality

Treatment Groups	Sperm Concentration (Counts x 10 <sup>6</sup> mil)		Sperm Motility (FMI) @ (cauda)	Sperm Abnormality (%)	
	caput	cauda		Head	Tail
Group I - Normal	337.15±27.61	419.31±11.41	149.92±12.75	5.23±0.34	7.84±2.01
Group II - 50 mg/kg	299.99±16.99	412.39±11.91	139.31±11.50*	9.09±0.19	8.23±1.71
Group III - 100 mg/kg	273.51±15.50*	365.01±15.11**	122.37±12.95*	12.45±0.69*	10.75±1.75*
Group IV - 150 mg/kg	268.45±13.12**	271.34±13.91***	119.15±7.94**	17.54±0.19*	14.45±1.34*

Data represented as mean ±SEM, (N=5). Significance between \*Normal control and extract treated group.; \*p <0.05, \*\*p <0.01, \*\*\*p <0.001.

Administration of ethanolic extract of *Microcosmus exasperatus* caused significant decrease in protein, albumin and globulin (Table 3). Low levels of protein are indicative of inhibition of spermatogenesis.<sup>48</sup> Decreased protein content reduces the growth rate of testis, development of spermatogonia to spermatocytes and the spermatogenic process.<sup>49</sup> Similar observations have been reported by earlier workers.<sup>50-51</sup> The reduction noted in the present study could also be due to the same mechanism of action. Serum urea and creatinine profile on treatment with the extract did not show any significant change indicating no toxic effect on the kidney. The liver marker enzymes showed only marginal variations indicating non toxic nature and absence of side effects on normal metabolism of the liver.

A reduction in the level of antioxidant enzymes CAT, GPX, GSH, SOD and GR were recorded on treatment with the extract in the present study (Table 4). Lipid peroxidation of the plasma membrane is prevented by SOD which scavenges extra and intra cellular anions. Conjugation with CAT and glutathione peroxidase is essential to act against H<sub>2</sub>O<sub>2</sub>.<sup>52</sup> The reduced level of enzymes might be due to the excess production of anions in response to the treatment with the extract. The inhibition of the action of these antioxidant enzymes may be because of an increased production of reactive oxygen species or that their decreased expression may lead to oxidative stress.<sup>53</sup> All these biochemical changes result in increased LPO bringing about reduction in the viability and motility of sperm leading to infertility.<sup>36</sup>

**Table 3:** Effect on biochemical profile

Parameter/ Groups	Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Urea (gm/dl)	Creatinine (gm/dl)	SGOT (u/L)	SGPT (u/L)	ALP (u/L)
Group I Normal	8.94±10.42	5.40±0.71	3.54±0.08	15.35±0.41	1.33±0.71	17.17±0.41	22.41±0.23	164.81±5.10
Group II 50 mg/kg	7.13±0.54	3.71±0.74	3.42±0.39	17.82±3.71	0.54±0.41	5.81±0.54	18.41±0.34	132.14±0.71
Group III 100 mg/kg	5.45±0.94	3.12±0.81	2.33±0.05	17.58±3.50	0.92±0.71	14.62±0.54	23.12±0.21	161.23±3.21
Group IV 150 mg/kg	5.96±0.94	3.50±0.79	2.45±0.24	16.52±5.80	0.91±0.51	12.41±0.13	16.31±0.24	181.34±0.71

Data represented as mean ±SEM, (N=5).

**Table 4:** Effect on the level of antioxidant enzymes

Parameter/ Groups	CAT (u/gm Hb)	GPX (U/L)	GSH (mmol/ml)	SOD (u/gm Hb)	GR (U/L)
Group I - Normal	7.64±0.29	0.345±0.04	11.43±2.01	22.41±1.41	19.75±1.74
Group II - 50 mg/kg	5.54±0.45	0.213±0.05	6.13±1.45*	16.51±2.13*	18.54±3.15
Group III - 100 mg/kg	4.73±2.13*	0.189±0.07*	4.51±1.91**	14.63±1.53**	15.21±1.31
Group IV - 150 mg/kg	3.95±1.54**	0.145±0.05*	3.41±1.43**	11.12±1.54**	14.54±2.71*

Data represented as mean ±SEM, (N=5). Significance between \*Normal control and extract treated group.; \*p <0.05, \*\*p <0.01.

Gonadotropin releasing hormone is produced and secreted from the hypothalamus. This hypothalamic peptide hormone regulates the synthesis and secretion of FSH and LH from anterior pituitary which controls the whole reproductive function. The secretion of testosterone in the Leydig cells is stimulated by LH. Low testosterone levels have been associated with decreased reproductive ability.<sup>54-56</sup> The reduction of testosterone results in male infertility. In the present study also decrease in the level of testosterone was observed which indicates that the extract is responsible for impairing the reproductive functionality of the treated animals. The results of the study show that administration of *Microcosmus exasperatus* extract caused an increase in LH concentration (Table 5). This observation may be due to suppression of testosterone induced by *Microcosmus exasperatus*. The decreased level of testosterone in turn brings about an increase in LH and FSH concentration in

order to stimulate the production of more testosterone.<sup>55</sup> This suggests that the hypothalamic cells responsible for the synthesis and release of gonadotropin releasing hormone function normally and correctly. The reduced level of testosterone may be due to a potential mechanism through which it is converted into estradiol - a precursor of estrogen. Hence in the present study it appears that the antifertility activity of *Microcosmus exasperatus* is mediated at the level of testis only and not at the level of hypothalamus and pituitary. The study showed a dose dependent increase in the level of estrogen. This might be due to the conversion of testosterone to estrogen.<sup>57-59</sup> This in turn is supported by the observation of a decrease in the level of testosterone. For male contraception, it is not necessary to stop spermatogenesis, but it is enough to eliminate the fertilizing ability of the spermatozoa by causing changes in the morphology or in the function of sperm.<sup>60</sup>



**Table 5:** Effect on Sex hormones levels and pituitary gonadotropins

Treatment Groups	Parameters			
	Testosterone (ng/ml)	LH/ICSH ( $\mu$ l/ml)	FSH ( $\mu$ l/ml)	Estrogen(pg/ml)
Group I - Normal	3.20 $\pm$ 0.31	3.15 $\pm$ 0.41	0.81 $\pm$ 0.07	29.42 $\pm$ 0.16
Group II - 50 mg/kg	1.56 $\pm$ 0.41	1.52 $\pm$ 0.21	1.34 $\pm$ 0.03	41.41 $\pm$ 0.75*
Group III - 100 mg/kg	1.23 $\pm$ 0.61*	3.53 $\pm$ 0.83	2.45 $\pm$ 0.07*	44.08 $\pm$ 0.85*
Group IV - 150 mg/kg	0.54 $\pm$ 0.26**	4.62 $\pm$ 0.41*	3.32 $\pm$ 0.07*	57.34 $\pm$ 0.81**

Data represented as mean  $\pm$ SEM, (N=5). Significance between \*Normal control and extract treated group. \*p <0.05, \*\*p <0.01.

**Table 6:** Effect on the Fertility of male albino rats

Groups	No. of male	No. of females	No. of pregnant females	No. of implantation	No. of viable fetuses	Total No. of resorption sites
Group I - Normal	5	10	8/10 (80%)	7.13 $\pm$ 0.84	9.16 $\pm$ 1.08	5
Group II - 50 mg/kg	5	10	5/10 (50%)*	6.05 $\pm$ 0.93	4.05 $\pm$ 0.99*	5
Group III - 100 mg/kg	5	10	3/10 (30%)**	4.11 $\pm$ 0.89*	3.05 $\pm$ 1.13**	4
Group IV - 150 mg/kg	5	10	1/10 (10%)***	2.91 $\pm$ 0.15**	2.85 $\pm$ 1.13**	2

Data represented as mean  $\pm$ SEM, (N=5). Significance between \*Normal control and extract treated group. \*p <0.05, \*\*p <0.01, \*\*\*p <0.001.

Dose dependent reduction in the number of pregnant females was observed on treatment with the ethanol extract of *Microcosmus exasperatus* (Table 6). In addition, the number of implantation, viable fetuses and resorption sites also showed a decrease. This could be due to failure of fertilization as indicated by reduction in parameters such as epididymis sperm concentration, reduced sperm motility and increased sperm abnormality. The testosterone concentration or androgenic synthesis alters spermatogenesis. Antispermato-genic effects of plant extracts and degenerative changes in the testes leading to failure in fertilization have been reported in previous studies. As ascidians are also sedentary like the plants, the same role may be applicable here also.

## CONCLUSION

It can be concluded that *Microcosmus exasperatus* is capable of suppressing the fertility of male rats without affecting the biochemical parameters, function of the hypothalamus and pituitary. Hence it is possible to develop a safe male contraceptive by further studies.

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