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Antigonadal Effects of *Terminalia arjuna* in Male Albino Rats

¹Alok Chattopadhyay, ²Ronojoy Sengupta and ¹Shaba Parveen

¹Department of Physiology, Harimohan Ghose College, J206, Paharpur Road, Kolkata 700024, India

²Department of Biotechnology and Bioinformatics, Sikkim Manipal University Learning Centre P-21A, Old Ballygunge Road, Kolkata 700019, India

Corresponding Author: Alok Chattopadhyay, Department of Physiology, Harimohan Ghose College, J206, Paharpur Road, Kolkata 700024, India

ABSTRACT

Terminalia arjuna is a well-known medicinal plant for its antidysenteric, antipyretic, astringent, gastroprotective, hypocholesterolemic and cardiogenic properties. Conversely, antispermatogenic effect of this plant has also been reported. The aim of this study is to evaluate the effects of bark extract of this plant on gonadal steroidogenesis, spermatogenesis and plasma gonadotrophin (FSH and LH) levels in male albino rats. Wistar strain adult rats were administered orally with alcoholic extract of its bark dissolved in distilled water at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days. Testicular steroidogenesis was evaluated by spectrophotometric assay of the activities of 17 β -hydroxysteroid dehydrogenase (17- β HSD) and Δ^5 -3 β hydroxysteroid dehydrogenase (Δ^5 -3 β HSD) and radioimmunoassay of plasma testosterone level. Plasma FSH and LH levels were also determined by radioimmunoassay. Spermatogenic activity was evaluated by counting the germ cells at stage VII of seminiferous cycle. Treatment for 21 days led to significant decrease in the activities of steroidogenic enzymes, plasma testosterone level and gametogenic activity. These parameters showed non-significant changes in the 7 and 14 days treatment group and no significant change was found in plasma gonadotrophin levels at any of the above durations. Though the exact mechanism of this antigonadal effect cannot be determined from the present study, one of the active constituents, β -sitosterol (a phytosterol) might be involved in this inhibition. Moreover, since treatment at the above dose and duration caused this effect, these data may have some clinical implication. Therefore, during its long-term medicinal use this adverse effect should be given due importance.

Key words: *Terminalia arjuna*, male rat, spermatogenesis, steroidogenesis, gonadotrophin

INTRODUCTION

Terminalia arjuna, a popular medicinal plant and a member of the family Combretaceae is found on the banks of rivers, streams and dry watercourses in sub-Himalayan tract and various other parts of India. Fruits, leaves and barks of this tree are well known for medicinal properties (Dhawan *et al.*, 1977). Bark extract have antidysenteric, antipyretic, astringent and cardiogenic properties. The powder of bark has been reported to be used as a diuretic in symptomatic hypertension (Chatterjee and Pakrashi, 1994). Alcoholic bark extract also possesses a gastroprotective property on diclofenac induced gastric ulceration (Devi *et al.*, 2007) and can act as antioxidant and hypocholesterolemic agent (Devi *et al.*, 2007; Gupta *et al.*, 2001). It has been reported to act as a spermicidal agent and additionally has semen coagulant properties in mice

(Dhawan *et al.*, 1977). Antispermatic action of *Terminalia arjuna* bark extract has also been reported (Jha and Dixit, 1986). It has been further reported that oral administration of alcoholic extract of the bark caused atrophy, altered contractile pattern of the vas deferens, decrease in testicular weight and impaired spermatogenesis with formation of multinucleated giant cells (Chauhan *et al.*, 1990). However, systematic study of this plant on the male gonadal activities is missing.

Thus, the present study was undertaken to investigate the effects of the bark extract of this plant on testicular and accessory sex organs' weight and spermatogenesis. Additionally, testicular steroidogenic enzyme Δ^5 - 3β hydroxysteroid dehydrogenase (Δ^5 - 3β -HSD) and 17β hydroxysteroid dehydrogenase (17β -HSD) activities as well as plasma levels of testosterone and gonadotropins (FSH and LH) have also been evaluated. The study, therefore, may reveal the detailed anti-gonadal properties of this bark extract on male rats.

MATERIALS AND METHODS

Animals: The experiments were carried out on 32 adult male albino rats of a laboratory-bred Wistar strain weighing 150-160 g. The animals were housed in a laboratory controlled environment ($27\pm 2^\circ\text{C}$, 98% humidity) and the lighting schedule was maintained at 12 h of light per day. All the rats were given standard laboratory food and water *ad libitum*.

Preparation of extract: The bark of *T. arjuna*, obtained from the campus of University of Calcutta, Department of Home Science in February. It was shadow-dried, powdered and soxhletted in 50% ethanol. Crude extract was collected after evaporating ethanol. To prepare doses, a weighed quantity of the dried extract was macerated with an equal quantity of gum acacia and suspended in distilled water.

Treatment schedule: The selected dose of *T. arjuna* was $150 \text{ mg kg}^{-1} \text{ b.wt. day}^{-1}$ (Chauhan *et al.*, 1990) and the suspension was administered orally with a feeding needle. The animals were divided into four equal groups among which group I was labeled as control group and the other three were treated groups. These three groups (Groups II, III and IV) received the bark extract at the above-mentioned dose for 7, 14 and 21 days, respectively while the control rats received the vehicle (gum acacia suspension in distilled water) alone. All the animals were sacrificed 24 h after the last treatment following protocols and ethical procedures. Blood samples for hormone assay were collected from the hepatic vein under light ether anaesthesia. Plasma samples were separated by centrifugation, frozen and stored at -20°C until assayed.

Body and organ weight: The body weight of all the rats were recorded on the first day of treatment (initial) and on the day of sacrifice (final). The testes and accessory sex organs (epididymes, seminal vesicle and ventral prostate) were dissected out, trimmed of fat and weighed in single pan electronic balance. The relative weights of organs were expressed per 100 g body weight.

Assay of steroidogenic enzyme activities: Testicular Δ^5 - 3β -HSD was assayed spectrophotometrically by the method of Talalay (1962) and subsequently modified by Chattopadhyay *et al.* (1999). Testis was homogenized in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1mM EDTA (ethylene diamine tetraacetic acid) at a tissue concentration of 100 mg mL^{-1} of homogenizing mixture. It was cold centrifuged at

10,000 rpm for 30 min. The supernatant (1 mL) was mixed with 100 μ L sodium pyrophosphate buffer (pH 8.9) and 30 μ g dehydroepiandrosterone, making the incubation volume 3 mL. Enzyme activity was assayed after adding 0.5 μ M NAD⁺ to the mixture in a spectrophotometer against a blank (without NAD⁺). One unit of enzyme activity is equivalent to a change in absorbance of 0.001 min⁻¹ at 340 nm. Testicular 17 β -HSD was assayed following the method of Jarabak *et al.* (1962) and subsequently modified by Sarkar *et al.* (1991). The same supernatant (1 mL) was mixed with 400 μ M sodium pyrophosphate buffer (pH 10.2) and 0.3 μ M testosterone to make the volume 3 mL. Enzyme activity was determined after adding 1.1 μ M NAD⁺ to the mixture in a spectrophotometer against a blank (without NAD⁺). One unit of enzyme activity is equivalent to a change in absorbance of 0.001 min⁻¹ at 340 nm.

Assay of hormones: Radioimmunoassay of plasma testosterone was carried out following the method of Jacobs (1974) using testosterone ¹²⁵I RIA Kit (ICN Biochemical Inc., Diagnostic Division, Costa Mesa, CA 92626, USA). Radioactivity was determined using the gamma counter (Model No. IC-4702, Electronic Corporation of India, Hyderabad, India). All samples were run in duplicate in a single assay to avoid interassay variation. The intraassay coefficient of variation was 6.5%. Concentrations of plasma LH and FSH were quantitated by RIA following 2nd antibody precipitation method. Carrier free ¹²⁵I for hormone iodination was obtained from Bhaba Atomic Research Centre (Mumbai, India). Pure rat LH (NIADDK-LH-1-6) and FSH (NIADDK-FSH-1-6) were iodinated using chloramine-T (Sigma Chemical Co., St. Louis, MD, USA) according to the method of Greenwood *et al.* (1963). The antisera to LH and FSH, NIADDK anti-r-LH-S-9 and NIADDK anti-r-FSH-S-11 were used at a tube dilution of 1:150,000 and 1:100,000 respectively. The sensitivities of the assays were 0.75 μ g L⁻¹ for LH and 1 μ g L⁻¹ for FSH. All the samples were assayed in duplicate and the intraassay coefficient of variation was <7%. Hormone concentrations were expressed in terms of National Institutes of Health (NIH) reference preparation RP-2.

Quantitative study of spermatogenesis: For histological study of spermatogenesis testis was fixed in Bouin's fixative after a small excision at each of the two poles and processed for section cutting, embedded in paraffin and 5 μ m sections routinely prepared using a microtome. Sections were stained by periodic acid Schiff technique and counterstained with hematoxylin. The relative number of each variety of germ cells at stage VII of the cycle of seminiferous epithelium, i.e., type A spermatogonia (ASg), preleptotene spermatocytes (pLSc), midpachytene spermatocytes (mPSc), step seven spermatids (7Sd) were counted according to the method of Leblond and Clermont (1952) and modified by Biswas *et al.* (2004). The relative numbers of each variety of germ cells were counted in 25 round tubular cross-sections at stage VII of the cycle in each rat. The nuclear diameter of different germ cells was measured using Leitz micrometer. All the nuclear counts (crude counts) were corrected for difference in tubular diameter by the formula of Abercrombie (1946).

Statistical analysis: All values were expressed as Mean \pm SEM. Data were analyzed by ANOVA (model 1 or fixed model) followed by Student's two tailed 't' test (Das, 1981) to test for differences between control and treated experimental groups. Differences were considered significant when p<0.05.

RESULTS

The study shows that there was no significant difference in body weight between the *T. arjuna* treated rats either for 7, 14 or 21 days of treatment and vehicle treated control rats (Fig. 1). Weights of testes and accessory sex organs (epididymes, seminal vesicle and ventral

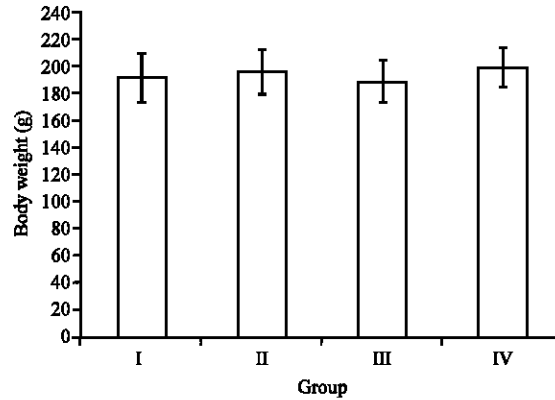


Fig. 1: Effect of *Terminalia arjuna* treatment at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days on body weight in male rats, Group I: Vehicle treated control group, Group II: *Terminalia arjuna* treatment for 7 days, Group III: *Terminalia arjuna* treatment for 14 days and Group IV: *Terminalia arjuna* treatment for 21 days

Table 1: Effect of *Terminalia arjuna* treatment at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days on testicular weight, accessory sex organs' weight in male rats

| Group | mg% b.wt. | | | |
|-------|----------------|---------------|---------------------------------|------------------|
| | Testicular wt | Epididymal wt | Seminal vesicle wt (with fluid) | Ventral prostate |
| I | 1474.66±32.93 | 545.65±23.82 | 472.61±22.81 | 226.28±14.18 |
| II | 1434.89±39.19 | 536.87±18.05 | 450.87±29.38 | 218.36±12.40 |
| III | 1422.39±36.16 | 521.17±17.44 | 446.34±26.78 | 212.42±15.65 |
| IV | 1360.44±28.16* | 489.12±16.55* | 384.16±18.98* | 192.16±13.40* |

Values marked with asterisks are significantly different from control values (values are Mean±SEM of 8 rats group⁻¹), ANOVA followed by Student's two tailed 't' test, where *p<0.05, Group I: Vehicle treated control group, Group II: *Terminalia arjuna* treatment for 7 days, Group III: *Terminalia arjuna* treatment for 14 days and Group IV: *Terminalia arjuna* treatment for 21 days

prostate), were found to be decreased significantly in 21 days treated group in comparison to control group. The testicular weight and seminal vesicle weight (mg% body weight) were reduced from 1474.66 to 1360.44 and 472.61 to 384.16, respectively (Table 1). The activity of key steroidogenic enzymes testicular 17β-HSD and Δ⁵-3β-HSD were significantly reduced in 21 days treated group in comparison with control though for 7 and 14 days group the results were statistically non-significant (Fig. 2a-b). Radioimmunoassay of hormones revealed a significant decrease in the plasma level of testosterone in 21 days treated group compared with control (Fig. 3), but the plasma gonadotrophin levels (FSH and LH) remain unchanged at all of these treatment groups in comparison to control (Fig. 4 and 5).

Quantitative study of spermatogenesis showed a significant decrease in counts of different stages of germ cells on the rats treated for 21 days. The counts for type A spermatogonia (ASg) and step 7 spermatids (7Sd) were decreased from 0.72 to 0.58 and 76.32 to 43.92 respectively (Table 2). Thus, the histological study revealed degeneration of different stages of sperms in the seminiferous tubules (in 21 days treated group). The immature spermatozoa which were lined up along the lumen side of the seminiferous epithelium in the control testis (Fig. 6a) were absent in most of the tubules at stage VII in this 21 days treated group (Fig. 6b).

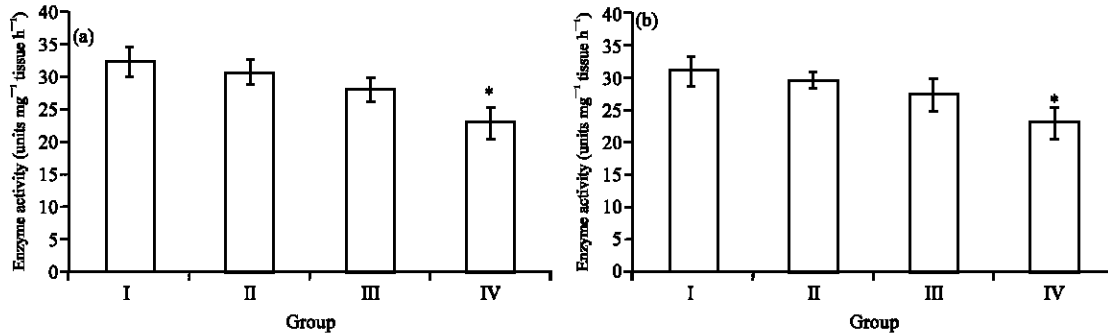


Fig. 2(a-b): (a) Effect of *Terminalia arjuna* treatment at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days on testicular Δ^5 -3 β HSD activity in male rats and (b) Effect of *Terminalia arjuna* treatment at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days on testicular 17 β HSD activity in male rats, Values marked with asterisks are significantly different from control values (Values are Mean \pm SEM of 8 rats group⁻¹), ANOVA followed by student's two tailed 't' test, where *p<0.05

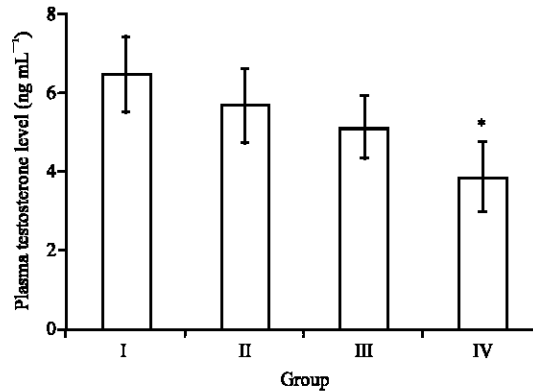


Fig. 3: Effect of *Terminalia arjuna* treatment at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days on plasma testosterone level in male rats, Group I: Vehicle treated Control group, Group II: *Terminalia arjuna* treatment for 7 days, Group III: *Terminalia arjuna* treatment for 14 days and Group IV: *Terminalia arjuna* treatment for 21 days, Values marked with asterisks are significantly different from control values (Values are Mean \pm SEM of 8 rats group⁻¹), ANOVA followed by student's two tailed 't' test, where *p<0.05

DISCUSSION

In the present study it was found that after treatment of *T. arjuna* (at the dose of 150 mg kg⁻¹ day⁻¹) for 21 days there were significant decreases in testicular Δ^5 -3 β -HSD and 17 β -HSD activities when compared with control. Since, these enzymes play important role in steroid hormone biosynthesis, the decreased levels of these enzymes after *T.arjuna* treatment, is therefore, a reflection of reduced testosterone synthesis (Muroso and Payne, 1979). The weight of testes and accessory sex organs are the indicators of a possible alteration in androgen status. Reduction in sex

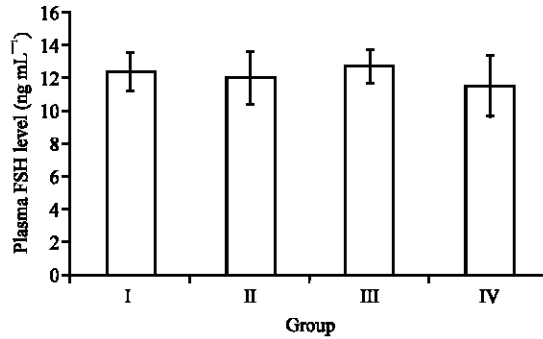


Fig. 4: Effect of *Terminalia arjuna* treatment at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days on plasma FSH level in male rats, Group I: Vehicle treated Control group, Group II: *Terminalia arjuna* treatment for 7 days, Group III: *Terminalia arjuna* treatment for 14 days and Group IV: *Terminalia arjuna* treatment for 21 days

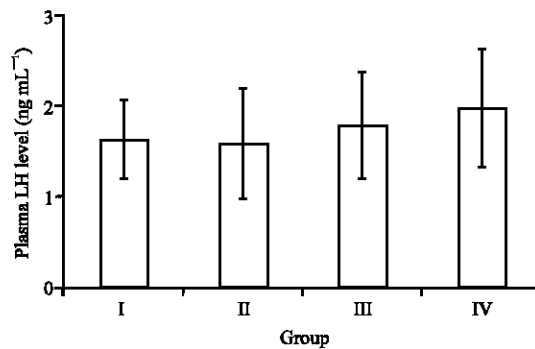


Fig. 5: Effect of *Terminalia arjuna* treatment at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days on plasma LH level in male rats, Group I: Vehicle treated Control group, Group II: *Terminalia arjuna* treatment for 7 days, Group III: *Terminalia arjuna* treatment for 14 days and Group IV: *Terminalia arjuna* treatment for 21 days

Table 2: Effect of *Terminalia arjuna* treatment at the dose of 150 mg kg⁻¹ day⁻¹ for 7, 14 and 21 days on spermatogenesis at stage VII of seminiferous cycle in rats

| Group | No. of germ cells | | | | | |
|-------|-------------------|-------------|-------------|-------------|-----------|----------------------|
| | ASg | pLSc | mPSc | 7Sd | mPSc: 7Sd | 7Sd degeneration (%) |
| I | 0.72±0.04 | 22.43±1.41 | 23.20±0.81 | 76.32±4.04 | 1:3.29 | 17.7 |
| II | 0.69±0.08 | 21.32±1.37 | 22.46±1.55 | 72.09±3.39 | 1:3.21 | 19.7 |
| III | 0.65±0.08 | 21.16±0.96 | 21.87±1.02 | 65.82±6.12 | 1:3.01 | 24.6 |
| IV | 0.58±0.06* | 18.72±1.02* | 19.52±0.84* | 43.92±6.62* | 1:2.25 | 43.7 |

Values marked with asterisk are significantly different from corresponding control values, Values are Mean±SEM of 8 rats group⁻¹ (*p<0.05), ANOVA followed by Student's two tailed 't' test, ASg: Type A spermatogonia, pLSc: Pre-leptotene spermatocyte, mPSc: Mid pachytene spermatocyte, 7Sd: Step 7 spermatid, Group I: Vehicle treated control group, Group II: *Terminalia arjuna* treatment for 7 days, Group III: *Terminalia arjuna* treatment for 14 days and Group IV: *Terminalia arjuna* treatment for 21 day

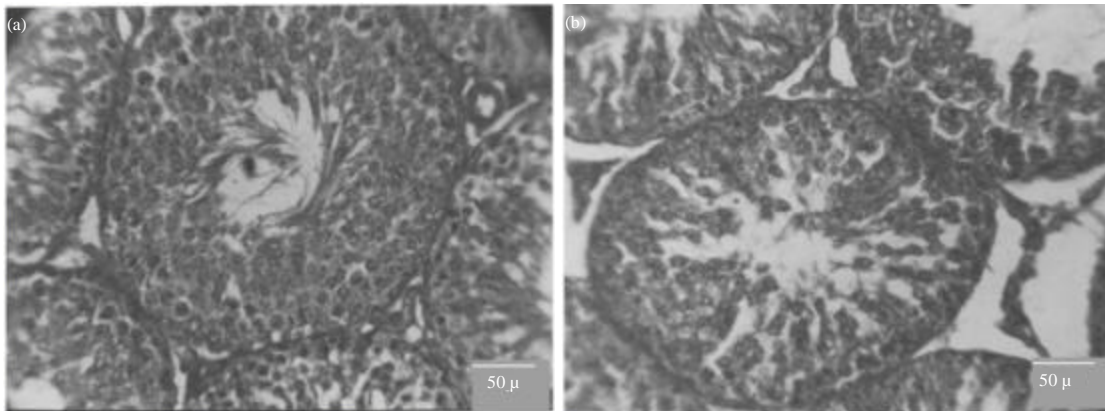


Fig. 6(a-b): (a) Photomicrograph of testicular section at stage VII of seminiferous cycle of vehicle-treated control rats (X 225) and (b) Photomicrograph of testicular section at stage VII of seminiferous cycle of rats treated with *Terminalia arjuna* (at the dose of $150 \text{ mg kg}^{-1} \text{ day}^{-1}$) for 21 days (X 225)

organ weight observed in the present study in the 21 days treated group suggests that androgen secretion was suboptimal or that testicular endocrine function had been compromised. Testicular size and weight are normally regulated by fluid secretion from Sertoli cells (Waites and Gladwell, 1982) and the production of sperms in the seminiferous tubules (Aman, 1970). Hence, testicular weight less than control values in this 21 days treated rats is probably due to degeneration of germ cells and Sertoli cells. This finding can be supported by previous observation where oral administration of this bark extract resulted in decreased testicular and accessory sex organs' weight (Chauhan *et al.*, 1990).

The quantitative analysis of the seminiferous epithelium at stage VII of the cycle revealed a significant decrease of different germ cells such as type A spermatogonia (ASg), preleptotene spermatocyte (pLSc), etc. in 21 days treated group in comparison with control. Theoretically, the mPSc: 7Sd ratio should be 1: 4 (Clermont and Morgentaler, 1955), but the same ratio became 1: 3.29 in the vehicle treated control rats. It indicates 17.7% spermatid degeneration. This ratio became 1: 2.24 in 21 days treated rats, indicating that during the process of spermatocyte to spermatid conversion, 43.25% of the cells degenerated. Previous reports of antispermatogenic effects of *T. arjuna* in rats (Jha and Dixit, 1986; Chauhan *et al.*, 1990) are thus confirmed by this present quantitative study of spermatogenesis.

In this study, there was no significant difference in plasma levels of FSH and LH in any of the treated groups compared to control. There is general consensus that spermatogenesis in rats requires a normal level of FSH and LH (Choudhary and Steinberger, 1975; Almiron *et al.*, 1984; Kula, 1988). At the same time, it has also been reported that the spermatogenic process is testosterone dependent (Steinberger, 1971; Biswas *et al.*, 1983) and testosterone along with other testicular androgens can initiate and maintain spermatogenesis (Santen, 1999; Weinbauer *et al.*, 2001). Therefore, in the 21 days treated group reduced testosterone level resulted in degeneration of germ cells and spermatogenic arrest. Again, it has been reported that a Sertoli cell secreted

protein (hSCP-80) controlled by pachytene spermatocytes (Register *et al.*, 1995) stimulates Leydig cell steroidogenesis (Onoda *et al.*, 1991). Thus, decreased counts of spermatocytes found in the 21 days treated group may lead to reduced steroidogenesis.

The exact mechanism by which *T. arjuna* bark extract treatment resulted in reduced gametogenesis and steroidogenesis in male rats cannot be evaluated from the present study. The active constituents present in this plant product include tannins, triterpenoid saponins (arjunic acid, arjunolic acid, arjungenin) some flavonoids, and β -sitosterol (a phytosterol) (Miller, 1998). Among these, β -sitosterol has been reported to decrease gonadal steroidogenesis in fish and diminish sperm counts in rats (Nieminen *et al.*, 2008). It has been further reported that this phytosterol may impede with spermatogenesis, oogenesis and gonadal steroidogenic system in zebrafish (Christianson-Heiska *et al.*, 2007). It causes disturbance to the reproductive system by changing sex ratios in this species (zebrafish) (Nakari and Erkomaa, 2003). Probably, hypocholesterolemic effect of this plant (Gupta *et al.*, 2001) resulted in decreased cholesterol (substrate) availability for steroidogenesis. Alternately, it may also inhibit the activity of cholesterol side chain cleavage enzyme P_{450sc} (MacLachy and Van Der Kraak, 1995) the key-enzyme converting cholesterol to pregnenolone (Elliott and Elliott, 2005). Thus, decreased levels of testicular Δ^5 - 3β -HSD and 17β -HSD and the resultant reduced plasma testosterone level found in this study might be due to the action of β -sitosterol. Further studies are required to elucidate the exact mechanism of reproductive dysfunction in male rats induced by oral administration of alcoholic bark extract of *Terminalia arjuna*. Moreover, during medicinal use of this bark extract, its adverse effect should be duly taken into consideration.

Therefore, it can be concluded that alcoholic bark extract of *Terminalia arjuna* at a particular dose and duration decrease testicular and accessory sex organs' weight, spermatogenic and testicular steroidogenic capacity inhibiting reproductive activity in male rats.

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