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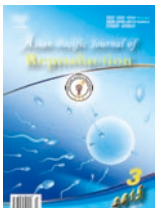

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Antifertility effect of aqueous-ethanolic (1:1) extract of the fruit of *Terminalia chebula*: Rising approach towards herbal contraception

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ABSTRACT

Objective: To explore the anti-fertility efficacy of aqueous-ethanolic (1:1) extract of fruits of *Terminalia chebula* (*T. chebula*). **Methods:** Aqueous-ethanolic (1:1) extract of fruit of *T. chebula* was administered orally at a dose of 60 mg/0.5 mL distilled water/day for 28 days. Different parameters were studied including body weight, relative weight of reproductive organ, sperm motility, sperm count, testicular cholesterol, plasma testosterone, testicular androgenic key enzymes such as Δ^5, 3β-HSD and 17-β-HSD, bio-markers of oxidative stress, toxicity study and histological analysis of the tissues. **Results:** The treated group showed a significant diminution in spermatogenic profile. On the other hand testicular cholesterol showed a significant elevation in *T. chebula* treated group and plasma testosterone was decreased significantly in comparison to control. The above said androgenic key enzymes were exerted a significant diminution in extract treated group. Anti-oxidative enzymes such as catalase and superoxide dismutase showed a significant reduction, and a significant elevation in the level of conjugated diene and thiobarbituric acid reactive substance was noted in treated group. GOT and GPT study of liver and kidney showed a non-significant change which confirmed the non-toxic nature of *T. chebula*. Histological study of testis of treated group exhibited significant reduction in seminiferous tubular diameter. **Conclusion:** The results of present experiment suggested that the aqueous- ethanolic (1:1) extract of fruit of *T. chebula* exerted a significant anti-spermatogenic effect in male rat.

1. Introduction

Population explosion is creating so many obstructions worldwide day by day. This overpopulation can be checked through biological means with special reference to modulation in the human fertility ability. Along with the advancement in the reproductive biomedicine different hormonal contraceptive pills are developing but all have side effects. Epidemiological studies showed that the oral hormonal contraceptives are inflating the risks of cerebral thrombosis, rise in serum triglyceride as well as cholesterol[1, 2], and it is also responsible for creating malignant tumors, abdominal pain, headache, diabetes, nausea and menstrual changes[3, 4]. Oxynol-9 which is present in a potent spermicidal agent has shown a tendency of inflammation and ulceration in genital organ and even the risk of HIV-I infection of its repeated use[5]. So, formulation of new herbal medicines has become a growing trend in modern ongoing experiments which includes the use of different plant parts extract having anti-spermatogenic activity but their exact mechanism of action is not cleared. Initiative has been taken globally to find out the efficacy of herbal product for male contraception[6]. A few

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herbal formulations have already been developed but their mode of action is still beyond our knowledge. A number of plants such as *Acalypha indica* (*A. indica*), *Praneem vici* (*P. vici*), *Carnica papaya* (*C. papaya*), *Alstonia scholans* (*A. scholans*), *Albizzia lebbeck* (*A. lebbeck*), *Mentha arvensis* Linn. (*M. arvensis*), *Jatropha curcus* (*J. curcus*), *Tinospora cordifolia* (Willd.) (*T. cordifolia*) etc. have been studied to search out their spermicidal property[7-10]. These herbal contraceptives are health friendly, easily available and also pocket friendly even in rural areas. Experiments also have been conducted previously in our laboratory regarding the antifertility effect of the leaf extract of *Stephania harnandifolia* (*S. harnandifolia*) [11, 12] and composite extract of *Acyranthus aspera* (*A. aspera)* and *Stephania hernandifolia* (*S. hernandifolia*) as herbal spermicidal agent by doing invitro study on human sperm [13-15]. *Terminalia chebula* (*T. chebula*) has a long term folk medicine reputation for the fertility management of male. In Northern part of India as well as in remote area in our state, this fruit is used as contraceptive medicine though the scientific basis of the action of this plant is beyond our knowledge. A scientific report has been published regarding the inhibition of hyaluronidase activity of human and rat spermatozoa *in vitro* and anti-spermatogenic activity in rats *in vivo* by *T. chebula*, a flavonoid rich plant[16]. Inspite of that there is no routine and scientific study about the male contraceptive efficacy of fruits of *T. chebula*. So, the present investigation was conducted to focus the antifertility effect of the aquous ethanolic (1:1) fruit extract of *T. chebula* following some bio-chemical and spermiological sensors.

2. Materials and methods

2.1. Preparation of plant extract

T. chebula was collected from local area and they were identified and authenticated by Botany department of our University. The fruits of *T. chebula* were dried, powder and extracted in aqueous-ethanol (1:1) at 37 ℃ for 48 hours. The extract was then filtered and the filtrate was dried in rotary evaporator.

2.2. Animals and treatment

Adult, proven fertile male Wistar strain rats, weighing (150±10) g of (80±5) days were selected. The rats were housed in cages under standard conditions [(12h light/12 h dark, (25 ±2) ℃] and were kept for 15 days for acclimation prior to experimentation. They were provided with standard chew and water *ad libitum*. Animals were divided into two groups. Each group comprised of 6 animals as detailed below:

Group I (Control): Animals were provided with 0.5 mL/100 g of distilled water per day.

Group II (Treated with *T. chebula* at the dose of 60mg): Animals were provided with oral administration of aqueous-ethanolic (1:1) extract of T. chebula at the dose of 60mg/ 0.5ml distilled water/100 g body weight per day for 28 days.

2.3. Routine sperm analysis

2.3.1. Sperm motility

Sperm motility was assessed by the method described by Zemjanis and would be evaluated microscopically within 2-4 minutes of their isolation from the cauda epididymis and later expressed as percentages[17].

2.3.2. Sperm count

Epididymal sperm count was obtained by mincing the four pairs of cauda epidydimis in distilled water and filtering through a nylon mesh. The spermatozoa would be counted by hemocytometer using Neubauer (Deep 1/10 mm, LABART, Germany) chamber described by Pant and Srivastava[18].

2.4. Estimation of the activity of androgenic key enzymes

2.4.1. Measurement of testicular Δ^5, 3β-hydroxysteroid dehydrogenase (HSD) activity

Testicular Δ^5, 3β-HSD was measured by standard method[19]. Decapsulated testicular tissue was homogenized carefully at 4 ℃ in a 20% spectroscopic grade glycerol containing 5 mM of potassium phosphate and 1 mM EDTA at a tissue concentration of 100 mg/mL of homogenizing mixture. This mixture was centrifuged at 10 000 g for 30 min and the supernatant was collected. 1 mL of supernatant was mixed with 1 mL of 100 μM of sodium pyrophosphate buffer, pH 8.9 (Loba Chemical Company, Mumbai, India), 40 μL of ethanol containing 30 μg of dehydroepiendosterone and 960 μL of 25 mg% bovine serum albumin (BSA) so that the volume of incubation mixture become 3 mL. Enzyme activity was measured after addition of 100 μL of 0.5 μM nicotinamide adenine dinucleotide (NAD) to the tissue supernatant mixture in spectrometer cuvette at 340 nm against a blank (without NAD). Optical density (OD) was recorded at 30 sec interval for 3 min. One unit of enzyme activity was the amount causing a change in absorbance of 0.001/min at 340 nm.

2.4.2. Measurement of Testicular 17β-hydroxysteroid dehydrogenase (HSD) activity

17β-HSD activities were measured biochemically[20]. Supernatant prepared for Δ^5, 3β-HSD activity was used here also. 1 mL of the supernatant was added with 440 μM sodium pyrophosphate buffer pH 10.2, 40 μL ethanol containing 0.3 μM testosterone (Sigma Chemical Company, St Loius, MO. USA) and 960 μL of 25 mg% of BSA that makes the incubation mixture a total of 3 mL. Enzyme activity was assessed after addition of 100 μL of 0.5 μM NAD to the tissue supernatant mixture in spectrometer cuvette at 340 nm against a blank (without NAD). OD was recorded at 30 sec interval for 3 min. One unit of enzyme activity was the amount causing a change

in absorbance of 0.001/min at 340 nm.

2.5. Analysis of biochemical sensors:

2.5.1. Estimation of testicular cholesterol level

Testicular cholesterol was estimated by a standard method [21]. In a centrifuge tube, 10 mL of alcohol – acetone mixture and 0.2 mL tissue homogenate prepared on phosphate buffer (pH 7.0) was taken. Tubes were immersed in a boiling water bath till the solvent begins to boil. They were then cooled at room temperature and centrifuged. The supernatant was collected and allowed to evaporate to complete dryness. The residue was dissolved in 2 mL of chloroform. Series of cholesterol standards were prepared. In the test tube marked as blank, 2 mL of chloroform was taken. In each tube of sample standard and blank, 2 mL of acetic anhydride sulfuric acid mixture was added and mixed thoroughly. All the tubes were placed in a dark place at room temperature for 15 min. Reading was noted at 680 nm. From standard curve, concentration of cholesterol in unknown sample was calculated.

2.5.2. Estimation of plasma testosterone

Plasma was obtained by centrifuging the collected heparinized blood. Plasma testosterone was measured according to the standard protocol of National Institute of Health and Family Welfare (NIHFW) [22], using the testosterone kit of EQUIPAR, USA. 25 μL of each standard or sample was dispensed into appropriate well followed by addition of 100 μL of enzyme conjugate containing horseradish peroxidase (HRP) and mixed. The strips were incubated for 60 min at 37 ℃. The reaction solution was decanted forcefully from all the wells followed by three washing. 100 μL of tetra methyl benzidine (TMB) substrate containing chromogen was added and after scheduled time the reaction was stopped by addition of stop solution supplied in the kit. The absorbance of standards and samples were read against the blank at 450 nm. Testosterone concentration in the sample was calculated based on the five standards supplied. Its cross reactivity with other androgens was 0.9%. Intra assay variation was 6.2%. Inter assay variation was omitted as all the samples were assayed at a time.

2.6. Oxidative stress related bio-sensors

2.6.1. Estimation of thiobarbituric acid reactive substance (TBARS)

TBARS standard method was followed[23]. Testis and sperm pellet were considered for the quantification of TBARS. Cauda epididymis was incised and washed in normal saline to liberate the sperm and centrifuged. The testis pellet were collected and was homogenized in 0.5 M Tris-HCl buffer solution (pH 7.0). The homogenate at the volume of 0.5 mL was mixed with 0.5 mL of normal saline and 2 mL of thiobarbituric acid- trichloro acetic acid (TBA-TCA) mixture, and then boiled at 100 ℃ for 10 min. This mixture was then cooled at room temperature and centrifuged at 4 000 g for 10 min. The whole supernatant was taken in spectrophotometer cuvette and OD was noted at 535 nm.

2.6.2. Estimation of conjugated diene (CD)

Quantification of the CD was performed biochemically[24]. Testis and sperm pellet were considered for the quantification of CD. The incised epididymis was washed in normal saline to liberate the sperm and then centrifuged. The testis and pellet were collected and was homogenized in 0.1M of ice-cold phosphate buffer (pH 7.4).The lipid was extracted with chloroform-methanol (2:1) mixture followed by centrifugation at 10 000 g for 5 min. The chloroform layer was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.5 mL of cyclohexane and the absorbance was noted at 233 nm to measure the amount of hydro-peroxide formed and was expressed in nM/mg of tissue.

2.6.3. Estimation of the activities of catalase

Catalase (CAT) activity was measured by a standard method[25]. Testis and sperm pellet were considered for the estimation of the level of CAT. For evaluation of catalase activity in sperm pellet, cauda epididymis was incised and washed in normal saline to liberate the sperm and centrifuged. The testis and pellet were collected and was homogenized in 0.5 M Tris-HCl buffer solution (pH 7.0). Homogenates were centrifuged at 10 000 g at 4 ℃ for 10 min. In a spectrophotometric cuvette, 0.5 mL of hydrogen peroxide (H_2O_2) and 2.5 mL of distilled water were taken and mixed well. Reading of absorbance was noted at 240 nm. 40 μL of supernatant from the homogenate after centrifugation was added and subsequent six readings were noted at 30 sec interval.

2.6.4. Estimation of the activity of the superoxide dismutase

For the measurement of superoxide dismutase (SOD) activity, standard protocol should be followed [26].

2.7. Assessment of toxicity parameters

For the assessment of metabolic toxicity, GOT and GPT activities of liver and kidney were estimated [27].

2.8. Histological studies

Testes were embedded in paraffin block, sectioned at 5 μm thickness and stained with haematoxyline and eosin. The prepared slides were observed under high power objective in a trinocular microscope, which was handled with a computer. Photograph of a particular field was taken. Seminiferous Tubular Diameter (STD) was measured with the "Dewinter caliper pro 3.0 software".

2.9. Statistical analysis

Statistical significance of difference in two variables i.e. treated group and control groups were evaluated by using two-tail *t*-

test[28]. Difference of data (Mean± SE, n=6), P<0.05 was statistically considered as significant.

3. Results

3.1. Body weight and relative weight of reproductive organs

After 28 days of oral administration of aqueous-ethanol (1:1) fruit extract of *T. chebula* to male rats, there is no significant alteration (P>0.05) in the body weight (Table 1). However, the relative weights of reproductive organs such as testis, epididymis, seminal vesicle were decreased significantly (P<0.05) in the treated group when compared with control (Table 1).

3.2. Sperm count and sperm motility

Sperm count of cauda epididymis was reduced significantly (P<0.05) after the treatment for 28 days when comparison was made with control (Table 2).

Sperm motility has shown a significant diminution (P<0.05) in treated group in respect to control group (Table 2).

3.3. Testicular cholesterol

Level of testicular cholesterol showed a significant elevation (P <0.05) in the group treated with *T. chebula* for 28 days in respect to control (Figure 1).

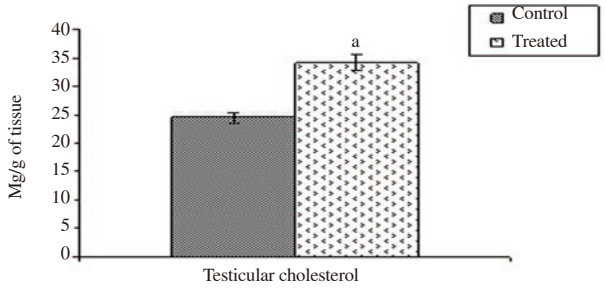

Figure 1. Aqueous-ethanol (1:1) fruit extract of *T. chebula* on testicular cholesterol.
Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test. Bar with superscript 'a' differ from control significantly, P< 0.05.

3.4. Plasma testosterone

Plasma level of testosterone was decreased significantly (P<0.05) in aqueous-ethanol (1:1) fruit extract of *T. chebula* treated group when compared with control (Figure 2).

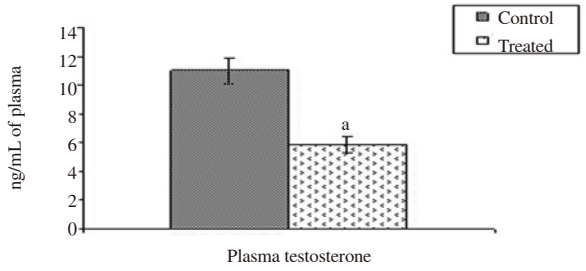

Figure 2. Efficacy of aqueous-ethanol (1:1) fruit extract of *T. chebula* on plasma testosterone.
Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test. Bar with superscript 'a' differ from control significantly, P<0.05.

3.5. Activity of testicular Δ^5, 3β–HSD and 17β–HSD

After 28 days of concern extract treatment, the activity of testicular Δ^5, 3β-HSD and 17β-HSD were exhibited a significant reduction (P <0.05) in comparison to control group (Figure 3).

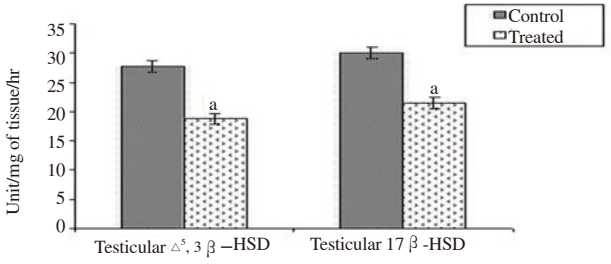

Figure 3. Effectiveness of aqueous-ethanol (1:1) fruit extract of *T. chebula* on androgenic key enzymes i.e. Δ^5, 3β-HSD and 17β-HSD.
Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test. Bars with superscript 'a' differ from control significantly, P< 0.05.

3.6. Activities of catalase and superoxide dismutase

Two important anti-oxidative enzymes are CAT and SOD. Activities of CAT in sperm pellet and testis had shown a significant diminution (P<0.05) in *T. chebula* treated group for 28 days in respect to control (Figure 4).

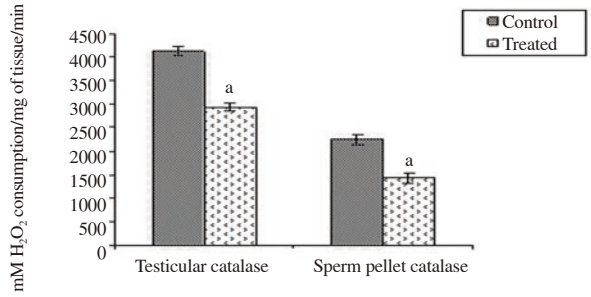

Figure 4. Activity of aqueous-ethanol (1:1) fruit extract of *T. chebula* on catalase in testis and sperm pellet.

Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test. Bars with superscript 'a' differ from control significantly, P< 0.05.

SOD activity in testis as well as sperm pellet had also shown a significant diminution (P<0.05) in treated group when comparison was made with control (Figure 5).

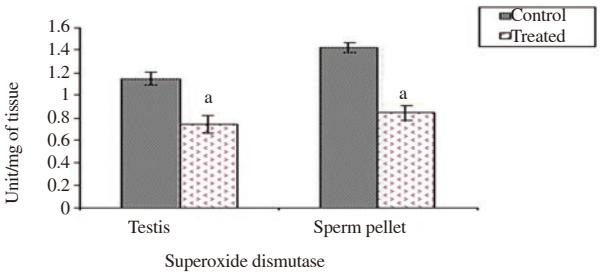

Figure 5. Impact of aqueous-ethanol (1:1) fruit extract of *T. chebula* on SOD in testis and sperm pellet.
Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test. Bars with superscript 'a' differ from control significantly, P< 0.05.

3.7. Levels of CD in sperm pellet and testis

CD levels in sperm pellet and testis after the treatment of aqueous-ethanol (1:1) extract of *T. chebula*, showed a significant increase (P <0.05) when compared with control (Figure 6).

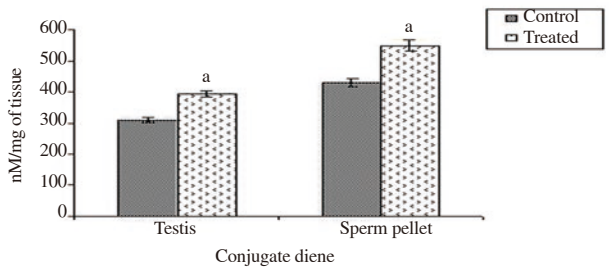

Figure 6. Effect of aqueous-ethanol (1:1) fruit extract of *T. chebula* on conjugated diene.
Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test. Bars with superscript 'a' differ from control significantly, P< 0.05.

3.8. Levels of TBARS in testicular tissues

Another free radical by product TBARS in the said tissues in the *T. chebula* treated group increased significantly (P<0.05) in respect to control (Figure 7).

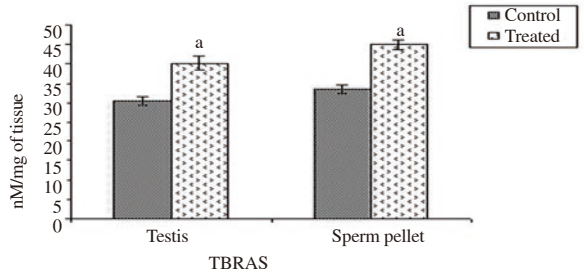

Figure 7. Activity of aqueous-ethanol (1:1) fruit extract of *T. chebula* on estimation of Thiobarbituric Acid Reactive Substance.
Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test. Bars with superscript 'a' differ from control significantly (P< 0.05).

3.9. Activities of GOT and GPT

GOT and GPT activities in liver and kidney of the animals treated with said extract did not exhibit any significant alteration (P>0.05) compared with control (Table 2).

4.0. Histological study

Treatment with aqueous-ethanolic (1:1) extract of fruit of *T. chebula* exhibited a significant diminution in the Seminiferous Tubular Diameter (STD) of the testis after the treatment of 28 days (Figure 8).

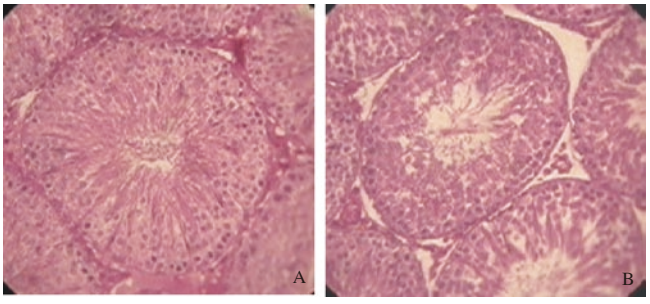

Figure 8. Histology of testis, 400 × (Haematoxylin-Eosin Stain).
Representative microphotograph of rat testis showing the difference in the seminiferous tubular diameter (STD) and sperm population in tubule between control rat (A) and *T. chebula* treated rat (B).

Table 1
Effect of *T. chebula* fruit extract on body weight and relative weight of reproductive organs.

Group	Initial body weight (g)	Final body weight (g)	Testiculo somatic index (g%)	Seminal vesiculo somatic index (g%)	Epididymal somatic index (g%)
Control	120.000±4.080	150.830±2.380	1.690±0.040	0.520±0.043	0.330±0.039
Treated	123.330±5.580	157.500±3.810	1.340±0.049[a]	0.330±0.031[a]	0.200±0.028[a]

Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test.Values with superscript 'a' in each vertical column vary from control significantly, P<0.05.

Table 2

Sperm count and sperm motility and level of GOT and GPT activities in liver and kidney after the treatment with *T. chebula* fruit extract.

Group	GOT activity (Unit/mg of tissue)		GPT activity (Unit/mg of tissue)		Sperm count (Million/mL)	Sperm motility (%)
	Liver	Kidney	Liver	Kidney		
Control	29.16±0.90	25.33±0.88	25.50±1.20	20.66±1.40	24.00±1.15	88.16±1.64
Treated	32.83±1.30	27.83±1.27	28.00±1.06	24.66±0.95	15.16±0.83[a]	61.00±3.04[a]

Data were expressed in terms of Mean± SEM (n=6) followed by 'two tail' t-test. Values were presented with superscript 'a' vary from control significantly, $P< 0.05$.

4. Discussion

The present study showed that the oral administration of aqueous-ethanolic extract of the fruit of *T. chebula* at a dose of 60 mg/ 100 g body weight per day results inhibition in spermatogenesis when treated for 28 days. The said extract out of other solvent extracts and the said dose are most promising which has been identified by our trial and error method in pilot work. The fruit extract had no growth hindering factor as there was no significant change in the initial and final body weight of treated group but the seminal vasculosomatic, testiculosomatic, epididymal somatic indices in treated group exhibited significant reduction in their weight when compared with control. This variation proved that weight of the organ may change on the basis of the treatment related effects and deviation in organ weight in different groups also supports the alteration in steroidogenesis[29, 30].

An individual's fertility status can be identified by assessing the sperm count and sperm motility which indicated the semen quality[31]. The downward deviation of said parameters proved the impaired male androgenesis[32]. Treated group showed a rise in testicular cholesterol that indicates the inhibition in testicular androgenesis[33] as cholesterol is the mother molecule or precursor molecule for male androgenesis[34]. Significant reduction in plasma testosterone in treated group supported inhibition in the steroidogenic enzyme activity.

Δ^5, 3β-HSD and 17β-HSD, the androgenic key enzymes also significantly decreased in the extract treated group of the fruit of *T. chebula* in respect to control and this is perhaps due to the inhibition in the secretion of pituitary gonadotrophins[35, 36]. Reproductive impairment due to the fruit extract resulted oxidative stress which is associated with imbalance between antioxidant defence system and production of reactive oxygen species (ROS) [37]. In this respect, we also measured the antioxidant enzyme activities in testis and sperm pellet. SOD and catalase are two antioxidative enzymes having free radical scavenging activity in male reproductive organs[38]. The present study showed a testicular impact by significant decrease in the SOD and catalase activity in the said testicular tissue. Increased level of free radical by-products such as CD and TBARS in the said tissues further supported the generation of ROS in the testicular tissue.

Non toxic effect of the aqueous-ethanolic (1:1) fruit extract of *T. chebula* was proved by the non significant alteration in GOT and GPT levels in compared to control as GOT and GPT are the indicators of metabolic toxicity[39]. Therefore it may be stated that the aqueous-ethanolic (1:1) fruit extract of *T. chebula* has antifertility effect without creating any toxicity on metabolic organs.

From this study, it may be concluded that aqueous-ethanolic (1:1) extract of fruit of *T. chebula* has potent ability to induce antifertility effect by decreasing spermatogenesis, activities of androgenic key enzymes, plasma testosterone and increasing the testicular cholesterol without creating hepato as well as reno toxicity. More information is required for better understanding about the anti-fertility effect of the concerned plant extract in male reproductive physiology. This study may develop a hope to the pharmaceutical industries in near future by introducing a herbal contraception in modern age of herbal drug technology.

Conflict of interest statement

We declare that we have no conflict of interest.

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