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Research Article

Pre-clinical Study of Hydro-ethanol (60:40) Extract of *Areca catechu* (L.) on Testicular Androgenesis in Albino Rat: An Approach for Herbal Male Contraceptive Development

Sukriti Hazra, Dibya Pal, Tanusree Mondal, Shibani Das, Debidas Ghosh*

Molecular Medicine, Nutrigenomics and Public Health Research Laboratory, Department of Bio-Medical Laboratory Science and Management, Vidyasagar University, West Bengal, India.

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ABSTRACT

The present study was designed to determine the anti-testicular activity of hydro-ethanol seed extract of *Areca catechu* (10, 20 and 40 mg) in a dose-dependent fashion in albino rats. Seminal vesicular fructose (SVF), oxidative stress sensors, gene expression, somatic weight, reproductive organo-somatic indices, spermiological, hormonal profile, and testicular histological examination for qualitative and quantitative investigations of spermatogenesis were observed. In compared to the placebo-treated control (PTC), the exposure of the said doses showed a statistically significant downward deviation in the spermiological, hormonal, SVF profile, the kinetics of testicular key androgenic dehydrogenase enzymes, and diameters of seminiferous tubules. Antioxidant enzyme kinetics were reduced significantly against PTC, and the quantity of malondialdehyde in male gonad and sperm precipitate was elevated in a significant level ($p < 0.05$). Expression of the apoptotic promoting gene in testis was increased ($p < 0.05$) and anti-apoptotic Bcl-2 gene expression exhibited a significant decrease ($p < 0.05$) in the treated group compared to the PTC. The alterations in the activities of phosphatases in hepatic tissue were not significant from the background of PTC, suggesting that the plant has no systematic undesirable effect in physiological processes. The observation highlighted that 10 mg dose as the threshold dose for imposition of such anti- testicular activity leading to male contraception.

Introduction

The world's largest problem right now is population expansion.[1] Overpopulation has many undesirable impacts on economic and environmental advancement. Contraception is the most widely used and well-known strategy for managing the expanding population. Female contraceptive options in the market, with hormonal and chemical contraceptives being more prevalent than male contraceptives. Male contraception could be a step towards family planning and have a major impact on the global reduction of unwanted pregnancies.[2] Many people in developing nations use herbal birth control solutions since using contraceptives have a number of negative effects, including tumor, hypertension, cerebral and cardiac stroke, and diabetes in respect to chemical contraceptive pills or tablets.[3] Herbal remedies have little or no negative effects, are inexpensive, and readily accessible. In this field of contemporary study, plant extracts possessing anti-spermatogenic properties have gained popularity. According to previous studies, antifertility activities have been noted in many traditional herbs like *Abrus precatorius, Cajanus cajan* (L.), *Acacia nilotica, Aristolochia indica*.[4] *Arecanut catechu* commonly known as betel nut.[5] The betel nut fruit has an oval or spherical form. Chewing the seeds of *A. catechu* is a popular habit in many communities of different states in our country especially in the eastern

***Corresponding Author:** Prof. Debidas Ghosh

Address: Molecular Medicine, Nutrigenomics and Public Health Research Laboratory, Department of Bio-Medical Laboratory Science and Management, Vidyasagar University, West Bengal, India.

Email ✉**:** debidasghosh999@gmail.com

Tel.: +91-9232690993

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zone of India and other countries like Bangladesh, Sri Lanka, and Pakistan.[6] In Buddhist scriptures, it is well written that *A. catechu* has many therapeutic activities.[7] Betel nut delivers some pharmaceutical properties for the rectification of inflammatory, ulcer, and diabetes.[6] The potential male contraceptive properties of *A. catechu* (seed) have not been thoroughly studied scientifically till now. The ultimate goal of this study was to assess the dose-dependent contraceptive ability for male of *A. catechu* seed.

MATERIALS AND METHODS

Plant Extract

A. catechu seeds were brought from local vendor of Midnapore town and confirmed by the expert of the Botany and Forestry department, Vidyasagar University, Midnapore- 721102, and the registered number of the said sample was "*A. catechu*/VU/Bio/12/20". The seeds were processed for drying in room temperature and then mashed. The aqueous-ethanol (60:40) solvent was used for dissolving the above seed powder at the weight/volume ratio of 100 g in 1 liter, the resulting solution was then stored at 25 to 28°C for 48 hours while being periodically shaken. Whatman NO-1 filter paper was used to filter the aforementioned solution. Using a rotary evaporator, the resultant solvent was allowed to dense, and the obtained powder (2.58 g) was stored in a shielded glass container for later use.

Animal Care

Mature male Wistar strain rats were kept in animal cages with appropriate lighting and darkness (light:dark=1:1, 25 ± 2°C) for 2 weeks to allow the experimental animals for acclimatization. The rats were acquired from a vendor licensed by the Committee for Control and Supervision of Experiments on Animals (CCSEA). Animal feed and drinking water were provided as per the demand of the animals. The protocol of the experiment was framed as per the approval of Institutional Ethics Committee (IEC) (Ethical clearance no VU/IAEC- I/DG-2/3-16/19. Dt.11.12.2019.). Total numbers of animals were categorized into 4 groups along with six rats in each group. Every day at 10:00 AM, oral gavage was used for treatment. To avoid potential nutrient-phytomolecule interactions, no meal was provided for two hours before and following the extract administration.

Rat Treatment

Placebo-treated control (PTC)

During the period of experiment distilled water (DW) was gavaged at the quantity of half mL/100 g of somatic weight (SW) of rat per day for 4 weeks.

Extract treated group (10 mg)

Each rat of this category was subjected for treatment at the quantity of 10 mg seed extract/half mL DW/100 g of SW/day for 4 weeks.

Extract treated group (20 mg)

Effective seed extract was ingested at the quantity of 20 mg/half mL DW/100 g of SW in each day for 4 weeks.

Extract treated group (40 mg)

The said seed extract was allow to charge orally at the quantity of 40 mg/half mL DW/100 g of SW in each day for 4 weeks.

Using euthanasia, required biological samples were collected from all of the rats through standard protocol on 29th day from the starting of the experiment. At intervals of 7 days, the body weights were also measured. Following the extraction of epididymal spermatozoa through a caudal epididymis incision, spermiological sensors were assessed. Blood was drawn into a heparinized syringe, centrifuged at 3000 X g, and serum was separated to determine the level of testosterone. Dissection was used to obtained testes and other male accessory sex organs. Right testis and liver of each rat were maintained at -20°C for the investigation of biochemical and genomic sensors while the left testis was placed in tissue fixative (Bouin's) for histological observation.

Sperm Count and Motility

Spermatozoa were counted microscopically using a Neubauer hemocytometer chamber, and the results were displayed as a count of spermatozoa in million per mL milliliters of suspension.[8] Normal saline (1-mL) was used for dispersing 100 µL of epididymal spermatozoa in a 10:1 ratio. Subsequently, a light microscope was used to count the motile spermatozoa, and percentages were computed following the counting of 150 sperm throughout the slides and it is was transformed into percentage count.[9]

Status of acrosome cap (SAC)

Sperms were aspirated from cauda epididymis using normal saline, and the sample was diluted at a ratio of 10:1 using a solution of D-glucose and phosphate buffer. Sperm suspension samples (25 µL) was applied on uniform gelatin-coated glass slides and allow for incubation for two hours at body temperature (37°C) in a wet chamber. The percentage of sperm with holes in their sperm caps was calculated and reported.[10]

Sperm swelling test

A solution of (SST) was made using sodium citrate and fructose at the quantity of 0.735 and 1 g respectively as per the validated method. Following a 2 hour incubation period, 100 µL of sperm suspension was added to 1 mL of HOS solution to maintain the ratio of 1:10. The value of this sensor was expressed as a percentage of sperm cells with coiled tails or swollen.[11]

Sperm viability test

Caudal epididymis was used for sperm collection by giving a smooth and fine incision followed by a gentle injection

of a fixed volume of physiological saline and its aspiration followed by its delivery in a petri plate. The numbers of viable and non-viable sperm were ascertained using the eosin–nigrosine staining method. On a transparent glass slide, 50 µL of diluted epididymal fluid was applied, along with eosin and nigrosine at the quantity of 50 and 100 µL, respectively. Then, using those produced slides and a 400X microscope, the viable sperm count was determined.[12]

Nuclear chromatin decondensation (NCD) test

The sperm precipitate was rinsed two times using borate buffer of 0.05 M and pH 9.0. After that, 900 µL of a solution containing 1% SDS and 6 mM EDTA were added in a container for their proper mixing. The preparation was mixed with 100 µL of sperm precipitation in above buffer. The reaction was ended using an equal volume of 2.50% glutaraldehyde. About 15 µL of the mixture and 5 µL of the required concentration of cytological dye were added in a micro fuse tube. The prepared mixture was used for smear on a glass slide and a cover slip was used to cover it. The sperms with uncoiled chromatin were noted and the value were expressed in percentages under a 400X magnificent light microscope.[13]

Kinetics of Testicular Key Androgenic Dehydrogenases

Standardized methods were used for testicular Δ5,3 β -hydroxysteroid dehydrogenase (HSD) and 17β-HSD kinetics assessment.[14,15] Testicular tissue was allowed for homogenization at weight/volume 100 mg/mL using phosphate buffer (5 mM) containing 20% analytical category of glycerol and EDTA (1 mM). After centrifugation for standard time as per method, the supernatant was used for noting the optical density at 340 nm and result was expressed as unit/mg of tissue/hour.

FSH and LH

Serum FSH and LH levels were measured using the immunoassay method. The hormonal kit (Wuhan Fine test, China) was utilized for this purpose.[16]

Oxidative stress markers

For assessing the kinetics of superoxide dismutase (SOD) and peroxidase in the testicular tissue and sperm precipitation, standard protocols were followed.[17,18] Phosphate buffer saline (pH- 7.4) of 0.1 M strength was used for homogenization and after centrifugation supernatant was used along with required chemicals for recording OD at 420 and 436 nm, respectively.

For quantification of malondialdehyde (MDA), the same supernatant was used along with required chemicals as per method and the OD was measured at 535 nm.[19]

Serum testosterone

Serum testosterone was measured using testosterone ELISA kit and solid-phase sandwich technique was followed.[20] Absorbance of calibrators and serum sample were recorded at 450 nm relative to the blank.

Testicular cholesterol

Testicular tissue was homogenized in neutral phosphate buffer. In a centrifuge tube 2 mL of said homogenate and 10 mL of alcohol-acetone combination were taken and allow to boil. Following that, they were centrifuged and then allowed to cool. The supernatant was evaporated until it was completely dry. Chloroform (2 mL) was used to dissolve the residue. A number of cholesterol standards were prepared. About 2 mL of chloroform was added to the test tube labelled as the blank. About 2 mL of the acetic anhydride sulfuric acid was added to each tube having the sample, standard, and blank, and thoroughly mixed. Then reading was taken at 680 nm. [21]

Fructose level in seminal vesicular fluid

Seminal vesicular fluid was deproteinized by using ZnSO4 and NaOH, then the fluid was diluted at 1:16 ratio and centrifuged. After separating the clear supernatant portion, the samples' and standard's optical density (OD) were measured at 470 nm in comparison to the blank.[22]

Real Time PCR Study

Complementary DNA was synthesized from isolated mRNA from male gonadal tissue using cDNA synthesis kit from Roche Diagnostics. The expression of pro-apoptotic Bax, anti-apoptotic Bcl2 and key androgenic dehydrogenases of testicular tissue was noted using qRT-PCR device.[23]

Toxicity Profile

Hepatic phosphatases (acid and alkaline) activities were recorded using standard methods.[24, 25]

Histological Study

Bouin's fixed testicular tissue sections were used for staining using hematoxylin and counter-stained by eosin (H&E) as per standard technique to observe the seminiferous tubular diameter and for the study of spermatogenic cycle at seventh stage.[26]

Periodic acid Schiff (PAS) staining

Semi-quantitative study of PAS positive material especially glycogen in testicular section was performed using standard protocol.[27]

Mass Spectrometric Analysis Followed by Liquid Chromatography (LC-MS) Study

The investigation of the various phytocompounds was carried out in the spectrometer using the scheduled detector and pump with vacuum digester and auto sampler. The standard method was followed for the identification of phytomolecules(s) with their mass, peak height, RT value, positive and negative ionization mode from phytomolecule library comparison.[28]

Statistics

Analysis of variance was studied and then two-tail "t"-test among multiple groups for comparison was followed to study the significant difference among the different conditions.[29]

RESULTS

Somatic Weight and Reproductive Organo-somatic Indices

There was no-significant difference ($p > 0.05$) in somatic weight among treated groups in contrast to PTC. On the other hand, testicular, seminal vesicular and epididymal somatic indices were decreased significantly ($p < 0.05$) in 20 and 40 mg *A. catechu* seed extract (ACSE) treated groups when compared to PTC though testiculo somatic index was decreased significantly at 10 mg treated group counter to PTC (Table 1).

Sperm Count and Motility

Normal sperm count for healthy fertile individuals as per WHO is >39 million/mL.[30] Treatment with ACSE, resulted in a significant reduction ($p < 0.05$) of sperm count compared to PTC (Table 2). WHO report published in 2010 that suggested human fertility is normal if the sperm mortality rate is greater than 40%.[31] After treatment with ACSE the value of sperm motility was decreased significantly ($p < 0.05$) in ACSE treated groups in respect to the PTC. The highest level of effectiveness in this instance was seen with a 40 mg dose of ACSE (Table 2).

SST and SAC

Above 58% of SST is considered normal by WHO guideline.[32] Since there was no reference value available for rat spermiological sensors, the said value was translated for rat's fertility assessment. After treatment with ACSE the SST positive sperm count was significantly decreased ($p < 0.05$) in ACSE-treated groups compared to the PTC (Table 2). The SAC of sperm was significantly ($p < 0.05$) deteriorated after 4 weeks of treatment with ACSE compared to PTC. The 40 mg showed maximum efficacy than others in this case (Table 2).

Sperm viability

Viable sperm, more than 58% consider as normal fertility state.[33] After the treatment with different doses of ACSE, the count of alive sperm was reduced significantly ($p < 0.05$) than PTC where 40 mg showed maximum efficacy in this case (Table 2).

Nuclear chromatin decondensation (NCD)

After ACSE treatment, the percentage of NCD was decreased significantly ($p < 0.05$) in ACSE-treated groups in contrast to PTC. The highest level of effectiveness in this instance was seen with a 40 mg dose ACSE (Table 2).

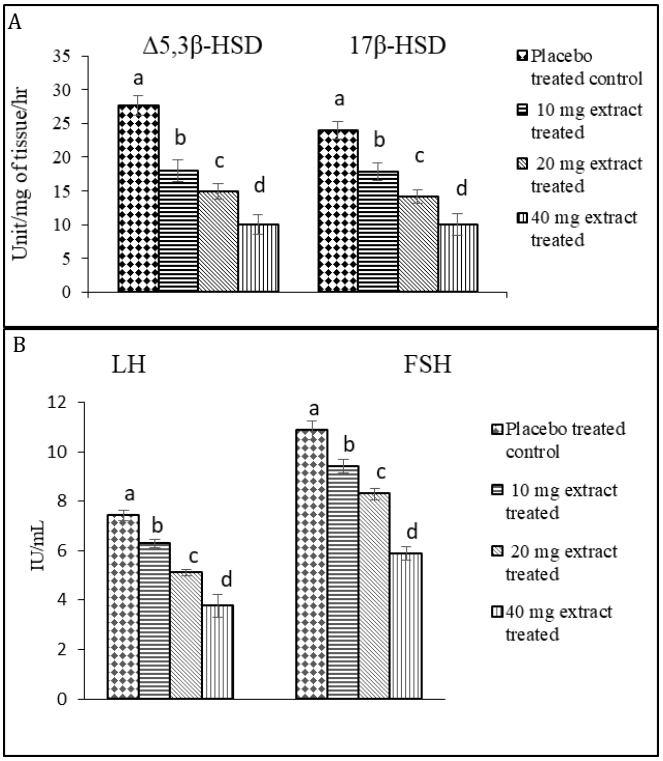

Fig. 1: Result of exposure at different doses of ACSE on the activities of (A) testicular Δ5,3β-HSD, 17β-HSD and (B) serum levels of LH and FSH. Variance analysis afterward Student's '*t*'-test were conducted for statistical analysis. All bars indicate Mean ± SEM (n=6). Bars with a, b, c, d superior letters contrast each other significantly, $p < 0.05$

Table 1: Results of exposure at several doses of ACSE on somatic weight and reproductive organo-somatic indices in experimental rats

Experimental group	*Initial body weight (g)*	*Final body weight (g)*	*Testiculo-somatic index (%)*	*Seminal vesiculo-somatic index (%)*	*Epididymal somatic index (%)*
PTC	132.66 ± 1.92[a]	151.83 ± 1.04[a]	1.8 ± 0.01[a]	0.49 ± 0.006[a]	0.55 ± 0.05[a]
10 mg extract treated	131.33 ± 2.04[a]	150.33 ± 1.40[a]	1.44 ± 0.006[b] (20.00%)	0.42 ± 0.01[a] (14.28%)	0.44 ± 0.01[a] (20.00%)
20 mg extract treated	130.16 ± 2.00[a]	150.5 ± 2.12[a]	1.22 ± 0.006[c] (32.22%)	0.36 ± 0.02[b] (26.53%)	0.37 ± 0.007[b] (32.72%)
40 mg extract treated	131.83 ± 3.08[a]	150 ± 2.54[a]	0.70 ± 0.05[d] (61.11%)	0.23 ± 0.01[c] (53.06%)	0.25 ± 0.002[c] (54.44%)

Variance analysis afterward Student's '*t*'-test were conducted for statistical analysis. All the digits in table indicate Mean ± SEM (n=6). Data with a, b, c, d superior letters contrast each other significantly, $p < 0.05$.

Kinetics of Testicular Key Androgenic Dehydrogenases

Activities of the said androgenic dehydrogenases were inhibited significantly ($p < 0.05$) by ACSE compared to PTC. Maximum inhibition was noted in the 40 mg ACSE-treated group in contrast to the 10 and 20 mg dose-treated groups (Fig. 1).

FSH and LH

Treatment with ACSE decreased FSH and LH levels significantly ($p < 0.05$) than PTC. This was maximally reduced in the 40 mg ACSE treated group (Fig. 1).

Oxidative stress markers in sperm pellets and testis

The SOD and peroxidase activities were significantly decreased ($p < 0.05$) by ACSE treatment compared to PTC. This activity was maximally reduced in 40 mg ACSE-treated group. Between the 10 and 20 mg ACSE-treated groups, the difference was not significant ($p > 0.05$) (Fig. 2).

After ACSE treatment at different doses, MDA was significantly enhanced ($p < 0.05$) in testicular tissue and sperm pellets than PTC. Here, 40 mg dose showed the maximum effect on oxidative stress generation in the male reproductive organs (Fig. 2).

Serum Testosterone, SVF and Testicular Cholesterol Levels

All ACSE doses significantly reduced ($p < 0.05$) serum levels of testosterone, SVF, and testicular cholesterol levels than PTC. The 40 mg ACSE showed maximum effect where no difference was noted between 10 and 20 mg of ACSE-treated groups (Fig. 3).

Real Time PCR Study

Testicular Bax expression was significantly increased ($p < 0.05$) along with Bcl-2, 17β-HSD and Δ5,3β-HSD expression were decreased in ACSE-treated groups than PTC. A maximum deviation in genomic expressions of said sensitive markers were noted in the 40 mg ACSE- treated group (Fig. 4).

Activities of ALP and ACP

In all ACSE dose-treated groups, phosphatases (acid and alkaline) activities did not differ significantly ($p > 0.05$) from the PTC (Fig. 5).

H&E Stain and PAS Stain

Significantly decreased ($p < 0.05$) in number of different generations of germ cells at stage VII and seminiferous tubular diameter (STD) was observed in ACSE-treated groups in contrast to PTC (Table 3 and Fig. 6).

Table 2: Results of exposure at several doses of ACSE on sperm parameters in experimental rats

Experimental group	*Sperm count (Millions/mL)*	*Sperm motility (%)*	*Sperm viability (%)*	*SST (%)*	*SAC (%)*	*NCD (%)*
PTC	26.68 ± 0.56^a	89.24 ± 2.45^a	79.42 ± 1.25^a	71.03 ± 1.63^a	80.78 ± 1.62^a	79.77 ± 0.55^a
10 mg extract treated	18.66 ± 1.56^b (30.05% against PTC)	63.50 ± 3.35^b (28.84% against PTC)	70.42 ± 2.04^b (11.33% against PTC)	56.02 ± 1.57^b (21.13% against PTC)	64.04 ± 1.89^b (20.72% against PTC)	66.52 ± 1.48^b (16.61% against PTC)
20 mg extract treated	14.74 ± 0.82^b (38.53%against PTC)	56.00 ± 0.81^b 37.24% against PTC)	56.38 ± 1.32^c (27.48% against PTC)	45.78 ± 2.86^c (35.54% against PTC)	52.68 ± 1.41^c (34.78% against PTC)	56.81 ± 1.30^c (28.78% against PTC)
40 mg extract treated	11.25 ± 0.72^c (57.83% against PTC)	40.03 ± 1.61^c (55.14% against PTC)	36.68 ± 1.68^d (54.19% against PTC)	35.22 ± 1.37^d (50.41% against PTC)	46.46 ± 1.40^d (42.48% against PTC)	37.71 ± 1.50^d (52.72% against PTC)

Variance analysis afterward Student's '*t*'-test were conducted for statistical analysis. All the digits in table indicate Mean ± SEM (n=6). Data with a, b, c, d superior letters contrast each other significantly, p<0.05.

Table 3: Results of exposure at several doses of ACSE on quantification of different generations of germ cells at stage VII of the spermatogenic cycle in testis and testicular STD in experimental rats

Experimental group	*ASg*	*pLSc*	*mPSc*	*7Sd*	*STD × 400 (μm)*
PTC	0.5 ± 0.01^a	21.48 ± 0.90^a	19.43 ± 0.24^a	67.34 ± 0.73^a	548.33 ± 2.04^a
10 mg extract treated	0.44 ± 0.01^b	17.19 ± 0.58^b	15.06 ± 0.43^b	43.89 ± 1.41^b	457.83 ± 1.13^b
20 mg extract treated	0.36 ± 0.01^c	13.97 ± 0.79^c	13.26 ± 0.47^c	34.31 ± 1.36^c	417.00 ± 1.61^c
40 mg extract treated	0.23 ± 0.008^d	8.24 ± 0.40^d	8.84 ± 0.38^d	21.64 ± 0.90^d	375.00 ± 1.26^d

Variance analysis afterward Student's '*t*'-test were conducted for statistical analysis. All the digits in table indicate Mean ± SEM (n=6). Data with a, b, c, d superior letters contrast each other significantly, p<0.05.

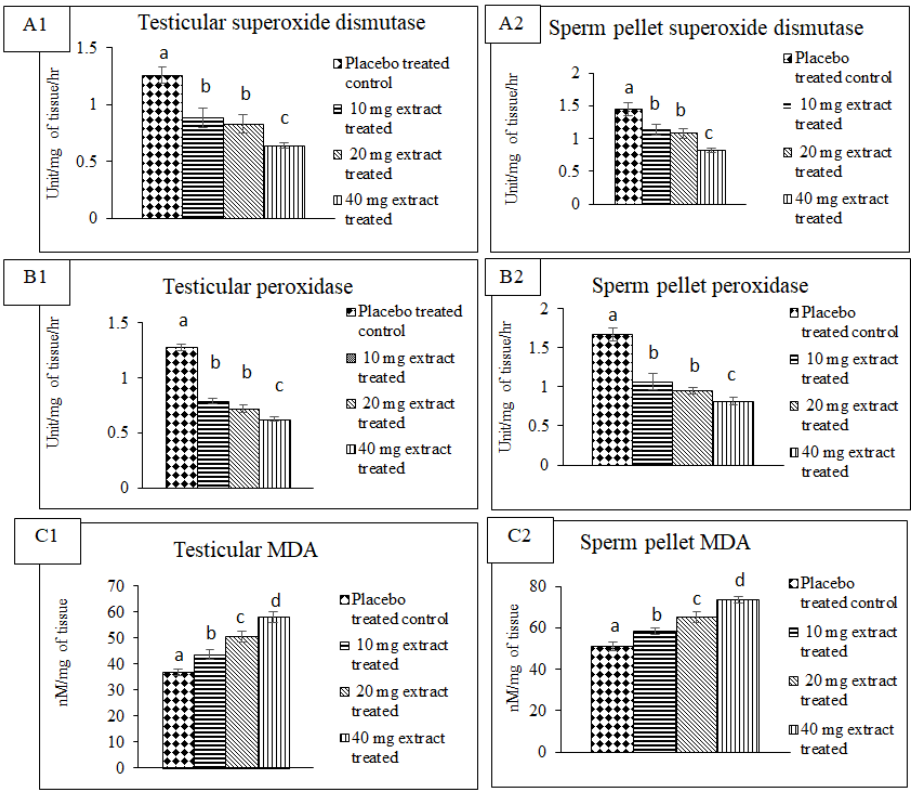

Fig. 2: Influence of exposure of ACSE at various doses on the activities of SOD in testicular tissue (A1) and sperm pellet (A2); activities of peroxidase in testicular tissue (B1) and sperm pellet (B2); and the levels of MDA in testicular tissue (C1) and sperm pellet (C2). Data were expressed as Mean ± SEM (n=6). Variance analysis afterward Student's '*t*'-test were conducted for statistical analysis. Bars indicate Mean ± SEM (n=6). Bars with a, b, c, d superior letters contrast each other significantly, p<0.05

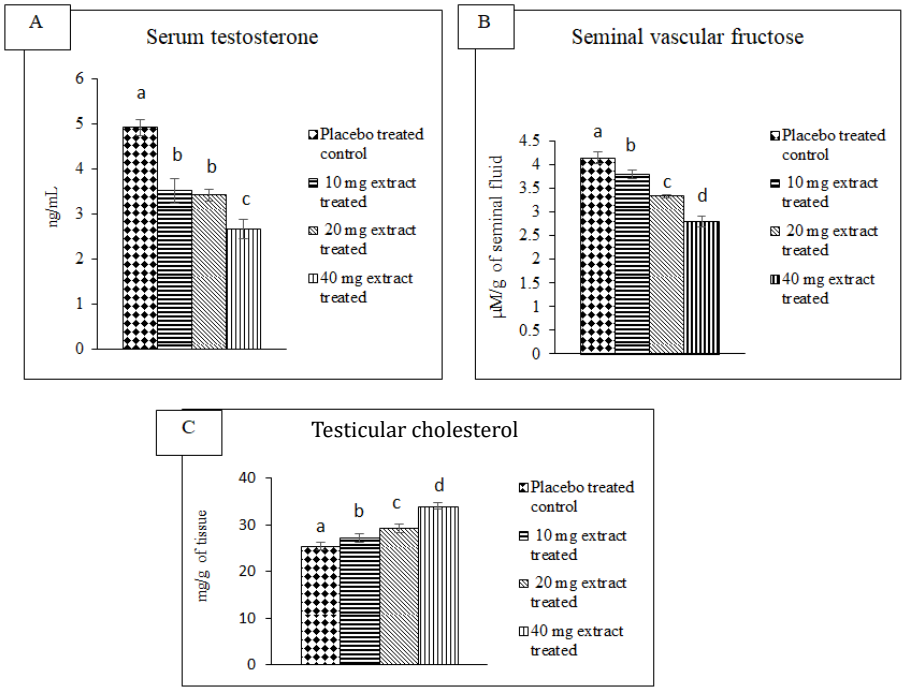

Fig. 3: Result of exposure at different doses of ACSE on the levels of (A) serum testosterone , (B) seminal vesicular fructose, and (C) testicular cholesterol in albino rats. Values are given as Mean ± SEM (n=6). Variance analysis afterward Student's '*t*'-test were conducted for statistical analysis. Bars with a, b, c, d superior letters contrast each other significantly, p<0.05

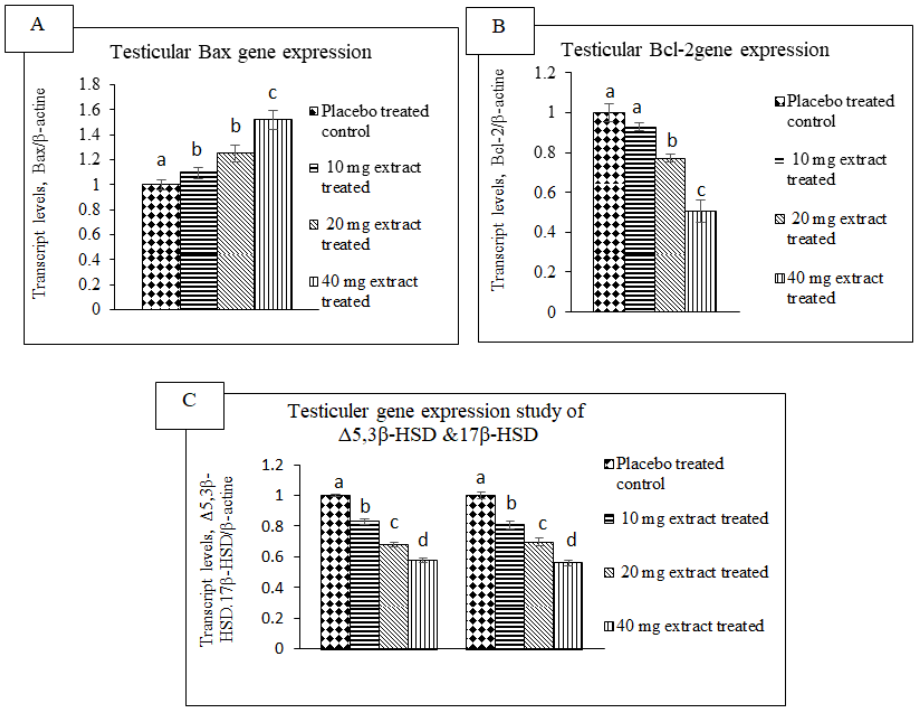

Fig. 4: Effect of ACSE on gene expression study of testicular (A) Bax, (B) Bcl-2, (C) Δ5,3β-HSD and 17β-HSD. Variance analysis afterward Student's '*t*'-test were conducted for statistical analysis. Bars indicate Mean ± SEM (n = 6). Bars with a, b, c, d superior letters contrast each other significantly, $p < 0.05$

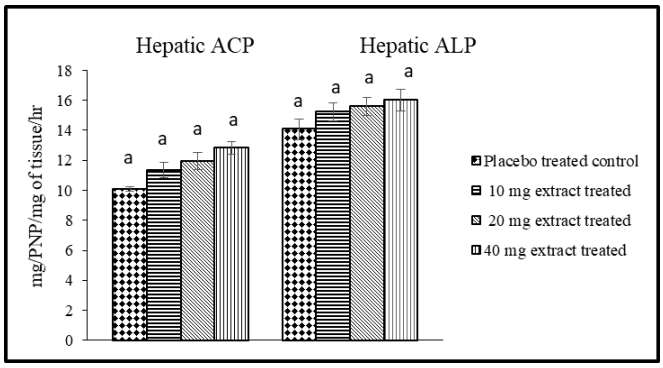

Fig. 5: Hepatic ACP and ALP activities in ACSE-treated rats at different doses. Variance analysis afterward Student's '*t*'-test were conducted for statistical analysis. All the bars indicate Mean ± SEM (n = 6). Data with same superscript (a) did not differ significantly from each other, $p < 0.05$

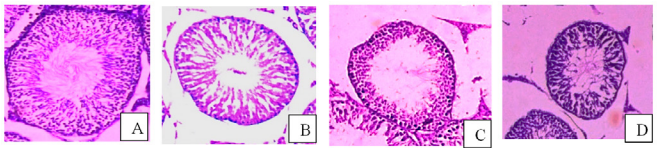

Fig. 6: Histology of testis 400 × (H & E Stain). (A) Representative microphotographs of testicular tissue showing the normal sperm population in qualitative aspect in seminiferous tubule along with the STD of PTC. (B-D) showed statistical diminution in sperm cell population along with the STD significantly at doses of 10, 20 and 40 mg of ACSE in contrast to the PTC

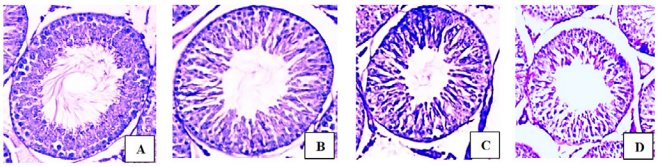

Fig 7: Periodic acid-schiff (PAS) staining of testicular sections of ACSE-treated rat. (A) PTC showed the normal intensity of PAS positive biomolecule in seminiferous tubule. (B-D) showed a remarkable inhibition in the intensity of PAS positive biomolecule

Table 4: Different types of compounds of ACSE were analyzed by LC-MS study

S. No.	*Compounds*	*Ion mode*	*Observed m/z*
1.	Galanthamine	$[M+H]^+$	287.6642
2.	Ferulic acid	$[M+H]^+$	194.4197
3.	Catechin	$[M+H]^+$	290.2206
4.	Pterosin B	$[M+H]^+$	218.4748
5.	Caffeic acid	$[M+H]^+$	179.9778
6.	Genistein	$[M+H]^+$	270.6466
7.	Pentadecanoic acid	$[M+H]^+$	242.5388
8.	Pterosin A	$[M+H]^+$	248.1864
9.	Arecoline	$[M+H]^+$	156.2071
10.	Formononetin	$[M+H]^+$	269.8395

The PAS staining showed decreased intensity of PAS positive biomolecules in ACSE-treated groups compared to PTC (Fig. 7).

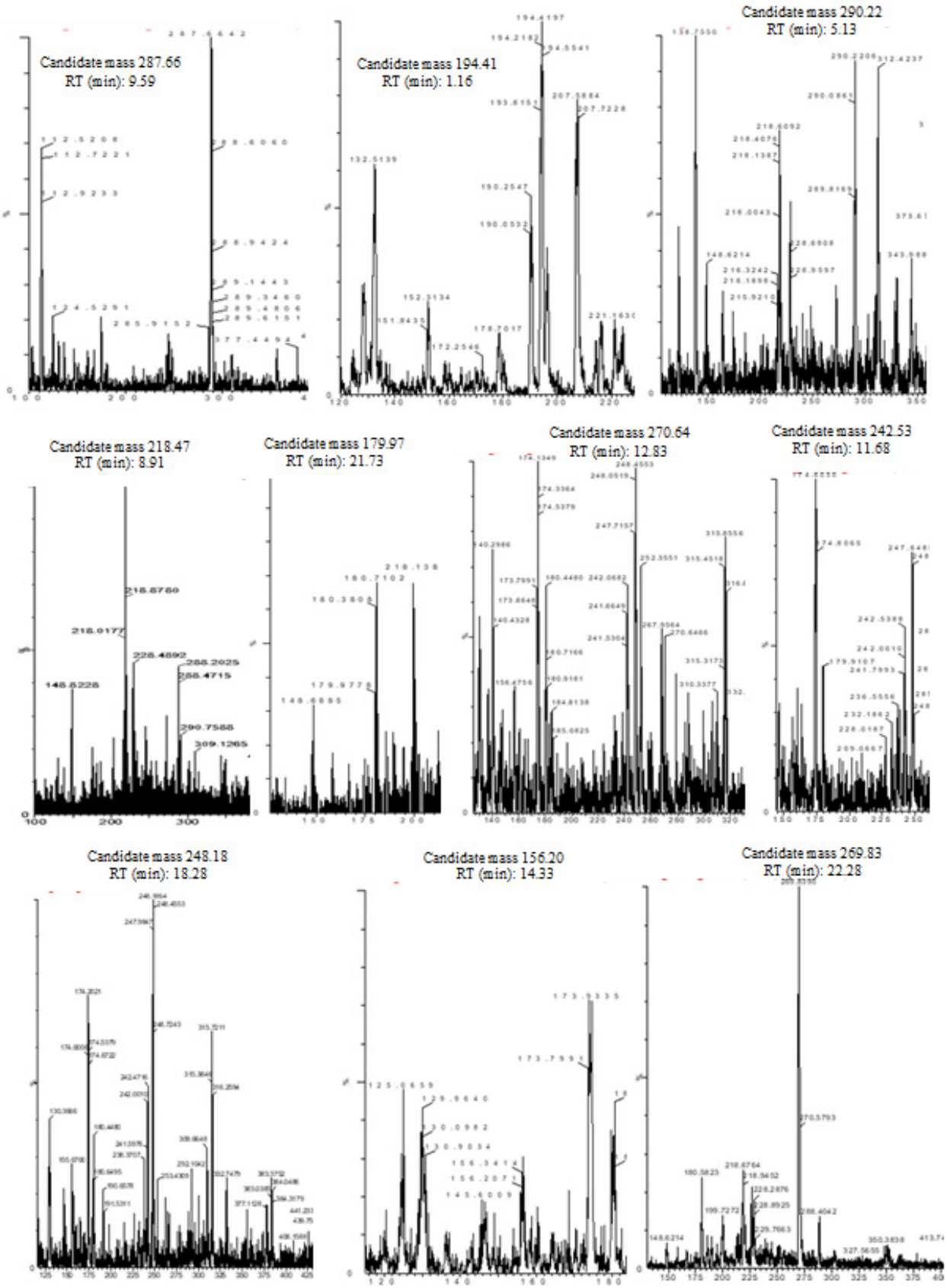

Fig. 8: LC-MS analysis of ACSE

Mass Spectrometric Analysis Followed by Liquid Chromatography

The phytochemical composition of ACSE was performed by using LC-MS showing 10 phytocompounds, presented in Table 4 and Fig. 8.

Discussion

The development of herbal contraceptives is a recent breakthrough in the scientific community. Following this general direction, an experiment was conducted to evaluate the impact of oral administration of *A. catechu* seed extract (ACSE) at varying doses on the testicular activity of albino rats. The extract was administered for 4 weeks as 28 days are required to complete one spermatogenic cycle wave. Reproductive indices showed significant weight loss, which suggests a reduction in testicular androgenesis because this organ's growth is androgen-dependent.[33] Following the administration of ACSE at said doses in comparison to the PTC, there was a drop in the sperm count with defective acrosomal cap status and a low response in the SST, indicating the deterioration of sperm membrane integrity. Sperm motility and count indicate the quality of the semen.[32] After the treatment with ACSE at different doses, the downregulation of such parameters is indicative of impaired androgenesis. A decreased number of epididymal sperms at the middle stage of the seminiferous epithelial cell cycle and a reduction in the number of specific germ cell generations following treatment with ACSE, which resulted in a decline in the spermatogenesis process because of a low amount of testosterone in the plasma.[34] Testicular cholesterol, a precursor of testosterone synthesis in treated groups, was increased which further suggests the deviation in androgenesis.[35] A remarkable PAS-positive biomolecule supporting the inhibition in androgenesis as testosterone has a positive effect on PAS-reactive biomolecule synthesis.[27] Along with this, spermicidal efficacies were again checked by plasma membrane intactness status, i.e., viability test, which might be due to the oxidative stress imposition by this plant, supported here by decreased kinetics of testicular key androgenic dehydrogenase enzymes.[36] Testosterone regulates the quantity of fructose in seminal plasma; a decrease in testicular androgenesis may be the cause of this decrease in fructose levels in seminal vesicles.[37] A decrease in antioxidative enzyme activities like SOD and peroxidase and a significant increase in the end product of lipid peroxidation in the testis and sperm precipitation are signs of oxidative stress (OS) imposition. Certain functional traits, such as NCD, also influence sperm fertility. Although sperm's chromatin is heavily packed and compact in structure, decondensation of this chromatin is crucial for the development of the pronucleus and the normal fertilization process.[38] The spermicidal effect of excessive OS production on germ cells results in sperm morphological and functional damage supported by spermiological assessment.[39]

After treatment with *A. catechu*, diminution in FSH and LH levels were noted. The ACSE may have some effects on the central nervous system, which can prevent neural stimulation necessary for pituitary gonadotrophin release. This results in a deficiency of pituitary gonadotrophins, which are necessary for both initiation and completion of spermatogenesis and steroidogenesis processes.[40] Up-regulated Bax expression and downregulated Bcl2 expression correlates the results of testicular dysfunctions.[41] Additionally, the anti-androgenic properties of said fractions are further confirmed here by decreased testicular Δ5, 3β-HSD, and 17β-HSD expression.[42,43] Hepatic ACP and ALP activities, which are markers of metabolic toxicity, were assessed to determine whether the administered doses of *A. catechu* had any adverse effect. This study demonstrated that the ACP and ALP activities in the treatment group described above did not deviate significantly compared to PTC group, indicating that the extract is not harmful to overall metabolism. Alkaloids, flavonoids, phenolic compounds, and other phytoconstituents were identified in ACSE from the LC-MS study. It is hypothesized that the bioactive phyto components in the seed extract could contribute to male infertility by inducing disruptions to the sperm cell membrane. Lipid peroxidation can negatively impact spermatozoa's ability to impose ROS and disturb the physiology of the sperm membrane. It can also negatively affect testicular androgenesis.[44]

From the dose specific study about spermiological sensors and translating the cut-off value of WHO from human to rat, it has been noted that 40 mg dose resulted the level of inhibition below the border line of fertility. The other two doses are not so effective due to less amount of phytomolecules needed for such inhibitory activities.

Conclusion

The study focused on ACSE has anti-testicular activity by inhibiting spermatogenesis and androgenesis without endangering the general metabolism. This investigation may provide signals to the herbal drug manufacturing industries for the development of male contraceptives using phytomolecules in the coming date, considered as an alternative medicine for family planning.

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