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Pre-clinical study on hypo-testicular activity of hydro-ethanol (60:40) extract of *Areca catechu* (L.) in albino rat: Dose-dependent response

Research Article

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Abstract

The study focused to assess the dose-dependent hypo-testicular activity of hydro-ethanol seed extract of *Areca catechu* (10, 20 and 40 mg/ 100 g body weight) in albino rats. For this purpose, body weight, reproductive organo-somatic indices, as well as motility, count, hypo-osmotic swelling and acrosomal cap status of sperm along with the quantity of testicular androgenic key enzyme activities, serum testosterone, oxidative stress sensors, seminal vesicular fructose (SVF), apoptosis linked gene expression study and histology of testicular tissue for spermatokinetics were covered here. All the said doses revealed a significant ($p<0.05$) downward deviation in spermiological profile, serum testosterone, SVF levels, activities of testicular Δ5,3β and 17β-HSD, seminiferous tubular diameter compared to the vehicle-treated control (VTC). In contrast to the VTC, activities of superoxide dismutase and peroxidase were decreased along with elevation in the levels of thio-barbituric acid reactive substances significantly ($p<0.05$) in testis and sperm pellet of extract-treated groups. Testicular pro-apoptotic Bax gene expression was elevated and anti-apoptotic Bcl-2 gene expression was diminished significantly ($p<0.05$) after extract treatment compared to the VTC. Non-significant changes ($p>0.05$) were noted in hepatic acid and alkaline phosphatase activities which focused the non-toxic characteristics of said extract in general. This study interpreted that, 40 mg showed drastic hypo-testicular efficacy cum necrotic changes compared to other doses though the therapeutic dose that cross the borderline of fertile sensors from spermiological indicators is 20 mg dose. Liquid chromatography-mass spectrometry analysis of the said extract showed that ten phyto-molecules belong to flavonoid, alkaloid and isoflavone groups mainly.

Keywords: Androgenesis, Apoptosis, Oxidative stress, Male contraceptive, Spermatokinetics, Spermiological profile.

Introduction

At present, population explosion has become the biggest issue all over the world (1). Over growing population has several adverse effects on environmental and economic up-gradation. Contraception is one of the very popular and well-known methods to control over growing population. Several types of contraception methods are available in the market such as hormonal and chemical contraceptives, where female contraceptives are more popular than male contraceptives, though the second variety has a significant impact on reducing unintended pregnancies worldwide and it could be a step toward family planning (2). In developing countries, lots of people use herbal products for birth controlling because regular and long-term use of contraceptive pills creates many adverse effects like cerebral and cardiac stroke, hypertension, diabetes and tumour (3). On the other hand, herbal medicine is easily available and pocket-friendly with no side effects. Herbal extracts with anti-spermatogenic activity have become a trend in this modern research field. According to previous studies many herbs have been shown to have antifertility activity like *Abrus precatorius* L. (Fabaceae), *Cajanus cajan* L. (Fabaceae), *Acacia nilotica* L. (Fabaceae) and *Aristolochia indica* L. (Aristolochiaceae) (4). *Areca catechu* L. (*A. catechu*) belongs to Arecaceae, commonly known as supari palm. The fruit of this plant is oval or round-shaped and called areca nut or betel nut (5). Chewing *A. catechu* seed coupled with betel leaf and betel quid is a common practice among a large number of people in many parts of India like West Bengal, Assam, Bihar and Odisha. Betel nut chewing is an ordinary routine in many parts of Asian countries like Pakistan, Bangladesh and Sri Lanka (6). According

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to Buddhist writing, it has some medicinal properties that are also mentioned in Sanskrit medical literature (7) such as anti-inflammatory, anti-ulcer and anti-diabetic (6). There is no detailed scientific research till now in the field of male sub-fertile cum contraceptive effects of *A. catechu* (seed). The investigation was designed to reveal the hypo-testicular efficacies of the hydro-ethanolic (H-E) extract (60:40) of *A. catechu* seed as a function of the applied doses.

Materials and Methods

Chemicals and Reagents

For extraction, ethanol was purchased from Narang Scientific Industries (Ambala, Haryana, India) Roche supplied the pure RNA tissue kit (New Delhi, India) and Apex (Apex Medicine Service, Kolkata, West Bengal) provided the primers for the genomic analysis. Other chemicals were procured from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India).

Extraction of Plant Part

Seeds of *A. catechu* were purchased from the seed market in Midnapore and verified by the taxonomists of the Botany department, Vidyasagar University (721102), West Bengal, India. The number of voucher specimen of the seed of said plant was "*A. catechu*/VU/Bio/12/20". Seeds were chopped and dried. One litre of hydro-ethanol (60:40) solvent was used to dissolve 100 g of dried seed powder and kept at room temperature for 48 hours with intermittent shaking. After that, the filtration of this mixture was done by Whatman NO-1 filter paper. The wine red-coloured solvent mixture was concentrated with the help of a rotary evaporator and 2.58 g air-dried powder was kept in an airtight glass container for future investigation.

Animal Housing

Adult Wistar strain male rats were purchased from the Committee for Control and Supervision of Experiments on Animals (CCSEA) authorised vendor and retained in animal cages under suitable conditions (12 hours light/ 12 hours dark, 25 ± 2˚C) for 10 days before experimentation to acclimatize the rats. Food and water were given in the required amount. This experimental research protocol has been followed by the Institutional Ethics Committee (IEC) and the obtained clearance number was- VU/IAEC-I/ DG-2/3-16/19. Dt.11.12.2019.). Rats were distributed into 4 groups and each group has 6 rats. Treatment was conducted orally by gavage every day at 10 A.M. No feed was provided for 2 hours of pre and post-extract administration period to overcome the nutrient-phyto molecule interaction if any.

Animal Treatment

- **Vehicle-treated control (VTC):** Distilled water (DW) was forcefully delivered by gavage at half mL in proportion to the rat's 100 g weight for 4 weeks.
- **10 mg extract treated (low dose):** This group was treated with 10 mg H-E extract of seed of *A. catechu*/ half mL DW in proportion to the rat's 100 g weight for 4 weeks.
- **20 mg extract treated (moderate dose):** The said extract of *A. catechu* was given at 20 mg dose/ half mL DW in proportion to the rat's 100 g weight for 4 weeks.
- **40 mg extract treated (high dose):** The above extract of *A. catechu* was provided at the dose of 40 mg/ half mL DW in proportion to the rat's 100 g weight for 4 weeks.

All rats were sacrificed by euthanasia after the completion of the experimental schedule. Body weight was also taken at the time of sacrifice. Spermiological sensors were evaluated after the collection of epididymal spermatozoa by incision of the caudal epididymis followed by its washing with phosphate buffer saline. To measure serum testosterone level, blood was collected in a non-heparinized syringe and serum separation was done by centrifugation of blood sample at 3000 g. Testes, epididymis and seminal vesicles were removed through dissection. Surface fluid and blood of the said organs were removed by using a tissue paper with proper care and very gently. Weights of those aforementioned organs were recorded after the removal of connective tissues from their surfaces. One testicle of rat have been put into the picric acid-based Bouin's fixative for histo-architechtural studies whereas other testicle and liver were preserved at -20˚C for genomic and biochemical analysis.

Sperm Concentration and Motility

Neubauer hemacytometer chamber was used to perform a microscopical count of spermatozoa and denoted as number of spermatozoa in a million per mL of epididymal suspension (8).

Epididymal spermatozoa (100 µL) were dispersed in normal saline (1 mL) for dilution at a 1:10 ratio. After that, the motile percentage of spermatozoa was counted by using a light microscope and the percentages were calculated after counting 100 or more than 100 sperm in microscopic fields (9).

Acrosomal Cap Status (ACS)

Fresh sperm were obtained from the epididymis and diluted with Phosphate buffer saline (PBS)-D glucose (1:10). Sperm samples (20 µL) were smeared on thin gelatin-coated slides and incubated at 37˚C for 120 mins. The sperm with holes in gelatin coat near the sperm cap, were counted and expressed in percentage (10).

Hypo-Osmotic Swelling (HOS) Test

Sodium salt of citrate (735 mg) and keto hexose (100 mg) were used to prepare the HOS solution. Diluted sperm (100 µL) was mixed with 1 mL HOS solution (1:10) and left for 2 hours incubation at 37˚C. Sperm cells with coiled tail or swelled were expressed in percentage value (11).

Androgenic Key Enzyme Activities

Standard methods were used for the assessment of catalytic potentialities of androgenic key enzymes

i.e., Δ5,3β and 17β-hydroxysteroid dehydrogenase (HSD) of testis (12,13). Potassium phosphate (5 mM) containing spectroscopy category glycerol (20%) and ethylene diamine tetra acidic acid (EDTA) (1 mM) were used for homogenization of the said tissue sample at the quantity of 100 mg tissue/mL of solvent. The homogenized substance was allowed for centrifugation at 10000 g for 30 mins. To study the catalytic potentialities of the said enzymes, supernatant was collected and 1 mL of it was used for this observation. The activities were expressed as unit/mg of tissue/hr after recording optical density (OD) by using UV Visible Spectrophotometer (Thermo Scientific; USA) at 340 nm at 30 sec interval for 3 mins.

Oxidative Stress (OS) Markers

The UV Visible Spectro Photometer (Thermo Scientific; USA) was used for estimating catalytic potentialities of superoxide dismutase (SOD) and peroxidase from testicle and sperm pellet. Said samples were homogenized at 50 mg of tissue strength/mL in 0.1 M phosphate buffer at neutral pH. Supernatant of this mixture was separated by centrifugation at 10000 g for 20 mins at low temperature (4˚C). From the prepared sample, 20 µL was transferred to spectrophotometric cuvette and then Tris buffer of 2.04 mL (pH 8.2) and pyrogallol of 20 µL were added and allowed for proper mixing. At half-minute interval for successive 3 mins, the absorbance was noted at the spectral wavelength of 420 nm (14).

For peroxidase assay, 0.1 mL supernatant fluid of above tissue homogenate was collected, mixed with 0.5 mL guiacol (20 mM) and 0.3 mL of 12.3 mM H_2O_2. Results were obtained by recording OD at 436 nm for 3 mins using UV Visible Spectro Photometer (Thermo Scientific; USA) (15).

For thiobarbituric acid reactive substances (TBARS) assay, centrifugation was conducted of the said homogenized samples at 10000 g for a short period (5 mins) at a low temperature (4˚C). Separated upper layer was utilized to quantify TBARS. Absorbances were recorded at 535 nm by using UV Visible Spectro Photometer (Thermo Scientific; USA) (16).

Estimation of Serum Testosterone

The kit for testosterone assay was procured from Lilac Medicare (P) Ltd, Mumbai, India. The concentration of testosterone in serum was quantified by Sandwich ELISA (MERCK; Germany) (17). Collected serum was placed into microwells. Standard at the volume of 25 µL and the sample of 25 µL were dispensed into the appropriate wells. Hundred µL of horseradish peroxidase (HRP)-conjugated enzyme was mixed and allowed for 1 hour incubation at 37˚C. The solvent of microwells was washed and repeated forceful decanting for several times by using washing buffer. The substrate of HRP was used as per the supplied kit. Then, 0.1 mL of stop solution was applied in the microwells for terminating the chemical conversion. The absorbance of calibrators and samples were measured against the blank at 450 nm using a differentiation filter at 630 nm.

Seminal Vesicular Fructose (SVF) Assay

The level of SVF was quantified from the seminal plasma fluid. Twenty µL seminal plasma was mixed with 220 µL of distilled water and deproteinized by adding 50 µL of ZnSO4, 50 µL of NaOH. This diluted seminal plasma was centrifuged at 400 g for 15 mins. The clear supernatant fluid was collected and applied to estimate fructose. The absorbances of standard and samples were measured using the same model of UV Visible Spectro Photometer at 470 nm against the blank (18).

Real-time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Study of Testicular Apoptotic Markers

"Transcriptor First Stand cDNA Synthesis Kit" was acquired from Roche Diagnostics and used to synthesize cDNA from the testicular tissue's extracted messenger RNA. Using qRT-PCR Light Cycler 480 II (Roche; Germany), the proapoptotic gene expression i.e., Bax and anti-apoptotic gene expression i.e., Bcl-2 in testicular tissue were examined (19).

Toxicity Profile

To assess liver toxicity, acid and alkaline phosphatase (ACP and ALP) activities were measured as per standard methods (20, 21).

For general toxicity assessment, serum glutamate transaminase (SGOT) and glutamate pyruvate transaminase (SGPT) activities were measured by a specific kit (Span Diagnostic Ltd. Surat; India) (22).

Histological Study

Bouin's solution was taken for fixation of testicular tissue in the histo-architechtural assay. Tissue sections were prepared in microtome at 5 µm thickness. Haematoxylin and Eosin (H & E) stains were used for processing the tissue sections according to the standard method (23).

Liquid Chromatography-Mass Spectrometry (LC-MS) Study

The Quattro Micro™API mass spectrometer (Waters, Milford, Massachusetts, USA) was used to conduct the LC-MS study of the different types of phytocompounds using the quarternary pump and a diode array detector (DAD). The LC device was associated with degasser, an autosampler and a fixed temperature-controlled column component which were connected to a detector (Waters 2998). Extract was injected in a C-18 column. The column's temperature was set to 40˚C. Acetic acid -water 90% denoted by A and 10% MeOH denoted by B were utilized as solvents. Within m/z 50 to 1200, spectra were captured in both +ve and -ve ionization modes (24).

Statistics

The statistical analysis of the collected data was done using "Multiple Comparison Students' two-tail '*t*'-test" followed by ANOVA (25) and a significant difference was considered here, $p< 0.05$.

Results

Rat's Body Weight and Reproductive Organo-somatic Indices

Non-significant difference ($p>0.05$) was recorded in the somatic growth of rat after extract treatment in comparison to the VTC. Testiculo-somatic, seminal vesiculo-somatic and epididymal-somatic indices were less in significant level ($p<0.05$) (18.49%, 11.11%, 21.56%) in 10 mg dose (27.74%, 26.66%, 31.37 %) in 20 mg dose and (58.38%, 53.33%, 54.90%) in 40 mg dose-treated groups respectively when compared with VTC (Table 1).

Sperm Concentration and Motility

According to WHO, the normal value of sperm count in human is >39 million/mL of semen in average. The count less than 50% is considered as one of the indicators of male infertility (26). The count less than 50% is considered as one of the indicators of male infertility. Similarly, motile sperm greater than 40% considered as normal fertility indicator in human (27, 28). As there is no such reference data available for rat sperm analysis, comparison was made with the WHO reference value of human sperm count and motility for fertility status assessment of rat's spermiological sensors in this experiment. Treatment with different doses, sperm count was significantly diminished ($p<0.05$) i.e., in 10, 20 and 40 mg doses compared to the VTC. Considering the WHO reference value and its translation to rat, cut-off value of fertility from the viewpoint of sperm count, crossed at the dose of 40 mg in comparison to the VTC ,though other doses are also close to that borderline which were indicated in parenthesis (Table 1).

After treatment with H-E extract of *A. catechu*, the value of sperm motility was decreased in 10, 20 and 40 mg significantly ($p<0.05$) in contrast to the VTC. Focusing the 40% cut-off value of sperm motility in respect to the VTC, for male fertility assessment the 20 and 40 mg doses of extract treatment crossed that cut-off value indicated in parenthesis. (Table 1).

HOS and ACS of Sperm

As per WHO guideline, human reference value of the HOS-positive sperm is above 58% considered as fertile (27). Comparison was done using the WHO reference value of the human HOS test for fertility assessment in rat as such cut-off values are not available in spermiological analysis. After treatment with said extracts of *A. catechu*, the percentages of HOS-positive sperm were significantly decreased ($p<0.05$) in 10, 20 and 40 mg dose-treated groups compared to the VTC. Considering the cut-off value of fertility from the purview of HOS in our study of rat's spermiological data, all the dose-treated groups crossed that cut-off values respect to the VTC level indicated in parenthesis (Table 1).

According to Chan et al., 1999 the normal value of ACS for human is more than 40% indicating normal fertile condition (29). In this study, significantly a lower percentage ($p<0.05$) of sperm responded to ACS after 28 days of treatment with *A. catechu* seed extract in all the said i.e., 10, 20 and 40 mg doses compared to the VTC (Table 1).

Table 1: Effects of treatment with different doses of H-E extract of *A. catechu* seed on body weight, organo-somatic indices and spermiological sensors in albino rats

Group	Initial body weight (g)	Final body weight (g)	Testiculo-somatic index (%)	Seminal vesiculo -somatic index (%)	Epididymal -somatic index (%)	Sperm count (millions/mL)	Sperm motility (millions/mL)	HOS (millions/mL)	Normal ACS (millions/mL)
VTC	134.5 ± 2.56[a]	154.33 ± 1.83[a]	1.73 ± 0.03[a]	0.45 ± 0.03[a]	0.51 ± 0.06[a]	25.02 ± 1.76[a]	22.06 ± 1.25[a]	17.29 ± 1.45[a]	19.61 ± 1.26[a]
10 mg extract treated	133.5 ± 2.40[a]	153.16 ± 1.57[a]	1.41 ± 0.02[b]	0.40 ± 0.02[a]	0.40 ± 0.01[a]	17.00 ± 0.57[b] (67.94 % ↓ against VTC)	10.49 ± 1.20[b] (47.55 % ↓ against VTC)	9.76 ± 1.02[b] (56.44 % ↓ against VTC)	11.30 ± 1.21[b] (57.62 % ↓ against VTC)
20 mg extract treated	133 ± 1.96[a]	153.83 ± 2.07[a]	1.25 ± 0.02[c]	0.33 ± 0.01[b]	0.35 ± 0.002[b]	15.13 ± 0.85[b] (60.47 % ↓ against VTC)	8.01 ± 1.05[b] (36.31 % ↓ against VTC)	7.20 ± 0.96[c] (41.64 % ↓ against VTC)	7.76 ± 1.21[c] (39.57 % ↓ against VTC)
40 mg extract treated	133.30 ± 4.11[a]	153.50 ± 2.40[a]	0.72 ± 0.07[d]	0.21 ± 0.02[c]	0.23 ± 0.01[c]	12.04 ± 0.99[c] (48.12 % ↓ against VTC)	4.61 ± 1.24[c] (21.16 % ↓ against VTC)	4.46 ± 1.37[d] (25.79 % ↓ against VTC)	5.85 ± 1.10[d] (29.83 % ↓ against VTC)

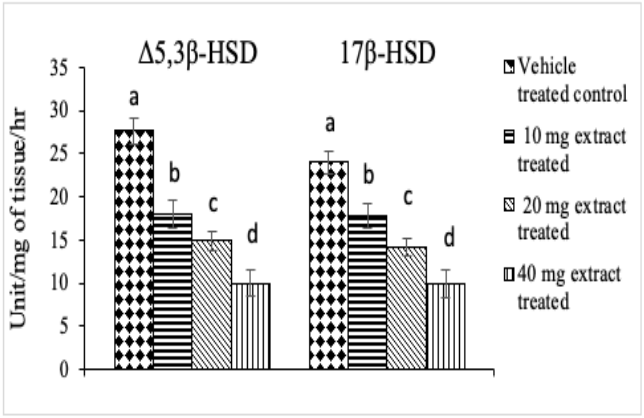
Data were expressed as mean ± SEM (n=6). "ANOVA followed by Multiple Comparison Student's two tail 't'-test". Values with different superscripts (a, b, c, d) in each column differ from each other significantly, $p<0.05$. Percentage given.

Testicular Δ5, 3β and 17β-HSD Kinetics

In comparison to the VTC, Δ5,3β and 17β-HSD catalytic potentialities of testis were reduced at significant level ($p<0.05$) in all the doses (25.77%, 35.10% in 10 mg dose-treated; 40.78%, 46.13% in 20

mg dose-treated; 58.38%, 63.77% in 40 mg dose-treated group respectively) (Figure 1).

Figure No 1: Catalytic potentialities of testicular Δ5,3β and 17β-HSD in different doses of H-E of *A. catechu*. Data were expressed as Mean ± SEM (n=6). "ANOVA followed by Multiple Comparison Student's two-tail '*t*'-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05



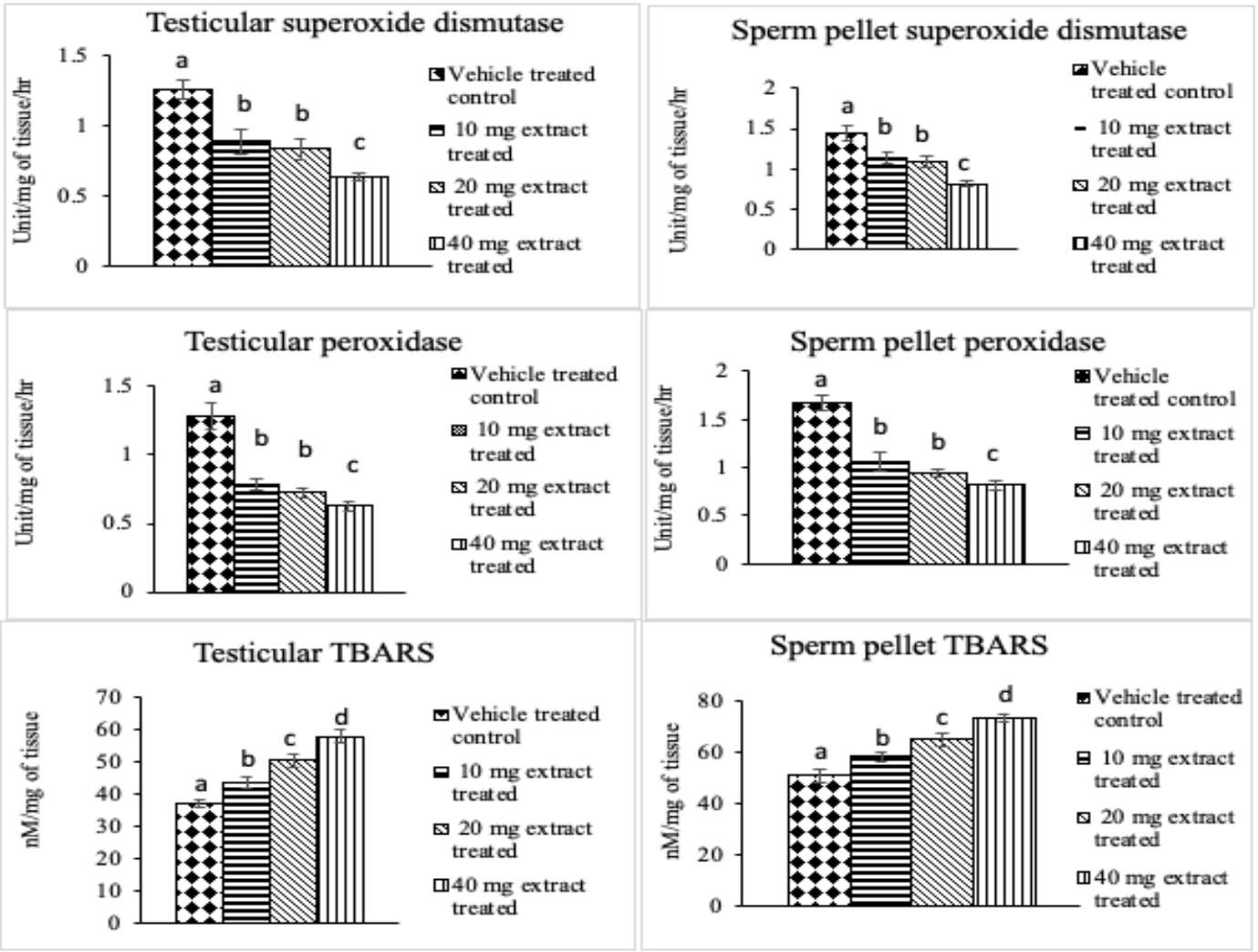
OS Markers

The activity of SOD in testicle and sperm pellet was inhibited in all the said doses i.e. (30.15%, 22.06% in 10 mg, 34.12%, 25.51% in 20 mg, 50.00%, 44.82% in 40 mg) of said extract treated groups significantly ($p<0.05$) in comparison to the VTC (Figure 2).

The catalytic potentiality of peroxidase enzyme in testicle and sperm pellet was significantly diminished ($p<0.05$) in all the doses of H-E extract i.e., in 10 mg (37.79%; 36.52%), 20 mg (43.30%; 43.11%) and 40 mg (51.18%; 50.89%) when compared to the VTC (Figure 2).

After the treatment with H-E extract of the said plant part at different doses, TBARS level was significantly elevated ($p<0.05$) in testicle and sperm pellet when compared to the VTC. The percentages of elevation in the quantity of TBARS in 10 mg dose were 17.91% in testis and 14.61% in sperm pellet when comparison was made with VTC. In 20 mg dose-treated group, the percentages of TBARS elevation were 28.51% in testis and 27.72% in sperm pellet, whereas, in 40 mg dose-treated group, the elevation levels were 56.87% in testis and 44.00% in sperm pellet when compared with the VTC (Figure 2).

Figure No 2: Impact of H-E extract of *A. catechu* on the oxidative stress markers in testicular tissue and sperm pellet. Data were expressed as Mean ± SEM (n=6). Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05. "ANOVA followed by Multiple Comparison Student's two-tail 't'-test".



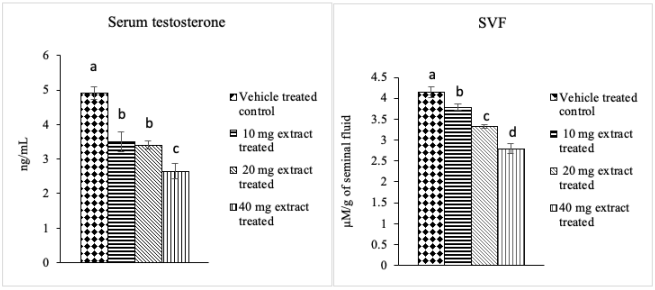
Serum Testosterone

Serum testosterone level was decreased significantly ($p<0.05$) in all doses of H-E extract treated groups (28.57% in 10 mg, 30.61% in 20 mg and 46.12% in 40 mg dose-treated groups respectively) as compared with VTC. Serum level of testosterone was significantly lowered ($p<0.05$) in 40 mg dose treated group when comparison was made with 10 and 20 mg dose treated groups (Figure 3).

SVF Quantification

The level of SVF was decreased in 10 mg (8.47%), 20 mg (19.61%) and 40 mg (32.44%) H-E extract treated groups significantly ($p<0.05$) against to the VTC (Figure 3). The level of diminution of this sensor among the dose-treated groups was significant ($p<0.05$) as the function of the said doses.

Figure No 3: Effect of H-E extract of *A. catechu* at different doses on SVF and serum testosterone levels in albino rats. Values were given as Mean ± SEM (n=6). Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05. "ANOVA followed by Multiple Comparison Student's two-tail 't'-test"



Bax and Bcl-2 Gene Expression

In comparison to the VTC, after 28 days of oral administration of the H-E extract, testicular Bax gene expression was elevated significantly ($p<0.05$) in 10 mg (9%), 20 mg (25%) and 40 mg (51%) treated groups but Bcl-2 gene expression was decreased in 10 mg (8%), 20 mg (23%) and 40 mg (49.5%) treated groups significantly ($p<0.05$) (Figure 4).

Activities of ACP, ALP, SGOT and SGPT

In all the said doses of H-E extract treated groups, the activities of ACP, ALP, SGOT and SGPT did not alter significantly ($p>0.05$) from the VTC (Figure 5).

Figure No 4: Effect of H-E extract of *A. catechu* on gene expression of testicular Bax and Bcl-2. Data were expressed as Mean ± SEM (n=6). "ANOVA followed by Multiple Comparison Student's two-tail 't'-test". Bars with different superscripts (a, b, c) differ from each other significantly, p<0.05.

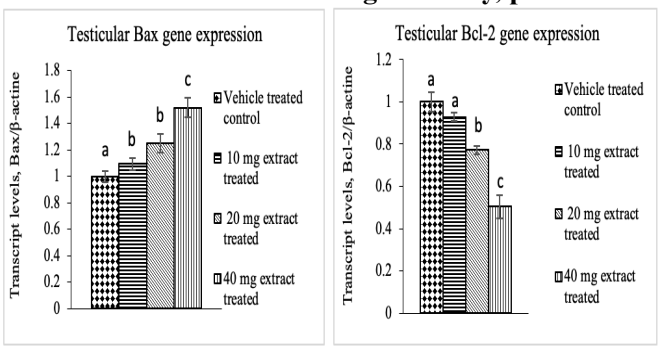
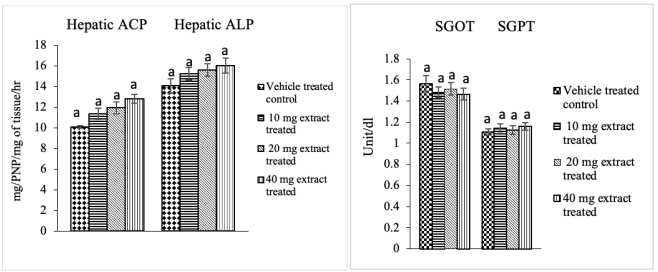


Figure No 5: Toxicity level in H-E extract treated rats. Values were expressed as Mean ± SEM (n=6). "ANOVA followed by Multiple Comparison Student's two-tail 't'-test". Bars with same subscript (a) did not differ from each other significantly, p>0.05.



Diameter of Seminiferous Tubule (DST) and Quantification of Germ Cell at Stage VII of Spermatogenic Cycle

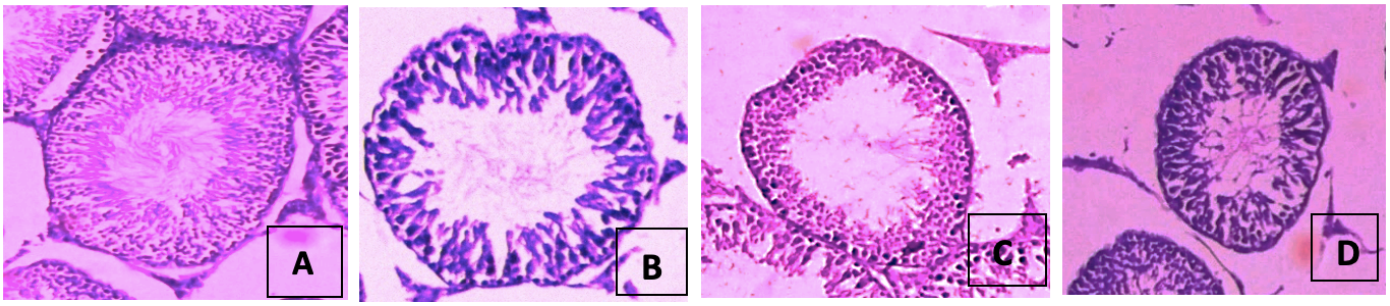
Significant decrease ($p<0.05$) in the numbers of different generations of germ cells at stage VII i.e., ASg, pLSc, mPSc, 7Sd, of seminiferous epithelial spermatogenic cycle and DST were noted in comparison to the VTC. The level of diminutions were at 10 mg dose (14.54%, 22.19%, 25.11%, 32.32%, 16.50%); 20 mg (27.27%, 39.47%, 41.26%, 46.25, 23.94%); and 40 mg dose (52.72%, 63.48%, 59.47%, 63.72%, 31.56%) of H-E (60:40) extract of seed of *A. catechu* treated groups when compared to VTC (Table 2 and Figure 6).

Table 2: Effects of different doses of H-E extract of seed of *A. catechu* on the number of different generations of germ cells at stage VII of the spermatogenic cycle and DST

Experimental group	ASg	pLSc	mPSc	7Sd	DST × 400 (μm)
VTC	0.55 ± 0.01^{a}	19.33 ± 0.17^{a}	18.95 ± 0.04^{a}	68.53 ± 1.33^{a}	546.33 ± 2.29^{a}
10 mg extract treated	0.47 ± 0.01^{b}	15.04 ± 0.04^{b}	14.19 ± 0.65^{b}	46.38 ± 1.58^{b}	456.16 ± 1.40^{b}
20 mg extract treated	0.40 ± 0.01^{c}	11.7 ± 0.14^{c}	11.13 ± 0.42^{c}	36.83 ± 1.16^{c}	415.5 ± 1.74^{c}
40 mg extract treated	0.26 ± 0.01^{d}	7.30 ± 0.14^{d}	7.68 ± 0.47^{d}	23.21 ± 1.82^{d}	373.88 ± 2.08^{d}

Values were expressed as Mean ± SEM (n=6). ANOVA followed by Multiple Comparison Student's two-tail '*t*'-test. Values with different superscripts (a, b, c, d) in each column differ from each other significantly, $p<0.05$.

Figure 6: Histology of testis, 400 × (H & E Stain). Representative microphotographs of testicular tissue showing the sperm population at stage VII of spermatogenic cycle in seminiferous tubule along with the DST of VTC (A). Testicular cross-section focusing on diminution in the size of DST along with qualitative aspect of sperm cell population at stage VII in respect to the VTC after the treatment with H-E extract of *A. catechu* at a dose of 10 mg (B). Testicular cross-section focuses on the diminution in sperm cell population at stage VII along with the DST in respect to the VTC of H-E extract of *A. catechu* at the dose of 20 mg (C). Testicular cross-section showed maximum diminution in sperm cell population at stage VII along with the DST at the dose of 40 mg in respect to the VTC as well as other doses after the treatment with said extract (D).



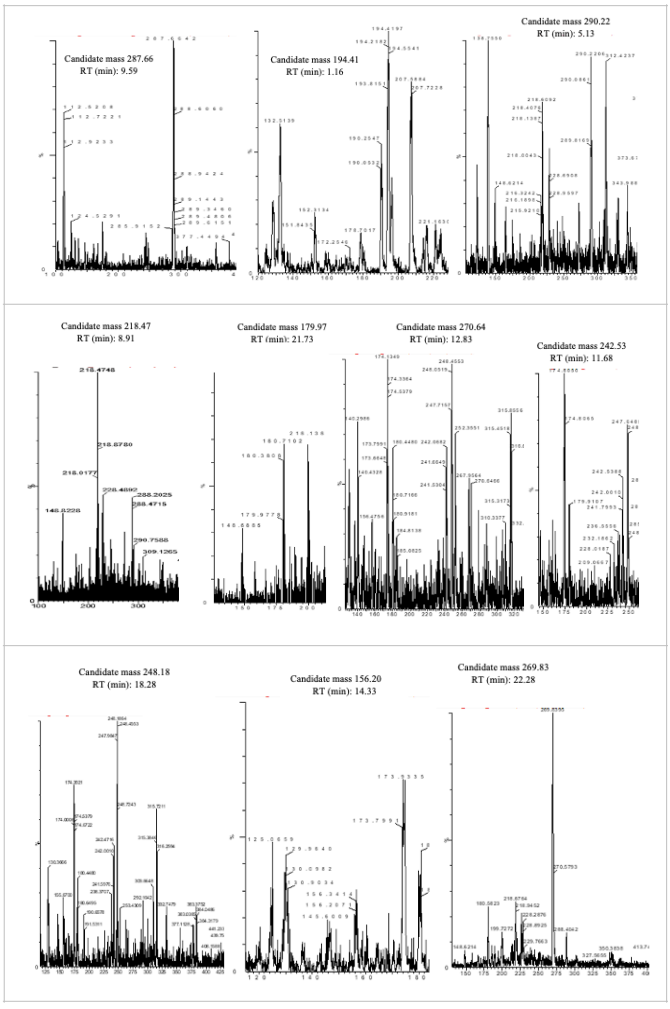
Phytomolecule present in H-E Extract of Seed of *A. catechu* analysed by LC-MS.

The quantitative analysis of hydro-ethanolic *A. catechu* seed extract was performed by using LC-MS. The LC-MS Spectrum of *A. catechu* seed extract having *m/z* with positive ion 287.6642, 194.4197, 290.2206, 218.4748, 179.9778, 270.6466, 242.5388, 248.1864, 156.2071, 269.8395. These data indicated *m/z* 287.6642 is galanthamine- an alkaloid, *m/z* 194.4197 identified as ferulic acid classified as a phenolic phytochemical, *m/z* 290.2206 is identified as catechin belongs to the group of polyphenols, *m/z* 218.4748 indicated the compound as pterosin B - this compound derived from sesquiterpenoid, *m/z* 179.9778 identified as caffeic acid, a type of polyphenol, *m/z* 270.6466 is identified as genistein, it's a class of compound known as isoflavones, *m/z* 242.5388 is pentadecanoic acid, a straight chain saturated fatty acid, *m/z* 248.1864 identified as pterosin A- and is sesquiterpenoids nature, *m/z* 156.2071 is arecoline, an alkaloid and *m/z* 269.8395 that is formononetin a flavonoid (Table 3 and Figure 7).

Table 3: Phyto-compounds of H-E extract from *A. catechu* seed by LC-MS study

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

Figure 7: LC-MS analysis of H-E extract of seed of *A. catechu*



Discussion

Herbal contraceptive development is a new trend in the scientific community. Going with this trend, the experiment was performed to determine the cause-effect relationship study of H-E extract treatment of *A. catechu* seed on testicular activity in albino rat at different doses. As single wave of the spermatogenic cycle in Wistar strain rats takes 12-14 days to complete,

so the treatment duration was fixed here for 28 days to cover two waves of the said cycle for proper imposing the effect of the extract on the germ cells proliferation, differentiation and its impact properly, accurately and correctly. Significant diminution in reproductive organo-somatic indices indicate a reduction in testicular androgenesis as the growth of these organs are androgen-dependent (30). A decreased number of sperm cells with defective ACS and HOS-positive indicate the degradation of sperm acrosome and plasma membrane potentiality after administration of *A. catechu* seed extract at different doses in contrast to the VTC. These sensors with low values are indicators of male infertility. Focusing the WHO cut-off value of spermiological sensors for maintaining the fertility in human and translating that data in rat from the percentage of diminution of the specific sensors, highlighted the promising male contraceptive potentiality of the said extract. Most of the spermiological sensors were below the cut-off value of WHO, a lower borderline for maintaining fertility which supported the male contraceptive potentiality of the said extract. Sperm count, motility, HOS-positive sperm and ACS of sperm all are involved to signify the quality of sperm cells for the ability to fertilize the oocyte (31). Downregulation of these parameters indicated the impairment of androgenesis after the treatment. Diminution in sperm concentration in epididymal washed sample along with a decreased quantification of concerned generations of sperm cells at stage VII of spermatokinetics in the seminiferous tubular germinal epithelium after the treatment with said extract which led to reduction in spermatogenesis process due to low plasma level of serum testosterone (32). This has been supported here by low level of androgenic key enzyme activities (33). The decrease in SVF level may be the result of a reduction in testicular androgenesis as androgenesis is an important regulator of fructose synthesis in seminal vesicle (34). The alternative possibility for the impairment in the testicular activity by the administration of different doses of *A. catechu* seed extract may be the cause of OS imposition in reproductive organs as ROS have a deleterious effect on testicular androgenesis and gametogenesis (35). In this respect, anti-oxidant enzyme activity was assessed in target tissues. Significant diminution in the activities of the antioxidative enzymes such as, SOD and peroxidase along with the significant elevation in the quantity of lipid oxidation-peroxidation chain reaction end products i.e., TBARS in testicles and spermatozoa's pellet were observed, which are the strong indicators of OS imposition. Excessive production of ROS has spermicidal effect on germ cell that causes sperm morphological destruction leading to a considerable drop in motile sperm percentages along with acrosomal dysfunction and differentiation in plasma sperm membrane architecture (36, 37). Generation of ROS after the extract treatment may be strengthened here by apoptotic gene expression status analysis as apoptotic induction is also under the control of ROS (38). Effective seed extract treatment at different dose levels poses downward expression of apoptotic inhibiting gene i.e., Bcl-2 gene but upregulation of the apoptotic favourable gene expression i.e., Bax gene in testis of treated rats caused to uplift the apoptotic sperm cell death rate which ultimately leads to testicular dysfunction (39). Whether the applied doses of *A. catechu* have any toxicity effect or not, hepatic ACP and ALP activities along with SGOT and SGPT activities were measured, the important and sensitive sensors of hepato-toxicity assessment. Non-significant differences in said sensors in above-mentioned extract administered groups against the VTC reflected the nontoxic nature of the extract in general. After the LC-MS study, it may be stated that alkaloids, flavonoids, isoflavones, terpenoids, phenolic compounds and other phytoconstituents were found in this seed extract. It is hypothesized that the seed extract contains bioactive photo-elements that may help to induce male infertility by causing sperm cell membrane infractions and by imposing injuries on sperm cells. The lipid peroxidation process may disrupt the physiology of the sperm membrane and adversely influence the ability of fertility of spermatozoa or may impose negative modulation on testicular androgenesis (40).

Among the three doses, the 40 mg (highest dose) resulted maximum efficacy. This may be due to the presence of concerned phyto-molecule in this seed extract at higher concentration at 40 mg dose. The minimum but effective dose indicated here was 10 mg but the optimum cum threshold dose was 20 mg which may be due to receptor binding optimization kinetics of the concerned phyto-compound(s).

Conclusion

It may be concluded that hydro-ethanol (60:40) seed extract of *A. catechu* has the ability to induce testicular hypofunction by reducing the spermatogenesis and androgenesis process without creating any metabolic toxicity. This research may provide the pharmaceutical industry to generate a hope for the promising male herbal contraceptive generation in the near future through the advancement of research in herbal contraception from the viewpoint of drug development from natural bio-resources.

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