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Preventive Role of Human Chorionic Gonadotrophin (hCG) or Vitamin E or Vitamin C on Ethyl Acetate Fraction of *Terminalia chebula* Mediated Antitesticular Activities

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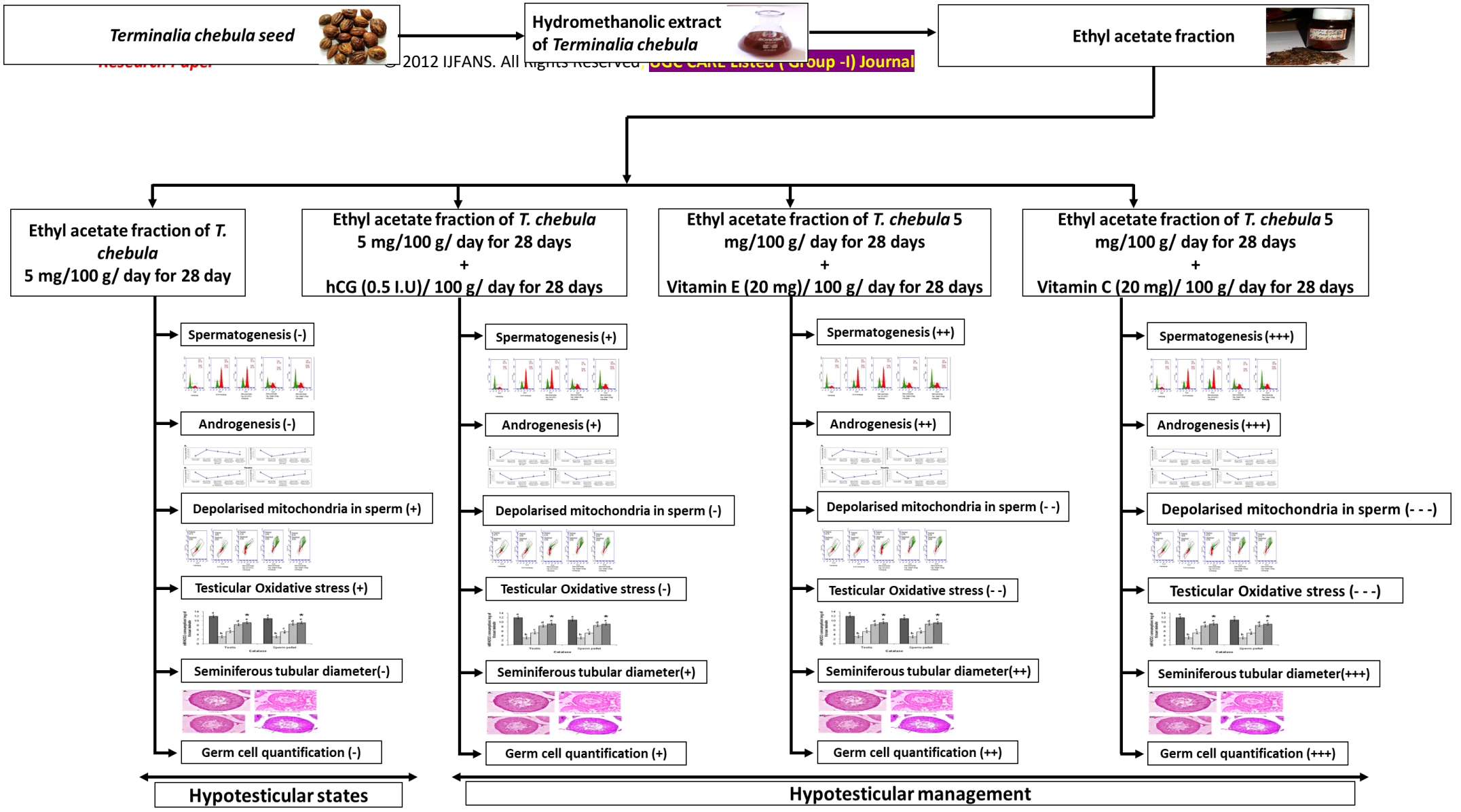
ABSTRACT

Background: Herbal treatment is one of the approaches of male contraceptives though its molecular action is not properly known. *Terminalia chebula* is one of such herbal component that has traditional reputation for antitesticular activity. **Objectives:** To evaluate the curative role of hCG (human chorionic gonadotrophin) or vitamin E or vitamin C against ethyl acetate fraction induced testicular hypo function. **Materials and Methods:** To know the mode of action of ethyl acetate fraction of *Terminalia chebula* towards hypotesticular activity and to find out the curative nature of hCG or vitamin E or C against hypotesticular activity, studies were conducted through spermatogenic, steroidogenic, androgenic and antioxidative enzyme analysis along with histological study. Count of different generations of sperm cells at middle stage i.e. stage VII of the cycle of spermatogenesis along with programmed cell death by genomic analysis were observed. **Results:** Dose of ethyl acetate fraction (5 mg/ 100 g body weight) focused significant negative deviation in spermatogenesis, androgenesis and decreased germ cells numbers at different generations with decreased seminiferous tubular diameter along with increased numbers of programmed death of sperm cells in Wistar strain male rats. Significant recoveries in all the parameters were noted near to the control level after supplementation with hCG or vitamin E or vitamin C. Out of these three supplements,

vitamin C was the most effective for this purpose. **Conclusion:** Effective fraction treatment with its effective dose level responsible for induction of hypotesticular function, which was recovered by supplementation of vitamin C or vitamin E or hCG where vitamin C was most effective in this purpose. This focus that direct effect of effective fraction of *Terminalia chebula* on testis is mainly responsible for hypotesticular activity induction as vitamin C supplementation which has both steroidogenic and spermatogenic corrective effects along with direct antioxidative role on sperms.

Keywords:

Anti-fertility, Ethyl acetate fraction, Flow-cytometer, Germ cell apoptosis, Spermatogenesis, *Terminaliachebula*.



Pictorial Abstract

INTRODUCTION

Uncontrolled and huge increment of the population imposing the extra burden to the community and which ultimately one of the main reasons for low economic state and population burden in different economically growing countries throughout the world. From the advancement of reproductive biomedicine, availability of several oral hormonal contraceptive pills and contraceptive methods for male are not always safe and not out of side effects. A task force was set up by World Health Organization to find out an oral herbal non steroidal contraceptive from different medicinal plants for male without any side effects to control the population explosion worldwide.[1-3] Molecular mechanism of action of available herbal contraceptives is beyond our knowledge. For that reason and as per guidelines of WHO, scientists and researchers are engaging to develop a male herbal contraceptive from an herbal origin which is cheap, eco-friendly and have less health hazards.[1-6] Progress and possibilities towards development of highly efficacious contraceptives were found among female than male contraceptives. Developing incipient contraceptives is essential for understanding male reproductive physiology. *Terminalia chebula* has long term folk medicinal reputation from ancient. Our previous study regarding the antifertility efficacy of different solvent fractions of *Terminalia chebula* well established its hypotesticular activity by suppressing the spermatogenesis and androgenesis process when treatment was conducted with effective solvent fractions and a polyherbal formulation was done previously to established the hypotesticular activity by using the seed extracts of *Terminalia chebula* and *Musa balbisiana*.[3,7] So, the current study was executed to know the molecular basis of action of effective fraction of the seed of *T. chebula* for hypotesticular activity induction as well as the curative role of hCG or vitamin E or vitamin C co-administration against hypotesticular activity on ethyl acetate fraction treated male albino rats.

MATERIALS AND METHODS

Ethyl acetate solvent fractionation from effective solvent extract

For preparation of ethyl acetate solvent fractionation (EA-Fr) from effective solvent extract, 600g pulverized seeds of *Terminalia chebula* (*T. chebula*) was dissolved in 2 litre of hydro-methanolic (H_2O: MeOH : 3:2) solution. After the preparation of hydro-methanolic extract of the seed of *T. chebula*, fractionation was conducted using ethyl acetate in a 5 liter separating flask. After that, ethyl acetate fraction was collected separately and dried under reduced pressure using rotary evaporator. Finally 2 liter of HM (3:2) extract of *T. chebula* afforded 750 mg of ethyl acetate fraction and then dried fraction was finally used to experimental animals.[8-11]

Animal acclimatization

Healthy and adult male rats of albino strain about 60 days having body weight about 140 ± 10 g were used for this experiment from our CPCSEA approved animal vendor 'Saha Enterprise'. Before using the animals in the study, experimental rats were preconditioned in animal facility centre for two weeks at 25 ± 2°C ambient room temperature, 44-55% of humidity and kept them in 12hr : 12hr in day-night in this cyclic condition to acclimate them. Standard feed and water *ad libitum* were provided to all the experimental animals. The directives of our Institutional Ethics Committee (IEC/6-15/C-6/16; Dated: 19.04.2016) were followed throughout the experiments.

Experimental design

Searching for the ameliorative effect of hCG, vitamin E or C on EA-Fr of *T. chebula* mediated hypotesticular activity, this experiment was conducted with five equal groups having six rats in each group. In that case the treatment was done by co administrating of EA-Fr with hCG or vitamin E or vitamin C at different treated groups along with EA-Fr treated group separately at 5 mg dose excluding the control group. After completion of acclimatization of the experimental rats, initial body weights were recorded. Oral treatment of the said fraction to the treated group was done daily at 9:00 AM for 28 days because one wave of spermatogenic cycle in Wistar rat takes 21 days to complete.[3] Ethyl acetate solvent fraction in dry form was dissolved in distilled water (d.w) and was administered orally at a dose of 5 mg/ 0.5 ml d.w/ 100 g body weight to the different experimental groups. Literature review and our previous pilot work helping us to select the different dose level of hCG or vitamin E or vitamin C for this recovery activity study against antitesticular activity of effective seed fraction of seed of *T. chebula*. Animals of vehicle treated control group were treated with distilled water. After completion of the treatment schedule, the results were compared to select the most effective recovery from antifertility activity after co-administration of hCG or vitamin E or vitamin C in comparison with the vehicle treated control.

Treatment protocol

Treatment through gavage was done with ethyl acetate solvent fractions of HM (3:2) extract of *T. chebula* at a dose of 5 mg/ 100 g body weight for 28 days to the experimental animals and EA-Fr with hCG (0.5 I.U) or vitamin E (20 mg) or vitamin C (20 mg) to co administered groups for single time of treatment/ day as per following treatment schedule.[12-16] The treatment time and food supply were followed as per previous experiments.

Vehicle treated control group: Rats were treated with distilled water by gavage only by 0.5 ml/ 100 g of body weight/ rat/ day for 4 weeks and considered as vehicle treated control group.

Ethyl acetate fraction 5 mg exposed group: Rats were treated with said fraction by 5 mg/ 0.5 ml distilled water/ 100 g body weight/ rat daily for 4 weeks at 9:00 A.M.

Ethyl acetate fraction 5 mg + hCG (0.5 I.U) exposed group: Rats were treated with the said fraction once daily at 9:00 A.M. at the above dose and hCG was injected intramuscularly at 5:00 P.M. once in a day at the dose of 0.5 I.U. (International Unit)/ 100 g body weight for 4 weeks.

Ethyl acetate fraction 5 mg + vitamin E (20 mg) exposed group: Rats were treated with the said fraction once daily at 9:00 A.M. at the same dose and vitamin E at 20 mg/100 g dose was provided at 5:00 P.M. through oral route by gavage for 4 weeks.

Ethyl acetate fraction 5 mg + vitamin C (20 mg) exposed group: Experimental rats were treated with EA-Fr once daily at 9:00 A.M. at the fixed dose and vitamin C at 20 mg/100 g dose was provided at 5:00 P.M orally through gavage for 4 weeks.

Sample tissue collection

Experimental animals were sacrificed on 29th day by euthanasia after 28 days of treatment protocol. Final body weight was measured before sample collection of all the animals. Different spermatogenic, genomic, biochemical and androgenic and histologic sensors, the assessment of different antioxidant profile and toxicity levels were assessed. Different organs like testes, liver, kidney and for spermatogenic profile assessment, epididymal sperms were

collected. Organs were preserved in -20° for different enzymatic and biochemical sensor analysis. For the assessment of serum testosterone and toxicity level, blood was collected from dorsal aorta of experimental rats. Right testes of each experimental animal were placed in 'Bouins' fixative for histological study.

Sperm count

By mincing and filtering the pairs of cauda epididymis in 1 ml of phosphate buffer saline (PBS), epididymal sperms (w/v) were obtained and counted using Neubauer chamber microscopically.[17]

Flowcytometric analysis of motile sperm

One drop of sperm suspension (prepared in 1 ml of PBS) was placed in a glass slide to count the motile sperm microscopically within 2-4 min of their isolation from caudal part of epididymis and result was denoted in percentages.[18]

Flowcytometric analysis of viable sperm

Live and dead sperm cells estimation was performed using BD cell viability kit. At about 1.8 ml of pre-warmed Tris buffer in a cell suspension of 1×10^6 cell/ml mixed with epididymal fluid and 2 ml of Thiazole Orange (TO), 2 ml of Propidium Iodide (PI) were added with that mixture and incubated at 37°C for 5 min and results were recorded using fluorescence activated cell shorter (FACS).[19]

Polarised/ depolarised sperm mitochondrial asessment by FACS

Mitochondrial membrane potential detection JC-1 kit was used for the assessment of sperm mitochondrial membrane integrity. In that case sample cell washing and staining were performed twice by diluting 50 µL of epididymal fluid with 500 µL of JC-1 working solution in a centrifuge tube followed by vortex and incubation. After that all the sample precipitates were washed with 1 X assay buffer and after discarding the supernatant, all the sample precipitates were resuspended in 500 µL of 1 X assay buffer for flowcytometric analysis. [20]

Androgenic key enzymes activity assessment

Standard protocol was performed to evaluate androgenic key enzyme activities.[21,22] Homogenization of testicular tissue samples at about 100 mg/ ml was performed in homogenizing mixture containing 5 mM of potassium phosphate, 20% glycerol and 1 mM EDTA. Both of these androgenic enzyme activities were evaluated of each testicular tissue samples by spectrophotometrically after collection of the centrifuged tissue supernatants at 340 nm wavelength of light.

Quantification of serum testosterone by Enzyme linked immunosorbant assay (ELISA)

According to the guidelines of National Institute of Health and Family Welfare (NIHFW) the level of serum testosterone was evaluated by kit method.[23] Concentration of serum testosterone in unknown sample was evaluated by solid phase immunoassay technique with the help of coated antibody in micro plate wells along with standards and enzyme conjugate provided in the kit. At the end of the whole enzymatic reaction after a certain time period the reaction was allowed to stop using stop solution and the optical density of each sample wells was measured using selective and differentiating filter of ELISA reader. Intra-assay variation 5.2% and no inter-assay variation due to measurement of all the samples at a time and 0.09%, 1.7% cross reaction levels were noted with other androgens like androstenedione and dihydrotestosterone.

Cholesterol assessment in testis

Cholesterol in testis was measured by enzymatic colorimetric method.[24] Centrifuged supernatants of testicular tissue homogenates at a tissue concentration of 50 mg/ ml of ice-cold phosphate buffer as homogenizing media was used here for testicular cholesterol estimation.

Catalase activity assessment

Homogenized and centrifuged supernatants of testicular tissue samples and sperm pellet were used for the estimation of the activity of catalase enzyme by standard protocol.[25] Optical density (OD) of each sample mixture in spectrometric cuvette that containing 0.5 ml of hydrogen peroxide, 2.5 ml of deionized water and 100 µL of centrifuged tissue homogenates were recorded at 240 nm wavelength of light using spectrophotometer at 30 seconds interval of time.

Assessment of antioxidative enzyme superoxide dismutase (SOD)

Inhibition in the percentage of pyrogallol auto oxidation measurement helps to evaluate the SOD enzyme activity of testicular tissue using standard procedure of Marklund and Marklund.[26] Homogenized and centrifuged testicular tissue samples were allowed to spectrophotometric analysis by mixing 2 ml of phosphate buffer with 20 µL of tissue supernatants and 20 µL of pyrogallol in the spectophometric cuvette and OD was measured by taking the readings at 420 nanometer wavelength of light.

Biochemical quantification of Thiobarbituric acid reactive substances

The level of TBARS was measured from the absorption of thioburbituric acid. The levels of TBARS quantification was performed by mixing homogenized centrifuged targeted tissue at about 0.5 ml with 0.5 ml of normal saline and make the sample volume at about 3 ml by adding 2 ml of TBA-TCA (thiobarbituric acid-trichloro acetic acid) mixture. Then the sample mixtures were allowed to boil, cool and centrifuged in room temperature and after that spectrophotometric analysis was performed for the estimation of TBARS level by taking the OD of the whole sample supernatants in the cuvette at 535 nm wavelength of light against blank. [27]

qRT-PCR study

Separation of total mRNA (messenger RNA) from testicular tissue and from this mRNA complementary cDNA preparation was done as per the instruction provided in “Transcriptor First Stand cDNA Synthesis Kit”. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) study of testicular androgenic key enzymes and apoptotic markers was performed by using selected primers in Light Cycler 480 II.[3,28,29]

Histological study

Measurement of the diameters of seminiferous tubule of stained (hematoxylin-eosin) testicular tissue sections was done by using DeWinter Calipro-3.0 Software. Micro photographs of particular fields were taken of stained testicular tissue section for histoarchitectural analysis of different generation of germ cells at stage VII of seminiferous epithelium as per the method described by Leblond and Clermont.[31]

Toxicological study

For the assessment of metabolic toxicity, hepatic and renal GOT and GPT activities were evaluated to established the safety profile of the effective plant fraction as per standard protocol.[32]

Statistics

For statistical analysis of data, ANOVA (analysis of variance) followed by 'Multiple Comparison Student's two tail t-test' was used here. In statistical analysis, level of significance was considered at the probability value 0.05. One way ANOVA was used here for data analysis of this experiment by investigating the effects of single independent variable on dependent one.[33]

RESULTS

To establish the antifertility efficacy of the effective seed fraction of *T. chebula* and for the comparison of the ameliorative efficacy of hCG or vitamin E or vitamin C against ethyl acetate fraction mediated hypotesticular activity followed by the co treatment study, this results were analysed statistically.

Spermatogenic profile

Ethyl acetate fraction treatment of experimental rats of different treated groups results significantly decreased ($p<0.05$) in the rate of sperm count (52.50%) and sperm motility (35.71%), which were increased significantly ($p<0.05$) after hCG or vitamin E or vitamin C supplementation along with ethyl acetate fraction treatment at about 26.31%; 47.36%; 73.68% in case of sperm count and 11.11%; 22.22%; 33.33% in case of sperm motility. Most significant recovery of both of these parameters was noted when supplementation was done with vitamin C than other co-administrated group (Figure 1 and 2).

Sperm viability through flow-cytometry

Effective fraction treatment for 28 days results significantly decreased ($p<0.05$) in live sperm cell population size about 58.51% and significantly increased ($p<0.05$) in dead sperm cell population size at about 157.87%. Human chorionic gonadotrophin or vitamin E or vitamin C supplementation along with ethyl acetate fraction treatment to the treated group significantly increased ($p<0.05$) the live sperm cell population size at about 19.64%; 67.36%; 103.50% and significantly decreased ($p<0.05$) the dead sperm cell population size at about 8.94%; 35.08%; 47.72%. Supplementation with vitamin C treated groups poses maximum recovery of this parameter to the control level than other co treated groups (Figure 3).

Sperm mitochondrial membrane potentiality by FACS

Significant increased ($p<0.05$) in depolarized (63.23%) sperm mitochondria and significant decreased ($p<0.05$) in polarized (39.87%) sperm mitochondria indicates low mitochondrial membrane potentiality of sperm when treatment with ethyl acetate solvent fraction. Different supplementations with hCG or vitamin E or vitamin C along with ethyl acetate fraction treatment helps to resettle of this parameter significantly ($p<0.05$) by increasing polarized (11.05%; 35.73%; 49.87%) sperm mitochondria and decreasing depolarized (3.60%; 40.90%;

30.81%) sperm mitochondrial membrane and maximum efficacy in this concern was noted with vitamin C supplementation (Figure 4).

Androgenic key enzyme activities

Percentage of inhibition in case of both these two androgenic key enzyme activities at about 54.28% and 56.25% after effective fraction treatment. Percentages of recovery to these enzyme activities at about 25%; 56.25%; 87.50% and 28.57%; 64.28%; 100% after supplementation with hCG or vitamin E or vitamin C to the fraction treated groups and most effective recovery was noted in EA-Fr + vitamin C administrated group than others (Figure 5).

Serum testosterone level

Effective fraction treatment results, 61.70% of significant diminution ($p<0.05$) to this hormonal level and it was resettled significantly ($p<0.05$) towards control level due to hCG (92.01%) or vitamin E (41.78%) or vitamin C (138.09%) supplementation and most effective result in this concern was noted after vitamin C supplementation (Figure 6).

Level of testicular cholesterol

Increased ($p<0.05$) level of testicular cholesterol (80%) due to EA-Fr treatment was resettled towards the control level significantly about 11.11%; 22.22% and 33.33% after co-administration of hCG or vitamin E or vitamin C to the experimental groups along with EA-Fr treatment and most effectively resettled after vitamin C supplementation than other groups (Figure 7).

Activities of antioxidant enzymes

Percentages of catalase (73.75% and 71.90%) and SOD (73.97% and 74.48%) enzyme activities were significantly ($p<0.05$) decreased in EA-Fr treatment, and these two enzyme activities significantly but little rectified by hCG (68.57%; 67.96%; 82.79%; 89.95%) supplementation intramuscularly whereas oral supplementation of vitamin E (165.39%; 177.66%; 125.58%; 124.40%) and vitamin C (192.38%; 197.41%; 219.53%; 224.88%) produced maximum rectification to these enzyme activities, and most effective result was noted after vitamin C supplementation (Figure 8 and 9).

Levels of TBARS

Significantly increased ($p<0.05$) in the percentages of free radical by product in testicular tissue 45% and sperm pellet 85.71% due to effective fraction treatment was significantly corrected ($p<0.05$) up to the level of control after hCG (12.5%; 12.82%) or vitamin E (25%; 25.64%) or vitamin C (35%; 35.89%) supplementations, most effectively in vitamin C supplementation than other co treated groups (Figure 10).

Testicular genomic expression

Upward regulation of pro-apoptotic Bax gene and downward regulation of anti-apoptotic Bcl-2 gene and down regulation of $\Delta 5$, 3β-HSD and 17β-HSD gene expression due to EA-Fr treatment was resettled significantly $p<0.05$ to the level of control after co-administration of hCG (22.22%; 115%; 100%; 95%) or vitamin E (35.55%; 226.31%; 200%; 186%) or vitamin C (60%; 326.31%; 300%; 272.72%) in all the experimental groups. Maximum efficacy was noted in this concern by co-administration of vitamin C than the other co treated groups (Figure 11A and11B).

Histological study

Co administration of different supplementation with hCG or vitamin E or vitamin C significantly $p<0.05$ increased the seminiferous tubular diameter (12%; 40%; 68%) and significantly $p<0.05$ increased the numbers of hormone sensitive germ cells at stage VII i.e. Asg 100%; 200%; 275%, pLSc 53.30%; 92.30%; 121.99%, mPSc 20.97%; 37.83%; 54.57% and 7sd 17.85%; 37.17%; 66.64% which were decreased due to the effective fraction treatment. Most effective recovery of both of these sensors was noted after vitamin C supplementation (Figure 12 and Table 1).

Toxicity level

No significant changes $p>0.05$ noted during toxicity level assessment by assessing hepatic and renal GOT (3.33%; 8%) and GPT (3.44; 8.69%) activities of experimental animals after effective fraction treatment for 28 days (Table 2).

DISCUSSION

Induction of hypotesticular function due to effective fraction treatment and to know its molecular mechanism of action, this present experiment was conducted. Concerned different parameters like routine spermiological sensors; spermatogenic profile assessment by flow cytometry; androgenic profile assessment and biochemical sensor analysis; antioxidative enzymes activities assessment; oxidative stress related biosensors assessment; genomic sensors analysis; histological study and toxicity level assessment were done. All these parameters help us to find out the most effective recovery from the hypotesticular activity induced by EA-Fr followed by hCG or vitamin E or vitamin C co-administration. Decreased sperm count, sperm motility after the fraction treatment with EA-Fr fraction of *T. chebula* interfering the fertilizing capacity in rats which support the antifertility activity of the fraction.[2] Androgenic key enzymes activities were decreased by which testosterone synthesis was diminished which ultimately the cause of the induction of hypotesticular activity.[2] Diminution in serum testosterone level and elevation in cholesterol level in testis due to EA-Fr treatment further supported the impaired male steroidogenesis.[2] Oxidative stress generation by decreasing antioxidant enzyme activity and increasing free radical-by-products in testicular tissue which ultimately lead to reproductive damage by diminishing androgenesis and steroidogenesis.[2,3] Testicular androgenic gene expression was performed here to know such inhibitory effect of EA-Fr containing phytomolecules responsible for hypotesticular activity development due to phytomolecule-gene interaction process for inhibition at gene level. Effective fraction treatment increased the pro-apoptotic and decreased the anti-apoptotic gene expression. Side by side, elevated rate of germ cell apoptosis resulted decreased in sperm count and viability. Low level of serum testosterone that results decreased diameter of seminiferous tubule and decreased number of germ cells in seminiferous epithelium.[30,31]

From this experiment it may be stated that, treatment with EA-Fr of *T. chebula* decreased steroidogenic enzyme activities, increased level of testicular cholesterol, increased oxidative stress by decreasing antioxidant enzymes activities and increasing free radical by product. Which ultimately diminished the androgenesis process by which testosterone synthesis was decreased and therefore spermatogenesis process was hampered which ultimately the cause of hypotesticular function. Increased rate of sperm apoptosis and decreased rate of androgenic gene expression responsible for primary hypotesticular activity induction may be due to presence of phytomolecules in EA-Fr. On the other hand we may say that, the EA-Fr treatment may have an effect on testicular oxidative stress imposition that generates free

radicals which may develop hypotesticular status. Side by side, this fraction may have some effect on pituitary for inhibition in gonadotrophin secretion.[2,3]

Supplementation of hCG or vitamin E or vitamin C resulted recovery in the above mentioned parameters towards the control level. From this result it may be stated that hCG, vitamin E or vitamin C have the anti-oxidative properties as well as have modulating capacity for pituitary gonadotropin i.e. FSH and LH secretion which ultimately increase the testosterone production and therefore hypotesticular activity may be managed.[12-16] Side by side supplementation of vitamin E or vitamin C also decreased the oxidative stress generation in testes which may be the another cause of testicular hypofunction correction. From the analysis of the percentage of recovery rate from different parameters it was focussed that co-administration of vitamin C is most effective than co-administration of hCG or vitamin E against EA-Fr mediated hypotesticular activity. Vitamin C is responsible for steroid biosynthesis by the stimulation of FSH and LH hormone which facilitates steroidogenesis process.[16,34] Free radicals scavenging activity is managed in testicular tissue by good non-enzymetic antioxidant properties of vitamin C. Moreover vitamin C has stimulatory effect on testicular androgenesis.[16] Non toxic effect of EA-Fr was proved by hepatic and renal toxicity assessment study. As, hCG and vitamin E are not so effective like vitamin C from the view points of androgenesis and oxidative stress recovery so it focused that phytomolecules present in this fraction mainly exerts its direct effect on testes by inducing oxidative injuries other than inhibition in pituitary gonadotrophin secretion for its hypotesticular function because hCG acting as LH.[12,13,35]

CONCLUSION

Recovery from effective fraction inducing hypotesticular activity was managed by the supplementation of hCG or vitamin E or vitamin C. Due to antioxidative, spermatogenic and steroidogenic corrective role of vitamin C than other two supplementation is more for the reduction of direct effect of effective fraction inducing hypotesticular activity.

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CONFLICT OF INTEREST

All the authors state that there are no conflicts of interest within this article.

AUTHORS’ CONTRIBUTION

PG (prabalk93@gmail.com) has conducted the experimental work, molecular genetics study and drafted the manuscript; PG (prietygupta1992@gmail.com) carried out histological study, biochemical assay, animal handling and genomic study; AT (adrijamid@yahoo.com) performed the statistical analysis, helped in manuscript drafting, Co-ordinated the histological work, flow cytometric study and helped for drafting the manuscript; DG (debidasghosh999@gmail.com) contributed for the designing of the study, concept of the work and edited the manuscript.

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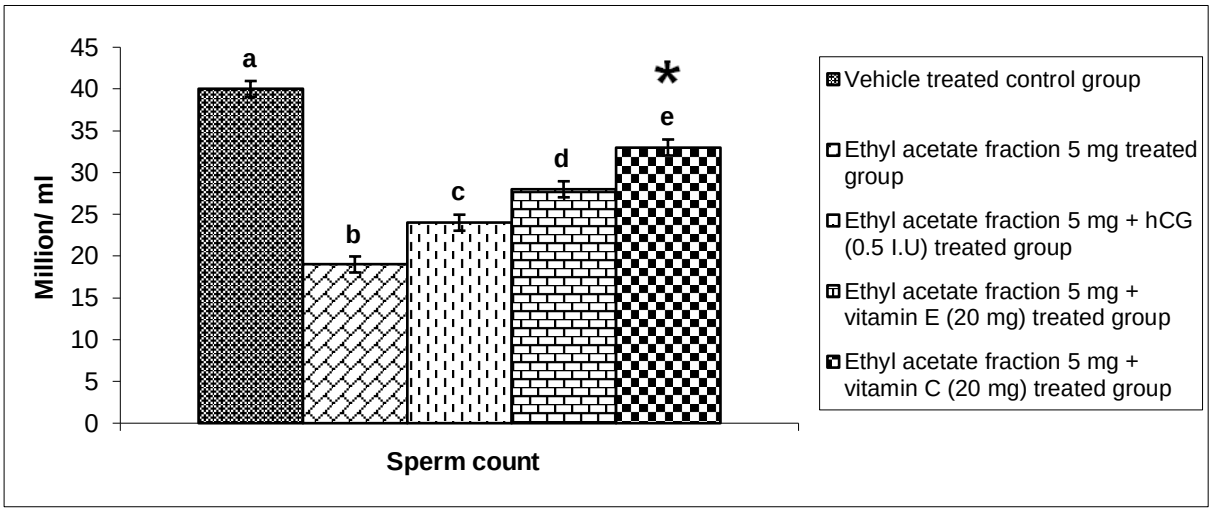

Figure 1:

Graphical representation of the bar diagrams of sperm count of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C treated groups in respect to the control group.

Ameliorative effect of hCG, vitamin E or vitamin C co administration on sperm count in *T. chebula*, EA-Fr treated male albino rats. Data of each bar diagrams were expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Bar diagrams with different superscripts (a, b, c, d, e) are differ from each other significantly at the level of $p<0.05$. Star mark (*) indicating bar shows most effective group of recovery from hypotesticular activity of EA-Fr of *T. chebula* than other treated groups.

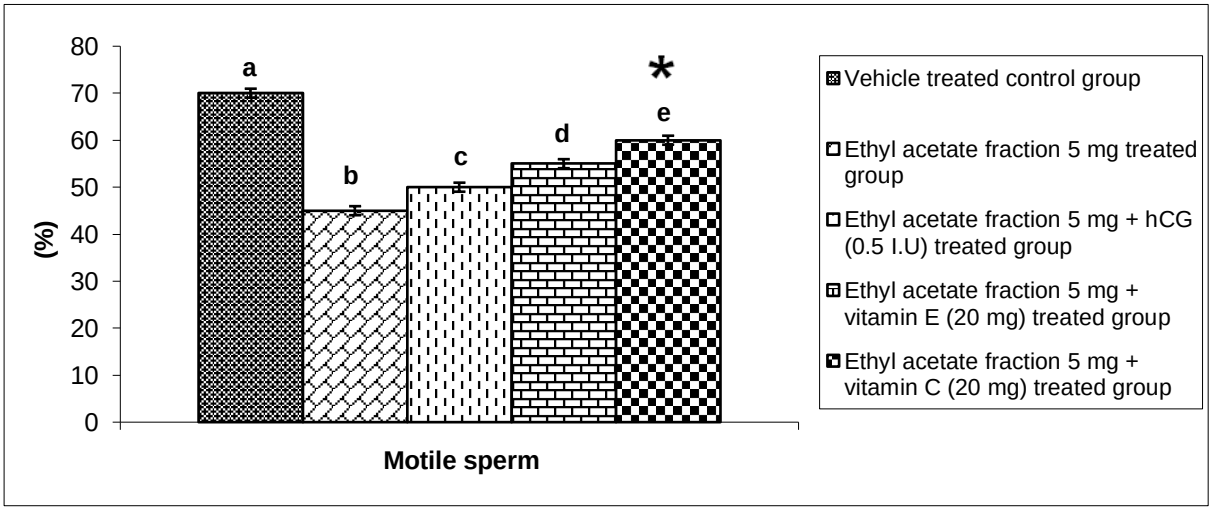

Figure 2:

Graphical representation of sperm motility of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C treated groups in respect to the control group.

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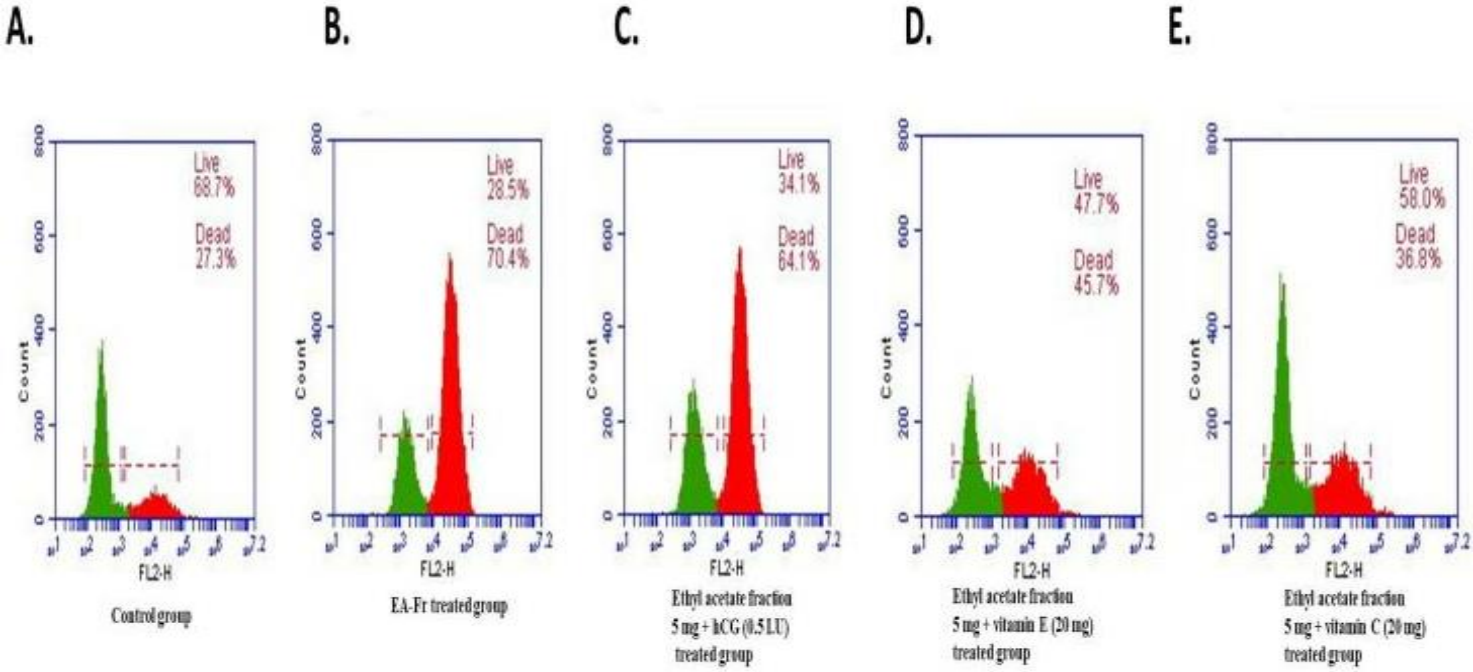
Recovery rate of hCG, vitamin E or vitamin C co administration on sperm motility in *T. chebula*, EA-Fr treated male albino rats. Each bar diagrams with data showing in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Bar diagrams with different superscripts (a, b, c, d, e) are differ from each other significantly at the level of p<0.05. Star mark (*) indicating bar shows most effective group of recovery from hypotesticular activity of EA-Fr of *T. chebula* than other treated groups.



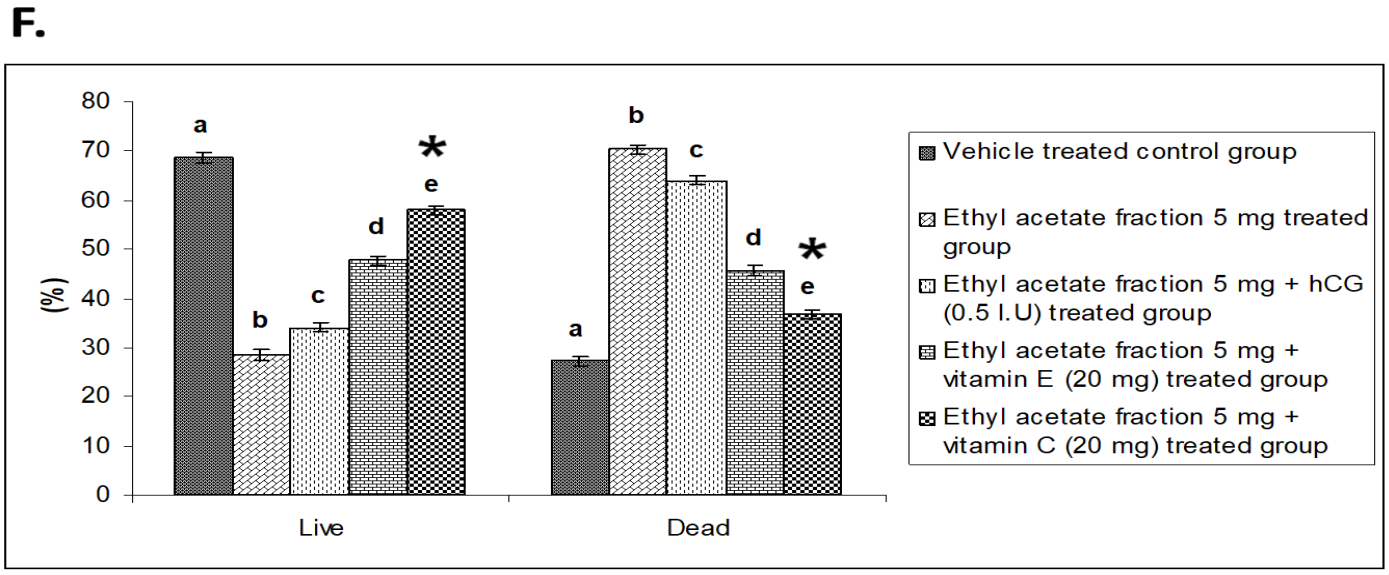


Figure 3:

Flow cytometric analysis of sperm cell viability of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C treated groups in respect to the control group.

Comparative analysis of sperm viability by flow cytometry in vehicle treated control and different treated groups of EA-Fr with the co administration of hCG or vitamin E or vitamin C (A) Vehicle treated control (B) Ethyl acetate fraction 5 mg treated group (C) Ethyl acetate fraction 5 mg + hCG (0.5 I.U) treated group (D) Ethyl acetate fraction 5 mg + vitamin E (20 mg) treated group (E) Ethyl acetate fraction 5 mg + vitamin C (20 mg) treated group. Picture (A-E) represents histogram of different treated groups of EA-Fr after co administration of hCG, vitamin E or C including control group. Picture (F) Bar diagrams of live and dead sperms in different treated groups after treatment with EA-Fr and co administration of hCG, vitamin E or C in respect to the control group. Bars with different superscripts (a, b, c, d, e) differ from each other significantly,

$p < 0.05$. FL2-H - Fluorescence channel 2- Height.

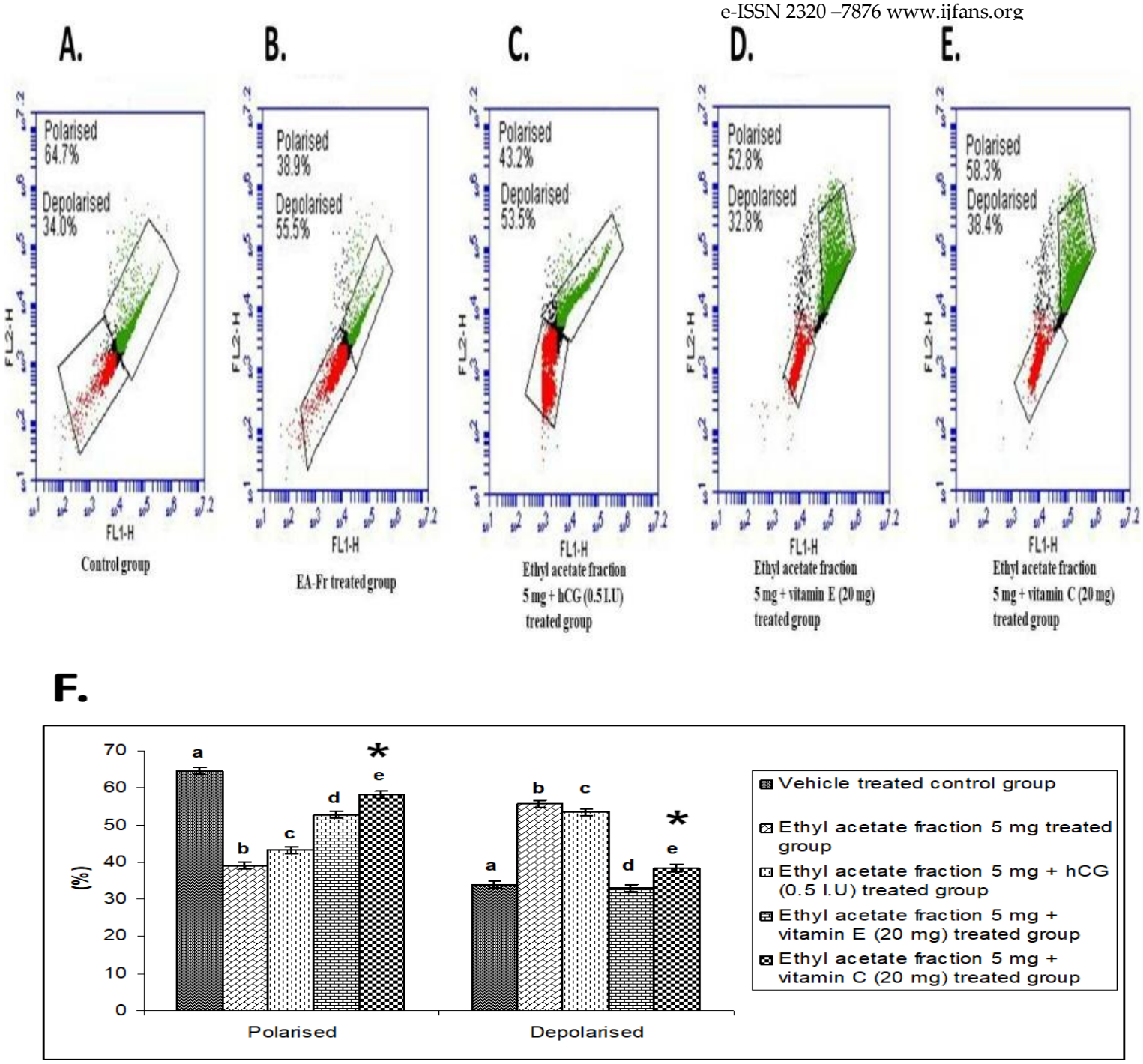


Figure 4:

Flow cytometric analysis of sperm mitochondrial status of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C treated groups in respect to the control group.

Comparative analysis of sperm mitochondrial status by flow cytometry in vehicle treated control and different treated groups of EA-Fr with the co administration of hCG or vitamin E or vitamin C (A) Vehicle treated control (B) Ethyl acetate fraction 5 mg treated group (C) Ethyl acetate fraction 5 mg + hCG (0.5 I.U) treated group (D) Ethyl acetate fraction 5 mg + vitamin E (20 mg) treated group (E) Ethyl acetate fraction 5 mg + vitamin C (20 mg) treated group. Picture (A-E) represents histogram of different treated groups of EA-Fr after co administration of hCG, vitamin E or C including control group. Picture (F) bar diagrams of polarised and depolarised sperms in

different treated groups after treatment with EA-Fr and co administration of hCG, vitamin E or C in respect to the control group. Bars with different superscripts (a, b, c, d, e) differ from each other significantly, p < 0.05. FL1-H - Fluorescence channel 1-Height

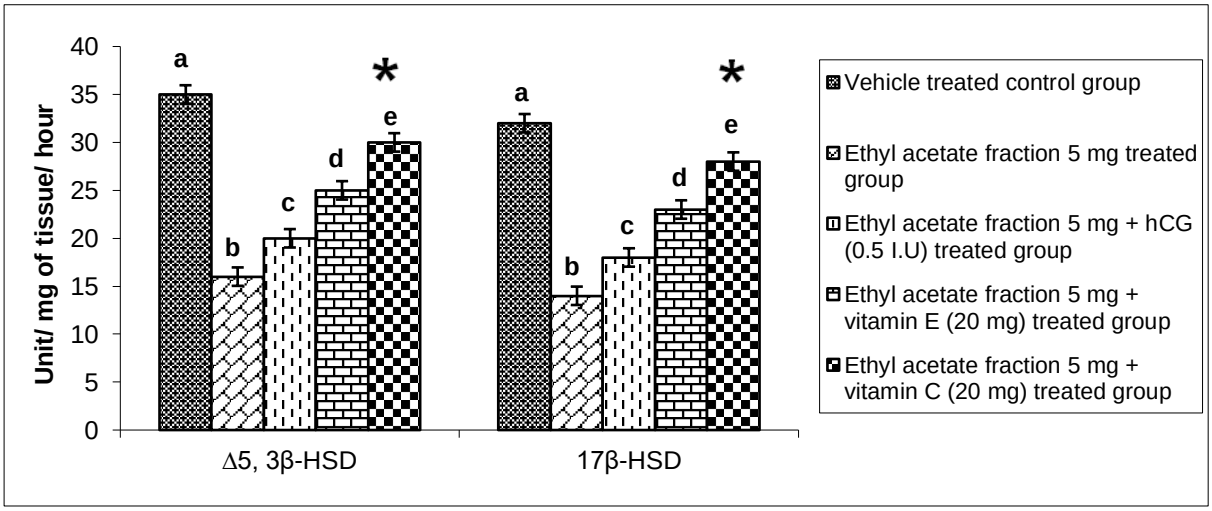


Figure 5:

Graphical representation of the bar diagrams of testicular androgenic key enzymes activities of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C co administrative groups in respect to the control group.

Resettlement of these testicular androgenic key enzymes activities towards control level after co administration of hCG or vitamin E or vitamin C in EA-Fr of *T. chebula* treated mature albino rats. Each bar diagram with data expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Values with different superscripts (a, b, c, d, e) on each bar diagram are differ from each other significantly, p<0.05. Star mark (*) indicates the most effective group of recovery from hypotesticular activity of EA-Fr of *T. chebula*.

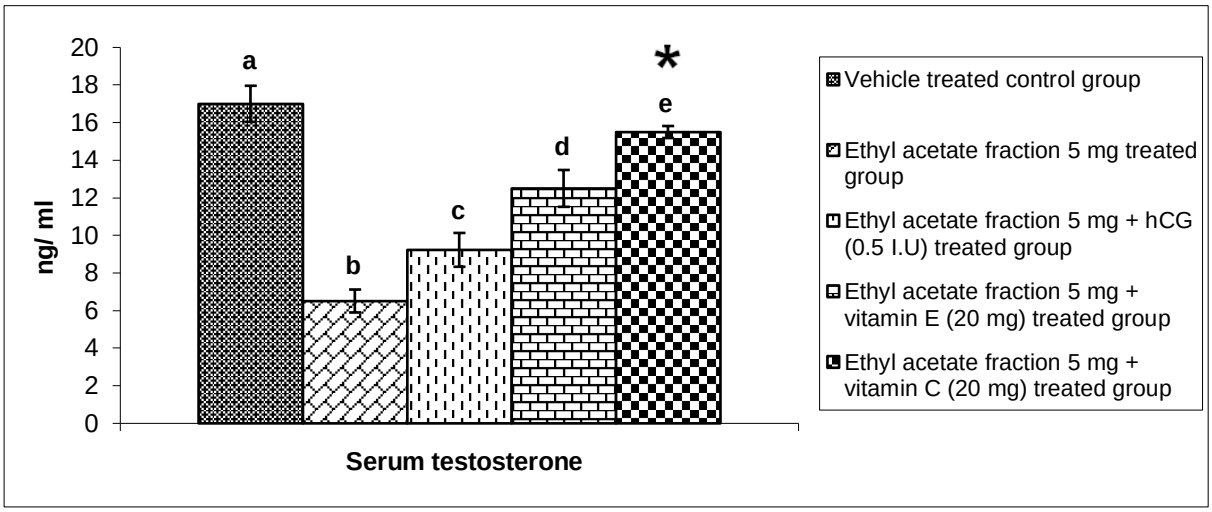


Figure 6:

Representation of serum testosterone level of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C administrative groups in respect to the control group.

Recoveries in serum level of testosterone towards control level after co administrating of hCG or vitamin E or vitamin C in EA-Fr treated rats. Data were expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Values with different superscripts (a, b, c, d, e) on each bar diagram are differ from each other significantly at the probability value of 0.05. Star mark (*) indicates the most effective group of recovery from hypotesticular activity of EA-Fr of *T. chebula*.

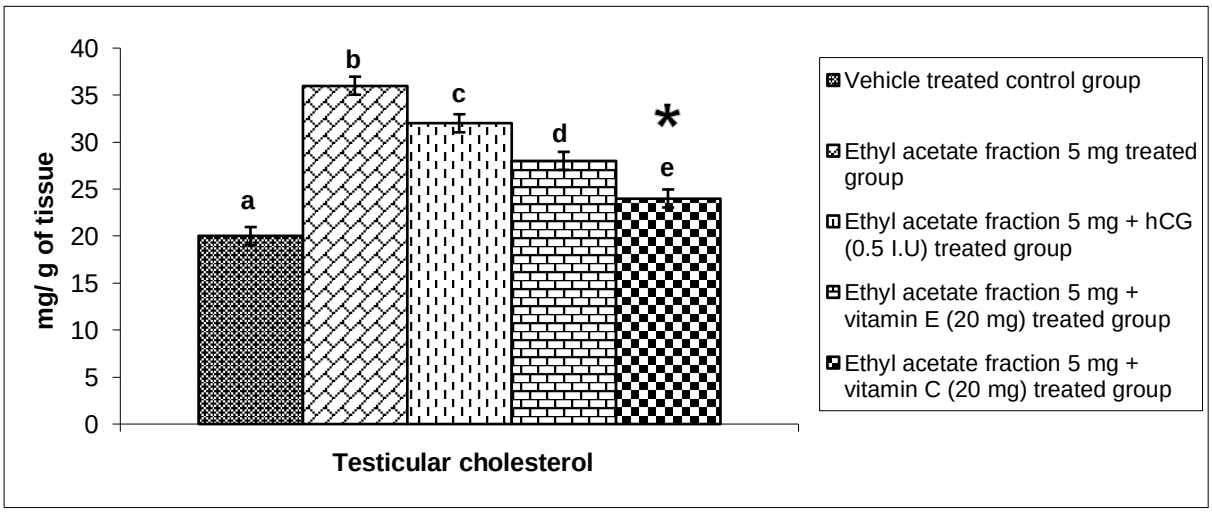


Figure 7:

Graphical representation of testicular cholesterol level of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C administrative groups in respect to the control group.

Comparative analysis of the testicular cholesterol level after hCG or vitamin E or vitamin C co administration in EA-Fr of *T. chebula* treated male albino rats. Data were expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA

followed by multiple comparisons "Student's two tail t-test". Bar diagrams with different superscripts (a, b, c, d, e) are differ from each other significantly, p<0.05. Star mark (*) indicates the most effective group of recovery from hypotesticular activity of EA-Fr of *T. chebula*.

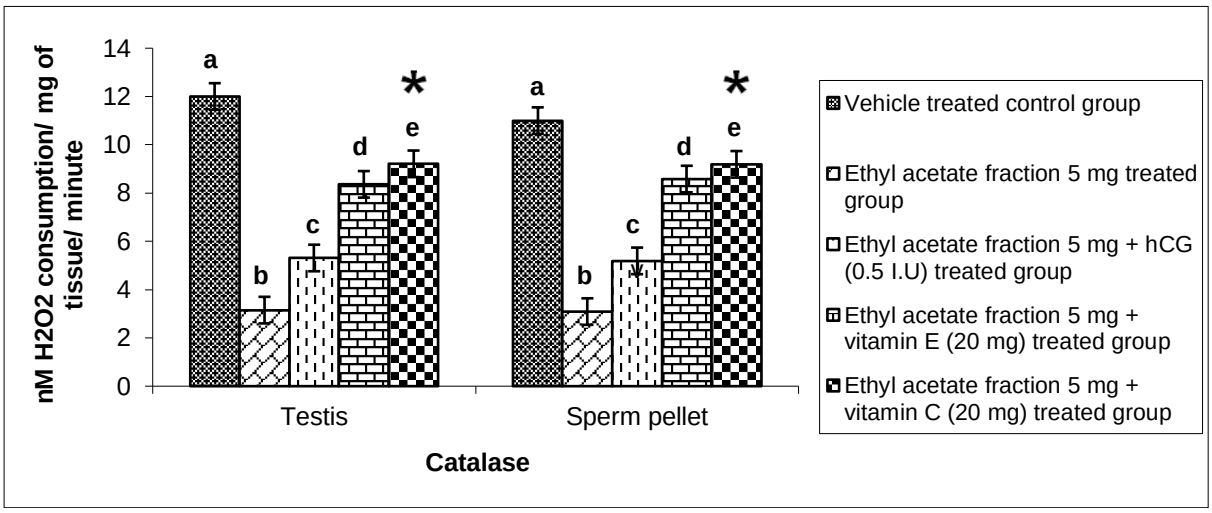


Figure 8:

Bar diagrams of antioxidative enzyme catalase of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C co administrative groups in respect to the control group.

Activity of catalase recovered towards control level after co administration of hCG or vitamin E or C in EA-Fr treated male albino rats. Data were expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Different values containing superscripts (a, b, c, d, e) on each bar diagram are differ from each other significantly, p<0.05. Star mark (*) indicates the most effective group of recovery from hypotesticular activity of EA-Fr of *T. chebula*.

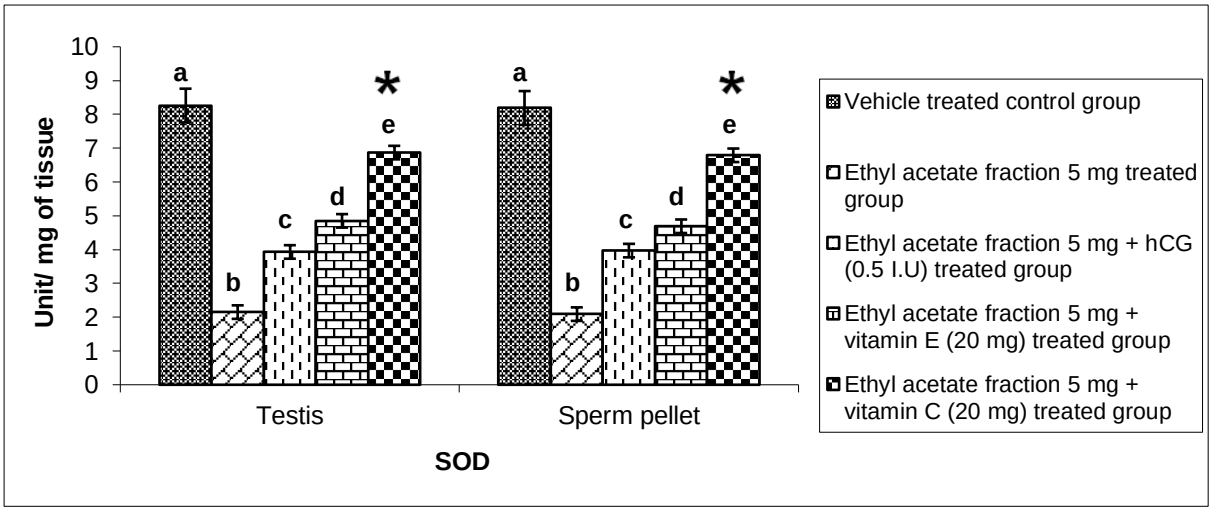


Figure 9:

Bar diagrams of antioxidative enzyme SOD of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C co administrative groups in respect to the control group.

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Activity of SOD recovered towards control level after co administration of hCG or vitamin E or C in EA-Fr treated male albino rats. Data were expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Different values containing superscripts (a, b, c, d, e) on each bar diagram are differ from each other significantly, p<0.05. Star mark (*) indicates the most effective group of recovery from hypotesticular activity of EA-Fr of *T. chebula*.

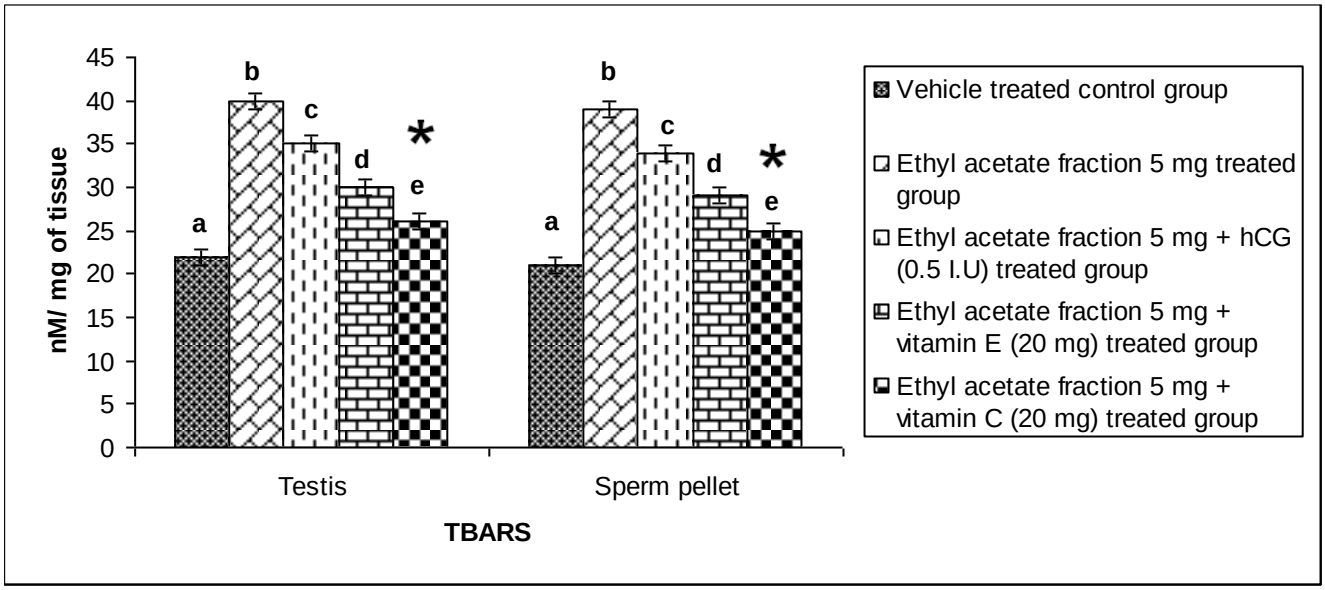
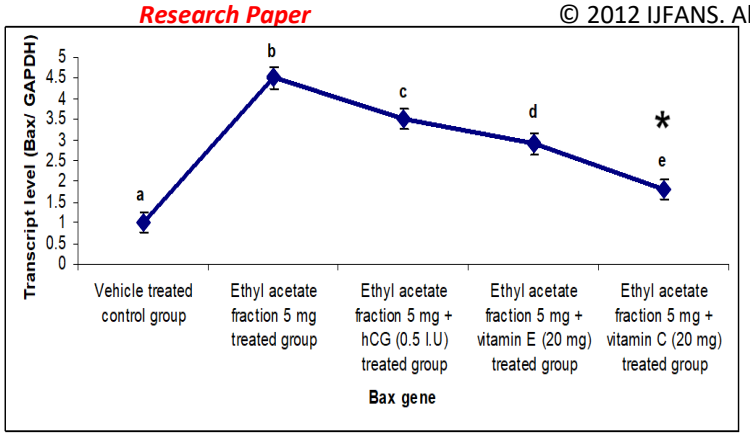


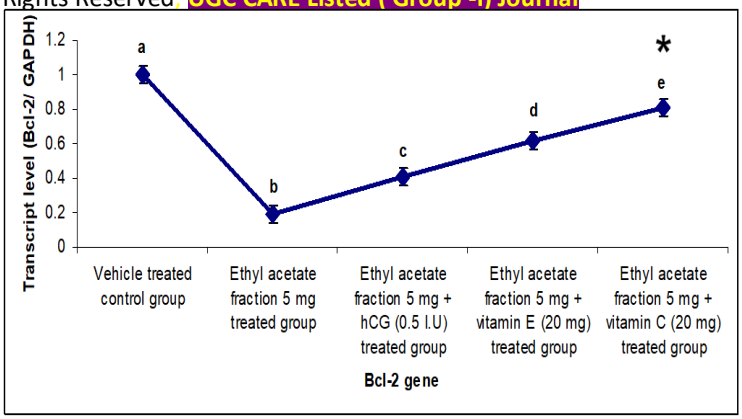
Figure 10:

Graphical Representation of bar diagram of TBARS of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C co administrative groups in respect to the control group.

Recovery level of testicular TBARS after hCG or vitamin E or vitamin C co administration in the EA-Fr treated matured male albino rats. Bar diagrams represents Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Bars with the values with different superscripts (a, b, c, d, e) are differ from each other significantly, p<0.05. Star mark (*) indicates the most effective group of recovery from hypotesticular activity of EA-Fr of *T. chebula*.

A.

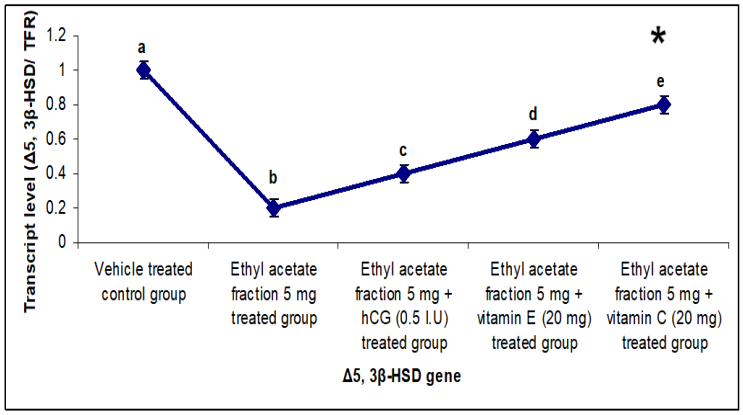


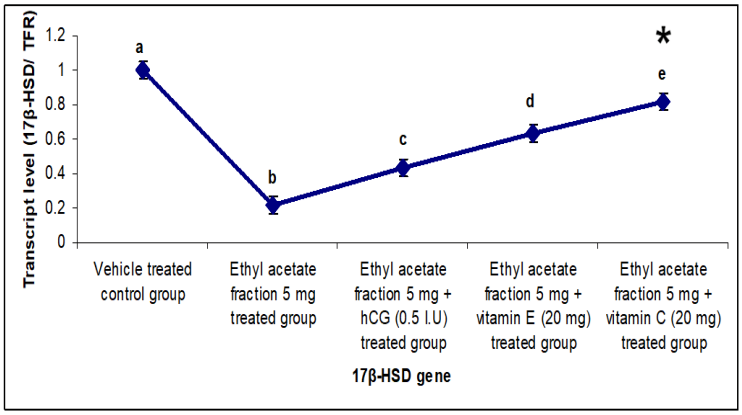


B.

Testis

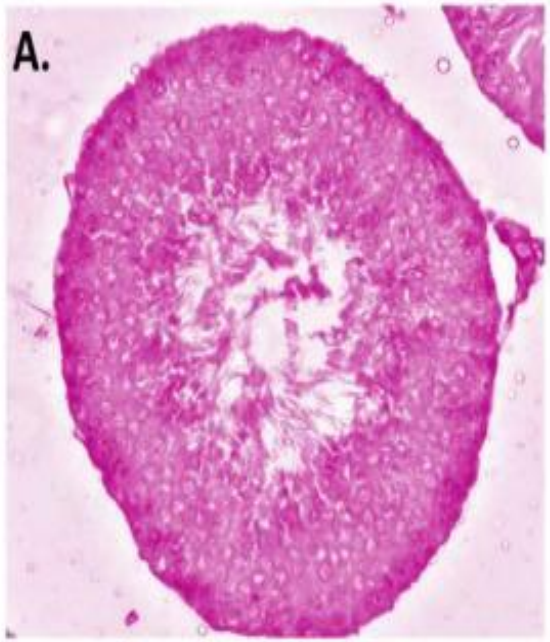
Figure 11A and 11B:



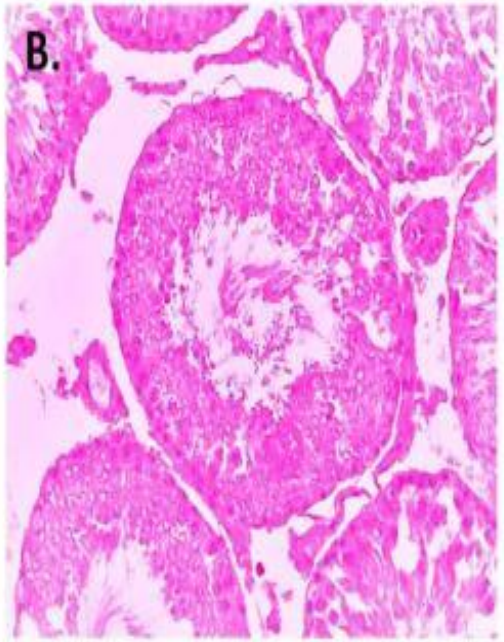


Line diagram of gene expression patterns of testicular Bax, Bcl-2, Δ5, 3β-HSD and 17β-HSD by qRT-PCR study of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C co administrative group in respect to vehicle treated control group.

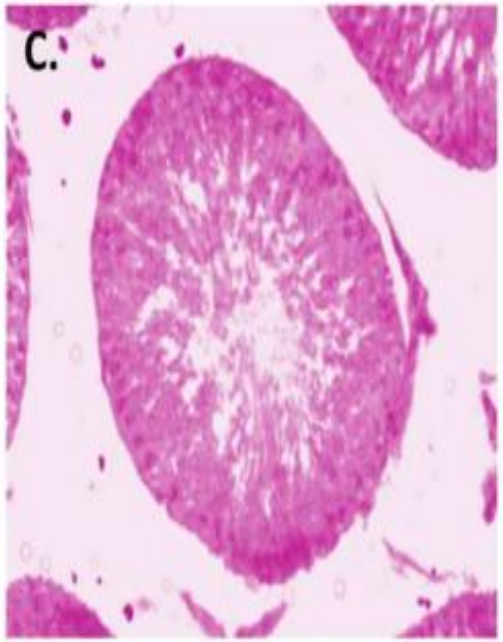
Ameliorative effects of treatment with hCG or vitamin E or vitamin C on testicular gene expression of EA-Fr of *T. chebula* treated rats. Mean values with standard errors expressed from the raw data's with the sample size of 6 (n=6) of each experimental group including control group. ANOVA followed by multiple comparisons "Student's two tail t-test" was followed here throughout the statistical analysis. Values with different superscripts (a, b, c, d, e) on each point of line diagram are differ from each other significantly, $p<0.05$. Star mark (*) indicates the most effective group of recovery from hypotesticular activity, induced after the treatment conduction with EA-Fr of *T. chebula*.



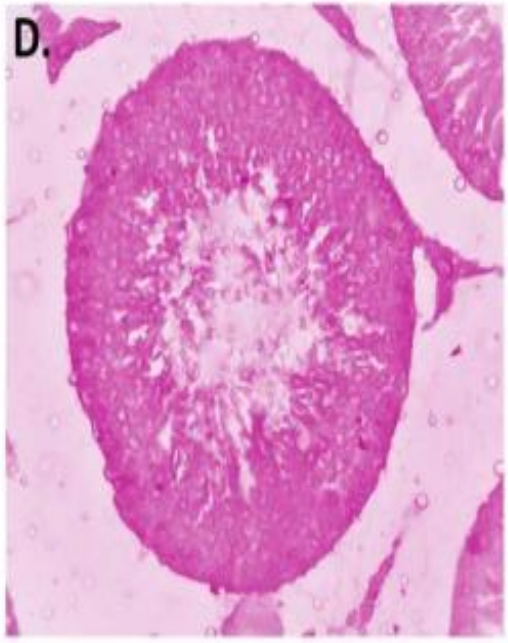
Control group



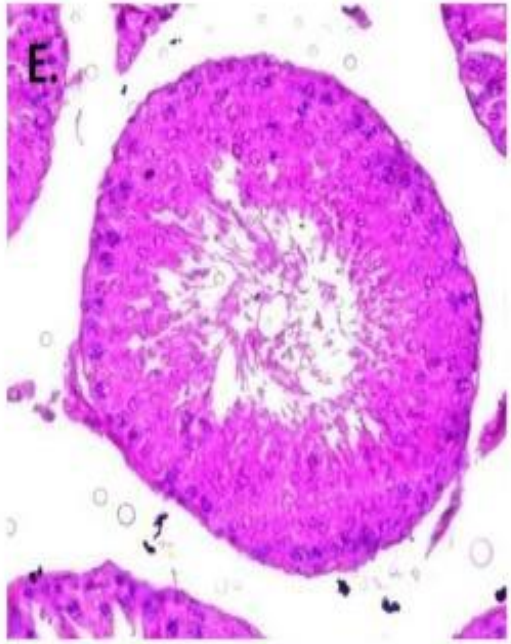
EA-Fr 5 mg dose treated group



Ethyl acetate fraction 5 mg + hCG (0.5 I.U) treated group



Ethyl acetate fraction 5 mg + vitamin E (20 mg) treated group



Ethyl acetate fraction 5 mg + vitamin C (20 mg) treated group

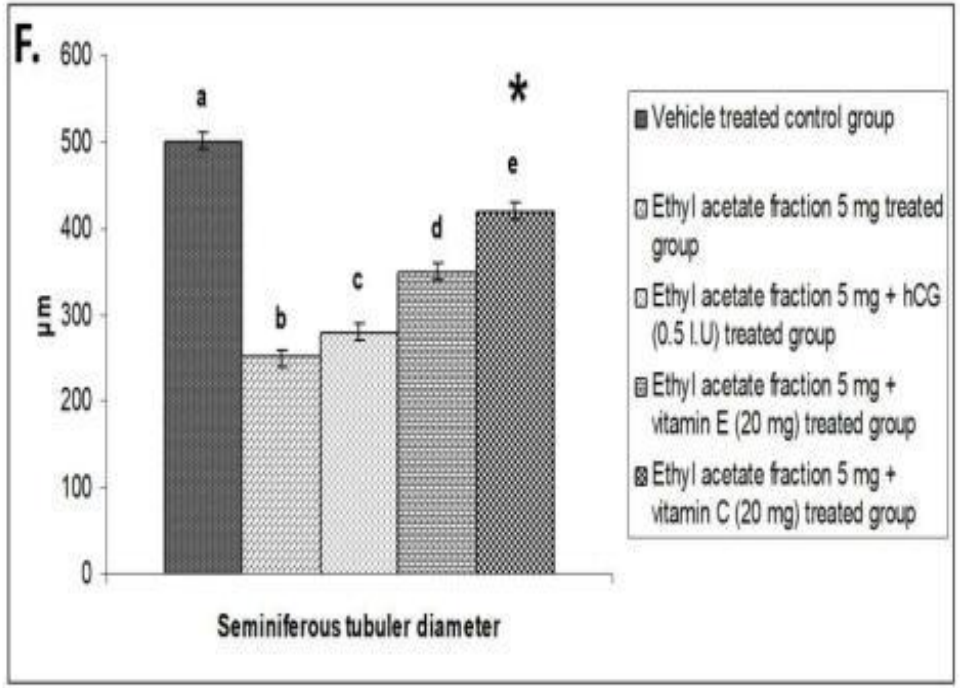


Figure 12:

Histopathological study of testicular tissue section of experimental rats of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C co administrative group in respect to vehicle treated control group.

[A] Represents microphotographs of testicular tissue section of control group.

[B] Testicular cross section after treatment with EA-Fr for 28 days.

[C] Testicular cross section after treatment with EA-Fr with the co administration of hCG.

[D] Testicular cross section after treatment with EA-Fr with the co administration of vitamin E.

[E] Testicular cross section after treatment with EA-Fr with the co administration of vitamin C.

[F] Bar diagrams of seminiferous tubular diameter of experimental groups.

Comparative analysis of corrective efficacies of hCG or vitamin E or vitamin C on seminiferous tubular diameter in EA-Fr treated male albino rats. Data were expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Bar diagrams with different superscripts (a, b, c, d, e) are differ from each other significantly at the level of $p<0.05$. Star mark (*) indicating the most effective group of recovery from hypotesticular activity induced by EA-Fr of *T. chebula*

Table 1:

Quantification of different generations of germ cells at stage VII spermatogenesis in EA-Fr treated groups and hCG or vitamin E or vitamin C co treated groups in respect to vehicle treated control group.

Groups	ASg	pLSc	mPSc	7Sd
Vehicle treated control	2.50 ± 0.06 [a]	23.51 ± 0.45 [a]	31.56 ± 2.10 [a]	73.59 ± 2.81 [a]
Ethyl acetate fraction 5 mg treated group	0.40 ± 0.03 [b]	9.23 ± 0.25 [b]	18.45 ± 1.26 [b]	40.32 ± 2.10 [b]
Ethyl acetate fraction 5 mg + hCG (0.5 I.U) treated group	0.80 ± 0.02 [c]	14.15 ± 0.51 [c]	22.32 ± 1.59 [c]	47.52 ± 2.90 [c]
Ethyl acetate fraction 5 mg + vitamin E (20 mg) treated group	1.20 ± 0.04 [d]	17.75 ± 0.54 [d]	25.43 ± 2.15 [d]	55.31 ± 3.45 [d]
Ethyl acetate fraction 5 mg + vitamin C (20 mg) treated group	1.50 ± 0.07 [e]	20.49 ± 0.61 [e]	28.52 ± 2.53 [e]	67.19 ± 3.75 [e]

Effect of EA-Fr of HM extract of *T. chebula*followed by co treated with hCG or vitamin E or C on different generation of germ cells at stage VII spermatogenic cycle. Data were expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Values with different superscripts (a, b, c, d, e) on each vertical column are differ from each other significantly, $p<0.05$.

Table 2:

Assessment of metabolic toxic effect of EA-Fr treated group and EA-Fr with hCG or vitamin E or vitamin C co administrative group in respect to vehicle treated control group.

Groups	Hepatic toxicity		Renal toxicity	
	GOT (Liver) (Unit/ mg of tissue)	GPT (Liver) Unit/ mg of tissue)	GOT (Kidney) Unit/ mg of tissue)	GPT (Kidney) Unit/ mg of tissue)
Vehicle treated control	30.00 ± 0.96 [a]	29.00 ± 0.96 [a]	25.00 ± 0.96 [a]	21.00 ± 0.62 [a]
Ethyl acetate fraction 5 mg treated group	31.00 ± 0.96 [a]	30.00 ± 0.88 [a]	27.00 ± 0.96 [a]	23.00 ± 0.72 [a]
Ethyl acetate fraction 5 mg + hCG (0.5 I.U) treated group	29.00 ± 0.96 [a]	31.00 ± 0.96 [a]	25.00 ± 0.96 [a]	22.00 ± 0.96 [a]
Ethyl acetate fraction 5 mg + vitamin E (20	30.00 ± 0.96 [a]	29.00 ± 0.96 [a]	26.00 ± 0.96 [a]	23.00 ± 0.99 [a]

mg) treated group				
Ethyl acetate fraction 5 mg + vitamin C (20 mg) treated group	29.00 ± 0.96 [a]	28.00 ± 0.96 [a]	25.00 ± 0.96 [a]	23.00 ± 0.72 [a]

Treatment with ethyl acetate fraction of *T. chebula* along with co administration of hCG or vitamin E or vitamin C to the experimental rats and comparison of their corrective potentialities on hepatic and renal toxicity study. Data were expressed in terms of Mean ± Standard error mean (SEM) (n=6). ANOVA followed by multiple comparisons "Student's two tail t-test". Values with same superscript (a) on each vertical column are not differ from each other significantly, $p>0.05$.