Original research article

Evaluation of hypotesticular activities of different solvent fractions of hydro-methanolic extract of the fruit of *Terminalia chebula* in Wistar strain adult albino rat: Genomic and flow cytometric approaches

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**A R T I C L E I N F O**

Article history:
Received 1 August 2017
Received in revised form 21 April 2018
Accepted 24 August 2018
Available online 19 September 2018

**Keywords:**
Hypotesticular activity
*Terminalia chebula*
Androgenesis
Spermiological study
Gene expression
Sperm cell apoptosis

**A B S T R A C T**

Fruit of *Terminalia chebula* Retz. (Combretaceae) has male contraceptive folk medicine reputation but its molecular aspect regarding hypotesticular activity is still in dark. The study focused the hypotesticular efficacy of the most potent fraction out of n-hexane, chloroform and ethyl acetate fractions of hydro-methanolic (3:2) extract of *Terminalia chebula* in connection with male herbal contraceptive development.

Treatment with above fractions of *Terminalia chebula* showed a significant diminution in the activities of androgenic key enzymes (Δ5p-HSD, 17p-HSD) and inhibition in serum testosterone level in compare to the control. Significant up regulation of testicular Bax gene and down regulation of Bcl-2 gene indicated the hypotesticular activity of these fractions. Flow-cytometric study focused a significant diminution in sperm viability and sperm mitochondrial status after the treatment with different fractions. Out of these, ethyl acetate fraction showed most promising hypotesticular effect without impairing any toxicity in general which highlighted that the fraction may contains antitesticular agent(s) in threshold levels compare to other fractions as it decreases spermiological, testicular genomic sensors and elevates sperm apoptotic sensors that may lead to male contraception.

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**Introduction**

Population explosion is one of the major problems throughout the world and it is the one of the priority area of research in all nations. To control human population, many contraceptive measures are considered but none is out of side effects. In India, family planning has been adopted as a primary tool to control population burden (Sharma et al., 2013). Contraceptive pills which are used in most of the couple for this purpose but excessive use of such pills is generating very adverse effects that sometimes lead to life threatening problems such as cerebral stroke, cardiac stroke, hypertension, abdominal pain, diabetes, nausea and irregularities of menstrual changes (Sabatini et al., 2011). Excessive use of Oxyynol-9 (N-9) which acts as a spermicidal agent widely available as a contraceptive which increases inflammatory, ulceration and increases the risk of HIV-1 infection (Fichorova et al., 2001). Till now no effective male contraceptive pill has been developed specially from herbal product though it is very much in need to develop a very effective male contraceptive pill without any side effect or low side effect as per direction of World Health Organisation (WHO).

In this connection, scientists are engaged in searching a cheap, eco-friendly and health hazardless contraceptive development based on herbal origin (Kannan et al., 2009). The use of medicinal plants for disease treatment has been supported by the traditional Indian systems of Ayurveda and Siddha (Beuscher et al., 1994). *T. chebula* commonly known as haritaki (Sanskrit and Bengali), black myrobalan, ink tree (English),

**Abbreviations:** Δ5p-HSD, Δ5, 3 beta-Hydroxy Steroid Dehydrogenase; 17p-HSD, 17 beta-hydroxy steroid dehydrogenase; T. chebula, *Terminalia chebula*; NH-Fr, n-hexane fraction; CH-Fr, chloroform fraction; EA-Fr, ethyl acetate fraction; FC, flow cytometry; TFR, transferrin receptor; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TO, thiazole orange; PI, propidium iodide; JC1, 55, 6,6-tetramethyl-6,0-tetraethylbenzimidazolcarbocyanine iodide; IEC, institutional ethic committee; qRT-PCR, quantitative reverse transcription polymerase chain reaction; FSC, forward scattered channel; SSC, side scattered channel; ANOVA, analysis of variance; SOP, standard operating procedure; SEM, standard error of mean.

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https://doi.org/10.1016/j.jab.2018.08.003
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harad (Hindi), is a medicinal plant that has been used from ancient period for disease management in Indian sub-continent along with South – East Asian region. Due to its medicinal use, it is listed on the top in Ayurvedic Materia Medica list (Rathinamoorthy and Thilagavathi, 2014). The dried fruits of *T. chebula* have been used to treat chronic fever, cough, diarrhoea, ulcer, asthma, sore throat, rheumatism, leucorrhoea, fungal skin infections, nervous irritability, etc (Aneja and Joshi, 2009; Dastur, 1962). “Triphala” is a very popular traditional herbal formulation where *T. chebula*, is used as chief ingredient this is also used for the management of diabetes (Singh et al., 2015), cancer, loss of appetite, and prescribed as cardio protective (Kaur, 2005).

Treatment with *T. chebula* resulted suppression in spermato genesis and fertilising abilities of spermatozoa (Gupta, 2017; Nair and Roopalatha, 2016; Srivastav et al., 2010). Our laboratory has previously reported the male contraceptive efficacy of hydroethanolic extract of *T. chebula* and most recently its efficient antifertility activity of aqueous extract of *T. chebula* and *Musa balbisiana* seed (*M. balbisiana*) in a composite manner (Ghosh et al., 2015b, 2017) has been reported. Both these research articles focused the antifertility effect of *T. chebula* extract which provided a positive outcome in this concern. In continuation with our previous study the present study has been designed to find out the effective fraction from the potent extract in this concern to reduce the bioburden of the systems from the phyto molecules present in the extract but not effective in this concern which is also the vision of WHO for herbal drug development.

**Materials and methods**

**Chemicals**

Chemicals and solvents were purchased from Sigma Chemical (St. Louis, MO, USA) or from Merck (KGaA, Germany) or from standard manufacturers.

**Plant material**

Fruits of *T. chebula* were purchased from local market of Midnapore town, identified by the Dept. of Botany and Forestry, Vidyasagar University (Midnapore-721102, West Bengal, India). A voucher specimen (VU/BMLSM/Cc-I/16) has been deposited in the herbarium of the same department. The fruits were dried and preserved in an air tight manner.

**Extraction and fractionation of plant material**

Hydro-methanol (HM) extract (3:2) of this plant material was prepared followed by its fractionation using n-hexane (NH) or chloroform (CH) or ethyl acetate (EA) as solvent as per standard method published by us (Panda et al., 2009).

**Selection of animals and animal care**

Twenty four healthy males, sexually matured and fertile Wistar strain rats weighing 130 ± 10 g were arranged for this experiment from institutional enlisted vendor, as well as CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) approved animal breeder “Saha Enterprise”. Animals were acclimatized for 15 days before starting the experiment. They were kept in room temperature of 25 ± 2 °C under light-dark cycle (12:12 h) for 7 days before experimentation. Standard food was supplied to animals daily and water *ad libitum*. The Institutional Ethic Committee (IEC) of Vidyasagar University approved the experiment (IEC Approval No-IEC/3-C-5/14; Dated-3/11/14). The guidelines given by the CPCSEA, Govt. of India, were strictly maintained.

**Experimental design**

Animals were divided into four groups on the basis of treatment schedule. Six animals were placed in each group. Their body weights were noted after 15 days of acclimatization. Individual fraction was administered to the specific group through oral route by gavage at 9.00 AM for 28 days. The duration of the treatment was fixed for 28 days because one wave of spermatogenic cycle takes 21 days to complete in Wistar strain rat.

- **Group I** [Vehicle-treated control]: Animals received only distilled water (0.5 ml/100 g body weight/day).
- **Group II** [n-hexane fraction (NH-Fr) treated group]: Animals were treated with NH-Fr of hydro-methanolic (3:2) extract (5 mg/0.5 ml distilled water/100 g body weight/day).
- **Group III** [Chloroform fraction (CH-Fr) treated group]: Rats were treated with CH-Fr of hydro-methanolic (3:2) extract (5 mg/0.5 ml distilled water/100 g body weight/day).
- **Group IV** [Ethyl acetate fraction (EA-Fr) treated group]: Animals were treated with EA-Fr of hydro-methanolic (3:2) extract (5 mg/0.5 ml distilled water/100 g body weight/day) for 28 days.

**Sample collection**

On 29th day of experimental schedule, all the animals were sacrificed using euthanasia after recording their body weight. Euthanasia is a painless or stress free death to the experimental rats without causing anxiety with minimum time lag phase by using an inhalation media i.e. carbon dioxide (CO₂) gas. Blood was collected from dorsal aorta (3 ml) using disposable syringe, transferred to the clot vial which contain clot activator (particles coated with water soluble polymers) for serum separation. It was preserved at −20 °C for serum testosterone assay. Liver, reproductive organs such as testes, epididymis of each animal were dissected out, cleaned with normal saline. Tissues were stored at −20 °C for enzymatic, bio-chemical and genomic analysis. Epididymal fluids were taken from caudal epididymis and processed for spermiological analysis in flow cytometer (FC).

**Sperm viability assessment by flow cytometer**

For quantitative analysis of live and dead sperms through Accuri C6 flow cytometer, BD Cell Viability Kit Cat No 349480 was used. Epididymal fluid was collected and mix with 1.8 ml of pre-warmed Tris buffer (1 × 10⁶ cell/ml of cell suspension). Then 2 μl of Thiazole Orange (TO) and 2 μl of Propidium Iodide (PI) were added and incubated at 37 °C for 5 min and data were collected by flow cytometer analysis according to Standard Operating Procedure (SOP) of this kit (Matyus et al., 1984).

**Sperm mitochondrial status assessment by flow cytometer**

Sperm mitochondrial status was assessed by Accuri C6 flow cytometer using JC1 (5, 5', 6, 6'-tetrachloro-1, 1', 3', 3'-tetraethylbenzimidazolcarbocyanine iodide) kit from BD Cat No 551302 and dimethyl sulfoxide solution. Fifty μl of epididymal fluid and 5 μl of JC1 staining solution were taken and then incubated in dark at 37 °C for 10 min. Five ml assay buffer was added, centrifuged at 1200 rpm for 5 min and then the supernatant was discarded. The cells in 500 μl were resuspended in assay buffer and data was collected after flow
cytometer analysis of the sample according to supplied SOP of this kit (Garner et al., 1997).

Assay of serum testosterone by ELISA

Level of serum testosterone was measured by solid phase-conjugated assay. The kit was purchased from Lilac Medicare (P) Ltd, Mumbai, India. The optical density was measured using standard method (Srivastava, 2001). The intra-assay variation was 5.2%. No inter-assay variation was considered here as all the samples were measured at a time. The cross-reaction level with androstenedione was 0.09% and dihydrotestosterone was 1.7%.

Assessment of testicular androgenic key enzyme activities

Testicular Δ5, 3β-HSD and 17β-HSD activities were assessed spectrophotometrically using standard methods (Jarabak et al., 1962; Talalay, 1962).

Gene expression study of Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA separation and complementary DNA implication

Testicular tissue was dissected out and stored immediately at −20 °C. Total mRNA was isolated from the tissue sample using kit of Roche Diagnostic. Preparation of cDNA was conducted from mRNA using “Transcriptor First Strand cDNA synthesis kit” of Roche Diagnostic (Ghosh et al., 2015a).

qRT-PCR study

For the gene expression study of testicular tissue, Light Cycler 480 II (Roche Diagnostics) was used for the assessment of Bax, Bcl-2, Δ5, 3β-HSD and 17β-HSD. Selected primers were arranged from Eurofins (Bangalore, India) and Xcelris Abellion Company (Gujarat, India). Denaturation at 95 °C for 15 min, annealing at 55 °C for 1 min and polymerization at 72 °C for 10 min were performed to conduct PCR reaction for 65 cycles. Reference gene, GAPDH (Glyceraldehyde 3-phosphate Dehydrogenase) was used for Bax.
and Bcl-2 gene expression but TFR (Transferrin Receptor), a reference gene was considered for \( \Delta 5, 3\beta \)-HSD and \( 17\beta \)-HSD gene expression study (Ghosh et al., 2015a).

Activity assessment of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT)

Hepatic GOT and GPT activities were measured for the metabolic toxicity assessment (Henry et al., 1960).

Statistical analysis

Analysis of Variance (ANOVA) followed multiple comparisons Student’s two tail t-test has been considered here to find out whether there is any significant difference among the groups at the level of probability, \( p < 0.05 \). Mean with standard error of mean (SEM) were considered for each group for such analysis (Sokal and Rohle, 1997).

Results

Sperm viability: Flow cytometric analysis

The percentage of dead sperm cell population was increased significantly \( (p < 0.05) \) in all the fraction treated groups i.e. in n-Hexane fraction (NH-Fr) (59.02%), chloroform fraction (CH-Fr) (55.47%) and ethyl acetate fraction (EA-Fr) (63.90%) of hydro-methanolic (3:2) extract of T. chebula in respect to vehicle treated control group. Most promising result was noted in EA-Fr treated group than other fraction treated groups (Fig. 1).

Sperm mitochondrial status assessment by flow cytometer

Depolarised sperm indicates low mitochondrial status of sperm. Number of sperm with depolarised mitochondrial membrane was increased significantly \( (p < 0.05) \) after treatment with the concerned fractions in respect to the vehicle treated control

![Fig. 2](image-url)
group. Percentage of depolarised sperm count was increased maximally in EA-Fr (106.53%) treated group when values were compared with NH-Fr (76.02%) and CH-Fr (74.09%) treated groups (Fig. 2).

Serum testosterone level

Significant ($p < 0.05$) diminution in the level of serum testosterone was noted in NH-Fr (28.90%), CH-Fr (29.68%) and EA-Fr (52.34%) treated groups as compared to the vehicle treated control group. After treatment with the EA-Fr, serum testosterone level was decreased significantly ($p < 0.05$) in respect to other fraction treated groups. There was no significant difference in the level of serum testosterone between NH-Fr (28.90%) and CH-Fr (29.6%) treated groups (Fig. 3).

Testicular androgenic key enzyme activities

Significant ($p < 0.05$) diminution in the activities of testicular $\Delta 5$, 3$\beta$-HSD and $17\beta$-HSD were noted after the NH-Fr (25.92%, 31.03%), CH-Fr (33.33%, 37.93%) and EA-Fr (44.44%, 44.82%) treated groups, in comparison with vehicle treated control group. Inhibition in the activities of these two steroidogenic key enzymes was noticed in significant level ($p < 0.05$) in the EA-Fr treated group in respect to the other two fraction treated groups (Fig. 4).

Testicular Bax and Bcl-2 gene expression study

Gene expression of testicular pro-apoptotic gene, i.e. Bax, was increased significantly ($p < 0.05$) in above said fractions treated groups by about 30%, 31%, 60% respectively where as anti-
apoptotic gene expression, i.e. Bcl-2, was decreased significantly (p < 0.05) by about 20% in NH-Fr, 35% in CH-Fr and 60% in EA-Fr when compared to the vehicle treated control group. Considering all the above-mentioned fraction treated groups, EA-Fr treated group showed maximum elevation in the level of Bax gene expression and diminution in Bcl-2 gene expression. No significant difference was noted in the level of above said gene expression between NH-Fr and CH-Fr treated groups (Fig. 5A).

**Gene expression study of testicular Δ5, 3β-HSD and 17β-HSD**

Levels of expression of testicular Δ5, 3β-HSD and testicular 17β-HSD genes were decreased significantly (p < 0.05) in NH-Fr (25%, 21%), CH-Fr (25%, 39%) and EA-Fr (59%, 58%) treated groups respectively when compared with the vehicle treated control group. Treatment with EA-Fr exhibited a significant downward deviation (p < 0.05) in the levels of said genes compared with NH-Fr and CH-Fr treated groups (Fig. 5B).

**Toxicity study**

Activities of liver GOT (5% in NH-Fr, 0% in CH-Fr and 5% in EA-Fr) and GPT (5%, 5%, 0% in NH-Fr, CH-Fr and EA-Fr) were not altered significantly (p > 0.05) among the fractions treated groups as well as in comparison with the vehicle treated control group (Fig. 6).

**Discussion**

The experiment was conducted to search out the most effective solvent fraction prepared from hydro-methanolic (3:2) extract of *T. chebula* for the induction of hypotesticular activities in connection with contraceptive herbal drug development. Among all the said fractions, the EA-Fr showed most remarkable results in this concern. This may be due to the presence of bio-active ingredients of *T. chebula* in sufficient levels in this fraction in comparison to the other fractions. These bio-active ingredients with contraceptive properties may able to inhibit the androgen syntheses which have been reflected by low level of serum testosterone as testosterone is the end product of androgenesis (Morris and Chailkoff, 1959). This has been supported by the low testicular steroidogenic key enzyme activities in EA-Fr treated group in comparison to the other fractions treated groups as Δ5, 3β-HSD and 17β-HSD are the key enzymes for androgenesis (Das et al., 2006; Murono and Payne, 1979). To find out the nature of such inhibition by the fraction based phytomolecules, the gene expression of testicular key androgenic enzymes i.e. Δ5, 3β-HSD and 17β-HSD were included. It has been noted that inhibition is at gene level which may be due to phytomolecule-gene interaction. Elevated level of testicular germ cell apoptosis was recognised after EA-Fr treatment following qRT-PCR study. Treatment with EA-Fr has focused a significant increase in the level of expression of pro-apoptotic marker i.e. Bax, while reduction in Bcl-2 expression, an anti-apoptotic marker, suggesting their important roles on apoptotic cell death which may be another cause of reduced sperm viability in this concern. So, the results pointed out the testicular germ cell apoptotic activity of the phytochemicals present in the said fraction. Simultaneously, these bio-active ingredients may destroy sperm morphology which was reflected by low mitochondrial status and low count of sperm viability by flow-cytometric analysis. Depolarisation of mitochondrial membrane was increased significantly in EA-Fr treated group in compared to other fraction treated groups which indicates the reduced sperm mitochondrial activity (Garner et al., 1997).

The EA-Fr of *T. chebula* has no metabolic toxic effect which has been focussed here from the non-significant alteration in the activities of hepatic GOT and GPT as these are important metabolic toxicity indicators (Akhter et al., 1990).

Regarding the mechanism of action of the effective fraction for hypotesticular activity of *T. chebula*, it may be hypothesized that the EA-Fr of hydromethanolic extract may contain the requisite amount of concerned phytomolecules for this purpose than other fractions. Moreover, the phytomolecule(s) may develop primary hypotesticular activity by exerting inhibitory actions on androgenic gene expression, sperm viability as well as sperm mitochondrial activity status along with elevation in the rate of sperm apoptosis by phytomolecule-androgenic and spermatogenic cross-talk via phytoingredient-gene interaction process.

The nature of phytomolecule(s) and their actual mechanism for such hypotesticular activities are not clear at present which is under process and hopefully would be unfolded from the subsequent studies in this line.

**Conclusions**

Ethyl acetate fraction of *T. chebula* has potent anti-testicular activity which will drive the scientific community to develop male herbal contraceptive from this fraction.

**Conflict of interests**

The authors have no conflict of interests to declare.

**Acknowledgements**

This research work was funded by DST, Govt. of India (File No: EMR/2014/000742) to conduct this work is gratefully acknowledged.

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