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Research Article

EFFECT OF UNSAPONIFIABLE FRACTION OF SEEDS OF *HYGROPHILA SPINOSA* T. ANDER ON TESTOSTERONE PRODUCTION OF RAT LEYDIG CELLS *IN VITRO*

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ABSTRACT

Objective: Seeds of *Hygrophila spinosa* (HS) T. Ander (Acanthaceae) are traditionally used as aphrodisiac and spermatogenic in Indian System of Medicine. Preliminary phytochemical screening of plant revealed the presence of triterpenoids and sterols in seeds. The study was planned to assess the effect of unsaponifiable fraction prepared from seeds of HS on isolated rat Leydig cells for testosterone (T) production using *in vitro* method.

Methods: Leydig cells were isolated from Wistar rats, aseptically, *in vitro* by collagenase cell dispersion method. Cells (2×10^6 cells/ml) were then incubated with a unsaponifiable fraction of HS (10, 100 and 1000 µg/ml dose levels in triplicate) in an incubator at 37°C under atmosphere of 95% CO_2 condition for 3 hrs in aseptic condition. Dehydroepiandrosterone was used as positive control in the study. The amount of testosterone secreted in culture media was estimated using high performance thin-layer chromatography (TLC). Benzene: Ethyl acetate (5:5% v/v) was employed as mobile phase and silica gel G F_{254} aluminum coated TLC plate as the stationary phase.

Results: The results indicated dose-dependent increase in testosterone concentration in test groups. Isolated rat Leydig cells treated with the test fraction showed increased amount of testosterone present in culture media as compared to that of control.

Conclusion: Unsaponifiable fraction prepared from seeds of HS showed ability to enhance biosynthesis of testosterone in isolated rat Leydig cells. *In vitro* studies showed that the fraction might act locally in testis on Leydig cells and stimulated testosterone synthesis.

Keywords: Aphrodisiac, Spermatogenic, *Hygrophila spinosa,* Testosterone.

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INTRODUCTION

The plant Kokilaksha is known as *H. spinosa* (HS) in Sanskrit literature belonging to Acanthaceae family and its seeds have been used in Ayurvedic preparations as *Vajikarana Aushadhi,* i.e., aphrodisiac and spermatogenic [1-3]. The plant has shown many pharmacological activities such as cooling, tonic, spermatorrhea, aphrodisiac, and spermatogenic [4]. Phytochemical study of this plant showed that seeds contain mucilage, sterols, unidentified alkaloids, fatty acids, minerals, and carbohydrates [5-12].

Literature survey revealed that this plant is not systematically screened for the claimed activity. Hence, unsaponifiable fraction prepared from seeds of HS was screened for testosterone production and release by *in vitro* method using isolated Leydig cells from male Wistar rats.

METHODS

Reagents and chemicals

All the solvents and chemicals used were of analytical grade and procured from Loba Chemicals, India. Materials and media used for *in vitro* studies were purchased from Sigma-Aldrich, USA, Acros Organics, India and Hi-media, India.

Plant materials

Whole plants of HS were collected in August from tribal area of Anand district, Gujarat, India. The plant samples were identified by taxonomist at J and J Science College, Nadiad, Gujarat, India. The specimen of the collected plant material was submitted to the department for future reference with specimen number 2011/NV/HS. Seeds were separated from the plants, dried under shade and milled using laboratory grinder to 70# powder. This powder was used for further extraction process.

Preparation of unsaponifiable fraction

Dried seed powder (4 kg) was extracted using 5000 ml of hexane (00160, Loba Chemie) in Soxhlet's extraction apparatus at 60°C for 48 hrs. Hexane extract was filtered and refluxed with sufficient quantity of 10% potassium hydroxide (05378, Loba Chemie) in methanol (00196, Loba Chemie) for saponification purpose. The content was removed and mixed with equal amount of water. The content was then partitioned with solvent ether (001040, Loba Chemie) to separate unsaponified matter. Ethereal extracts were pooled and passed through anhydrous sodium sulfate to remove moisture present. All ethereal portions were mixed together and evaporated to dryness using rotary vacuum evaporator (Heidolph, Germany) at 30°C. The yield of unsaponifiable fraction was determined and found to be 1.2% w/w. The fraction was then subjected to TLC studies for detection of sterols and triterpenoidal compounds present.

Detection of sterols and triterpenoidal compounds

The unsaponifiable fraction was evaluated for the presence of sterols and triterpenoidal compounds using TLC. Post chromatographic derivatization was performed using Liebermann-Burchard reagent [13]. Optimized mobile phase employed to separate these compounds on silica gel coated TLC plates (silica gel G 60 F_{254}, Merck) was hexane:ethyl acetate:methanol:glacial acetic acid (6:4.5:0.5:0.2 v/v/v/v) with saturation time of 10 minutes.

Animals

Protocols for *in vitro* studies were approved by the Institutional animal Ethics Committee (IAEC) constituted as per the norms of Committee for the Purpose of Control and Supervision of Experiments on Animals. The protocol numbers assigned were RPCP/IAEC/2013-14/R29, RPCP/IAEC/2011-12/R7 respectively. Healthy male Wistar rats of weight 250-350 g were used in the experiments. Rats were received

from Anand Agricultural University, Anand, Gujarat, India. All animals were housed at 25°C±2°C, with a relative humidity of 75%±5%, under 12 hrs light/dark cycle. A basal diet and water was provided *ad libitum*.

In vitro studies

Preparation of rat Leydig cells

Male animals were sacrificed and testes were dissected aseptically. Testicular Leydig cells were isolated by adopting collagenase dispersion method as described previously [14-15]. Briefly, testes were decapsulated and placed in digestion medium (0.005% trypsin inhibitor, 0.001% DNAase, 0.025% collagenase and 0.1% BSA in medium 199) at 34°C for 20 minutes in shaking water bath, set at 90 cycles/minutes shaking movement. Digestion was terminated by addition of separation buffer (0.071% sodium bicarbonate, 0.21% HEPES, 0.025% trypsin inhibitor, and 1% BSA in medium 199). Tubes were capped tightly, inverted few times and kept at 4°C for 10 minutes in the refrigerator. Seminiferous tubules settled at bottom and supernatant was separated. Clear supernatant was carefully siphoned off and collected from the top with Pasteur pipette. The supernatant was then centrifuged at 1500 g for 10 minutes at 4°C in cooling centrifuge. The cell pellet obtained at bottom was collected in centrifuge tubes and kept in the incubation medium (0.01% trypsin inhibitor and 1% BSA in medium 199). Purity of isolated Leydig cells was checked using positive staining of 3 β-hydroxysteroid dehydrogenase (HSD). Leydig cell suspension was diluted using incubation medium to set Leydig cell concentration of 2×10^6 cells/ml. The viability of cells was checked using trypan blue cell exclusion method [16].

Effect of unsaponifiable fraction on testosterone production using rat Leydig cells

In vitro experiment was divided into four groups. (1) Blank: Incubation medium without isolated Leydig cells. (2) Control: 1 ml isolated Leydig cells (concentration of 2×10^6 cells/ml) were mixed with incubation medium to make final volume up to 1 ml. (3) positive standard: 1 ml isolated Leydig cells were mixed with 0.1 ml of 10, 100, and 1000 µg/ml concentration of dehydroepiandrosterone (DHEA) diluted up to 2 ml using incubation medium. (4) Test: 1 ml isolated Leydig cells were mixed with 100 µl of 10, 100, and 1000 µg/ml concentration of unsaponifiable fraction diluted up to 2 ml using incubation medium. The cells were incubated for 3 hrs under atmosphere of 95% CO_2 condition using CO_2 incubator at 37°C. The content was centrifuged and the supernatant was separated [17,18]. The supernatant was portioned with 1 ml chloroform. The chloroform layer was separated and subjected to high performance thin layer chromatography (HPTLC) analysis to estimate the amount of testosterone. The amount of testosterone was determined quantitatively by adopting reported methodology with incorporation of minor modifications (Stahl E., 1969). Briefly, 50 µl of sample solution was spotted on silica gel G F_{254} aluminum coated TLC plate. Plates were developed using benzene:Ethyl acetate (5:5 v/v) as mobile phase at 27°C±5°C [13]. The sample solution was spotted on the plate in the form of a narrow band (6 mm × 0.45 mm) using Linomat-V semiautomatic spotter. Quantitative analysis was performed by scanner 4 using reflectance absorbance mode at 254 nm using deuterium lamp. The data were integrated using win-CATS software.

Statistical analysis

Results are expressed as mean±standard error of the mean. The difference between the means was determined by one-way analysis of variance followed by Dunnett's test. In all statistical tests, a value of $p<0.05$ was considered significant. All analysis was performed using Microsoft Excel 2007.

RESULTS AND DISUSSION

Preliminary phytochemical studies

Preliminary TLC studies of unsaponifiable fraction of HS showed the presence of triterpenoidal and phytosterols. The plate was subjected to post chromatographic derivatization using Liebermann-Burchard reagent. TLC pattern as shown in Fig. 1 suggested the presence of triterpenoidal and phytosterols.

Fig. 1: Thin-layer chromatography profile of unsaponifiable fraction of *Hygrophila spinosa* after derivatization with Liebermann-burchard reagent

In vitro studies

Effect of alkaloidal fraction on testosterone release by Leydig cells in vitro

Leydig cells isolated from rat testis were incubated with DHEA as well as with different concentrations of test fraction, separately. The amount of testosterone present in culture media was estimated using HPTLC by adopting reported methodology. The results (Fig. 2) indicated dose-dependent increase in testosterone concentration in test groups. *In vitro* studies showed that the fraction might act locally in testis on Leydig cells and stimulate testosterone synthesis.

Testosterone is produced by Leydig cells in testes through biochemical process known as steroidogenesis. DHEA is an intermediate, which is converted to testosterone through enzyme 17-β-HSD. DHEA, being terminal precursor in biosynthesis, was thus selected as a positive control (Samanta *et al*., 2015).

Excess DHEA available to Leydig cells, thus, enabled cells to produce a higher amount of testosterone as shown in Fig. 2. Comparatively higher amount of testosterone estimated in culture media containing isolated Leydig cells treated with test fraction revealed the ability of fraction to stimulate β-HSD itself.

Testosterone is converted to dihydrotestosterone (DHT) by enzyme 5 α-reductase. The higher amount of DHEA was found to stimulate 5 α-reductase activity in one of the clinical studies (Stomati *et al*., 2000). Activation of 5 α-reductase would be resulted into conversion of testosterone into its active metabolite DHT. This might be the probable reason responsible for showing lower amount of testosterone, as estimated in culture media containing Leydig cells treated with 1000 µg/ml concentration of DHEA. This type of phenomenon was also observed in the case of cells treated with 1000 µg/ml of unsaponifiable fraction.

CONCLUSION

The seeds of HS are traditionally used as aphrodisiac and spermatogenic. Phytochemical studies revealed the presence of triterpenoids and sterols the seeds. Unsaponifiable fraction prepared from seeds of HS was evaluated for testosterone production *in vitro* by isolated rat Leydig cells model.

In vitro studies confirmed that the fraction stimulated isolated Leydig cells to secrete more amount of testosterone as compared to

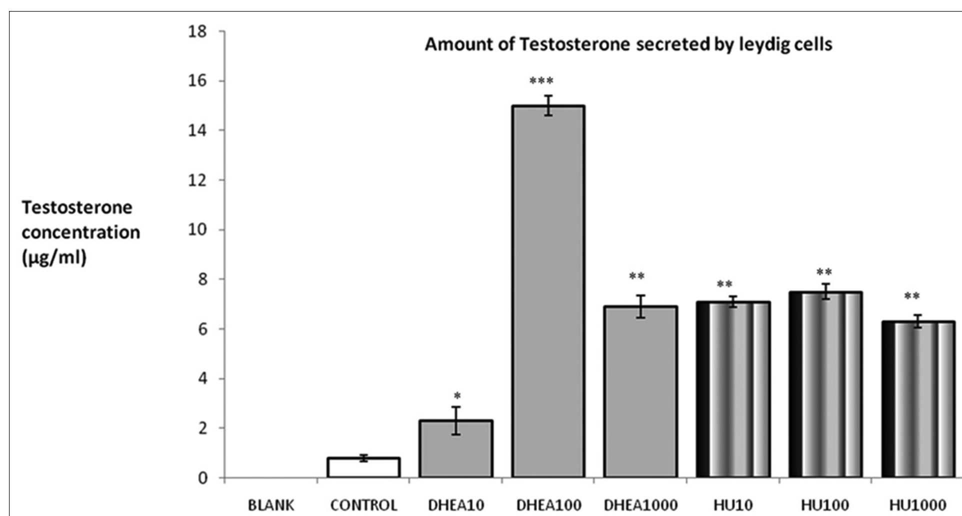

Fig. 2: Effect of test fraction on amount of testosteone secreted by Leydig cells *in vitro.* HU10: 10 µg/ml concentration of unsaponifiable fraction of *Hygrophila spinosa.* HU100: 100 µg/ml concentration of unsaponifiable fraction of *Hygrophila spinosa.*HU1000: 1000 µg/ml concentration of unsaponifiable fraction of *Hygrophila spinosa.* n=3, values are mean±standard error of the mean, *p<0.05, **p<0.01, ***p<0.001 as compared to respective control

control. DHEA was used as positive control in the set of experiments. It was concluded that the fraction might activate β-HSD or stimulate expression of β-HSD in Leydig cells. It was concluded from the studies that fraction might stimulate testosterone synthesis or release from Leydig cells, which in turn responsible for spermatogenic as well as aphrodisiac potential of the plant.

Further studies are needed to establish mechanism through which fraction stimulated testosterone level *in vitro*. The studies may be extended to investigate effect of fraction on gonadal axis, which controls synthesis of testosterone and has a role to play in induction of libido.

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