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RESEARCH ARTICLE

Assessment of Testosterone Biogenesis Enhancement by a Lupeol Derivative Separated from Seeds of *Hygrophila spinosa* T. Ander *in vitro*

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Abstract

The present study aimed towards assessing the isolated lupeol derivative *in vitro* for testosterone biogenesis enhancement by the action on isolated rat leydig cells. Studies were carried out to separate a novel bioactive molecule from an Indian medicinal plant, *Hygrophila spinosa*, used as an aphrodisiac and spermatogenic for years. Unsaponifiable bioactive fraction, prepared from seeds of the plant, was subjected to isolation of phytoconstituents by column and flash chromatography. The isolated compound was characterized by spectral studies as lupeol methyl ether. Later, it was subjected to *in vitro* studies using isolated leydig cells from rat for its action. Different amounts of isolated compound were incubated with leydig cells for 3 hours and concentration of testosterone after incubation was considered as a parameter. Dehydroepiandrosterone was used as positive control in the studies. The results indicated that the isolated compound was active and enhanced the testosterone biogenesis, as evident from increased amount of testosterone found in a medium treated with compound. This was the first report included isolation, chemical characterization and biological activity of Lupeol methyl ether from *Hygrophila spinosa* T. Ander.

Keywords: *Hygrophila spinosa*, Lupeol, Leydig Cells, Steroids, Tri-terpenoids, Testosterone

1. Introduction

Hygrophila spinosa T. Ander (HS), is a spinous herb (Acanthaceae) and known to possess aphrodisiac and spermatogenic properties. Ayurvedic literatures also indicated that the seeds of the plant increase the quality of semen[1–3]. The seed powder of the plant is included in numbers of herbal preparations used to treat debilities related to male reproductive system. Chemical investigations revealed the presence of steroidal compound (asterol I, II, III, and IV) and alkaloidal compounds (asteracanthine and asteracanthicine) in the seeds[4]. Previously, alcoholic extract of seeds showed aphrodisiac activity in male rats with evident from stimulated mounting behaviour in dose dependent manner[5]. In 2016, Vyas and Raval also reported aphrodisiac and spermatogenic activity of alkaloidal fraction prepared from seeds of the plant *in vivo* and *in vitro* models using rats as experimental animals[6]. Steroidal and triterpenoidal fraction (unsaponifiable) also showed stimulant action on isolated rat leydig cells on testosterone production *in vitro*[7].

However, no sufficient scientific evidences are available citing compounds responsible for the acclaimed activity. Hence, reverse pharmacognosy approach was used to isolate and quantitative estimate of compound from bioactive steroidal and tri-terpenoidal fraction prepared from seeds of *Hygrophila spinosa* T. Ander by column chromatographic methods[8]. We employed

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column chromatography and flash chromatography methods to isolate compound from bioactive steroidal and tri-terpenoidal fraction of the seeds. This was the first report which included isolation, chemical characterization and biological activity triterpenoidal derivative from the seeds.

2. Materials and Methods

2.1 Apparatus, Reagents, Solvents and Chemicals

Soxhlet's extraction apparatus; consisting of extraction chamber, round bottom flask and condenser, was used for extraction of the plant material. Flash chromatographic system (Isolera-I, Biotage) was used for separation of compounds from fraction. Rotary vacuum evaporator (Heidolph, Germany) was used to concentrate the extract while preparing triterpenoidal fraction from seeds. Reagents, solvents and chemicals used in the studies were of analytical grade purchased from LOBA Chemie, India. Media and other materials for *in vitro* studies were procured from Hi-Media, India, Acros organics, India and Sigma Aldrich, USA.

2.2 Plant Materials

The seeds of HS were collected from tribal region. Seeds were identified and authenticated by Taxonomist, J and J College of Science, Nadiad and certificate of authentication was issued with voucher (Reference No.:RPCP/2011/NV/HS) submitted to Pharmacognosy department. The seeds were cleaned manually to remove adhering dirt and sand. They were crushed in laboratory grinder and subjected to drying at room temperature under shade for 15 days and subsequently in tray dryer for 3 days at 60°C and then milled to 70 # using laboratory grinder. The powder was stored in airtight container for further process of extraction.

2.3 Preparation of Unsaponifiable Fraction (HSU) from Seeds

Dried seed powder (4 kg) was extracted using 5000 ml of hexane (00160, LobaChemie) 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, LobaChemie) in methanol (00196, LobaChemie) for saponification purpose. The content was removed and mixed with equal amount of water. The content was then partitioned with solvent ether (001040, LobaChemie) 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 biological studies and isolation studies.

2.4 Isolation and Characterization of Compound from Bioactive Fraction

Chromatographic methods such as supercritical fluid chromatography, flash chromatography and column chromatography are generally used to separate and isolate phytoconstituents from medicinal plants effectively[9]. Thus studies were planned to separate triterpenoidal phytoconstituents present in the bioactive fraction[7] using chromatographic techniques.

2.4.1 Isolation of Compounds using Column Chromatography

1g HSU was dissolved in 10 ml of chloroform and then mixed with 5 g silica (60-120#) for column chromatography. Glass column (25 mm diameter, 90 cm long) was packed with silica (60-120#, 50 g) slurry, prepared in hexane. The mixture was evaporated to dryness using water bath. Unsaponifiable fraction loaded on silica was subjected to column chromatography to isolate individual compound/s. Elution was started with 500 ml of chloroform. Subsequently elution was carried out by gradual increase in concentration (0.5%, 1%, 2%, 5%, 7%, 9% and 10% v/v) of methanol (100 ml of each fraction). Flow rate was set to 10 to 15 drops per minute during elution. Each fraction was concentrated using rotary vacuum evaporator and subjected to TLC. Fractions with similar TLC profile were combined together. The fractions collected from column chromatography were subjected to flash chromatography for further separation and purification of triterpenoidal compound/s[10].

2.4.2 Isolation of Compounds from Sub Fractions using Flash Chromatography

The fractions showing similar TLC profile collected using column chromatography were subjected to flash chromatography (Biotage Isolera™ One flash chromatographic system) for isolation of individual constituent. It was loaded in SNAP KP-Sil plastic cartridge contained with 25 g silica as stationary phase. Elution in flash chromatography was carried out with mobile phase of n-Hexane: Ethyl acetate (linear gradient system) with flow rate of 20 ml/min. Elutes were

scanned continuously at 205 and 288 nm throughout the experiment. The fractions (each of 20 ml) were collected using fraction collector in test tubes, unless a drift observed in absorbance at the set wavelengths. Each fraction was concentrated and subjected to TLC, individually. The fractions yielded single spot on TLC were mixed together and evaporated to dryness. One triterpenoidal compound (U1~200 mg) was isolated from sub-fraction of HSU.

2.5 Purity Assessment

Purity of U1 was confirmed by performing TLC studies using three different mobile phases. Plates were sprayed with anisaldehyde-sulphuric acid reagent. Sample solution (1000 ng/spot) yielded sharp, single spot in three different mobile phases. TLC pattern of U1 in three different mobile phases are shown in shown in Figure 1.

2.6 Spectral Studies of Isolated Compound

U1 was subjected to spectral studies using Infrared (IR), Nuclear Magnetic Resonance (1H NMR) and Mass (MS) spectroscopy. The sample pellet consisting of sample and anhydrous potassium bromide was placed in sample holder and IR spectrum was recorded in range of 4000 cm^{-1} to 400 cm^{-1} using IR spectrophotometer (Nicolet 6700, Thermo scientific). IR spectrum of U1 was compared with that of IR spectrum of lupeol as plant was reported to contain triterpenoidal compounds earlier. ^{1}H-NMR ($CDCl_3$) spectroscopy was performed on a Bruker-Avance II spectrometer (500 MHz). Electro spray mass spectroscopy (ES-MS) analyses were performed using API-2000 MS-MS (MDS SCIEX, Toronto, Canada) mass spectrometer. The probable structure of U1 was elucidated by comparing recorded spectroscopic data with the reported data of lupeol in previous reports[11].

2.7 *In vitro* Studies

Leydig cells were isolated from rat testes as per the method described earlier[7-8]. Isolated leydig cells were incubated with 1, 10, 100, 500 and 1000 µg/ml concentration of U1, prepared in Dimethyl Sulphoxide (DMSO). DMSO served as blank while DHEA solution in DMSO served as positive control. The experiments were performed by adopting the protocol described earlier[7,8]. Amount of testosterone was estimated, after incubation, in medium treated with different concentration of isolated compound using HPTLC method in silica gel G 60 F_{254} pre-coated aluminium plates with resolution carried out using benzene: ethyl acetate (5:5 v/v) as a mobile phase[7]. The results are shown in results section.

3. Results and Discussion

3.1 Isolation of Triterpenoidal Compound from HSU

HSU was subjected to column chromatography. Fractions were collected and monitored for presence of triterpenoidal compounds using TLC separation followed by derivatization with Anisaldehylde-sulphuric acid reagent. Fractions having similar TLC separation pattern of triterpenoidal compounds were mixed together and subjected to flash chromatography for isolation of individual phytoconstituents. Flash chromatography was performed on BiotageIsolera™ One flash chromatographic system using SNAP KP-Sil plastic cartridge (50 g silica gel) with mobile phase: Hexane: Ethyl acetate (linear gradient system) with flow rate of 10 ml/min. The fractions were collected using fraction collector, operated to collect all the fractions at 205 nm and 288 nm. The test tube was changed automatically, when desired quantity was filled in a test tube or a drift valley was detected by detector in signals while scanning in elutes at 205 and 288 nm. Gradient for methanol proportion was set using trial and error. Each fraction was collected, concentrated and subjected to TLC to detect the presence of triterpenoidal compounds. Fractions with similar TLC profile were combined together. The fractions containing single spot on TLC were mixed together and evaporated to dryness. One compound (U1~200 mg) was isolated from bioactive HSU.

3.2 Purity Assessment of Isolated Compound

Purity assessment of isolated compound was performed to ascertain the chemical purity of isolated constituents as well as to rule out presence of trace quantity of impurities.

The purity was ensured by performing TLC studies. TLC is solid liquid chromatography where the compounds are separated on basis of their relative absorption on stationary phase when allowed to run through mobile phase. The compounds are present as solid samples on TLC (solution is applied as band and dried) and are detected as solid in situ. This unique feature makes TLC a highly sensitive method and it is employed routinely for the purpose.

As shown in Figure 1, three distinct mobile phases were used to perform TLC studies to separate traces of chemically distinct compounds from U1. The plates were observed in day light, at 254 nm and at 366 nm. Sharp single spot on TLC confirmed that isolated constituent U1 was pure and devoid of any possible impurity.

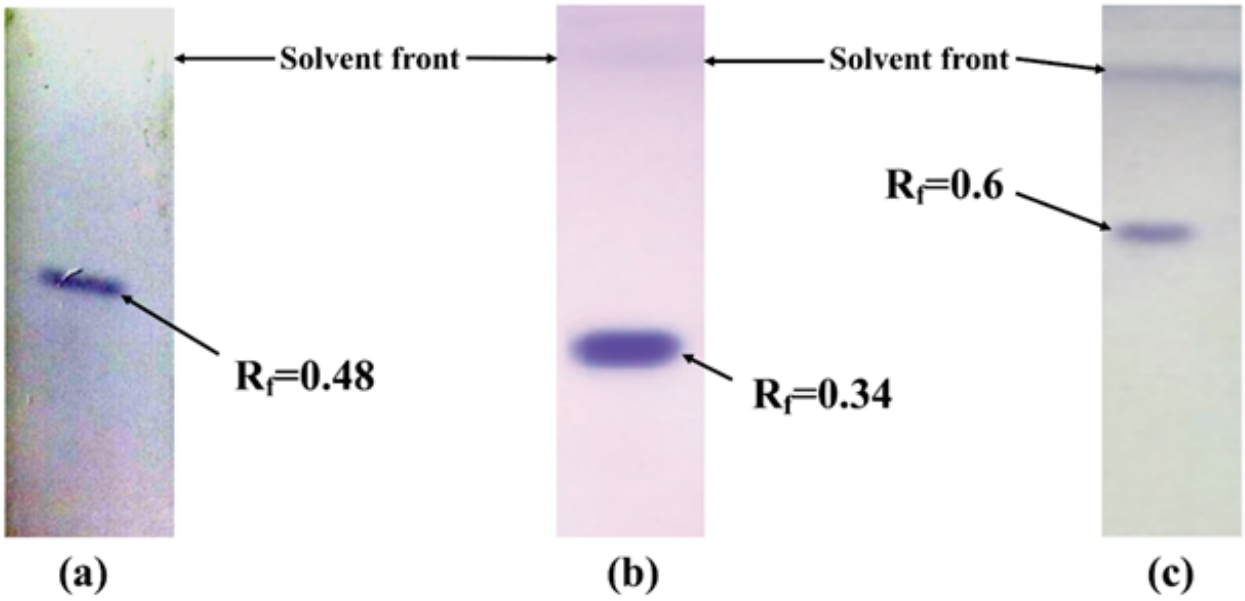

Figure 1. Purity assessment of isolated compound U1 in three different mobile phases. **(a).** Pet. ether: ethyl acetate: methanol: glacial acetic acid (6:4.5:0.5:0.2 v/v/v/v) **(b)**. Pet. ether: ethyl acetate: methanol (6:3:0.4 v/v/v) **(c).** Toluene: ethyl acetate: methanol (7:3:0.5 v/v/v).

3.3 Spectral Characterization of Isolated Compound

The structure of isolated and purified compound was elucidated using IR, 1H NMR and MS spectral studies[12].

IR (KBr disc): 2935.88 cm^{-1} (Aliphatic C-H str, Branched), 2866.33 cm^{-1} (Aliphatic C-H str), 1650 cm^{-1} (C=C str), 1593.67 cm^{-1} (Aliphatic C–C str), 1460.05 cm^{-1} (C-O-C str).

IR spectrum showed all characteristic wavenumbers corresponding to functional groups as reported for lupeol. IR spectrum showed the distinct presence of C-O-C stretch (1460.05cm^{-1}) indicating probable presence of Ether in the structure of U1.

1H NMR (500 MHz, $CDCl_3$) δ ppm: 0.76, 0.83, 0.88, 0.94 (s, -CH_3); 2.3 – 2.4 (s, 1H, CH_3-CH_2-CH_2); 3.1 -3.2 (q, 2H, CH_2-OCH_3); 4.5 – 4.7 (t,2H of Olefinic Proton); 7.2-7.3 (t, 1H, CH=CH_2).

NMR spectrum showed all characteristic δ values for peaks as reported for lupeol. Recorded NMR spectrum showed chemical shift at 3.1-3.2, corresponding to 2 hydrogensof CH_2-O-CH_3. This suggested the presence of ether group in lupeol structure.

ESI-MS m/z [M+H]+440.9; calcd. 440.74398, $C_{31}H_{52}O$; m/z 427.1 $[M\text{-}CH_3]^+$

Molecular weight of lupeol 427.1 g/molwhile mass spectrumof U1 showed base peak at m/z 440.9 ($[M+H]^+$~440.9). A peak was obtained at m/z 427.1 too. This suggested removal of methyl group from U1, and formation of lupeol molecular ion.

Considering results of comparative spectral studies, structure of U1 was predicted as Lupeol Methyl Ether as shown in Figure 2.

3.4 *In vitro* Studies

The results of *in vitro* studies showed that DHEA stimulated testosterone synthesis. It confirmed the ability of isolated leydig cells to synthesize more testosterone when provided suitable atmosphere. As shown in Figure 3, the alteration in testosterone concentration in U1 treated cells as compared to control was statistically significant at 500 and 1000 µg/ml of U1. The activity of HSU and U1 when compared, it was note that at concentration HSU treated leydig cells culture contained more amount of testosterone as compared to U1. The comparative studies confirmed that the activity of HSU was not solely due to the presence ofU1 but also other constituents present might act up on leydig cells too. It is revealed from *in vitro* studies thatU1 acted locally on leydig cells and stimulated testosterone synthesis. It was proposed that U1, being triterpenoidal moieties, could be taken up as precursors in testosterone synthesis and increased testosterone production by isolated rat leydig cells.

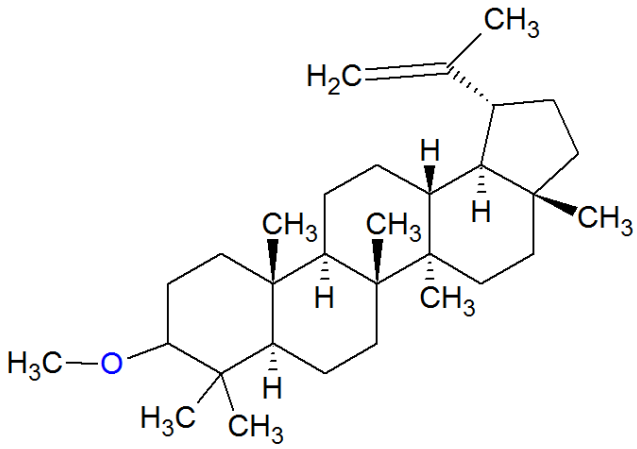

Figure 2. Probable structure of U1 (Lupeol Methyl Ether)(Calculated mol wt. 440.74398 g/mol).

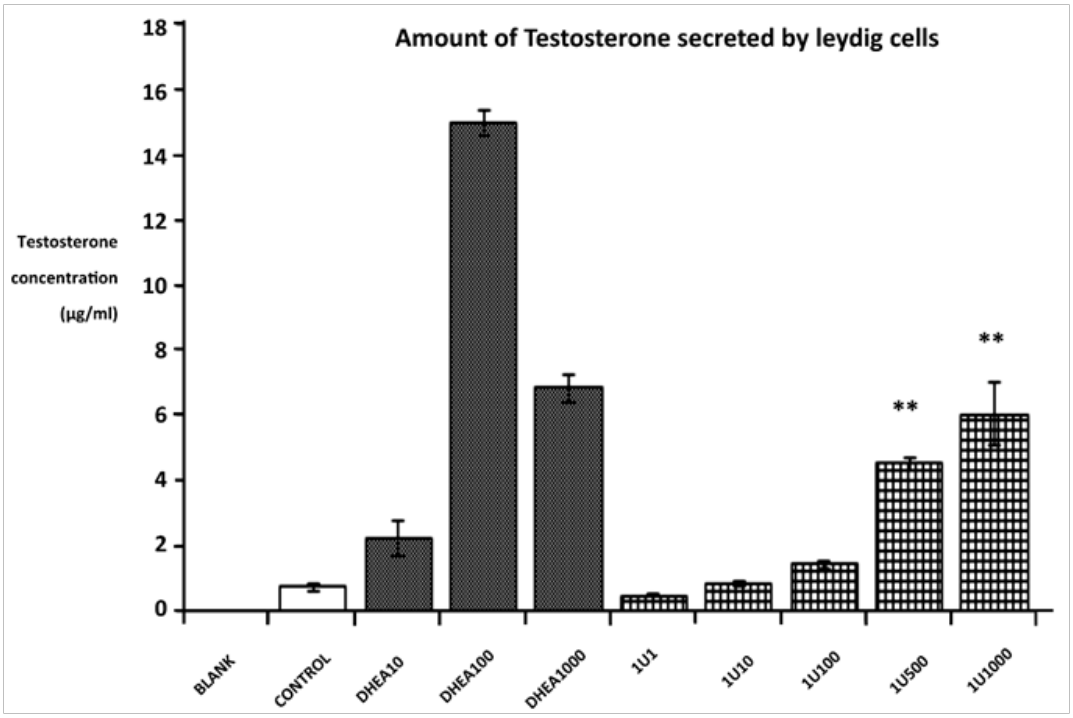

Figure 3. Results of *in vitro* studies of isolated compound U1.

4. Conclusions

It was concluded from the studies that HSU might act on leydig cells due to presence of triterpenoidal moieties. Triterpenoidal compounds have structure similarity with steroids and it was proposed that these compounds might act as steroidal precursors in testosterone synthesis. One triterpenoidal compound was isolated from bioactive fraction. Chemical characterization of compound revealed that the compound was lupeol methyl ether. This is the first report for presence of lupeol methyl ether in the plant. The studies also provided potential lead structure to be used to develop future drug candidates with spermatogenic and aphrodisiac potential.

5. Funding

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6. Conflict of interest

Authors declare no conflict of interest.

7. Authors' Contribution

Dr. Niraj Vyas - Experimentation, Chromatography experiments, data compiling, writing and reporting, paper writing; Experiments on extraction and TLC studies; **Dr. Kanan Gamit** - Biological Evaluation; **Dr. Manan Raval** - Conceptualization, methodology execution, data analysis using software and interpretation, paper reviewing and editing, funding acquisition; **Dr. Samir Patel** - Interpretation of spectra, structure determination.

8. List of Abbreviations and Symbols

HS: *Hygrophila spinosa* T. Ander
HSU: Unsaponifiable fraction
°C- Degree Celsius
g- Gram
#- Mesh size
mm-Millimetre
cm- Centimetre
%- Percentage
ml- Millilitre
v/v- Volume by volume
TLC-Thin layer chromatography
ml/min- Millilitre per minute
mg-Milligram
nm- Nanometre
ng-Nanogram
U1- Isolated compound
IR- Infrared spectroscopy
NMR- Nuclear Magnetic Resonance spectroscopy
MS- Mass Spectroscopy
MHz- Megahertz
DMSO - Dimethyl Sulphoxide
DHEA- Dehydroepiandrosterone
HPTLC- High Performance Thin Layer Chromatography
µg/ml= Microgram per millilitre

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