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Original Article

Extraction, Quantification and Anti-Cancer Activity of Important Secondary Metabolite "Shatavarin-IV" from *in vitro* Callus Culture of *Asparagus racemosus* Willd.

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ABSTRACT

Objectives: This study investigates the *in vitro* efficient pathway for the callus induction from nodal explant and isolation of the Shatavarin-IV from the callus extract from fresh callus and Dry callus. The effect of Shatavarin IV was examined on the Human lung carcinoma cell line. Gene expression study was carried out using a specific primer to conform weather it is anticancer compound or not. **Materials and Methods:** In the present study, 2,4-D and Kn were used for the direct callus induction. Callus culture, organogenesis and cell culture are used to harvest secondary metabolites. Quantification of Shatavarin-IV was performed using a TLC scanner at a wavelength of 425 nm. HPTLC analysis was performed to quantitatively determine the presence of Shatavarin-IV in Fresh Callus (FC), Dry Callus (DC) and Root Extract (RE). The mobile phase used for HPTLC analysis was ethyl acetate: methanol: water: formic acid (7.5:1.5:1:0.2 v/v). Extraction was carried out using 80% methanol and the extract was filtered. The extract was then analyzed using HPTLC methods. Further, the effect of Shatavarin-IV was studied on the human lung carcinoma cell line NCI-H23. The end result of cell cytotoxicity was analyzed using MTT dye. To, confirm whether the cell expressed an apoptotic gene or not RTPCR studies were been carried out. **Results:** The best hormonal combination for callus induction from nodal explant was found 11.31 µM 2,4-D combined with 4.64 µM Kn on MS as basal media. Shatavarin-IV accumulation in dry callus was 0.0305% w/w, wild-grown plant root extract showed 0.01732% w/w and in fresh callus presence of Shatavarin-IV was 0.0012% w/w. IC_{50} of the Shatavarin-IV was 0.8 Micromolar dissolved in DMSO. Gene expression study reflects the multifold increase of the BAX and decrease of BCL2, concluded interpreted as Shatavarin-IV causes apoptotic gene expression. **Conclusion:** It was concluded that, Shatavarin IV reduced the expression of BCL2 and increase BAX expression which positive sign that Shatavarin IV act as an anticancer compound. Shatavarin IV can be isolated from the *in vitro* callus culture, which can be alternative of whole plant that was exploited for its medicinal properties. (Schematic Abstract).

Keywords: Dry Callus, Fresh Callus, Shatavarin-IV, Gene expression, Phylloclade, Antiproliferative.

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INTRODUCTION

Plant-derived medicines have been screened by humans since ancient times.[1] Medicinal properties of the plant metabolites are identified followed by isolation of the principal component and characterized for commercial production.[2-4] The world still depends on such drugs that are only produced by plants.[5,6] Major active metabolites of plant-based drugs are alkaloids, terpenoids, flavonoids, polyamines and saponins.[7]*In vitro* approach for the production of cells with *A. racemosus* Willd. is known for its tuberous root and the presence of steroids in it.[8,9] Steroidal saponin is a unique metabolite of this plant along with alkaloids, flavonoids and terpenoids.[10,11] Shatavarin IV has molecular formula $C_{45}H_{74}O_{17}$ and the presence of three monosaccharides including deoxyhexose amongst which two are ß-D-Glucopyranoses and one α-rhamnopyranose.[12] The root is the most important medicinal part of the *A. racemosus* which contains Shatavarin-I, IV and V.[13-16] Root extract had immunoadjuvant potential.[17,18] Conventionally extract of root was used to treat diarrhoea and dysentery due to its anti-diarrhoeal activity,[19] conjunctivitis,[20] spasm, chronic fevers and rheumatism,[21] anti-anaphylactic,[22] adaptogen,[23,24] anti-leishmanial activity,[25] anti-stress,[26,27] anti-ulcer,[28,29] anti-bacterial,[30] radioprotective agent,[31] Immunostimulatory,[32] Hypoglycemic activity,[33] Increased breast milk output.[34] Antiobesity, antihyperglycemic and antidepressive potentials on rat model.[35]Recent biotechnological trends are flux towards the development of bioreactors that can manipulate plant cell totipotency and elicit it to produce secondary metabolites. Callus culture and cell culture of

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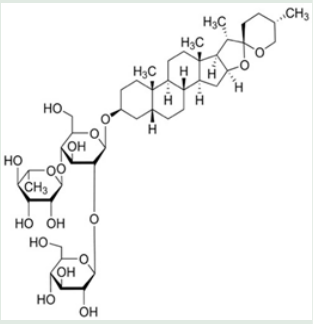
plant cells in an optimized bioreactor are promising technologies that are used nowadays to produce secondary metabolites production.[36,37] These plant cell base bioreactors are useful for the industrial-scale production of useful secondary metabolites.[38-40] Many secondary metabolites in nature are isolated from plant species which cannot be synthesized artificially because of their complex structure or lack of understanding of biosynthetic pathways.[41,42] If unorganized cell culture shows the presence of secondary metabolite with or without elicitors and genetic modification, then it could allow us to produce it on a large scale using the biotechnological approaches.[43-45] The use of elicitors in *in vitro* culture proved beneficial to increase the concentration of secondary metabolites.[46,47] The use of root powder in the manufacturing of Ayurvedic drugs increases its demand. Through the plant tissue culture technique, cell culture of the plant cells is the alternative strategy to minimize the exploitation of medicinal plants. Shatavarin-VI was present in the tuberous parts. So, every time plant needs to be uprooted from its habitat and sacrificed for medicinal-based market products. Loss from its natural habitat can add it to the list of threatened species in some areas.[48] *A. racemosus* contains steroidal glycosides, shatavarin, shatvarol and many medicinally important compounds in the roots as the most important part of this plant; it can be produced using callus culture.[37] In the present investigation comparative study of the Shatavarin IV was performed. The present investigation provides the amount of Shatavarin IV accumulation in the callus which was developed using 2,4-D and Kn which is high-frequency callus mass production in grams.



CAS Number: 84633-34-1, Product Name: Asparanin B ≥90% (LC/MS-ELSD) Product Number: SMB00155, Product Brand: SIGMA, CAS Number: 84633-34-1, Molecular Weight: 887.06

MATERIALS AND METHODS

Plant Material and Collection

A. racemosus mother plant was grown in Sardar Patel University Botanical Garden, Vallabh Vidyanagar, Gujarat. Nodal explant collected from a 4-year-old mother plant. From the tip 5th-6th of a node is used as an explant. The voucher specimen (DVB 2328) was deposited at the Department of Biosciences, Sardar Patel University, Gujarat, India.

Chemical reagents

2,4-D: Duchefa Biochemᵇᵥ, Netherland; D0911.0250); Kn: Hi-Media*, India; NaOH (Hi-Media*, India); HCl (Hi-Media*, India); Clerigel ™ Super (Gellan Gum) (Hi-Media*, India); 3% (w/v) sucrose (Hi-Media*, 90701 Extrapure AR, ACS);

HPLC grade methanol (Merck, 61860725001730, US); TCL Silica gel 60 F_{254} was used (20 X 20 cm, Sigam-Aldrich Canada); Glass syringe (Hamilton-Banaduz, Schweiz); MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5 diphenyltetrazolium bromide) Merck, EMD Millipore Corps. US, LOT 3770285/, 475989-1 g; DAMS (SDFCL Che. Ltd., Product Code: 20323 L05, India); MEM medium (AL047S-500 mL, Hi-Media*, India), $HgCl_2$ (SRL Pvt. Ltd., India).

Cell Culture

The human lung carcinoma cell line NCI-H23 was procured from NCCS, Pune. Cell culture was maintained in RPMI-1640 medium with 10% (v/v) FBS and 1X antibiotic; cDNA Kit (Thermo Scientific Verso, AB 1453A, US); Syber Green (Thermofisher Scientific A25742, US), RPMI 1640 Medium (Thermofisher Scientific, 11875093, US); FBS (Catalog number: A5256701, US). Primers were prepared form the website: https://ncbi.nlm.nih.gov/

Surface sterilization

Explant washed in running tap water for up to 5 min. Tween 20 (Hi-Media, India) used 20 drops/L for 15 min with autoclaved distilled water to minimize contamination followed by distilled water wash. Explant exposed to 0.1% (w/v) $HgCl_2$ (SRL Pvt. Ltd., India) for 5-8 min followed by the 3X sterile distilled water wash; further 70% (v/v) EtOH exposure for 30 sec was given followed by sterile distilled water wash three times (2-3 min). Explants were cut into a nodal segment of 2-3 cm in size.

Media Preparation

MS medium was used as basal media for *in vitro* studies. 3% (w/v) sucrose (Hi-Media*, 90701 Extrapure AR, ACS) was used as a carbon source in media preparation. Auxins and cytokinin stock solution (w/v) (2,4-D: Duchefa Biochemᵇᵥ, Netherland; D0911.0250) (Kn: Hi-Media*, India). The growth regulators were added in a medium of definite concentration followed by adjusting pH 5.6-5.8 using 0.1 N NaOH (Hi-Media*, India) and 0.1 N HCl (Hi-Media*, India) before making the final volume with distilled water. 0.25% (w/v) Clerigel ™ Super (Gellan Gum) (Hi-Media*, India) was added and uniformly saturated by microwave digestion. The liquid media were distributed into sugar tubes of 20×150 mm (Borosil) rimless (20 mL), Phyta Jar (Tarson) of 250 mL capacity and Sterile plastic Petri plate of 110 mm diameter. Non-absorbent cotton plug wrapped with Muslin cloth. The culture medium was sterilized by autoclaving at 121°C at 15 Psi (1.06 kg m^{-2}) for 15-20 min.

Cultural Conditions

The culture was incubated in a plant tissue culture laboratory at 23-25°C temperature and 50-70% relative humidity under a 16-h

photoperiod with a light intensity of 55 µmol m^{-2} s^{-1} (Philips, India). The sterile condition was maintained in the plant tissue culture laboratory.

Preparation of extract

In vivo, tubers were collected from the Sardar Patel Botanical Garden. Roots were thoroughly washed to remove dust particles and kept for drying at room temperature at 25±5°C. Dried roots were powdered and further extraction was carried out in 80% Methanol (Hi-media) made diluted with water (80 mL MeOH+20 mL H_2O). 1 g powder was kept in 100 mL 80% hydro MeOH solvent for 24 hr in an incubator shaker at 28°C at 120 rpm. The extract was centrifuged and the supernatant was collected and filtered with Whatman™ filter paper No.1 (prior to further experiment) (Supplementary Figure 1).

Fresh and dried callus extract

7-8-week-old callus culture was weighed and crushed in analytical grade methanol (Sigma -Aldrich) using mortar and pestle. For dry callus extract, the callus was dried at room temperature (25±5°C) and weighed. To record the final dry callus weight, callus weight is measured till its water content completely evaporates and weight becomes constant after 7 days. For extraction same procedure of repeated as performed above in the *in vivo* root.

Sample and Standard Preparation for HPTLC

All the extract was filtered with a 0.22-micron sterile filter and centrifuged at 12000 rpm before being used as the sample in the HPTLC. Standard was prepared in methanol with 1 mg/mL concentration.

Chromatographic condition

The chromatographic evaluation of Shatavarin-IV was carried out by applying the extracted sample and standard on a pre-coated aluminium silica plate for HPTLC. Readymade TCL Silica gel 60 F_{254} was used (20x20 cm, Sigam-Aldrich Canada). Samples and standards were applied by CAMAG Linomat V sample applicator using a 100 µL glass syringe (Hamilton-Banaduz, Schweiz) on a 20x10 cm TLC plate. The extract sample and standard were applied on a TLC plate with a size of 10 mm band width using a syringe with a continuous flow of nitrogen gas, the application rate was 150 nL s^{-1}. TLC chamber was filled with 40 mL mobile phase and kept for saturation of 10-15 min, 20 mL was kept in a TLC plate containing trough and 20 mL in another trough. Ethyl acetate: methanol: water: formic acid in the ratio of 7.5:1.5:1:0.2 v/v is used as a mobile phase to separate bands. The Y-axis position of the application was kept at 15 cm and the distance of the first band application X position was kept at 25 cm. After the development plate was kept for drying, the TLC bands were visualized by derivatization using an anisaldehyde-sulfuric acid reagent, spraying of reagent followed by heating at 120°C for 8-10 min in oven. Quantification was done by CAMAG TLC Scanner 3 (1.14.30), with absorption mode and reemission type at the 425 nm wavelength. The slit dimension was 4.00x0.30 mm, Micro at the scanning speed of 20 mm/s with 100 µm resolution/step.

Method of Validation

The method was developed and validated by evaluating linearity, precision and reproducibility. The method was optimized under the norms of the International Conference on Harmonization (ICH). The setup system precision was analyzed by repeatedly measuring the peak area of the Shatavarin-IV standard three times of the same band (500 ng/spot). Intraday and inter-day precision of Shatavarin-IV was experimented with using three

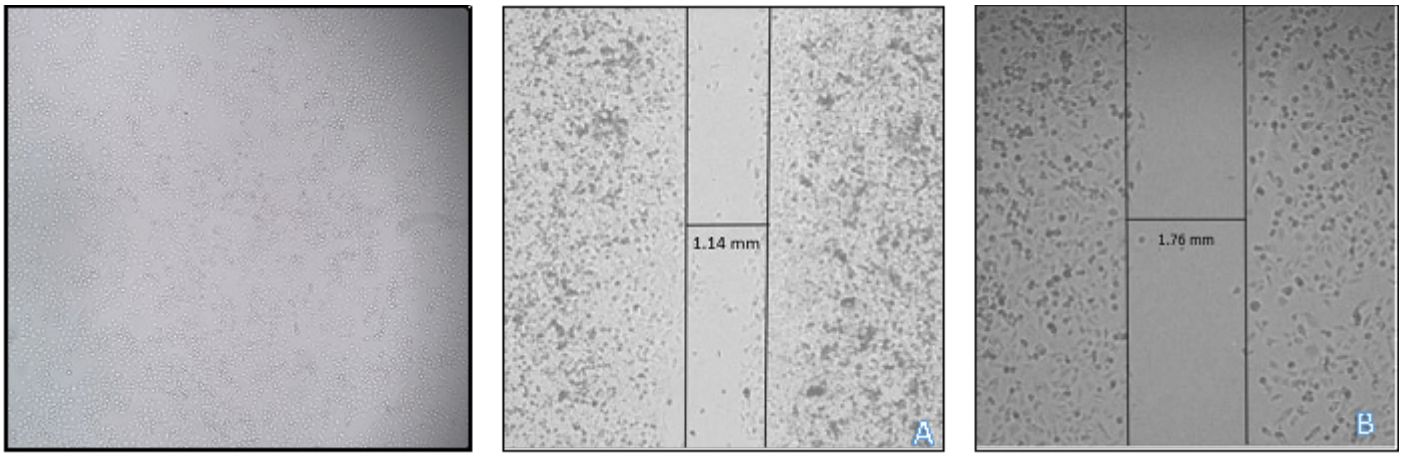


Figure 1: (i) Cell culture was maintained in RPMI-1640 medium with 10% FBS and 1X antibiotic. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 level. (ii) Representation of inhibition of cell proliferation using scratch assay. Cell proliferation is qualitatively assessed by visual inspection and quantitatively by measuring gap width. (A) At 24 hr post scratching, cells without treatment have started migrating and covering the wound. Width of the remaining scratch is 1.14 mm, (B) At 24 hr post scratching, Shatavarin-IV treated cells still have not started migrating. Width of the remaining scratch is 1.76 mm.

spots of 500 ng/spot applying on TLC on the first day followed by successive three days. The Limit of Detection (LOD) is the minimum concentration of standard that shows the area of peak threefold more than the signal-to-noise ratio. The Limit of Quantification (LOQ) was determined by the concentration of metabolite that shows the area of peak tenfold more than the signal-to-noise ratio. The accuracy of the detection was calculated by applying the standard in a replicate (n=3) for three days. The analyte amount in TLC was analyzed through the scanner at 425 nm and it was calculated in the Relative Standard Deviation (%RSD) of chromatogram peaks.

Statistical Analysis

Each experiment was conducted with 10 replicates three times. Data recorded were calculated with the statistical software SPSS (Version 20, SPSS Inc. Chicago, USA). The data were analyzed with one-way ANOVA to compare the effect of different hormone combinations on callus induction from nodal explants.

Cell culture

The human lung carcinoma cell line NCI-H23 was procured from NCCS, Pune. Cell culture was maintained in RPMI-1640 medium with 10% (v/v) FBS and 1X antibiotic. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 level (Figure 1 (i)).[49]

Evaluation of cytotoxicity

NCI-H23 cells were seeded into flat bottom 96-well plates at the density of $5x10^4$ cells/well. Cells in the well were treated with different concentrations of Shatavarin-IV in the range of 0.1, 0.2, 0.4, 0.6, 0.8 and 1 µM and incubated for 24 hr to measure the cytotoxicity. After treatment 20 µL MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5 diphenyltetrazolium bromide) was added to each well and incubated at 37°C for 4 hr. Formazan crystals were dissolved using 200 µL of DMSO and the intensity of colour was measured at 570 nm wavelength using a microplate reader (Infinite M200pro, Nanoquant Multimode reader, Tecan).

Scratch assay

A scratch assay was executed to check the antiproliferative property of Shatavarin-IV on cell line NCI-H23. To experiment approx. Cells were seeded in each well plate. Once the cells reached around 70% confluence, a scratch was made on the monolayer. The scratch was made in a straight line on the monolayer with a 100 µL pipette tip. Cells were washed thrice with PBS and the gaps were measured in each well. Three wells were kept as control without treatment and in three wells, Shatavarin-IV treatment was given for 24 hr. After incubation, cells were observed under a phase contrast microscope. The cells that migrated to the scratched surface were observed under an inverted microscope after 24 hr.

Gene expression analysis

Cells treated with Shatavarin-IV for 24 hr are used for RNA isolation. RNA quality and quantity were checked and further RNA was used for cDNA synthesis (Bio-Rad iScript cDNA). qPCR was performed using the SYBR Green mix. GAPDH was used as a reference gene Experimental data was analyzed by CT value and fold change expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Primers were designed using Primer 3.0 software (Table 1).

RESULTS

Induction of callus

In *in vitro* conditions explants can be propagated through two major pathways, direct or indirect organogenesis. Direct organogenesis is a prominent way to acquire truly genetically identical plants and in indirect organogenesis, there have been probabilities of change in the genetic makeup of somatic cells of the plant which are often termed "somaclonal variation".[50,51] Proliferation and multiplication of shoots through the nodal part of the twig were mostly used as an explant to produce true-to-type plants, which are devoid of genetic variation in comparison to the mother plant.

In presented experimental studies, MS medium supplemented with 2,4-D 11.3 µM combined with 2.32 µM Kn was proved best which produced approx. 5 g of callus after 4 weeks after inoculation. Callus weight was measured in each concentration (Table 2). Juvenile twigs are used as explants, which show higher proliferation as compared to the other explants such as internode and mature node. Callus proliferation starts from the node, as meristematic tissue was actively divided due effect of suitable growth regulators. Callus was induced faster in the range of 4.52-11.3 µM of 2,4-D in a period of within 2 weeks, confirmed with bulging around the nodal region of the explants (Figure 2). Juvenile explants having underdeveloped phylloclades, shows the highest callus percentage, in the range of PGRs combination having 11.3 µM 2,4-D with Kn (2.32-4.64 µM) (Table 2). Further some callus cultures were kept for multiplication on the same media for up to 6 weeks which allowed the callus to proliferate up to a maximum of 11-12 g (Supplementary Figure 2).

HPTLC analysis (Quantitative determination of Shatavarin-IV from the FC, DC and RE)

The method performed at present was validated as per ICH (International Council of Harmonization). In the present method, the standard linear curve of Shatavarin-IV was achieved by applying a standard solution on a TLC plate in the range of 100-1000 ng/spot (Figure 3). A calibration curve was plotted based on peak area against the concentration and the same as peak height against the concentration. The quantification of Shatavarin- IV in the DC, FC and RE was performed by comparing it with a standard chromatogram. The limit of detection 10 ng

and the limit of quantification 30 ng were analyzed by the three and ten times the noise level, respectively (Tables 3 and 4). The mobile phase experimented in the presented study was chosen after different solvent combinatorial trials.

TLC bands (8 mm) of sample and standard showed maximum sharp separation in ethyl acetate: methanol: water: formic acid in the ratio of 7.5:1.5:1:0.2 v/v. Development of a TLC plate of 20 cmx10 cm was carried out in the twin-trough glass chamber which was saturated with mobile phase before developing the TLC plate for 10-12 min. The TLC plate was dried and sprayed with an anisaldehyde sulfuric acid reagent followed by a heating plate at 120° C for 5 min. TLC plates were scanned for densitometric scanning using *CAMAGA* TLC scanner III in WinCATS© software in absorbance-reflectance mode at 425 nm for further analysis. Peaks were observed on standard bands having an R_f value of 0.42.

The purity of the standard marker was assessed by comparing spectra at the start point of the peak, the maximum height of the peak and the end of the peak. The chromatogram of the sample and standard was exactly overlayed. The calibration plot was linear with a correlation coefficient (r^2) of 0.98 (Shatavarin-IV) interpreted as having a good linear relation between the peak area and the concentration. The calibration curve equation obtained from linear regression was Y=2316+9.768*X (X indicates the concentration of standard Shatavarin-VI and Y indicates peak area) (Supplementary Figures 3-5).

The quantification method was evaluated by applying the same amount (500 ng) on TLC in replicate (n=3). The precision stability and recovery were expressed in relative standard deviation (RSD%) (Tables 3 and 4).

Table 1: List of the Primer.

Gene	Polarity	Primer sequence	Length
Bax	FORWARD	GCTGGACATTGGACTTCCTC	20
	REVERSE	CTCAGCCCATCTTCTTCCAG	20
Bcl2	FORWARD	TCCACCTGGATGTTCTGTGC	20
	REVERSE	ACAGGAACCCTCCCTCTGTT	20
E Cad	FORWARD	TGGACCGAGAGAGTTTCCCT	20
	REVERSE	CGACGTTAGCCTCGTTCTCA	20

Table 2: Effects of auxin and cytokinins with their various concentrations on *A. racemosus* nodal explants. Results were recorded after 4 weeks.

PGRs/ Concentration			Callus induction %	Texture of callus	Fresh Weight of callus in Gram
Sl. No.	2,4-D	KN			
1	2.26	0	**	Compact	0.88±0.30^{i}
2	4.52	0	***	Compact	1.67±0.12^{gh}
3	6.78	0	***	compact	1.79±0.11^{h}
4	9.04	0	***	Compact, Nodular	2.23±0.19^{fg}
5	11.3	0	***	Compact, Nodular	3.56±0.27^{bc}
6	2.26	2.32	****	Compact,	1.89±0.06^{gh}
7	4.52	2.32	***	Compact, Nodular	3.20±0.13^{bcd}
8	6.78	2.32	***	Compact, Nodular	3.00±0.13^{cde}
9	9.04	2.32	***	Compact, Nodular, Organogenic	3.77±0.17^{b}
10	11.3	2.32	****	Compact, Nodular, Organogenic	4.97±0.26^{a}
11	2.26	4.64	**	Compact	1.51±0.13^{h}
12	4.52	4.64	***	Compact	2.78±0.13^{def}
13	6.78	4.64	***	Compact, Nodular, Organogenic	2.69±0.19^{ef}
14	9.04	4.64	****	Compact, Nodular, Organogenic	3.31±0.20^{bcd}
15	11.3	4.64	****	Compact, Nodular, Organogenic	4.62±0.23^{a}

Percentage of explants that formed callus at the node and base each nodal explant: *0-25%, **26-50%, ***51-75%, ****76-100%.

Values represent mean±std error. Means followed by the same letter within each column are not significantly different according to Duncan's multiple range tests at $p \leqq 0.05$. Data were recorded after 4 weeks days. All experiments were performed on MS (Murashige and Skoog 1962) medium. *2,4-D*: 2,4-dichlorophenoxyacetic acid, *Kn* kinetin (6-furfurylaminopurine).

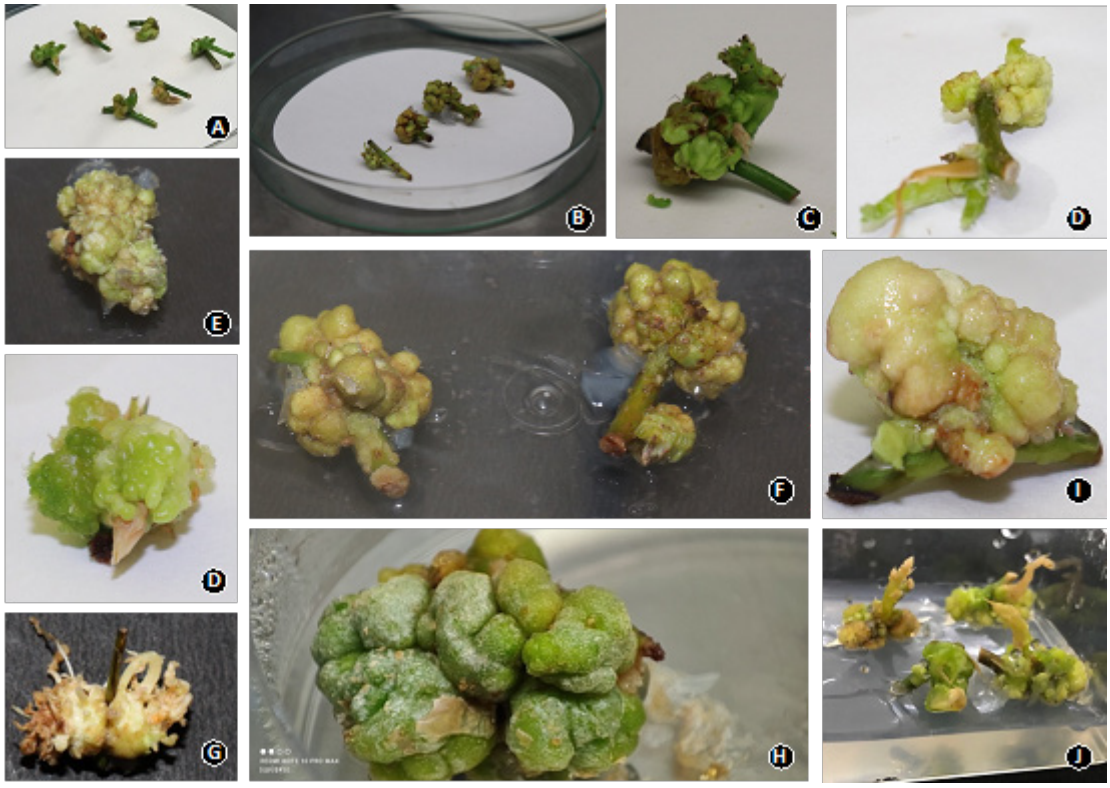


Figure 2: Callus induction using nodal explant: A. Nodal explant after 2 weeks (2,4-D 11.3 µM+Kn 2.32 µM) B. Nodal explant after 4 weeks (2,4-D 11.3 µM+Kn 2.32 µM) C. Nodal explant after 4 weeks inducing organogenic green colour callus (2.5 mg/l+4.64 µM) D. Induction of callus from the base of nodal explant after 3 weeks (2,4-D 6.78 µM+Kn 2.32 µM) E. Pro-liferation of callus after first subculture in 2,4-D 22.6 µM (Sub cultured from 2,4-D 22.6 µM after 4 weeks; the image was taken after 2 weeks after incubation). F. Proliferation of callus in 2,4-D 4.52 µM+Kn 4.64 µM. G. Nature: hard nodal ex-plant showing growth of shoot and callus (2,4-D 6.78 µM+Kn 4.64 µM; 3 weeks). H. Callus after the first subculture in the same medium (2,4-D 11.3 µM; Image was taken after 4 weeks subculture). I. Induction of callus after 4-week culture (2,4-D 11.3 µM+Kn 4.64 µM). J. Induction of callus (2,4-D 11.3 µM+Kn 4.64 µM).

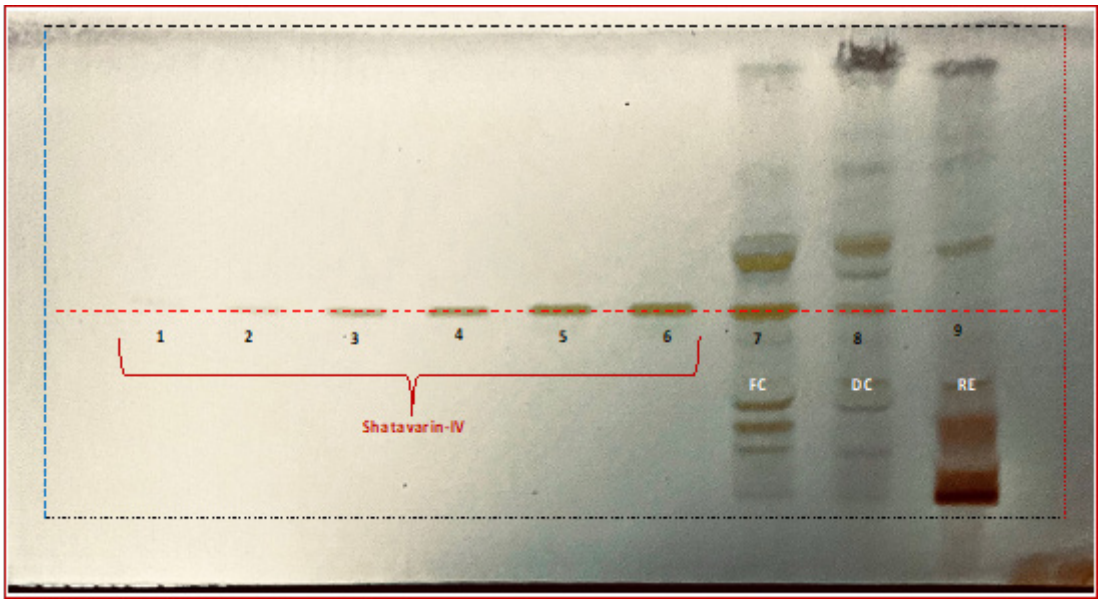


Figure 3: TLC plate with a standard band (1-6) and Fresh callus (7), Dry callus (8) and Root extract (9).

Effect of Shatavarin-IV on NCI-H23 cell line

During apoptosis, the mitochondria gene plays a crucial role in Programmed cell death. During apoptosis, expression of BCL2 and BAX is important as they are markers for apoptosis, BAX is pro-apoptotic and BAX is an anti-apoptotic marker. In cancerous cells, BCL2 activity is increased in comparison with BAX, which remains lower. After exposure of Shatavarin-IV to NCI-H23, 0.8 µM concentration showed IC_{50} after 24 hr incubation (Supplementary Figure 6). Expression of BCL-2 was found to be lower in the present study while in comparison the BAX expression was observed a 1.34±0.0027fold increase (Figure 4).

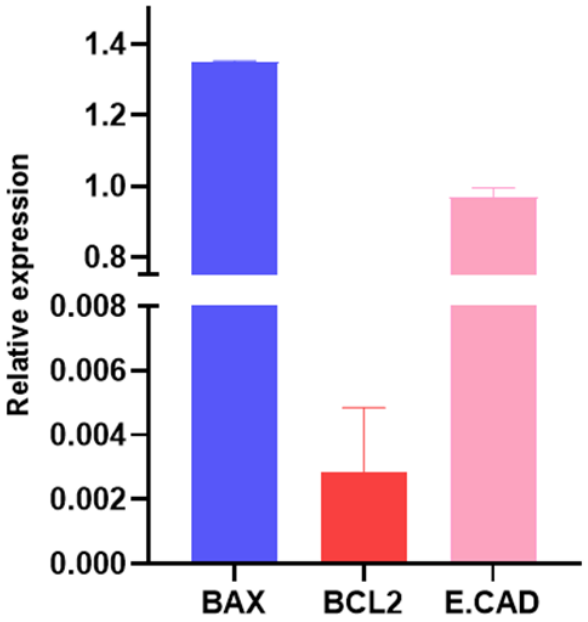


Figure 4: Gene expression fold change after 24 hr of Shatavarin-IV treatment to NCI-H23 cell line.Fold change in gene expression was analysed using qPCR. Gene expression of BAX,BCL2 and E.CAD was normalised to β-Actin.Statistical analyses were performed on the data.Values represent the Mean±SEM. ($p \leq 0.0001$).Fold change in gene expression in comparison to untreated (U.T.) NCI-H23 cells.

After the treatment with Shatavarin IV for 24 hr in the NCI-H23 cell line it was observed that gene expression was altered. BCL2 levels were lowered and BAX levels were higher due to Shatavarin treatment given to the NCI-H23 cells. BCL2 is an antiapoptotic protein in cancer; its high expression makes cells resistant to cell death.[52] Cancer cells that lose their characteristics through downregulation of proliferative and EMT markers go through programmed cell death. The study carried consideration of the distinct antiapoptotic and apoptotic markers for mitochondria, BCL-2 and BAX. When compared to BCL2, the expression of BAX was shown to be upregulated 1.34±0.0027 and BCL2 to be downregulated 0.0028±0.002 on the exposure of Shatavarin IV for 24 hr. Many of the chemotherapy formulations work on the down-regulation of BCL2 to escort the cells to apoptosis. A high level of BCL2 and a low level of BAX is a characteristic of tumor cells which aids in developing resistance against apoptosis.[53,54] Downregulation of BCL2 induces mitochondrial-dependent cell death. Wang *et al.* (2019)[55] also observed IGF-IR-mediated mitochondrial-dependent apoptosis in colon cancer on knockdown of IGF-1R. Many of the chemotherapy formulations work on the down-regulation of BCL2 to escort the cells to apoptosis.[55,56] Cell proliferation and metastasis state of tumor is associated with persistent EMT. In tumor cells transit from EMT to MET, it may lead to cancer cell death. *E. cadherin* is an epithelial marker and its low expression is observed in cancer cells which allow cells to proliferate. Increased fold change in *E. cadherin* expression is achieved by the treatment of Shatavarin, that may lead to altered EMT and anoikis of the cancer cells. Upregulation of *E. cadherin* transcript was found higher by 0.969±0.026 fold which is statistically significant. NCI-H23 cell line loss its metastasis properties via gaining anchoring properties on the expression of *E. cadherin* that leads to anoikis in NCI-H23 cell line cells.

Table 3: **The Limit of Detection (LOD) and Limit of Quantification (LOQ) was determined using a signal-to-noise ratio.**

Standard	Linear Rane (ng/spot)	Regression equation	R^2	LOD (ng)	LOQ (ng)
Shatavarin-IV	100 ng-1000 ng	Y = 2316+9.746 * X	0.98195	10 ng	30 ng

Table 4: **Reproducibility.**

Standard	Precision	Concentration/Spot	Concertation detected	Mean	RSD %
Shatavarin-IV	Intraday	500 ng	512	507.3	1.2
			506		
			504		
	Inter day	500 ng	518	506	1.1
			507		
			501		

Shatavarin IV treated cells inhibit the proliferation of cancer cells. After 24 hr of incubation of NCI-H23 cells with Shatavarin IV, the rate of cell proliferation was observed low which resulted in a distinct gap with formed scratch (Figure 1 (ii)). The observed scratch closure of NCI-H23 cells in the control condition was 1.55 mm, whereas the closure was 1.90 mm in Shatavarin IV treated NCI-H23 cells. Data suggests that on exposure to Shatavarin IV, cells lose the characteristic feature of uncontrolled proliferation, which results in an uncovered scratch after the treatment of Shatavarin IV for 24 hr.

DISCUSSION

In the present study callus culture establishment was done using 2,4-D and Kn on MS solid media without any additives. The experiment aims to produce maximum callus mass and check the amount of the Shatavarin-IV from it. HPTLC is the simplest, fast and reliable technique for the analysis of secondary metabolites. Using HPTLC with having suitable solvent system Shatavarin IV was easy to separate from the crude and quantify.[6] In the present experiment, Shatavarin-IV accumulation dry callus was 0.0305% w/w (0.39803 µg of Shatavarin-IV in 1305 µg), wild-grown plant root extract showed 0.01732% w/w (0.0866 µg Shatavarin-IV in 500 µg) and in fresh callus presence of Shatavarin-IV was 0.0012% w/w (1.14857 µg Shatavarin-IV in 10 mg). Pise *et al.*,[37] induce callus using node as explant which is used to produce cells sus-pension NAA (5.37 µM), 2,4-D (4.52-9.04 µM), BAP (2.22 µM), Kn (2.32 µM) culture for the secondary metabolite production. Reddy *et al.*, [56] reported 0.5 g of callus using 17.75 µM BAP+0.537 µM NAA, while Patel and Patel[57] used the same hormonal combination of 0.537 µM NAA alone and 5.37 µM NAA+2.21 µM BAP. Peise *et al.*,[37] reported 11.48±0.61 mg g^{-1} Shatavarin- IV in liquid media composition having 2,4-D combination with Kn and KH_2PO_4 as additives. Peise *et al.*, [37] reported 1.1% (11-12 mg mL^{-1}) Shatavarin-IV content in *in vitro* as in comparison 0.05% to 0.08% (0.5 mg mL^{-1}) Shatavarin-IV content found in roots. The conducted study showed the accumulation of Shatavarin-IV was higher in callus culture grown in solid MS media as compared to roots from the field-grown plant.

As compared to the present study, Mitra *et al.*,[58] experimented with a Shatavarin-IV rich fraction on MCF-7 (human breast cancer), HT-29 (human colon adenocarcinoma) and A-498 (human kidney carcinoma) cell lines using MTT assay. Kabir *et al.*,[59] synthesized silver nanoparticles from *A. racemosus* roots and exposed them to human Glioblastoma Stem Cells (GSCs) and Ehrlich Ascites Carcinoma (EAC) cells, in the reported study of gene expression TLR9, NF_kB, TNFa Tumor necrosis factor, p21 and IKK (IkappaB kinase) genes were elevated and PARP, EGFR (epidermal growth factor receptor), NOTCH2 (Neurogenic locus notch homolog protein 2), mTOR (mammalian target of rapamycin) and STAT3 (Signal Transducer And Activator of Transcription 3) genes decreased in GSCs cells, as *in vivo* study EAC tumor growth rate was reduced with increase life span and restored the haematological parameters. The NCI-H23 cell line was a type of lung adenocarcinoma. Shatavarin-IV showed cytotoxic activity on the NCI-H23 cell line with a 1.34±0.0027 fold increase in the BAX gene. BAX expression was higher in the adenocarcinoma than in squamous cell lung carcinomas.[60] The present study takes the side to predict that Shatavarin -IV is an anticancer metabolite present in the *in vitro* of *A. racemosus*.

Developing world, we are still dependent on drug products derived from a plant. This ultimately affects any medicinal plant population in their natural habitat. The modern plant biotechnology field has the waste scope of cell culture *in vitro* conditions. *In vitro*, cell culture of plants is considered to be the future of plant metabolite production, which fulfils the demand of the pharmaceutical sector.[61,62] Secondary metabolites can be harvested by the *in vitro*-grown culture through organogenesis, cell culture or callus culture.[63-67] These studies help to develop efficient protocols for the mass production of the phytochemical constituents through the cell culture in the Bioreactor and hairy root production.

CONCLUSION

In present study successfully produced callus cultures from *A. racemosus* and were able to isolate Shatavarin-IV from the callus. The amount of Shatavarin-IV obtained from the callus was higher compared to that extracted from the roots of wild-grown plants. Shatavarin-IV exhibited cytotoxic effects on human lung carcinoma cells (NCI-H23 cell line). Gene expression analysis suggested that Shatavarin-IV may induce apoptosis (programmed cell death) in cancer cells. This study suggests that *A. racemosus* callus culture can be a sustainable source of Shatavarin-IV for potential cancer treatment. Shatavarin-IV demonstrates promise as an anti-cancer agent, warranting further investigation. The study optimized a method for isolating and quantifying Shatavarin-IV using HPTLC (high-performance thin-layer chromatography). The research also explored the effects of Shatavarin-IV on cell proliferation and gene expression in cancer cells.

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CONFLICT OF INTEREST

All the authors declare no conflict of interest. All authors have seen and approved the final version of the manuscript being submitted. This article is original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

ABBREVIATIONS

DC: Dry Callus; **FC:** Fresh Callus; **RE:** Root Extract; **DMSO:** Dimethyl Sulphoxide; **2,4-D:** 2,4-Dichlorophenoxyacetic acid; **TDZ:** Thidiazuron; **Kn:** Kinetin; **NAA:** Naphthaleneacetic Acid; **BAP:** 6-Benzylaminopurine; **EtOH:** Ethanol; **MeOH**: Methanol; **HPTLC:** High-performance thin layer chromatography; **MS:** Murashige and Skoog; **TLR9:** Toll like receptor; **NF_kB:** Nuclear factor kappa B; **PARP:** Poly(ADP-ribose) polymerase; **EAC:** Ehrlich ascites carcinoma cells; **TNFa:** Tumor necrosis factor; **IKK:** IkappaB kinase; **EGFR:** Epidermal growth factor receptor; **NOTCH2:** Neurogenic locus notch homolog protein 2; **mTOR:** Mammalian target of rapamycin; **STAT3:** Signal Transducer and Activator of Transcription 3.

AUTHOR CONTRIBUTIONS

Plant tissue culture and extraction studies were carried out by Dinesh S Vasava, Abhishek Tote and Keyur Sindhava. Cell line studies were carried out by Dinesh S Vasava and Poonam N Bhagriya. HPTLC analysis studies were carried out by Dinesh S Vasava and Nitin Solanki. Poonam Bhagriya helped to write and edit the manuscript and to develop cell line protocol writing.

SUMMARY

It was found that, Shatavarin IV can be used as anticancer molecules as per study primarily on cell line, showed reduction in the expression of BCL2 and increase BAX expression. *In vitro* callus culture can be a alternative source of Shatavarin IV and can be isolated, which can be alternative of whole plant that was exploited for its medicinal properties.

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Supplementary Figures

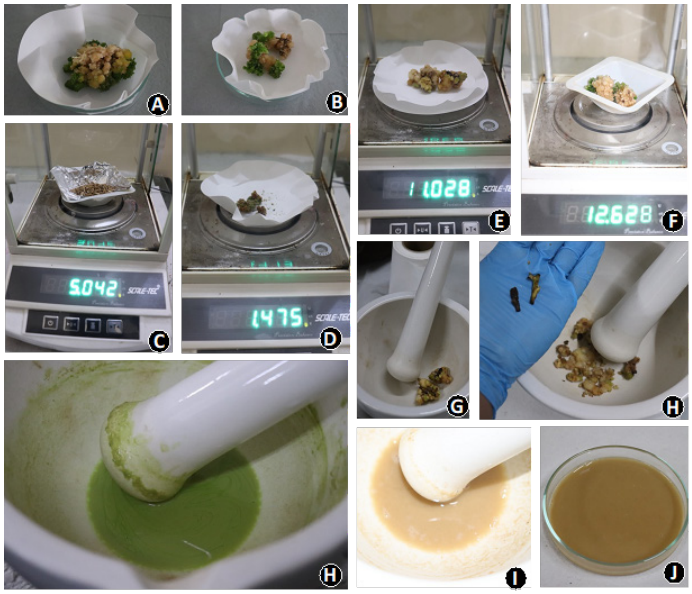

Figure 1: A, B, E and F: Fresh Callus weight; C, D: Dry callus weight; G, H and J: Crushing using mortar pestle; J: collected in to Petridis.

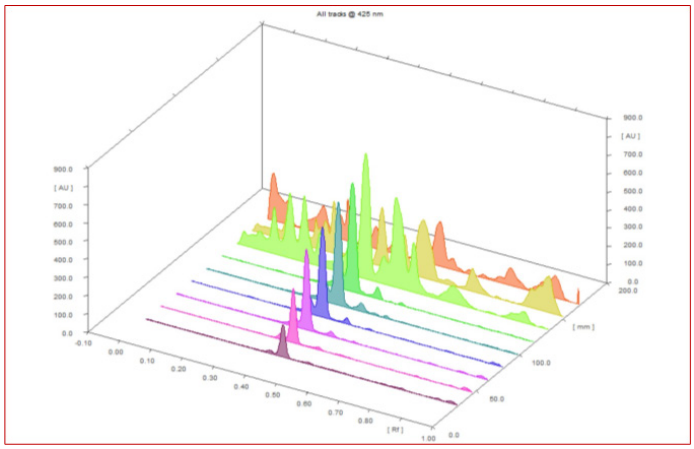

Figure 3: Chromatogram of Standard Shatavarin-IV with FC,DC and RE.

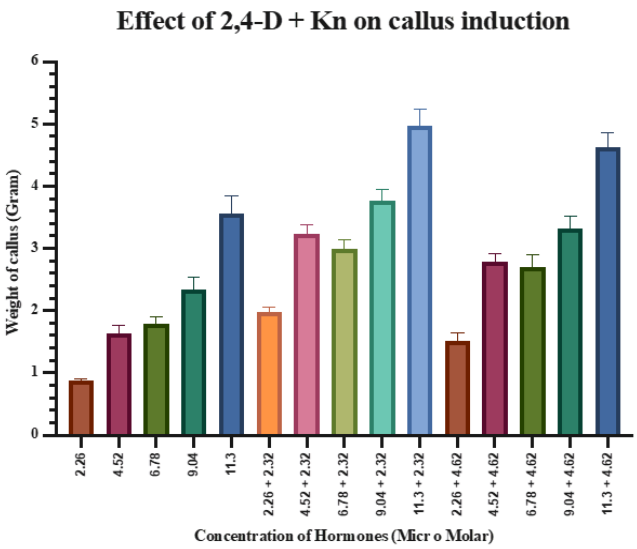

Figure 2: Graphical representation of the effect of hormones on the weight of the callus.

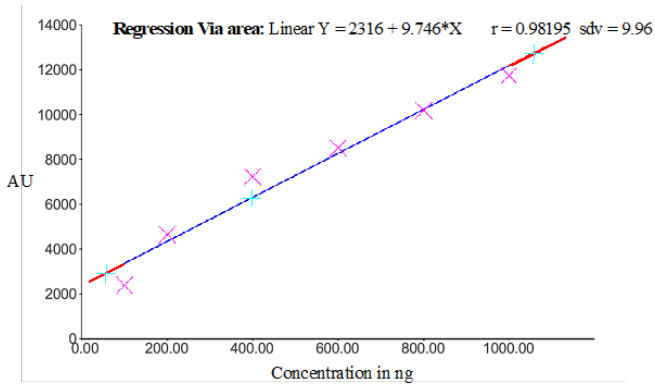

Figure 4: Calibration curve of Shatavarin-IV.

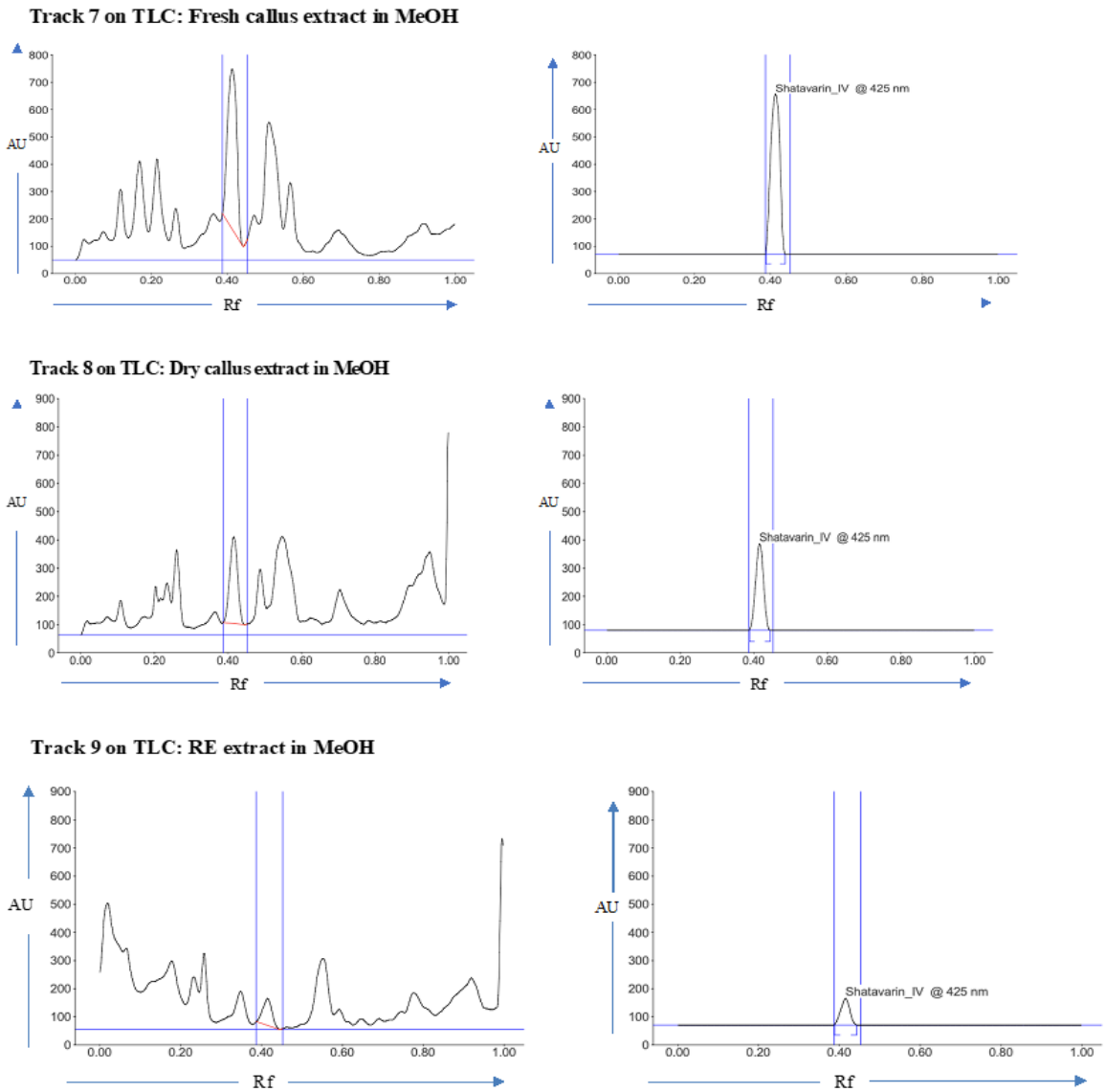

Figure 5: Track 7, Track 8 and Track 9 showed a comparative presence of Shatavarin-IV in Fresh callus, Dry callus and Root extract.

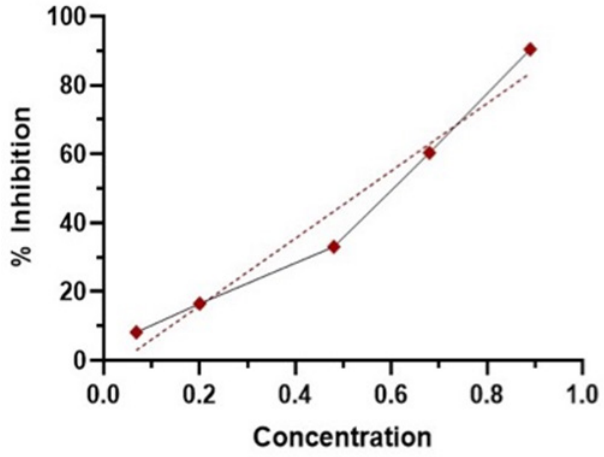

Figure 6: Effect of Shatavarin –IV on the proliferation of NCI-H23 cells after 24 hr of incubation. NCI-H23 cells were treated with Shatavarin–IV in different concentrations growth inhibition of cells was detected by MTT assay (0.8 Micromolar).