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In Vitro Culture, Isolation and Cytotoxic Effect of Solasodine from *Solanum Trilobatum* Linn.

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Abstract: ***The plants of the family Solanaceae is used widely for drugs and medicines. This is because they contain powerful alkaloids in their cells. A rapid and efficient in vitro plant regeneration method was developed for S. trilobatum from leaf explants. Solasodine was isolated from in vitro shoots and the field grown plant (control plant) and compared with the standard using TLC, UV-vis spectral analysis and IR. Cytotoxicity of solasodine was also done using DLA cells. Maximum of 32 shoots was regenerated from the leaf explant. Solasodine isolated was purified and compared with the standard and established the identity. The increasing demand of medicinal plants and indiscriminate destruction of medicinal plant flora has encouraged the application of biotechnological approach to produce the biologically active compounds. The finding of this study suggests that the isolation of compounds from in vitro shoots is much faster and easier and can be compared with the field grown plant and can be used as an alternative for the isolation of the valuable phytochemicals.***

Keywords: *In vitro* culture, *S. trilobatum*, DLA cells, Solasodine, Cytotoxicity

1. Introduction

India is endowed with a unique wealth of biota, which includes a large number of medicinal and aromatic plants, and many of them are rare and endemic. For centuries, plants have been an important source of drugs. The World Health Organization estimates that approximately 80% of the world's population relies primarily on traditional medicines as sources for their primary health care [1]. The medicinal use of plants is actually due to the presence of active components that are effective for human body in many ways [2]. Among the medicinal plants, about one thousand plant species were found to have anticancer potential out of 250, 000 plant species existing on earth [3]. Bioactivity guided isolation technique is the key to the discovery of some important anticancer agents like paclitaxel from *Taxus brevifolia* and camptothecin from *Camptotheca acuminate* [4]. Genus *Solanum (Solanaceae*) is rich in steroidal glycoalkloids, an important group of secondary metabolites. These compounds have the potential to be the starting material for synthesis of steroidal drugs. In majority of solanaceous plants, solasodine occurs as aglycone part of glycoalkloids, which is a nitrogen analogue to sapogenins Solasodine, can readily be converted to 16-dehydropregnenolone a key intermediate in the synthesis of steroid drugs eg. Progesterone, cortisone etc. *Solanum* plants and their cell and tissue cultures contain valuable steroidal alkaloids such as solasodine [5]. Solasodine is present in a number of *Solanum* species of the family Solanaceae, such as *S. khasianum*, *S. xanthocarpum*, *S. nigrum*, *S. gracile*, *S. laciniatum*, *S. lycopersicum*, etc. A number of traditional herbs containing solasodine have been used in the Indian System of medicine. Solasodine has been reported to have anticancer, insecticidal and anti-accelerator cardiac activities. Because of pharmaceutical importance, its isolation and quantitative determination was achieved by several workers using variety of analytical techniques.

Solanum trilobatum Linn. (Solanaceae) is a climbing shrub found in Deccan Peninsula. It is an important medicinal plant. The leaves contain rich amount of calcium, iron, phosphorus, carbohydrates, protein, fat, crude fiber and minerals. The decoction of various parts of the plant is used in chronic bronchitis. Roots used for consumption, berries and flowers are used for cough and leaves are cooked and eaten as a vegetable [6]. The plant is used as medicine for vomiting of blood and bilious matter, phlegmatic rheumatism, several kinds of leprosy. It is also antibacterial, antifungal, anti-mitotic [7-9]. Antitumour property possessed by the plant extracts was also reported [10]. An abundance of healing properties are found in Solanidine, Solasodine and α–Solanine from *Solanum trilobatum*. *In vitro* culture and cytotoxic effect of steroids from *Solamun trilobatum* was also studied [11].

Various members of this genus have been in focus for *in vitro* regeneration because of their high medicinal value owing to the presence of β-solanargine, solasodine and other closely related glyco-alkoloids [12]. Because of the many scientific, economic and ecological advantages of plant tissue culture, it is nowadays considered an important strategy for *in vitro* production of bioactive compounds for drug and food industries [13, 14]. The present investigation aims to do the preliminary phytochemical analysis micropropagation, isolation, identification and cytotoxic analysis of solasodine obtained from *in vitro* shoots and compared with field grown plant (control plant).

2. Material and Methods

S. trilobatum fruits were obtained from Ayurveda Research Institute, Poojapura, Thiruvananthapuram, India. Fruits were washed and surface sterilized using 0.1 % mercuric chloride for 10 min and washed in sterile distilled water for 3 times 5 min in each wash. Fruits were opened aseptically and seeds were inoculated on MS basal medium [15]. After 14 days,

the leaf and shoot tip explants obtained from the seedlings were inoculated on MS medium supplemented with BA, kinetin, IAA, alone or in different concentrations and combinations (Table I). The cultures were maintained under 16-18 h photoperiod (2500 lux) at 25± 1^0C. Shoot or callus initiation was observed from the explants after 4 wks in the respective media. The shoots regenerated from the different leaf explants inoculated on MS medium with IAA 0.1mg/l and Kin 2.5mg/l were selected for further multiplication.

2.1 Preparation of Extracts

The shoots of mature plant maintained in the field and the *in vitro* shoots in IAA 0.1mg/l and Kin 2.5mg/l is chopped, dried in shade and powdered. About 10g of the powder from each source was extracted (soxhlet) exhaustively with petroleum ether (60-80˚C). The extract was filtered and stored in refrigerator as stock solution. A part of the residue of the petroleum ether soluble portion was saponified by refluxing for 1 hour with 0.5 N alcoholic KOH and the unsaponified steroid fraction was extracted with diethyl ether. The steroid fraction was further fractionated by TLC using the solvent system hexane, diethyl ether and acetic acid (34: 6: 0.8) and spots were visualized in an iodine chamber.

2.2 Identification of the components

The steroid rich component from the samples and the standard of the solasodine was co-chromatographed and the solasodine was identified by spraying the chromatogram with 25g of antimony trichloride, dissolved in 75 ml chloroform after spraying, dried the chromatogram for 10 minutes. The one of fraction from the samples and the standard of the solasodine developed dark violet co lour. The R_f value of the samples were compared with that of the standard.

2.2.1 Isolation of the sample

The steroidal bands developed were marked in the TLC plate and the marked portion was scraped using thin spatula and eluted with diethyl ether. After the removal of sedimentary silica by centrifugation and filtration and the supernatant was collected and concentrated in a vacuum evaporator. All the seven fractions were used for further studies. The eluted solasodine fraction was subjected to confirmatory tests using the IR and UV spectrum. The isolated crystalline fractions of solasodine from the samples and standard were subjected for IR spectra. For UV spectrum the spots corresponding to Rf value of the standard solasodine were scraped using thin spatula and eluted with methanol. After the removal of silica by centrifugation, diluted with methanol and the ahsorbancy was taken at 210 nm and compared with that of standard to establish the identity.

2.2.2 *In vitro* cytotoxicity

The *in vitro* cytotoxicity was measured using Dalton's Lymphoma Ascites (DLA) cells. Inbred strains of swiss albino mice of body weight between 10-23gm were selected from the animal house and maintained under standard conditions. The DLA cells were collected from the stock and maintained and propagated in the peritoneal cavity of Swiss albino mice. In this procedure 0.8 ml physiological saline, 10^6 cells per ml in 0.1 ml physiological saline and 0.1 ml of different steroid fractions of *S. trilobatum* were incubated for 3 h at 37° C. To this cell suspension, 0.1 ml of trypan blue (0.1 %) was added. Cells were loaded into a haemocytometer and the number of stained cells was counted and calculated the percentage of dead cells per ml.

3. Results and Discussion

Direct shoot regeneration was obtained from the leaf explants inoculated on MS medium supplemented with various combination and concentrations of auxin and cytokinins.

The *in vitro* leaves inoculated on MS medium enriched with 1, 2, 3 mg/l BA or Kin alone and 0.05, 0.1, 0.3 and 0.5mg/l IAA in combination with 1, 1.5, 2 and 2.5mg/l BA or Kin were reported in the present study (Table 1).

Table1: Response of leaf explants on MS medium supplemented with IAA and BA or KIN after 30 days

Growth regulators			Percentage of callusing/explant	Mean number of shoot initials	Mean number of shoots
Auxin	Cytokinin				
IAA	BA	KIN			
-	1	-	-	-	8.02±0.2
-	2	-	-	-	9.36±0.5
-	3	-	-	-	10.66±0.1
-	-	1	-	-	9.01±0.5
-	-	2	-	-	20.58±0.9
-	-	3	-	-	29.50±0.1
0.05	1.0	-	-	12.30±0.5	-
0.05	1.5	-	-	14.81±0.2	-
0.05	2.0	-	-	25.36±0.2	-
0.05	2.5	-	-	16.11±0.9	-
0.1	1.0	-	-	10.55±0.4	2.36±0.2
0.1	1.5	-	-	11.75±0.6	5.30±0.6
0.1	2.0	-	-	11.97±0.5	8.89±0.4
0.1	2.5	-	-	12.20±0.6	10.22±0.1
0.3	1.0	-	-	4.01±0.8	-
0.3	1.5	-	-	5.87±0.9	-
0.3	2.0	-	-	5.99±0.5	-
0.3	2.5	-	-	9.08±0.1	-
0.5	1.0	-	70	11.65±0.4	-
0.5	1.5	-	70	15.94±0.8	-
0.5	2.0	-	80	26.31±0.2	-
0.5	2.5	-	90	18.84±0.7	-
0.05	-	1.0	-	15.77±0.3	-
0.05	-	1.5	-	25.35±0.3	-
0.05	-	2.0	-	36.78±0.3	-
0.05	-	2.5	-	50.53±0.7	3.19±0.05
0.1	-	1.0	-	-	11.01±0.6
0.1	-	1.5	-	-	19.98±0.1
0.1	-	2.0	-	-	27.33±0.4
0.1	-	2.5	-	-	32.05±0.4
0.3	-	1.0	70	10.04±0.1	1.77±0.7
0.3	-	1.5	70	17.11±0.1	1.24±0.5
0.3	-	2.0	80	26.05±0.6	3.48±0.5
0.3	-	2.5	90	12.22±0.5	1.50±0.1
0.5	-	1.0	60	2.26±0.9	-
0.5	-	1.5	50	5.05±0.4	-
0.5	-	2.0	50	5.81±0.1	-
0.5	-	2.5	50	2.0±0.3	-

Shoots initiated from the petiolar end after 15 days, the entire leaf surface produced shoot buds (Fig.1). The leaf explants inoculated on MS medium with 0.05mg/IAA and 2mg/l BA produced more than 25 shoot initials from the upper surface of the explants (Fig.2) But in 0.1 mg/l IAA with 2.5 mg/l BA produced only 10 healthy shoots with 3 cm and few shoot initials. Lower concentration of IAA and BA resulted the production of 10-12 small shoot initials from the petiolar end of the explants. At higher concentration of 0.5 mg/l IAA and BA, the entire surface of the leaf explants produced callus with shoot initials.

Figure 1: Shoots initiated from the petiolar end after 15 days

Figure 2: 0.5 mg/l IAA and BA

Figure 3: 0.5 mg/l IAA and 2.5mg/l Kin

The petiolar end and the leaf tip, numerous shoot initials were produced in the medium with 0.1mg/l IAA and 2.5mg/l Kin. After 20 days, numerous shoot initials were produced from the entire surface of the explants and more than 32 shoots obtained per explants after 30 days (Fig 3). MS medium with 0.05 mg/l IAA and 2.5 mg/l Kin produced more than 50 shoot initials with few long shoots. The explants on MS medium with 0.3mg/l IAA and 2mg/l Kin produced brown callus along with 26 shoot initials after 30 days. The explants on MS medium with 3mg/l Kin alone 29 shoots /explants after 30 days (Table 1)

The shoots obtained from various concentration and combinations of auxins and cytokinins were transferred to MS medium augmented with 0.5 mg/l NAA, root initiation was obtained after 14 days, 24 white roots with an average length of 4.5cm were obtained. The plantlets were then washed thoroughly to remove traces of agar. They were transferred to the autoclaved vermiculite in plastic cups and covered with polythene bags for retaining humidity. The plantlets were kept in the culture room and were gradually transferred to the room temperature. After two weeks new leaves emerged, the plantlets were transferred to soil in pots. The plant reached a height of 40 cm with 10-12 leaves after 45 days. After 75 days, the regenerated plant reached a height of 75 cm with new sprouting branches. The plant showed the appearance of the climber similar to the normal plant. The *in vitro* raised plant flowered with in one year. The regenerated plants were successfully maintained in the field with 80% survival. The plants were transplanted to square pits in the field at Department of Botany, Kariavattom, Thiruvanathapuram, India.

For the phytochemical screening of *S. trilobatum* steroid rich fraction on separation by TLC clearly showed that control and in *vitro* shoots showed seven fractions. The R_f values for each fraction was calculated and designated as FI-FVII for the studies (Fig.4) (Table 2).

Table 2: Fractionation of steroids in *Solanum trilobatum*

Fraction	Control shoot (field grown plant)	*In vitro* shoots
F-I	0.090	0.090
F-II	0.151	0.151
F-III	0.243	0.243
FIV	0.315	0.315
F-V	0.455	0.455
F-VI	0.576	0.576
F-VII	0.939	0.939

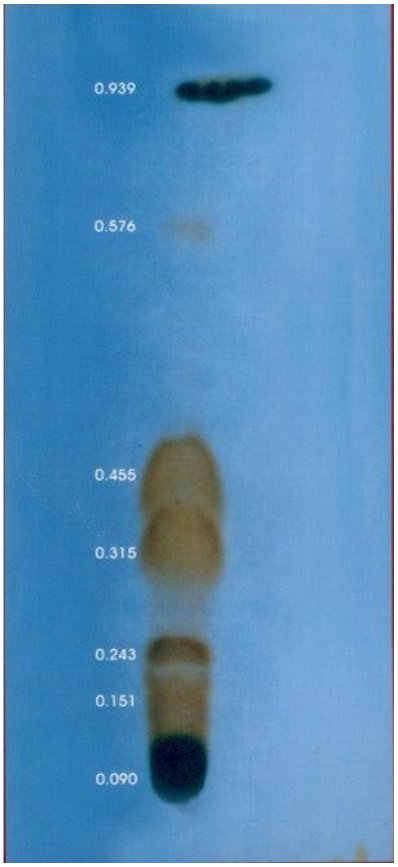

Figure 4

The separated steroids were identified using different spraying agents characteristics of the isolated compound. For the isolation of solasodine the authentic sample of solasodine was co-chromatographed with the sample and R_f value were compared with that of standard after spraying with antimony chloride in chloroform and was identified as dark violet spot appeared with R_f 0.243 and was corresponding to that of standard (Fig 5). The spot was identified as solasodine (fraction III).

Figure 5

For IR spectrum, the isolated fraction after the removal of silica gel by repeated centrifugation, filtration and evaporation and cooled for crystallization appeared as colorless needle like crystals were obtained (Fig.6) Using the standard the sample was identified as solasodine (Fig7&8). Quantification and identification were carried out using Uv-vis spectrophotometer. The spectral analysis revealed λ µg max at 385nm for solasodine which is same as that of standard. The cytotoxic potential of the seven steroidal fractions varied in DLA cells at 40µg/ µl, 60 µg/ µl, 80 µg/ µl and 100 µg/ µl, the cytotoxic potential of solasodine from control plant and *in vitro* plant was calculated and the percentage of cell death of *in vitro* shoots can be compared with the control plant (Table 3).

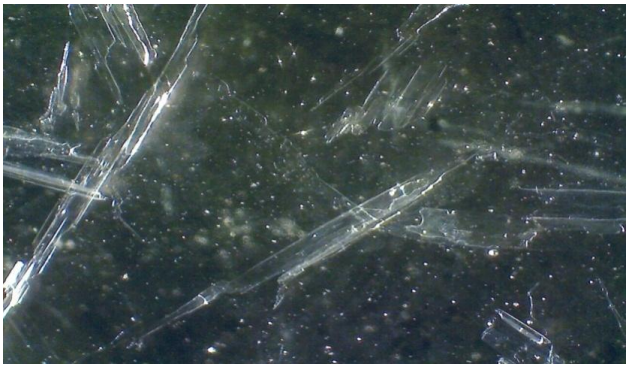

Figure 6: Colourless needle like crystals

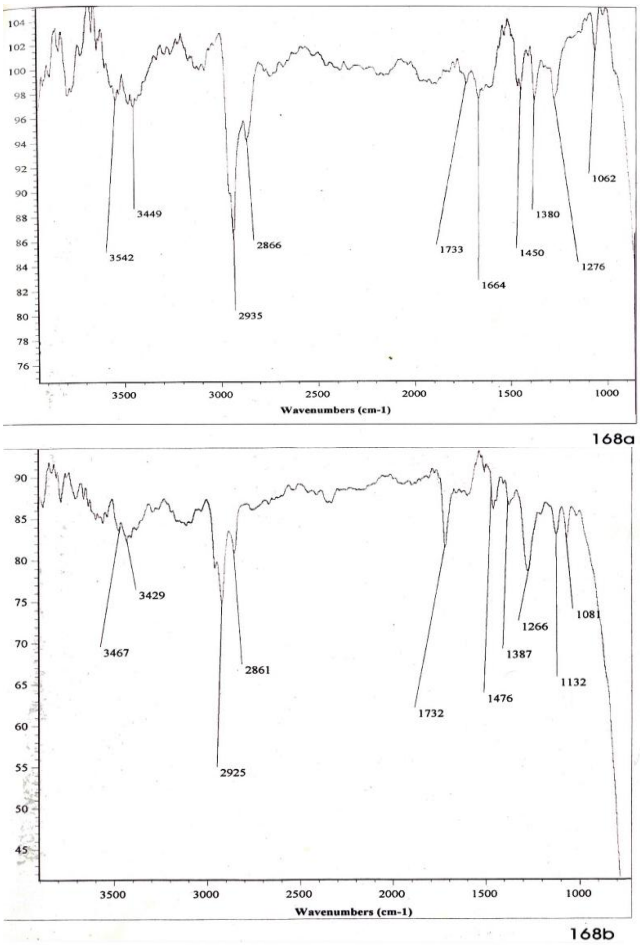

Figure 7 & 8: IR spectrum for standard and the sample

Table 3: Cytotoxicity of solasodine in control plant and *in vitro* plant

Sample	Fraction	Percentage of cell death			
		40	60	80	100
Control shoot	FIII	32.02±0.64	49.36±0.51	67.98±0.48	83.35±0.94
In vitro shoots	FIII	30.35±0.54	44.87±0.09	61.77±0.54	77.79±0.49

Plant cell and tissue culture can provide new means for the economic production of medicinal plants or bioactive chemical that they produce [16]. The concentration of growth regulators and the presence of additives play a key role in the *in vitro* synthesis of secondary metabolites [17]. Plant secondary metabolites are currently the subject of much research interest, but their extraction as part of phytochemical or biological investigations presents specific challenges that must be addressed throughout the solvent extraction process. Detection and analysis of secondary metabolites have important role in understanding the quality and quantity of the medicinal plant and role of tissue culture technology in drug industry. Bioassay is the first necessary step for the drug discovery process. *In vitro* cytotoxicity assays with cultured cells were widely used to test the antitumour activity of the plant extracts [18]. Recent developments in the screening of secondary metabolites and their mode of action and the unraveling of the active principle take a new deviation. The anti-tumor activity of four Ayurvedic herbs viz. *Curcuma longa*, *Ocimum sanctum*, *Tinospora cordifolia* and *Zizyphus mauritiana* were evaluated using Dalton Lymphoma ascites (DLA) tumor model in Swiss Albino mice [19]. *Piper longum*, *Semecarpus anacardium*, and whole plant of *Achyranthus aspera* have been screened for their cytotoxic constituents, fractions of petroleum ether extract showed the significant cytotoxicity [20]. Anti cancer property of *Viburnum foetens* was studied to evaluate potential of plant extract against breast cancer cell line [21]. *S. trilobatum* was found to be an important medicinal plant with seven steroids. In the present study, solasodine was identified with in *vitro* cytotoxic activity on DLA cells. The high *in vitro* response of this species raised the status for further biotechnological manipulations. The solasodine profile of normal plants was also similar to the solasodine to the *in vitro* plants. The solasodine isolated from *in vitro* shoots and their cytotoxic activity was similar to the mature control plant.

4. Conclusions

The present study revealed high *in vitro* response of leaf explants. About 32 plants were regenerated from single explant of *Solanum trilobatum*. Standardization of tissue culture technique provides continuous supply of in *vitro* shoots for the extraction of Solasodine . The solasodine and its cytotoxicity obtained from the *in vitro* shoots can be compared with that of the normal plant. This can be exploited for the use in the pharmaceutical industry. However further studies required for the proper drug development.

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