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Research Article

Quantification of Solasodine in Callus and Suspension Cultures of *Solanum xanthocarpum* Schrad. & Wendl. by RP-HPLC

*Zafar Rasheeduz, Humayun Parisa

Tissue Culture Laboratory, Faculty of Pharmacy, Jamia Hamdard University, New Delhi-110062, India

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ABSTRACT

In the present investigation, an attempt has been made to develop callus and suspension cultures from different organs of the plant and a modified, simple, rapid, accurate, robust and selective HPLC method for the determination of solasodine in natural leaf, stem, root, callus and suspension cultures of leaf, stem and root of the plant to check the possibility of increased content of solasodine in cultures. The callus and suspension cultures of different organs of *Solanum xanthocarpum* were developed using different combinations and concentration of 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), 6-benzyl adenine (6BA) and kinetin. The analytes were separated on ODS Hypersil C-18 column (250 mm × 4.6 mm, 10 µm particle size) using an isocratic elution of methanol – water (65:35) buffered with 20 mM phosphate (pH - 3.5) as the mobile phase with a flow rate of 1 ml min^{-1} and wavelength was absorbed at 205 nm by using U.V. detector. The calibration curve showed good linearity over a range of 0.5 – 500 µg/ml and the mean recoveries ranged from 99.26 to 99.92%. The stem callus developed on MS basal medium supplemented with 2,4-D, IAA, Kinetin and NAA (1.5ppm each) showed the maximum amount of solasodine (0.062% w/v) when compared with natural stem (0.048% w/v) and its suspension cultures (0.027% w/v). The increased amount of solasodine was quantified and validated for good linearity, precision, accuracy and robustness by HPLC.

Keywords: Callus cultures, HPLC, Solasodine, *Solanum xanthocarpum*, suspension cultures.

INTRODUCTION

Solanum xanthocarpum Shrad. and Wendl. belongs to family Solanaceae, in English it is called as yellow berried nightshade and in Hindi as kateli. Its synonymn is *Solanum surratense* Burm.f. (Solanaceae). It is one of the members of the Dasamula (ten roots) of the Ayurveda[1].

It is distributed throughout India as a wild annual herbaceous plant particularly in hilly regions and valleys which comprised of 90 genera and 2000-3000 species[2] and also occurs in Malacca and Ceylon, South East Asia, Malaya, Tropical Australia and Polynesia[3].

The plant is used in cough, bronchial asthma[4], chest pain, leprosy, skin diseases, scabies, in wound healing[5] and cardiac diseases[6]. The plant extract possesses antipyretic, anthelmintic, carminative, stomachic, febrifuge, laxative, rejuvenating and aphrodisiac properties. Stems, flowers and fruits are bitter, carminative. Root decoction used as febrifuge, diuretic and an expectorant[7]. Leaves are used in muscle pain when applied locally and its juice in rheumatism while mixed with black pepper[8]. Dried fruit extract of the species possesses anti-inflammatory activity[9].

The plant *Solanum xanthocarpum* possesses anti-fertility[10], anticancer[11], anti-inflammatory[12], anti-asthmatic[13], anthelmintic[14], anti-allergic[15], anti-diabetic[16], anti-oxidant[17], anti-pyretic and antimicrobial activities[18].

Solasodine is the principal constituent of *Solanum xanthocarpum*, a steroidal glycoalkaloid, an N-analogue of diosgenin and used as the starting material for the synthesis of steroid hormones like corticosteroids, anabolic steroids, etc[19].

From the literature review, it reveals that a few research work reported on pharmacognostic studies on fruits[20] and on tissue culture studies of *Solanum xanthocarpum* which includes roots and shoots differentiation[21], steroids synthesis by hormonal control[22], shoots induction and formation of plantlets from leaf, stem and root[23], callus differentiation[24], establishment of static callus cultures from fruit explants and the maximum biomass of callus was produced by using three different medias, i.e. BW, BMS, BMSKS[25], mesophyll protoplasts from plantlets[26], plant regeneration and organogenesis[27], multiple shoot induction and plantlet establishment[28], micropropagation[29]. However, in the present investigation, the callus and suspension cultures were developed on different organs of the plant by using new hormonal combinations and concentrations which has not been reported earlier and the estimation of solasodine is carried out by a modified and an improved method as compared to the previous one.

In the present study an attempt has been made to develop calli from different parts of the plant and to find out the potential of the cultures for the biosynthesis of Solasodine and determination of its content in natural leaf, stem, root and their different cultures using RP – HPLC.

MATERIALS AND METHODS

**Author for Correspondence*

Fig. 1: Aseptically grown seedlings of *Solanum xanthocarpum* (21 days old)

Fig. 2: Maintained Stem Callus with 2,4-D + Kinetin (1.5ppm each) for 95 days old

Fig. 3: Maintained Root Callus with 2,4-D + Kinetin (1.5ppm each) for 95 days old

Fig. 4: Maintained leaf calli with 2,4-D + Kinetin (1.5ppm each) for 120 days old

Plant materials and chemicals

The plant *Solanum xanthocarpum* was widely present in the forests of Faridabad which was procured and grown in the Herbal Garden of Jamia Hamdard and the authentification of the plant was done from NISCAIR, Pusa Road, New – Delhi. The authentication of the plant species (*Solanum xanthocarpum* Shrad & Wendl.) was done by Chief Scientist, Raw Materials Herbarium and Museum, NISCAIR, Pusa Road, New – Delhi and the voucher specimen of the plant was deposited in Herbarium of NISCAIR (Ref. NISCAIR/RHMD/Consult/-2013-2272/52). All the solvents, i.e. methanol and water were of HPLC grade (Fischer Scientific, Mumbai, India) and other reagents and chemicals were of analytical grade (Merck Limited, Mumbai, India). Solasodine (96% purity) was purchased from MPBio, Solan (India).

Sterilization and Development of Cultures

The immature leaves and viable seeds from the ripe berries were taken for the germination and were subjected to surface sterilization by washing with teepol and then with double distilled water 3-4 times then aseptically treated with different concentrations and different contact times of chemical sterilants like mercuric chloride with concentration (0.05, 0.1%) and contact time (5, 6, 8 and 10 mins.), with different concentrations of ethanol (96, 97%) for contact time (5, 7, 8 and 10 mins.) and sodium hypochlorite with concentration (0.1, 0.02, 0.2%) with contact time (5, 7, 8, 9 mins.).

The explants (leaves, stems and roots of seedlings) were aseptically transferred to a sterile beaker and washed with sterile double distilled water four to five times to remove the traces of chemical sterilants. Under aseptic conditions, the surface sterilized seeds were then transferred into the sterile petri plates containing cotton and filter paper beds and were kept in a B.O.D. Incubator (Yorko, Delhi) in light and dark cycle (16 hrs light and 8 hrs dark) at a temperature of 25 ± 2^{0}C for germination.

The surface sterilized leaves (1mm length) were transferred into the culture tubes containing the MS media[30] supplemented with various combinations and concentrations of the plant hormones which are given below:

A. MS + 2,4-D + IAA (1.5ppm each)
B. MS + 2,4-D + Kinetin (1.5ppm each)

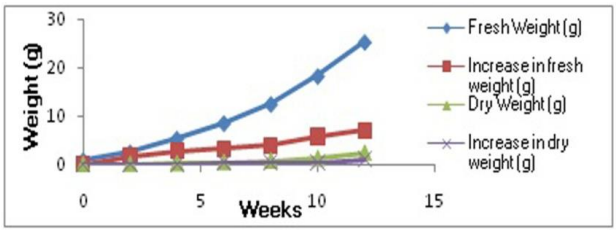

Fig. 5: Growth Kinetics of Leaf callus A (MS + 2,4-D + Kinetin – 1.5ppm each)

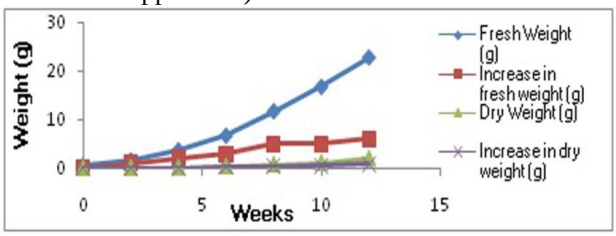

Fig. 6: Growth Kinetics of Stem Callus B (MS + 2,4-D + IAA + Kinetin + NAA- 1.5ppm each)

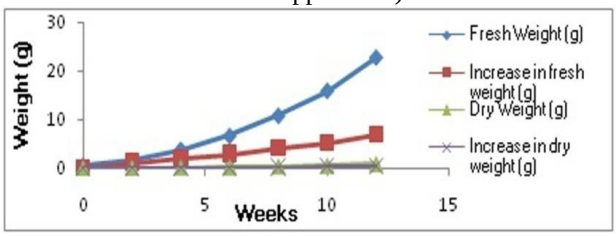

Fig. 7: Growth Kinetics of Root Callus B (MS + 2,4-D + IAA + Kinetin + NAA- 1.5ppm each)

C. MS +2,4-D + NAA (1.5ppm each)
D. MS + IAA + Kinetin (1.5ppm each)
E. MS + 2,4-D + 6BA (1.5ppm each)
F. MS + 2,4-D + IAA + Kinetin + 6BA (1.5ppm each)
G. MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each)
H. MS +2,4-D + Kinetin + NAA
I. MS + NAA + Kinetin + BA
J. MS + 2,4-D + IAA + 6BA (1.5ppm each)
K. MS + 2,4-D + 6BA + NAA(1.5ppm each)
L. MS + IAA + BA (1.5ppm each)
M. MS + 2,4-D (1.5ppm each)
N. MS + Kinetin (1.5ppm each)
O. MS + BA (1.5ppm each)
P. MS + IAA(1.5ppm each)
Q. MS + NAA(1.5ppm each)
R. MS + IAA + NAA(1.5ppm each)
S. MS + NAA + 6BA (1.5ppm each)
T. MS + NAA + Kinetin(1.5ppm each)
U. MS + Kinetin + 6BA(1.5ppm each)
V. MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each)

Similarly, the seedlings of the germinated seeds were transferred to the MS medium in culture tubes with sterile forceps supplemented with various combinations and concentrations as given above.

After the initiation of callus on leaf explant in 21-23 days, they were aseptically separated from the mother explants and the calli initiated on stem and roots of seedlings in 27-29 days and in order to develop an independent calli, they were transferred to the same medium on which they were initiated. The subculturing was regularly done at an interval of 3 weeks.

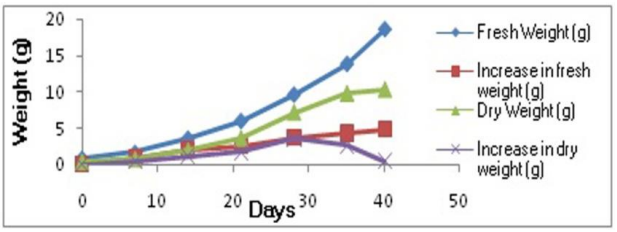

Fig. 8: Growth Kinetics of Leaf Suspension B (MS + 2,4-D + IAA + Kinetin + NAA- 1.5ppm each)

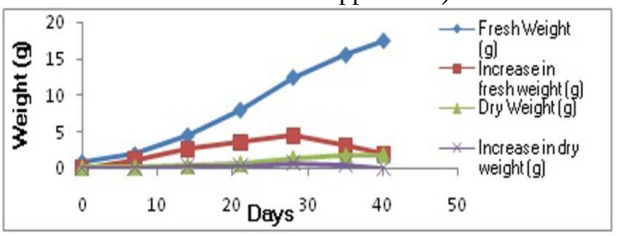

Fig. 9: Growth Kinetics of Stem Suspension B (MS + 2,4-D + IAA + Kinetin + NAA- 1.5ppm each)

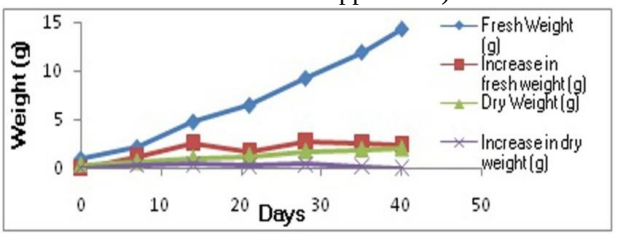

Fig. 10: Growth Kinetics of Root Suspension B (MS + 2,4-D + IAA + Kinetin + NAA- 1.5ppm each)

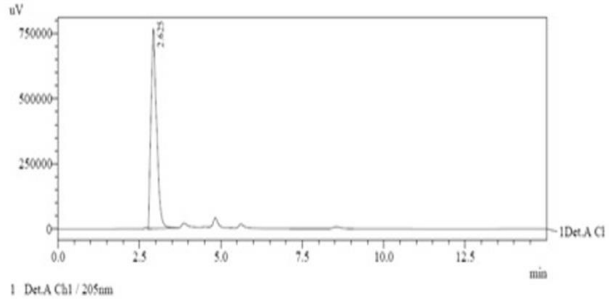

Fig. 11: HPLC Chromatogram of Standard compound (Solasodine) at 205 nm

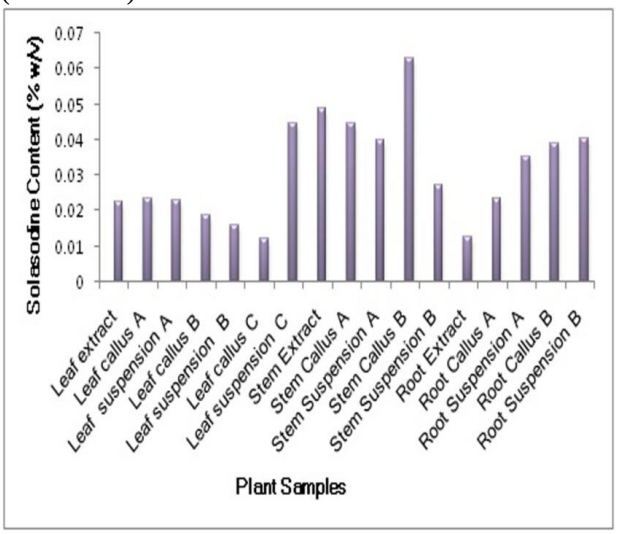

Fig. 12: Graphical representation of Solasodine in different samples

The developed calli were chopped down and transferred

Table 1: Qualitative chemical tests for the presence/absence of various phytoconstituents

Class of compounds	Leaf	Root	Fruit	Stem	Leaf Callus A	Leaf Callus B	Leaf Callus C	Root Callus A	Stem Callus A	Root Callus B	Stem Callus B
Alkaloids	+	+	+	+	+	+	+	+	+	+	+
Amino -acids	-	-	+	+	-	-	-	-	+	-	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+	+	+
Phenolics	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+	+	+	+
Coumarins	+	+	+	+	+	+	+	+	+	+	+
Tannins	-	+	-	+	-	-	-	+	+	+	+
Saponins	-	-	-	-	-	-	-	-	-	-	-
Mucilage	-	-	-	-	-	-	-	-	-	-	-
Proteins	-	-	+	+	-	-	-	-	+	-	+
Anthraquinone glycosides	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	-	-	-	+	-	-	-	-	+	-	+
Terpenoids	+	+	+	-	+	+	+	+	-	+	-

Keys: (+): Present and (-): Absent
Leaf callus A: Callus developed on MS + 2,4-D + Kinetin (1.5ppm each),
Leaf callus B: Callus developed on MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each)
Leaf callus C: Callus developed on MS + 2,4-D + NAA (1.5ppm each)
Root Callus A: Callus developed on MS + 2,4-D + NAA (1.5ppm each)
Root Callus B: Callus developed on MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each)
Stem Callus A: Callus developed on MS + 2,4-D + NAA (1.5ppm each)
Stem Callus B: Callus developed on MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each)

aseptically in MS medium supplemented with the similar hormonal combinations which showed best results in the previous experiment. The sub-culturing during the maintenance was routinely done after every three weeks.

For the establishment of suspension cultures, the fresh friable callus (3g) of leaf, stem and root (1-3 weeks old) were used for the initiation of suspension cultures by inoculating into 250 ml Erlenmeyer flask containing 60 ml of MS liquid media supplemented with growth hormones having same concentration and combinations as they were used in case of callus cultures with 3% sucrose. The flasks were agitated at 100 rpm on the Rotary Shaker (Yorko, Delhi) at 25 ± 2^{0}C maintaining the photoperiod (16/8hr light-dark cycle) with a relative humidity of 70%. The cultures were harvested after six weeks of growth by filtering culture through filter papers; the media were separately pooled, frozen and lyophilized.

The growth kinetics was studied by determining the increase in fresh weight and dry weight after every three weeks for callus cultures and for suspension cultures after every seven days.

In order to find out the presence/absence of different types of primary and secondary metabolites, general chemical tests were carried out as reported by Zafar et al[31]. The extracts of natural leaf, stem and berries, leaf callus A, B, C, root callus A, B, stem callus A and B were tested for the detection of different metabolites (Table 1).

Analysis of Solasodine by RP-HPLC method

Quantitative Analysis of Solasodine is done by a reported method of Kittipongpatana N et al[32] with modification.

Preparation of mobile phase

The mobile phase was prepared by mixing Methanol and Water buffered with 20 mM phosphate buffer and 0.5% OPA was added in the ratio of 65:35 (pH – 3.5). The mobile phase was degassed by sonication and filtered through vacuum filtration assembly (0.45 µm membrane filter) just before the HPLC analysis.

Preparation of Stock and Standard Dilution

Standard stock solution of Solasodine (100 µg/ml) was prepared as follows:

3 mg of standard was weighed accurately and dissolved in 30 ml of methanol (HPLC grade) and the solution was stored at a temperature of 4 ^{0}C protected from light. Working standard solutions were obtained freshly by diluting the stock solutions in methanol during analysis. The dilutions were prepared by serial dilution method.

Preparation of Samples

1 gm of each of powdered natural leaf, stem, root and their calli and suspension cultures (Leaf A and B, stem A and B, root A and B) dried at 60 ^{0}C were extracted with 25 ml of methanol separately, filtered and filtrate were concentrated and they were subjected to hydrolysis with 1 M HCl under reflux for three hours so as to remove the sugar residues. The hydrolysed material was cooled and the residue was dissolved in methanol and final volume made upto 10 ml and filtered through 0.2 µm membrane filter (Gelman Science, India). The samples were analyzed by HPLC for quantitative estimation of Solasodine by Shimadzu model HPLC equipped with quaternary LC-2010 CHT, O.D.S. Hypersil C_{18} Column (250 mm × 4.6 mm, 10 µm particle size), variable wavelength programmable UV/VIS detector and built-in L.C. solution software was used for routine drug analysis.

Table 2: Concentration of Solasodine in each sample and corresponding peak area

S.NO.	Sample Name	Peak Area	Conc. of Solasodine (mg/ml)	%w/v of Solasodine
1	Leaf extract	2143658	2.278	0.0227
2	Leaf callus A	2225834	2.366	0.0236
3	Leaf suspension A	2152647	2.288	0.0228
4	Leaf callus B	1780713	1.893	0.0189
5	Leaf suspension B	1513854	1.609	0.0160
6	Leaf callus C	1150175	1.223	0.0122
7	Leaf suspension C	4180003	4.443	0.0444
8	Stem Extract	4582493	4.871	0.0487
9	Stem Callus A	4196808	4.460	0.0446
10	Stem Suspension A	3738620	3.974	0.0397
11	Stem Callus B	5923601	6.296	0.0629
12	Stem Suspension B	2584096	2.746	0.0274
13	Root Extract	1186914	1.262	0.0126
14	Root Callus A	2197015	2.335	0.0233
15	Root Suspension A	3328871	3.538	0.0353
16	Root Callus B	3662223	3.893	0.0389
17	Root Suspension B	3790730	4.029	0.0402

Keys: Leaf callus A, Leaf Suspension A: Callus and Suspension culture developed on MS + 2,4-D + Kinetin (1.5ppm each), Leaf callus B, Leaf Suspension B: Callus and Suspension culture developed on MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each)
Leaf callus C, Leaf Suspension C: Callus and Suspension culture developed on MS + 2,4-D + NAA (1.5ppm each)
Root Callus A: Callus developed on MS + 2,4-D + Kinetin (1.5ppm each)
Root Suspension A: Suspension culture developed on MS + 2,4-D + NAA (1.5ppm each)
Root Callus B, Root Suspension B: Callus and Suspension culture developed on MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each), Stem Callus A: Callus developed on MS + 2,4-D + Kinetin (1.5ppm each)
Stem Suspension A: Callus and Suspension culture developed on MS + 2,4-D + NAA (1.5ppm each)
Stem Callus B, Stem Suspension B: Callus and Suspension culture developed on MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each)

Table 3: Intra and Inter-day Precision

Conc. of Solasodine (µg/ml)		Mean Area	SD	%RSD
Intra-day	5 µg/ml	107968.7	574.48	0.532
	10 µg/ml	218287.7	1583.84	0.725
	50 µg/ml	925257.6	2990.59	0.323
	100 µg/ml	1818045.0	863.21	0.047
	200 µg/ml	3647601.3	14847.57	0.407
	500 µg/ml	9218949.3	11652.35	0.126
Inter-day	5 µg/ml	108685.7	1095.882	1.008
	10 µg/ml	218606.3	698.04	0.319
	50 µg/ml	928866.3	1216.16	0.130
	100 µg/ml	1815570.0	3269.04	0.180
	200 µg/ml	3660743.6	16099.14	0.439
	500 µg/ml	9221521.6	27828.34	0.301

Abbreviations: SD: Standard Deviation, RSD: Relative Standard Deviation

Method Validation

In method validation, the parameters performed were linearity, precision, accuracy, recovery, robustness and limit of sensitivities as per the ICH guidelines[33].

RESULTS AND DISCUSSION

In the present research work, the seeds from the riped berries of *Solanum xanthocarpum* were taken and kept for germination. The seed germination done in the month of May and July. The chemical sterilant; sodium hypochlorite (0.1% concentration) showed 90% germination with 7 minutes contact time was found to be favourable than the other two sterilants (mercuric chloride and ethanol). The seeds were germinated within 21 days (Figure 1).

Callus initiation and maintenance from seedling

Table 4: Recovery Studies

Sample	Initial Conc. of the drug in sample (mg/ml)	Conc. of standard added after dilution (mg/ml)	Amount Recovered (mg/ml)	% Recovery	Mean Recovery (%)	RSD (%)
Leaf Callus (2,4-D + Kinetin)	2.366	0.05	2.394	99.08		
	2.366	0.10	2.459	99.72	99.49	0.359
	2.366	0.15	2.508	99.68		
Leaf Suspension (2,4-D + Kinetin)	2.288	0.05	2.335	99.87		
	2.288	0.10	2.380	99.66	99.77	0.106
	2.288	0.15	2.433	99.79		
Stem Callus (2,4-D + Kinetin)	4.460	0.05	4.490	99.55		
	4.460	0.10	4.549	99.76	99.76	0.21
	4.460	0.15	4.609	99.97		
Stem Suspension (2,4-D + NAA)	3.974	0.05	4.021	99.93		
	3.974	0.10	4.072	99.95	99.91	0.042
	3.974	0.15	4.119	99.87		
Root Callus (2,4-D + Kinetin)	2.335	0.05	2.369	99.32		
	2.335	0.10	2.427	99.67	99.58	0.229
	2.335	0.15	2.479	99.75		
Root Suspension (2,4-D + NAA)	3.538	0.05	3.568	99.44		
	3.538	0.10	3.615	99.36	99.51	0.195
	3.538	0.15	3.678	99.73		
Leaf Callus (2,4-D + IAA + Kinetin + NAA)	1.893	0.05	1.925	99.07		
	1.893	0.10	1.979	99.29	99.35	0.327
	1.893	0.15	2.037	99.71		
Leaf Suspension (2,4-D + IAA + Kinetin + NAA)	1.609	0.05	1.646	99.21		
	1.609	0.10	1.707	99.88	99.64	0.374
	1.609	0.15	1.756	99.83		
Root Callus (2,4-D + IAA + Kinetin + NAA)	3.893	0.05	3.935	99.79		
	3.893	0.10	3.979	99.65	99.68	0.095
	3.893	0.15	4.027	99.61		
Root Suspension (2,4-D + IAA + Kinetin + NAA)	4.029	0.05	4.053	99.36		
	4.029	0.10	4.119	99.75	99.53	0.198
	4.029	0.15	4.158	99.49		
Stem Callus (2,4-D + IAA + Kinetin + NAA)	6.296	0.05	6.321	99.60		
	6.296	0.10	6.354	99.34	99.55	0.198
	6.296	0.15	6.429	99.73		
Stem Suspension (2,4-D + IAA + Kinetin + NAA)	2.746	0.05	2.778	99.35		
	2.746	0.10	2.838	99.72	99.55	0.187
	2.746	0.15	2.884	99.58		
Leaf Callus (2,4-D + NAA)	1.223	0.05	1.271	99.84		
	1.223	0.10	1.319	99.69	99.62	0.257
	1.223	0.15	1.364	99.34		
Leaf Suspension (2,4-D + NAA)	4.443	0.05	4.479	99.68		
	4.443	0.10	4.539	99.91	99.74	0.149
	4.443	0.15	4.576	99.63		

The germinated seedlings were transferred to the MS media supplemented with the different combinations and concentrations of growth hormones in the culture tubes as mentioned in experimental part. However, only three combinations, i.e. MS + 2,4-D, + Kinetin, MS + 2,4-D + NAA and MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each) showed initiation of callus on stem of seedling as well as on roots. The result indicated that different types of auxins play important role in the initiation of callus on stem and root while; the cytokinin requirement was also significant in the concentration of 1.5ppm.

Later, the calli were transferred to the MS medium independently in the same hormonal combinations and concentrations in which they were initiated to study the growth kinetics.

The growth of the callus was satisfactory on MS + 2,4-D + Kinetin (1.5ppm each), MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each) and was not satisfactory on MS + 2,4-D + NAA (1.5ppm each). The developed calli showed vigorous growth on MS + 2,4-D + Kinetin (1.5ppm each) and MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each) on stem and root of seedling. The different calli after development were maintained upto 95 days. It was observed that the callus maintained on MS + 2.4-D + Kinetin (1.5ppm each) showed light brown coloured growth which turned to dark brown in colour which later

Table 5: Robustness

Conc. of Solasodine	Variable	Retention Time (%RSD)	Peak Area (%RSD)
50 µg/ml	Mobile Phase Composition	0.402	0.237
	Wavelength	0.497	0.355
	Flow Rate	0.418	0.362
100 µg/ml	Mobile Phase Composition	1.39	0.135
	Wavelength	0.696	0.196
	Flow Rate	0.953	0.115
200 µg/ml	Mobile Phase Composition	0.775	0.598
	Wavelength	0.666	0.322
	Flow Rate	0.566	0.501

RSD: Relative Standard Deviation

on turned to light and dark brown coloured on stem and root respectively [Figure 2 and Figure 3], whereas the callus on MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each) showed rapid growth on root and stem, the colour turned to cream and light brown respectively which later changed to light brown in root callus and stem callus changed to dark brown coloured. On the same hormonal combination as it was initiated which further confirmed that a combination of three types of auxins (1.5ppm each) alongwith kinetin is not only essential for initiation of callus but also for maintenance where, it showed a rapid growth on stem and root callus.

Callus initiation and maintenance from leaves

The surface sterilization of leaves was obtained with 0.1% of mercuric chloride at 7 minutes contact time which showed no contamination and no browning, whereas the sodium hypochlorite showed contamination in the explants (leaves) with both the concentrations.

MS medium supplemented with all the different hormone combinations and concentrations as mentioned above in materials and methods showed no initiation of callus except MS + 2,4-D + Kinetin (1ppm each), MS + 2,4-D + NAA (1ppm each), MS + 2,4-D + IAA + Kinetin (1ppm each), MS + 2,4-D + IAA + Kinetin + 6BA (1ppm each), MS + 2,4-D + IAA + Kinetin + NAA (1ppm each) but not satisfactory and later no growth was observed.

Then the concentration of the hormones were increased in MS medium supplemented with the different hormone combinations such as MS + 2,4-D + IAA (1.5ppm), MS + 2,4-D + Kinetin (1.5ppm each), MS + 2,4-D + NAA (1.5ppm each), MS + 2,4-D + IAA + Kinetin (1.5ppm each), MS + 2,4-D + IAA + Kinetin + 6BA (1.5ppm each) and MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each). With all the mentioned hormonal combinations, excellent initiation of callus was observed except one hormone combination, i.e. MS + 2,4-D + IAA (1.5ppm each). The independent calli were developed on the same hormonal combinations and vigorous growth was observed with three hormone combinations; MS + 2,4-D + Kinetin (1.5ppm each), MS + 2,4-D + NAA (1.5ppm each) and MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each) which were maintained further upto 120 days. It was observed that the callus maintained on MS + 2,4-D + Kinetin (1.5ppm each) showed dark brown coloured mass of cells which later turned to white and light brown in colour (Figure 4).

The callus on MS + 2,4-D + NAA (1.5ppm each) showed increase in mass which turns to light brown coloured mass from greenish yellow coloured friable and soft callus. While the callus on MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each) in the 5^{th} and 6^{th} passage of subculture shows light brown coloured compact mass of cells. The growth kinetics studies of leaf callus (Figure 5), stem (Figure 6) and root (Figure 7) were done for 12 weeks.

Thus, it may be stated that the initiation of callus was obtained with the auxin and kinetin concentration (1ppm) which is reduced concentration as in callus initiation. However, for maintenance when only 1.5ppm of each hormone was used, it showed a profused growth of callus on leaf.

In Suspension cultures, all the above combinations were tried as done in the callus cultures. The three hormone combinations showed initiation i.e. MS + 2,4-D + Kinetin (1.5ppm each), MS + 2,4-D + NAA (1.5ppm each) and MS + 2,4-D + IAA + Kinetin + NAA (1.5ppm each) and the satisfactory growth was observed in all these combinations. The suspension cultures were maintained upto 42 days and the growth studies of leaf (Figure 8), stem (Figure 9) and root suspension (Figure 10) were carried out for 6 weeks.

Qualitative analysis was done for the purpose of detecting the presence/absence of various plant constituents by performing the chemical tests in natural leaf, root, fruit and stem and different calli samples, i.e. leaf callus, root callus and stem callus of different hormonal combinations. It was found that the natural leaf and leaf callus contains alkaloids, flavonoids, carbohydrates, phenolics, steroids, coumarins and terpenoids. The natural root and root callus showed the presence of alkaloids, flavonoids, carbohydrates, phenolics, steroids, coumarins, tannins and terpenoids. While the natural stem and stem callus extract contains alkaloids, amino – acids, flavonoids, carbohydrates, phenolics, steroids, coumarins, tannins, proteins and cardiac glycosides, whereas the natural fruit showed the presence of alkaloids, amino – acids, flavonoids, carbohydrates, phenolics, steroids, coumarins, proteins and terpenoids which are presented in (Table 1).

Quantitative analysis of Solasodine carried out by RP – HPLC method. For the quantification of different explants and their callus extract samples, this method was employed as reported by Kittipongpatana N et al. with modification. They have already reported a HPLC method for the

quantification of solasodine in *Solanum aviculare*. This method was used for quantification of solasodine but the results obtained, i.e. retention time and the peaks were not symmetrical. This was probably due to the instrument in which Kittipongpatana N et al have used Milton Roy HPLC system (Riviera, FL, USA) consisting of a Constametric 3000 series isocratic pump, a Rheodyne injector (Rheodyne L.P., Cotati, CA, USA) and a Spectromonitor 3100 variable wavelength UV/VIS detector in which they tried with Econosil C-18, Alltima C-8 and C-18 columns (250 × 4.6 mm i.d.; 5 µm particle diameter; 100 Å average pore size). In the present investigation, Shimadzu HPLC model equipped with quaternary LC-2010 CHT, O.D.S. Hypersil C-18 column (250 × 4.6 mm i.d.; 10 µm particle diameter) and variable wavelength programmable UV/VIS detector was used and retention time and peaks were obtained with C-18 column while, the concentration of the mobile phase was also different which was 65:35 while, in the earlier report, it was 70:30. The chromatogram of standard Solasodine showed the retention time of 2.625 mins. which is less than the reported one. The chromatographic peaks of standard and different samples were sharp and uniform with non-significant variations in retention time (Figure 11). Overall, the maximum amount of solasodine was found to be present in stem callus B (0.0629%w/v) followed by natural stem extract (0.0487%w/v) and then stem callus A (0.0446%w/v) (Table 2). The results obtained are also shown as bar graph (Figure 12).

As the method developed was different from the earlier one, the validation of the procedure was carried out as per the ICH guidelines and is discussed below:

Linearity and Sensitivities

The calibration curve was plotted by injecting six different concentrations of standard compound, i.e. 5-500 µg/ml of Solasodine. The linear graph was obtained with coefficient correlation, i.e. $R^2 = 0.999$. The linearity was evaluated by calculating the slope, y intercepts and correlation coefficient (r) using a least squares regression equation. A signal-to-noise ratio of 3 and 10 is considered as acceptable for determining limit of detection (LOD) and quantitation (LOQ). The results obtained for LOD and LOQ for the solasodine were found to be 0.5 µg/ml and 1 µg/ml respectively which renders the proposed method sensitive for the quantification of Solasodine.

Precision

Intra-day and inter-day precision of six different concentrations (5, 10, 50, 100, 200 and 500 µg/ml) was done. It was observed in the range of 0.047 - 0.725% and 0.130 - 1.008% for intra and inter-day respectively, which is expressed in terms of %RSD and it exhibits the good precision of the method (< 2%). The results are depicted in (Table 3).

Recovery

The samples were spiked with the three different concentration levels of the standard solution, i.e. low, medium and high concentration and were analyzed in triplicate. The average recoveries of all the samples were found acceptable and was in the range of 99.35% to 99.91% and all the RSD values were in the range (<3%) which indicates the good accuracy of the method and are presented in (Table 4).

Robustness

The parameters which were changed for the determination of robustness were mobile phase composition (67:33), flow rate (1.02 ml/min) and wavelength (210 nm). The relative standard deviations (%RSD) of peak area and retention time of Solasodine were calculated for each parameter and were found to be less than 2 (%RSD>2) which confirmed the robustness of the method given in (Table 5).

CONCLUSION

In the present investigation, an attempt was made to develop the biomass for the production of Solasodine *in vitro* conditions, its quantification and validation with good linearity, precision, accuracy and robustness is reported for the first time by RP-HPLC method in this plant. The isocratic method is simple, rapid, easy, robust and reproducible and is an improved method as reported earlier by Kittipongpatana N et al. However, further work to increase the content of Solasodine in callus as well as in suspension cultures is under progress using abiotic, biotic elicitors and by genetic transformation using *Agrobacterium tumourogenesis*.

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