Production of therapeutically important coumarin-scopoletin from suspension cultures of *Abutilon indicum*

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Abstract
Abutilon indicum (L.) (Indian mallow) belonging to the family Malvaceae is native to tropical and subtropical regions. The important phytochemicals of A.indicum include coumarins viz. scopoletin, scoparone, abutilin-A, saponins and flavonoids known for their pharmacological activities like demulcent, aphrodisiac, laxative, diuretic, sedative, astringent, expectorant, tonic, anti- inflammatory, anthelmintic, analgesic and antiulcer agent. In the present study, callus was initiated from the leaf explants of A. indicum in-vitro grown plantlets which were further used to initiate cell suspensions in MS liquid media supplemented with 2,4-D and kinetin at concentrations 2 mg.L^{-1} and 1 mg.L^{-} respectively at an aeration of 120rpm. Growth kinetic studies revealed maximum biomass accumulation on the 9th day of culture, while scopoletin production reached its peak on the 12th day.

Sucrose concentration played a vital role in enhancing biomass accumulation with 4% sucrose taking a higher edge in comparison to 3% sucrose. The HPLC analysis of A. indicum suspensions at different stages of growth curve has revealed higher accumulation of scopoletin (13.86-fold) during stationary phase.

Keywords: *Abutilon indicum*, Callus, Suspensions, Coumarins.

Introduction

The *Abutilon* genus of *Malvaceae* family consists of about 150 annual or perennial herbs, shrubs and small trees; widely distributed in the tropical and subtropical regions of America, Africa, Asia and Australia.[26] *Abutilon indicum* commonly known as Thuthi/Atibala, is a woody shrub[8] reputed to be used for remedial purposes in Siddha system of medicine against piles, jaundice, leprosy and ulcer.[30] The leaf juice mixed with jaggery is used as antidote for snakebite.[19]

The seeds are used to treat puerperal disease, urinary disorders, chronic dysentery and fever.[9,13,28] The root and bark are used as diuretic[5] and anthelmintic. The fruit has been used to treat piles, gonorrhoea and cough.[6,23]

A. indicum, a rich source of the pharmaceutically important coumarin-scopoletin has been selected for *in vitro* and cell culture establishment in order to facilitate manipulation for enhanced secondary metabolite production.

The valuable secondary metabolite production using plant cell or organ cultures seems to be a reliable biotechnological procedure, when compared to the extraction from entire plant material. This process can be carried out by selecting superior cell lines of stable nature and thorough understanding of secondary metabolite synthesis pattern. Though, phytochemical production from plant cell cultures has been attempted earlier, issues regarding slow growth rate and low productivity are major concerns[10].

It is convenient to isolate secondary metabolites in polymeric form from plant cell cultures when compared to intact plant tissues.[1,16,22] The number of secondary metabolites present in plant cell cultures can be further enhanced by various tissue culture techniques like *in vitro* establishment genetic transformations, elicitation etc.

In the present study, the production of coumarin-scopoletin was carried out using suspension cultures of *A. indicum*.

Material and Methods

Materials: *A. indicum* seeds (Rajadhani Agro farms), MS media (Himedia), phytohormones (Duchefa Biochemie, Netherlands), Scopoletin (Sigma) and HPLC-grade chemicals (Hi media).

Establishment of *in vitro* cultures: The seeds of *A. indicum* have been thoroughly washed before surface sterilization with 0.1 % mercuric chloride for 5 min and then finally washed with sterile distilled water (8–10 rinses). They were then transferred to semisolid MS media supplemented with gibberellic acid (GA3; 1 mg.L^{-1}). The obtained seedlings were sub-cultured on MS basal media for *in vitro* establishment.

Callus induction: Different explants (leaf, stalk, node and root) obtained from *in vitro* grown plantlets of *A. indicum* were cultured on MS media supplemented with varying concentrations (0.125–1.00 mg. L^{-1}) of cytokinins [kinetin (Kn), zeatin (Zn), thidiazuron (TDZ), benzyl aminopurine (BAP)] and auxins [2,4-Dichloro phenoxy acetic acid (2,4-D), Indole acetic acid (IAA), naphthalene acetic acid (NAA)]; for callus induction. The cultures have been incubated under standard culture conditions [25 ± 2 °C; 16/ 8 h (light/dark) regime with 40–50 µmol m^{-2} s^{-1} light].

Initiation of Suspensions: Friable callus was transferred to MS liquid media supplemented with 2,4–D and Kn with 4% sucrose concentration before incubation on an orbital shaker at 120rpm under standard culture conditions. The suspension cultures of *A. indicum* were allowed to grow for acclimatization (10 days) in order to obtain uniform growth.

Growth studies: The uniform suspension of *A. indicum* was used to study the growth pattern. A cell mass of 3 g was transferred to 100 ml flasks containing 30 ml of MS liquid media with same media composition. The increase in biomass was assessed along with the coumarin content by harvesting the suspensions every 3 days, until the culture reaches decline phase. The biomass and scopoletin content in the suspensions were expressed as gram fresh weight (gFW) and microgram per gram fresh weight (µg.gFW^{-1}) respectively.

Quantification of Coumarins

Extraction: The scopoletin was dissolved in HPLC (high performance liquid chromatography) grade methanol (Himedia) at a concentration of 1 mg ml.$^{-1}$ The samples (gFW) were macerated into a fine suspension in HPLC grade methanol and filtered using a 0.22 µm membrane filter.

HPLC: The HPLC system (Shimadzu—LC10AT VP series) was equipped with a supelco column (250 × 4.6 mm, C18, ODS with particle size of 5 µm) and a UV–Vis detector with a flow rate of 1 ml.min.$^{-1}$ Scopoletin was detected at 300 nm wavelength around 4.6 min using a mobile phase of methanol and water (55:45) along with 0.1 % acetic acid.[25]

Statistical Analysis: Results calculated from the triplicate data were expressed as mean ± standard deviation. The obtained data were compared by the least significant difference test ($P \leq 0.05$) by using statistical analysis system (SAS, ver. 9.1).

Results and Discussion

***In vitro* establishment:** *A. indicum* seeds after surface sterilization with 0.1% mercuric chloride and 48 h soaking in water were transferred to MS media with GA3 (1 mg.L^{-1}) for germination. GA3 being a natural regulator in seed germination processes stimulates the production of hydrolytic enzyme to break the seed coat barrier.[24,29] The seeds of *A. indicum* are equipped with a thick and tough seed coat wherein GA3 present in the culture media stimulates the seed germination necessary for the establishment and maintenance of cultures.[4] The seeds germinated after two weeks of incubation period (Fig.1) were further cultured on MS basal media for maintenance.

Initiation of callus: The plantlets were grown in MS basal media until they reached a length of two internodes approximately. The explants like leaf, stalk and node were cultured on MS media supplemented with different plant growth regulators for initiation of callus (Table 1). The growth of callus improved, when leaf explants were cultured on MS media with 2,4-D and Kn at concentrations of 2 mg. L^{-1} and 1 mg.L^{-1} respectively (Fig. 2). The quick response of leaf explants for callus induction in comparison to nodal segments has been reported earlier in *A. indicum*[12] and other plant species.[17]

The induction and propagation of callus cultures using 2,4-D has been reported in various plant species *viz. A. galangal,*[15] *A. paniculate,*[27] *G. sylvestre*[2] etc. Inclusion of kinetin along with 2,4-D for successful callus propagation was reported in other plant species.[3,11] The callus subculture was carried out every 15 days on the same media combination.

Cell suspensions: The suspension cultures of *A. indicum* were initiated by transferring 3week old friable callus into MS liquid media having 2,4- D (2 mg.L^{-1}) and kinetin (1 mg.L^{-1}) with 4% sucrose which was similar to the media employed for callus induction. The effect of sucrose for enhanced callus growth and shoot formation was reported earlier in citrus hybrids[7] comparable to the results obtained in the present study.

Fig. 1: *In vitro* established *A. indicum* plantlets on MS basal media

Table 1
Initiation of callus from *A. indicum* leaf explants on MS media augmented with different phytohormones

Phytohormones (mg.L^{-1})		Response*
2,4-D	**Kn**	
0.25	--	--
0.50	--	--
1.00	--	+
1.00	0.25	+
1.00	0.50	++
1.00	1.00	+++
2.00	1.00	++++

+ poor; ++ moderate; +++good; ++++very good.
*****response recorded after 4 weeks of culture.

Fig. 2: *A. indicum* Callus initiation and propagation on MS media fortified with 2,4-D (2 mg.L^{-1}) and Kn (1 mg.L^{-1})

The callus was initially transferred to MS liquid media with the same combination of phytohormones in order to obtain synchronous cultures along with acclimatization of the cells in liquid media conditions. These cultures were grown for about 10 days at 120rpm and 25±2^{0}C under 16/8 h light/dark conditions. These suspensions were then filtered in aseptic conditions to obtain single synchronous cells. Suspension culture cells are suitable to operate as a system, as large numbers of cells growing in an aqueous environment under defined conditions are exposed to synchronized treatments .[28]

Growth Kinetics: In order to evaluate the growth kinetics of *A. indicum* in suspensions, 3 g (10% wv^{-1} inoculum) of isochronous cells were transferred to 30 ml of MS liquid media with same combination of phytohormones. The cultures were maintained in an orbital shaker at standard culture conditions. The experiments were conducted in batch mode in 100 ml conical flasks (Fig. 3).

The growth pattern of *A. indicum* suspension cultures were evaluated by harvesting the cultures at regular interval of 3 days, till the cultures reached the decline phase i.e. for a period of 27 days.

The biomass was calculated after the deletion of excess moisture and expressed as gram fresh weight (gFW). The doubling of cells was observed during the log phase, while the stationary phase was observed from 9th day to 21st day, after which there is a decline phase (Fig. 4). Maximum biomass of 18.35 gFW was observed at the end of log phase i.e. on 9th day of culture.

Scopoletin quantification: Biomass (1g) was ground in 10 ml methanol using mortar and pestle to obtain uniform homogenate. Then the solution was filtered with Whatmann filter paper to obtain a clear solution and the cell debris was discarded. The filtrate was dried and dissolved in 1 ml HPLC grade methanol and filtered through 0.22 µM syringe filters before injecting into HPLC. The data obtained with the sample was analysed by comparing the peak area to that of the standard scopoletin (Fig. 5).

Scopoletin was detected at 300 nm wavelength around 4.6 min using a mobile phase of methanol and water (55:45) along with 0.1 % acetic acid.[21] The content of scopoletin in suspension cultures of *A. indicum* was analyzed parallelly along with the growth curve and expressed as µg.gFW.$^{-1}$ The scopoletin was found to be at its peak i.e. 65.01 µg.gFW^{-1} on the 12th day. The maximum production of scopoletin on the 12th day was found to be 13.86-fold higher when compared to the initial day (4.69 µg.gFW^{-1}) content (Fig. 6).

The accumulation of secondary metabolites in other species was found to be maximum during the stationary phase of culture i.e. ajmalicine and serpentine in *Catharanthus roseus*[16,18].

The 0.65-fold lesser amount of scopoletin accumulation in suspensions of *A. indicum* in comparison to the previous report of 99.20 µg.gFW^{-1} in embryogenic calli[14] is more than compensated by the reduction in time period from 6 weeks to less than 2 weeks.

Conclusion

Scopoletin is an important secondary metabolite found in the plant *A. indicum* attributed with many pharmacological properties like antioxidant, anti-inflammatory, anti-bacterial and neuroprotection. The suspensions offer a unique advantage for continuous production of phytochemicals which can be further manipulated for enhanced accumulation using techniques like elicitation.

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Fig. 3: Cell suspensions of *A. indicum* in shake flasks containing MS liquid media fortified with 2,4-D (2 mg.L^{-1}) and Kn (1 mg.L^{-1})

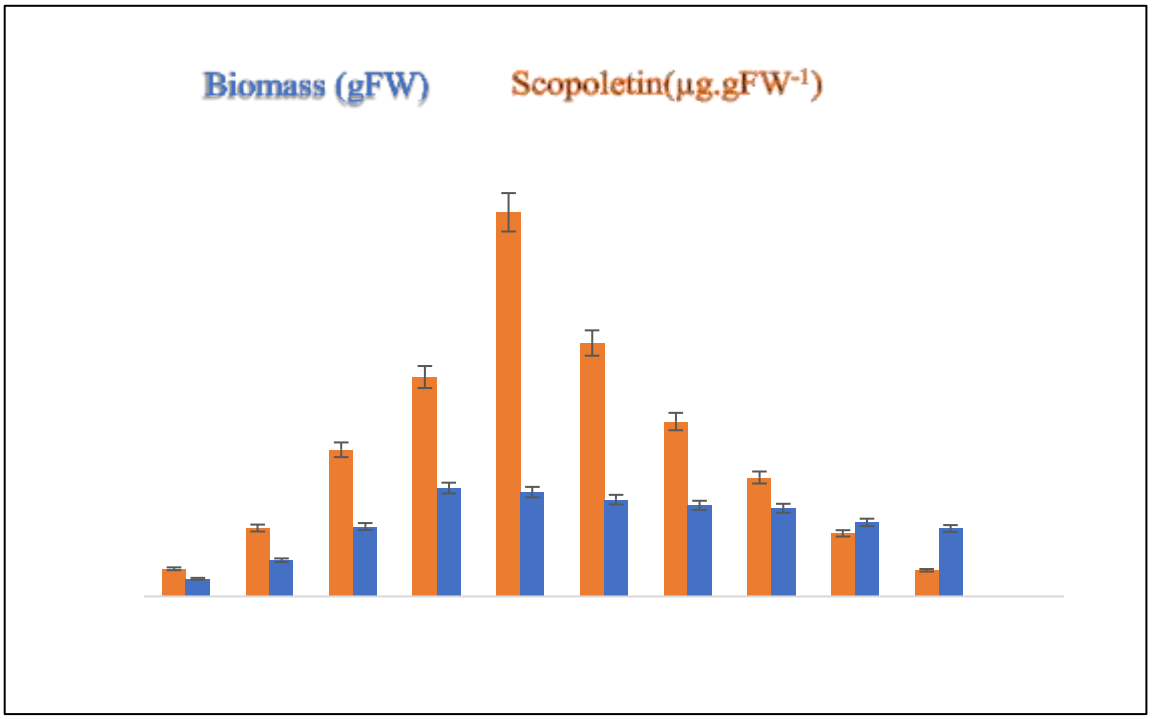

Fig. 4: Scopoletin production with respect to biomass accumulation in *A.indicum* suspensions

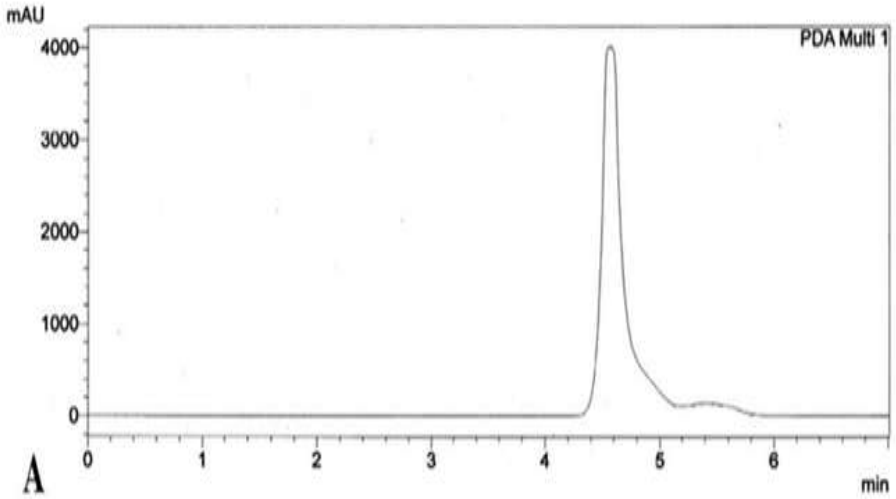

Fig. 5: HPLC graph of Scopoletin standard

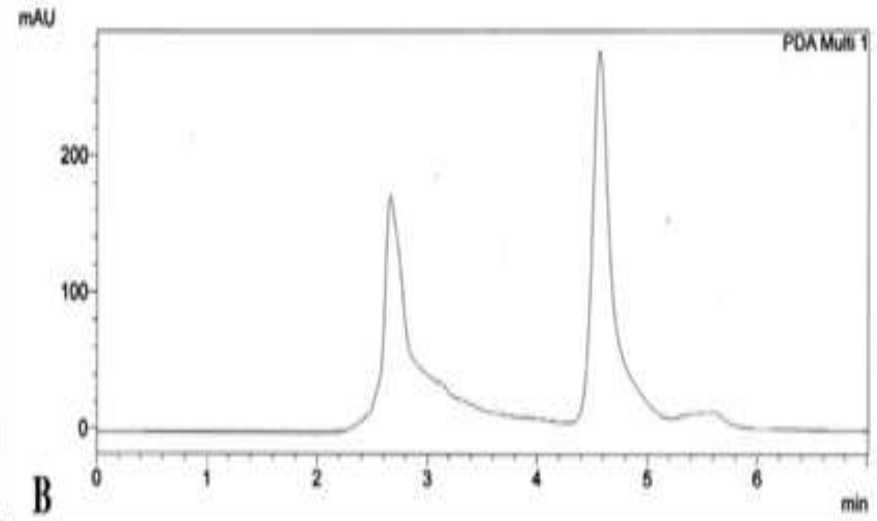

Fig. 6: Detection of Scopoletin in the suspensions of *A. indicum*

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