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Full Length Research Paper

Cell suspension culture of *Arnebia euchroma* (Royle) Johnston – A potential source of naphthoquinone pigments

Ravi Kumar[1], Nandini Sharma[2], Sonia Malik[3], Shashi Bhushan[1]*, Upendra Kumar Sharma[2], Devla Kumari[2], Arun Kumar Sinha[2], Madhu Sharma[1] and Paramvir Singh Ahuja[1]

[1]Division of Biotechnology, CSIR-Institute of Himalayan Bioresource Technology, Post Box No - 6, Palampur- 176061, Himachal Pradesh, India.
[2]Division Natural Plant Products, CSIR-Institute of Himalayan Bioresource Technology, Post Box No - 6, Palampur- 176061, Himachal Pradesh, India.
[3]Departmento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, 13083-970, Campinas, Brazil.

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The roots of *Arnebia euchroma* contain naphthoquinone red pigments, which have been used as colorant in food, cosmetics and as a dye for textile industries. Besides, these have medicinal and pharmaceutical properties too. Cell suspension culture serves as an alternative for secondary metabolite production. In this study, various growth factors such as inoculum build up process, phosphate source and *in situ* extraction methods were investigated on naphthoquinone pigment yield in *A. euchroma* cell suspension cultures. Effect of direct (solid to liquid) and in-direct (liquid to liquid) inoculum build up process was clearly evident, as direct inoculum registered 19.53% higher pigment yield than in-direct. Increased pigment yield was recorded in media having potassium phosphate sources (KH_2PO_4 and K_2HPO_4) rather than sodium (NaH_2PO_4) or ammonium ($(NH_4)H_2PO_4$). *In situ* extraction resulted in significantly higher yield of naphthoquinone derivatives (637.15 mg L^{-1}) than control (369.38 mg L^{-1}), along with higher volumetric and specific productivities. The density of liquid paraffin also influenced the *in situ* extraction. HPLC chromatograms showed acetylshikonin as the major naphthoquinone derivative in the quantified fraction. The results of present investigation revealed that optimized growth factors have significantly enhanced the yield (up to 72%) of naphthoquinone pigments.

Key words: Boraginaceae, cell culture, inoculums, paraffin, pigment, shikonin.

INTRODUCTION

Arnebia euchroma, commonly known as 'Ratanjot' of Boraginaceae family grows in wild (Figure 1A) at an altitude of 4000 to 4200 m a.m.s.l. in the Himalayan region. Its roots (Figure 1B) are a good source of red naphthoquinone pigments. These metabolites have commercial importance as natural colorants in food, cosmetics, textiles and exhibit various medicinal and pharmaceutical properties (Papageorgiou et al., 1999; Babula et al., 2009). The naphthoquinone pigments extracted from *Arnebia* species have antimicrobial, anti-inflammatory, anti-viral, anti-tumor, cardiotonic and contraceptive properties (Chen et al., 2002; Shen et al., 2002; Singh et al., 2003). These derivatives also

*Corresponding author. E-mail: sbhushan@ihbt.res.in. Tel: 91-1894-233339. Fax: 91-1894-230433.

Abbreviations: BAP, 6-Benzylaminopurine; **IBA,** indole-3-butyric acid; **PHW,** liquid paraffin heavy weight; **PLW,** liquid paraffin light weight; **DI,** direct inoculum; **IDI,** in-direct inoculums.

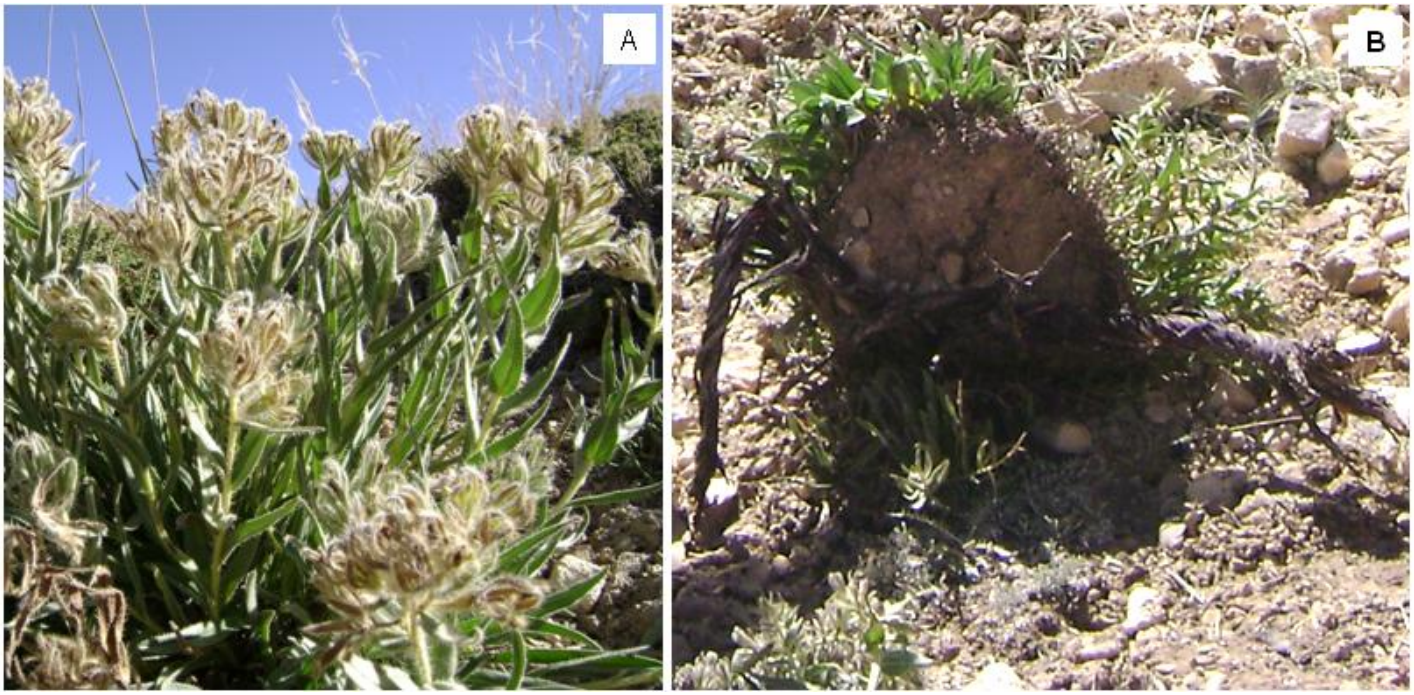

Figure 1. *Arnebia euchroma* A) plant, B) roots.

exhibit insulin like activity by inhibiting phosphatase and tensin homologue deleted on Chromosome 10 (PTEN) and protein tyrosine phosphatases (Nigorikawa et al., 2006). Studies have also revealed specific *in vivo* and *in vitro* antitumor effects of acetylshikonin (Xiong et al., 2009). It was found that this metabolite initiate tumor cell apoptosis processes. Acetylshikonin has been regarded as a wide spectrum therapeutic agent combating cancer.

In recent years, various methods and bioresources are being explored for the production of naphthoquinonones through cell culture technology. It provides a viable alternative over whole plant cultivation for the production of secondary metabolites. Advantages of cell suspension cultures for production of secondary metabolites include supply of product independent of the availability of plant, climate and geographical location. The possibility of synthesising novel compounds other wise not present in nature is an added attraction of cell culture systems (Kutney, 1997). Under *in vitro* conditions, a number of physical and chemical parameters such as medium pH, temperature and nutrients influence the yield of secondary metabolites (Malik et al., 2011). Cell wall polysaccharides (endogenous or exogenous) especially agar-agar (used as gelling agent in tissue culture medium) and pectin have been reported to influence the yield of secondary metabolites (Papageorgiou et al., 1999). Inorganic phosphate concentration of the medium is believed to influence secondary metabolism. Phosphate depleted medium enhanced the yield of cinnamoyl putrescine as well as the activity of key enzymes required for metabolite synthesis and suppressed the cell growth (Knobloch and Berlin, 1981). Electron microscopy studies have revealed that naphthaquinone pigments were synthesized in cytosol as lipid vesicles, and later transferred to outer periphery of plasma membrane for excretion into medium (Tsukada and Tabata, 1984). This deposition may be responsible for cell growth inhibition by interfering with membrane permeability during cultivation, for which *in situ* extraction method can be employed. Keeping in view, the present studies were aimed at enhancing yield of naphthoquinone pigments in *A. euchroma* cell suspension cultures employing different strategies such as different inoculum types, inorganic phosphate sources, and *in situ* extraction methods.

MATERIALS AND METHODS

Plant material and establishment of cell suspension cultures

A. euchroma plants procured from KIBBER (trans Himalayas; 4200 m a.m.s.l.) were used for the establishment of *in vitro* shoot cultures. Murashige and Skoog (1962) medium (MS) supplemented with 6-benzylaminopurine (BAP, 10 µM) + indole-3-butyric acid (IBA, 5 µM), 0.8% (w/v) agar and 3% (w/v) sucrose was used for callus induction from leaves of *in vitro* grown shoots. High pigment producing callus line 'A-46' (7yrs old) was used to initiate cell suspension cultures with 10% inoculum density in 250 ml Erlenmeyer glass conical flask containing 50 ml of liquid MS medium with supplements as described above. The medium was autoclaved at 121°C for 20 min. The callus cultures were kept under dark in culture room and suspension cultures in an incubated

shaker (Kühner, Switzerland) with 100 rpm rotation speed at 25±2°C temperature. The callus and suspension cultures were maintained by regular sub-culturing at 15 and 10 days interval, respectively.

Naphthoquinone pigment production in suspension culture

A. euchroma cells (at 10% inoculum density) from callus culture were transferred to 250 ml Erlenmeyer conical flasks containing 50 ml pigment production medium, that is, liquid M9 medium (Fujita et al., 1981) but without addition of plant growth regulators (10 µM IAA and 10 µM Kinetin). The cell suspensions were kept in incubated shaker under similar culture conditions as described for establishment of cell suspension cultures.

Inoculum type

Two different inoculum; i) direct inoculum (DI; solid to liquid), and ii) in-direct inoculum (IDI; liquid to liquid) were used. In DI, *A. euchroma* cells from established solid callus cultures were inoculated in liquid M9 medium. In case of IDI, cells from established *A. euchroma* suspension cultures after five consecutive sub-culturing at 10 day interval were used for inoculation in Liquid M9 medium.

Phosphate sources

Different phosphate sources, namely, NaH_2PO_4 (sodium di-hydrogen phosphate), KH_2PO_4 (Potassium di-hydrogen phosphate), K_2HPO_4 (di-potassium hydrogen phosphate), $(NH_4)H_2PO_4$ (ammonium di-hydrogen phosphate) at the concentration of 19 mg L^{-1}each (as per M9 medium) were used. The inoculum type used was DI, as optimized in previous experiment.

In situ extraction

Two types of *in situ* extraction solvent, that is, liquid paraffin heavy weight (PHW) and liquid paraffin light weight (PLW) were used for extraction of naphthoquinone pigments during cultivation in liquid M9 medium. Chemically, paraffin is a non-polar alkane hydrocarbons (C_nH_{2n+2}).

Liquid paraffin is a mixture of chemically inert heavier alkanes. The properties of two kinds of liquid paraffin used are given in Table 1. The solvent was added to the medium in the ratio of 1:5. The cultures kept in incubated shaker and the conditions were same as described for establishment of cell suspension cultures. The types of inoculum and phosphate source used in this experiment were DI and KH_2PO_4 (19 mg L^{-1}) respectively (as optimized in earlier experiments).

Analytical methods

Cell biomass and product kinetics

The biomass yield of cell suspension culture was measured at two day interval. The cells were harvested by sieving suspension cultures through 45 µm nylon mesh, washed twice with distilled water and placed on Whatman-4 filter paper for removal of excess moisture and weighed. The cells were dried at 60°C till reached to constant weight. The dry weight was expressed in gram dry weight per liter (g L^{-1}DW).

Average biomass growth rate (AGR) = $(X_{max} - X_i) / X_i \,.\, t = D^{-1}$

Volumetric productivity of naphthoquinone derivatives (Q_p) = $(Y_p) / t \,.\, v$ = mg l^{-1} D^{-1}

Specific Productivity of naphthoquinone derivatives (q_p) = $(Y_p) / X_{max} \,.\, t$ = mg g^{-1} D^{-1}

Where: X_i = initial dry weight of cells (g), X_{max} = maximum dry weight of cells (g), Y_p = pigment yield, t = culture period (days), v = volume of the medium (L)

Spectrophotometric analysis of naphthoquinone pigments

In growth factor optimization experiments, that is, inoculum build-up process, inorganic phosphate source and *in situ* extraction, naphthoquinone pigments were quantified as free shikonin through spectrophotometric analysis. Pigments were recovered as a blue aqueous layer using potassium hydroxide (KOH) with slight modifications as described earlier (Yazaki et al., 1998). These were extracted from the cells (1g FW), with iso-amyl alcohol and converted into free shikonin by addition of 1 ml of 2.5% potassium hydroxide (KOH). However in case of *in situ* extraction, 1 ml of 2.5% KOH was added in 100 µl shikonin derivatives containing liquid paraffin. The optical density (OD) of the extracted KOH fraction was measured at 620 nm using SPECORD 200, Analytikajena (Germany). The measured OD value was calculated based on standard curve prepared by using ChromaDex shikonin standard (Mol. wt. 288.30) procured from Life Technologies (India) Pvt. Ltd. Delhi.

Characterization of naphthoquinone pigments through HPLC analysis

The samples (10 replicates at two day interval) drawn from experiment conducted with optimized growth factors (DI, KH_2PO_4 and *in situ* extraction) were analyzed through HPLC following protocol described earlier (Sharma et al., 2008). In brief, acetonitrile: methanol (95:5 v/v) at flow rate of 0.6 ml/min in an isocratic elution was used as mobile phase. Detection wavelength was set at 520 nm and the injection volume of samples was 20 µl. HPLC (Waters model 600E system, Waters, Milford, MA, USA) equipped with a reverse phase Purospher®-Star RP-18e column (250 × 4.6 mm I.D., 5 µm, Merck, Darmstadt, Germany), a photodiode array detector (Waters 2996), an inline-degasser AF (Waters), 717 plus auto injector (Waters) and Waters empower software was used for analysis. HPLC grade solvents (acetonitrile and methanol) were procured from E. Merck (Mumbai, India) and samples were filtered through 0.45 µm membrane filters (Millipore, Germany) prior to use.

Statistical analysis

The cell culture experiments were conducted under a completely randomized block design (CRD) and the data was processed using 'Statistica' software. Significant differences between treatment means were based on Duncan's test as per requirement. Under different experiments, each treatment had ten replicates.

RESULTS AND DISCUSSION

Effect of inoculum type on pigments production

The inoculum used from solid cultures (DI) registered

Table 1. Properties of organic solvent used for in situ extraction of naphthoquinone pigment.

Parameter	Paraffin heavy weight	Paraffin light weight
Solubility	Insoluble in water and alcohol, soluble in ether and chloroform	Insoluble in water and alcohol, soluble in ether and chloroform
Colour	Water white	Water white
Density at 20°C g ml^{-1}	0.860-0.890	0.830-0.860
Kinematic Viscosity @ at 37.8°C, cs	≥ 64	≥ 30

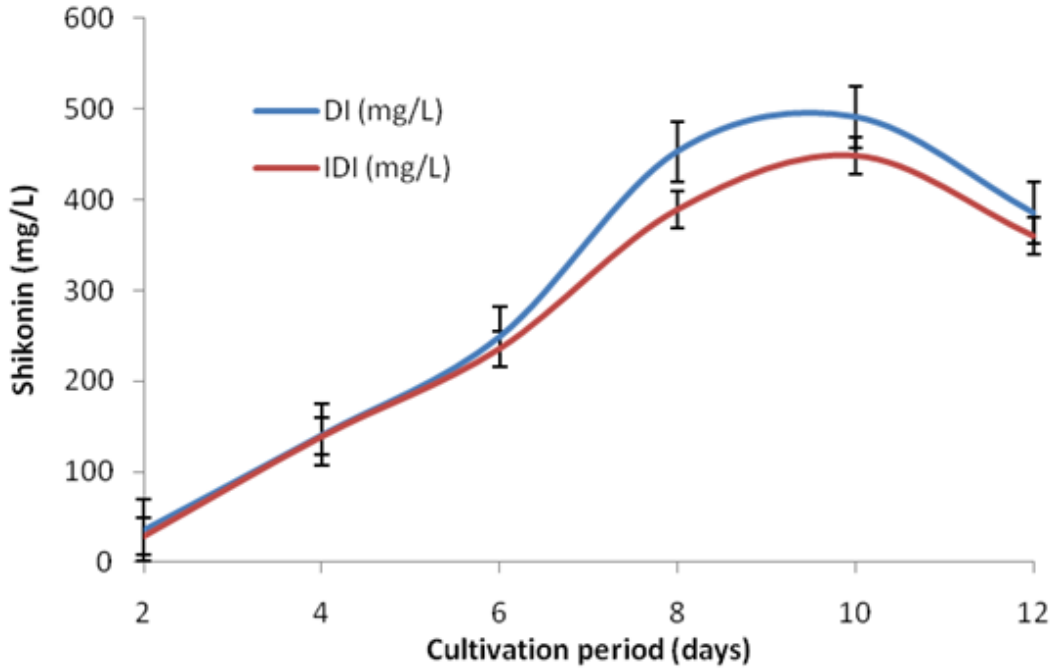

Figure 2. Effect of inoculum build-up process on naphthoquinone pigment yield (± SD).

significantly higher pigment yield (490.75 mg L^{-1}) in liquid M9 medium than IDI (447.92 mg L^{-1}) on 10th day of the cultivation period (Figure 2). The total increase in DI was 1.09 fold, about 10% higher than IDI. The increase in pigment yield from DI inoculum might be due to agar in medium, which is a polysaccharide, thereby discerning the role of cell wall polysaccharide in secondary metabolism. The pectic molecules present in agar

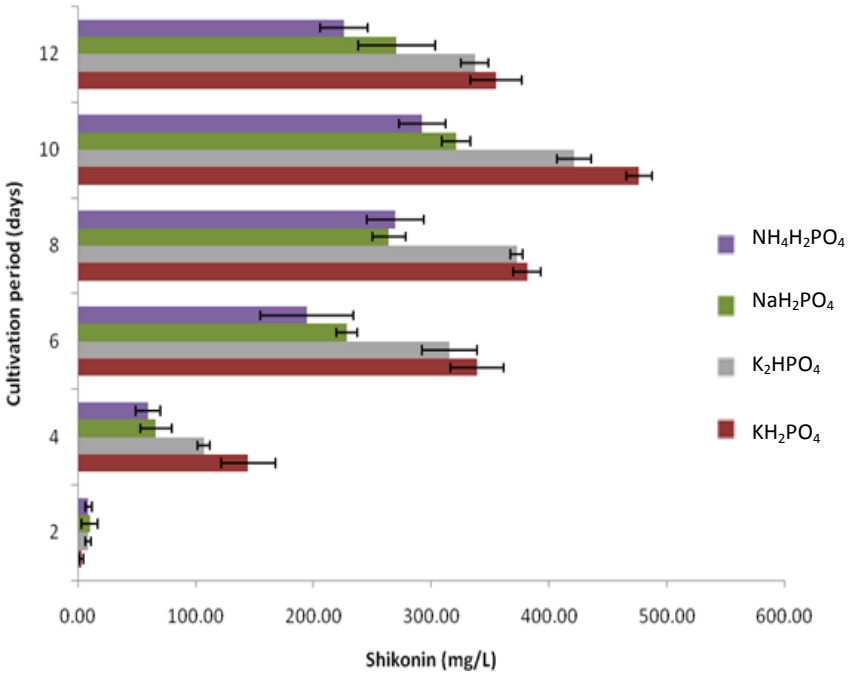

Figure 3. Effect of phosphate source on naphthoquinone pigment yield.

have earlier been reported to act as elicitor for secondary metabolism (Papageorgiou et al., 1999). Also, there are reports on addition of plant or microbial cell wall polysaccharides to increase the yield of secondary metabolites. Results are in confirmation with earlier reports on higher shikonin derivative yield from *L. erythrorhizon* callus culture as compared to cell suspension cultures (Hara et al., 1987).

Effect of phosphate sources on pigment yield

The phosphate concentration in medium is suggested to be one of the most important nutritional factor affecting cell growth and secondary metabolite yield (Saito and Mizukami, 2002). The M9 medium is a low-buffered media. It contains NaH_2PO_4 as the sole phosphate source and therefore it is pertinent to compare effect of different phosphate sources, namely, sodium, potassium and ammonium on yield of naphthoquinone pigments. The results revealed a significantly higher yield of pigments in culture medium having KH_2PO_4 (524.10 mg L^{-1}) as phosphate source on 10th day of cultivation period (Figure 3). The higher yield from potassium phosphate source suggested the stimulatory role of potassium on secondary metabolism. Interestingly, NaH_2PO_4 as a source of phosphate, reported in M9 medium (Fujita et al., 1981) for shikonin production from *L. erythrorhizon,* recorded significantly lower yield than KH_2PO_4 in present study. The medium having ammonium based phosphate source recorded lowest metabolite yield as compare to others, thus indicating inhibitory role on secondary metabolite yield. In taxoid production also, a two-stage approach has been advocated in cell cultures of *Taxus chinensis*, first to get higher cell biomass in modified MS medium (20 mM ammonium) and then transfer the cells to low ammonium concentration medium (Zhou and Zhong, 2009). Thus, the results further verify the inhibitory role of ammonium in secondary metabolite production.

Effect of *in situ* extraction on pigment yield

Liquid paraffin with varying kinetic viscosity and density (paraffin heavy weight (PHW) and paraffin light weight (PLW)) was used as organic solvent for *in situ* extraction of naphthoquinone pigments. It was evident from the results that *in situ* extraction with both types of solvents has resulted in higher pigment yield, that is, 49.1 and 72.5% respectively in PLW and PHW than control (Table 2). In early production phase, PLW containing medium had slightly higher metabolites yield. However, later on PHW was found to be more efficient in extraction of naphthoquinone pigments, as significantly higher shikonin content (637.15 mg L^{-1}) was recorded as compared to PLW (552.5 mg L^{-1}) on 10th day of cultivation in M9 medium. This may be attributed to higher kinematic viscosity and density of PHW. The higher volumetric and specific productivity has clearly

Table 2. Effect of *in situ* extraction on cell biomass and pigment production (± SD).

Treatment(s)	AGR (D^{-1})	Shikonin yield (mg L^{-1})	Q_p(mg $L^{-1}D^{-1}$)	q_p(mgg$^{-1}D^{-1}$)
Control	0.068 ± 0.018	369.38± 31.89	34.34 ± 14.48	0.045 ± 0.10
Paraffin light weight (PLW)	0.061 ± 0.015	552.50± 74.40	47.80 ± 21.59	0.044 ± 0.012
Paraffin heavy weight (PHW)	0.056 ± 0.013	637.15± 58.10	71.92 ± 20.59	0.072 ± 0.012

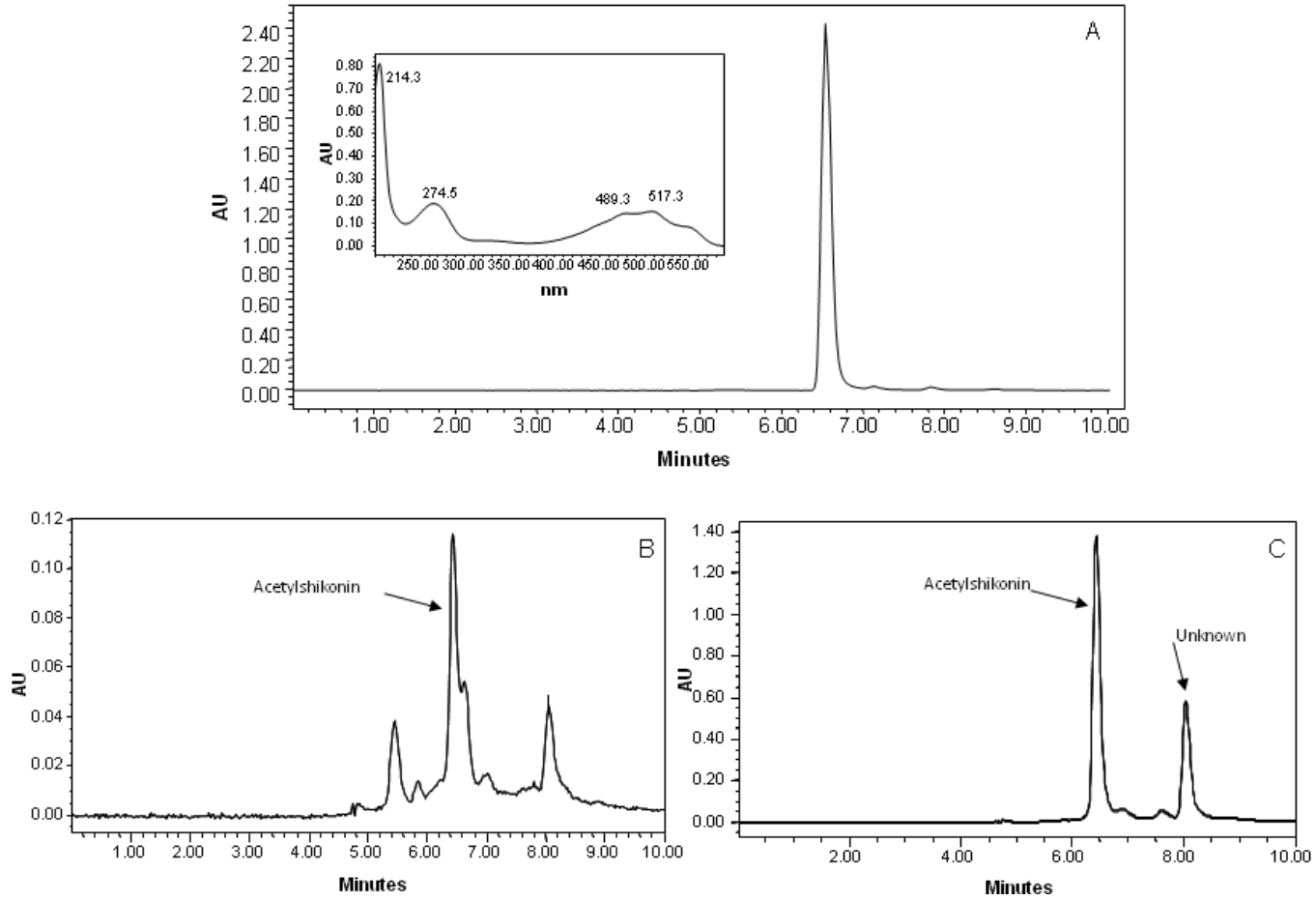

Figure 4. HPLC chromatogram: A) standard acetylshikonin, B) 5th day old culture, and C) 10th day old culture.

demonstrated the efficient extraction of these metabolites by organic solvent. Similar observations in *A. euchroma* cell suspension cultures have been recorded by Fu and Lu (1999), however, the maximum shikonin yield obtained was 245.68 mg L^{-1} by simultaneous use of fungal elicitors and n-hexadecane as *in situ* organic solvent. A decrease in cell biomass was evident from average growth rate (AGR) in two-phase culture than control (Table 2). This decrease may be attributed to poor gaseous exchange, which can suppress cell growth because of reduced anabolism. In case of taxol production, *in situ* solvent (tested range 5 to 20%) extraction stimulated the metabolite yield, but resulted in significantly lower biomass growth (Wang et al., 2001).

Characterization and quantification of naphthoquinone pigments

Characterization and quantification of naphthoquinone derivatives in methanol extract was achieved under the chromatographic conditions as mentioned in materials and methods. 20 µl injections were performed without any loss of resolution. The main derivative identified by matching its retention time and spectra with standard was acetylshikonin in cell suspension cultures. HPLC chromatograms of the quantified fraction at different time interval are depicted in Figure 4A to C. The metabolite was detected at 6.47 min retention time (Figure 4A). The data obtained reveals that metabolite production commence

with the visual indication of pigment formation after two days interval (Figure 4B) and reached to its maximum (139.49 mg L^{-1}) at the end of 10th day (Figure 4C) in shake flask. The purity of this naphthoquinone derivative was found to be greater than 98 % at respective intervals.

Conclusions

Direct inoculum build-up process enhanced the yield of naphthoquinone pigments in cell suspension cultures of *A. euchroma*. Potassium containing phosphate sources found to have positive effect on the yield of pigments as compared to ammonium and sodium. High-density liquid paraffin (PLW) as *in situ* extraction solvent along with other optimized growth factors significantly increased the yield of naphthoquinone pigments upto 72%. High yield and purity of acetylshikonin in the light of recent study on *in vivo* and *in vitro* antitumor effects will be of immense importance to nutraceutical and pharmaceutical industries.

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