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Shikimic Acid (Tamiflu Precursor) Production in Suspension Cultures of East Indian Sandalwood (*Santalum album*) in Air-lift Bioreactor

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Abstract

Shikimic acid is the key precursor for industrial synthesis of the potent neuraminidase inhibitor, oseltamivir (Tamiflu) that has tremendous importance in the treatment of flu. Plant and microbial sources are the only sources of shikimic acid. We report, suspension cultures of Indian Sandalwood tree (*Santalum album* L.) grown in air-lift bioreactor and shake-flask cultures as alternative and renewable resource of shikimic acid. Hot aqueous and ethanolic preparations of biomass and spent media yielding shikimic acid and quinic acid were further characterized by TLC and LC-ESI-MS/ MS analyses. Suspension cultures in Erlenmeyer shake flask and air-lift bioreactor of 50 mL and 2 L volumes, yielded 0.07 and 0.08 % (w/w) shikimic acid, respectively, in 2-3 weeks. Significantly, we propose that alternative plant cell cultures, sans rigorous genetic manipulation can be exploited commercially for shikimic acid production.

Highlights

- Shikimic acid, the sole molecule used in industrial synthesis for commercial production of Tamiflu has limited sources.
- East Indian Sandalwood (*Santalum album*) cell suspension cultures grown in shake flask and air lift bioreactors accumulate shikimic acid and their derivatives both in biomass and spent media.
- Shikimates and quinates were detected by combined TLC and LC-MS/ MS approaches.
- Sandalwood cell suspension culture offer itself as an alternative source for production of 0.07-0.08 % (w/w) of shikimic acid in 2-3 weeks.
- An inexpensive natural bioresource for shikimic acid is proposed.

Keywords: air-lift bioreactor; bird flu; Santalum album; shikimic acid; Tamiflu

Introduction

Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) (Eijkman, 1985), is a high value chemical constituent, essentially extracted in commercial scales from the matured pods of Chinese star anise (*Illicium verum* L.) (Bohm, 1965), the starting material for chemo-synthesis of neuraminidase inhibitor, GS4104 (oseltamivir phosphate). Sold as Tamiflu® (Roche Pharmaceuticals), it is the sole orally administered drug available for prevention and treatment of Influenza A virus strain H5N1 infections, bird flu. Synthetic routes suited for commercial-scale production of oseltamivir are established (Singer et al., 2007; Yarnell, 2005). The WHO emphasizes to study shikimic acid supply bottleneck in the lights of influenza pandemics (WHO, 2006). Even today, the major commercial supply is from Chinese star anise, but in short supplies due to the sudden worldwide demand, and is still

expensive (Enserink, 2006). *Illicium verum* L. is grown only in four provinces in China and over-exploitation is endangering the species. The related 50 species of the genus *Illicium*, possess a neurotoxic sesquiterpene lactone, anisatin (Lederer et al., 2006). Shikimic acid content in star anise seeds varies with time of harvest of pods, handling and storage and a host of similar factors (Farina and Brown, 2006). No doubt, to obtain shikimic acid in kilogram quantities, apart from chemo-synthesis and extraction, the metabolic engineering (fermentation) approaches are accomplished in microbes. Blocking the aromatic amino acid biosynthesis through designing of shikimate kinase or EPSP synthase deficient strains has shown to be effective in production of higher titers of shikimic acid in *Escherichia coli*, *Bacillus subtilis* and *Citrobacter freundii* (Kramer et al., 2003). These fermentation processes are potential and conceivable supply pool for the future global market for shikimic acid.

Feasibility of transformation of quinic acid (derivatives) to shikimic acid (derivatives) and vice-versa (Alves et al., 1999) by chemical (Shinada et al., 1998) and enzymatic means (Gamborg, 1967) has also been explored. Excellent review on the analytical, isolation and purification of shikimic acid from plant and microbial resources is available (Bochkov et al., 2012). Several plants, especially the woody species are projected as potential alternatives to Chinese star anise, which yields 2-7 % w/w of shikimic acid (Enrich et al., 2008). Recently, leaves of the gymnosperm *Ginkgo biloba* were proposed as alternative resource for shikimic acid (Usuki et al., 2011). Roche uses 90 % of star anise production for production of Tamiflu (Ortiz and Clauson, 2006).

Stock piling of the drug by many countries compounds the problem. Hence, an in vitro resource for shikimic acid production using plant cells shall be of wide health-care biotechnology interest. Thus, we explored cell culture systems in East Indian sandalwood *Santalum album*, famous for its sesquiterpenoid essential oil, to produce shikimic acid in suspension culture in shake flask and air-lift bioreactors. Furthermore, India produces roughly around 2000 tons of heartwood per year (Ral, 1990), with an estimated market volume of more than $1 billion (Viswanath et al., 2008). Besides, sandalwood already finds proven applications as antibacterial, antifungal (Dikshit and Hussain, 1984), anti-cancer, anti-hypertensive, and anti-pyretic (Desai et al., 1991), antiviral (Benencia and Courreges, 1999) and anti-*Helicobacter pylori* (Ochi et al., 2005) in various forms and formulations across traditional medicinal systems and in laboratory scale experiments. This is probably, the first report on the production of shikimic acid from plant suspension cell culture and significantly, without tinkering with the cellular machinery with inhibitors, precursors or any undesirable rigorous genetic manipulation approaches.

Experimental Procedures

Plant materials

The plant materials studied were defined as follows; callus (CA), in vitro grown highly proliferating cell line (IIT KGP/ 91), yielding friable mass of single cells, grown on solid MS media (Murashige and Skoog, 1962) or solid Woody Plant Medium (WPM) (Lloyd and McCown, 1981) supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) (1 mg L^{-1}), harvested 14 d post subculture. The friable callus biomass so obtained was sub cultured in the same medium every fortnight in a ratio of 1: 5 of old suspension and fresh medium. Somatic embryos: In vitro grown embryos from callus, grown on solid media, supplemented with indole-3-acetic acid (IAA) (0.5 mg L^{-1}) and 6-benzylaminopurine (BAP) (0.5 mg L^{-1}), harvested 2 weeks of induction into embryogenic media from callus line mentioned

above. Both the above cultures were maintained in dark at 25±2 °C with 50-60 % relative humidity. In vitro seedlings: in vitro grown somatic seedlings, obtained from somatic embryos, grown on solid media supplemented with IAA (1 mg L^{-1}), indole-3-butyric acid (IBA) (1 mg L^{-1}) and gibberellic acid (GA) (0.5 mg L^{-1}), harvested 6 weeks after induction of somatic embryogenesis. Seedlings were maintained under white fluorescent light of 1000-1500 lux (16 h light/ 8 h dark cycle) at 25±2 °C with 50-60 % relative humidity. Vegetative twigs, from a field grown 6 year old tree, YT (young tree) and a field grown 13 year old tree growing in the Indian institute of Technology, Kharagpur, India campus were analyzed for a comparative study.

Maintenance of suspension cultures, growth rate and biomass estimation

Embryogenic suspension cultures were grown and maintained in Erlenmeyer conical flasks of 250 mL capacity with 50 mL liquid MS or WPM medium containing the growth regulator 2, 4-D (1 mg L^{-1}) and sucrose (30 g L^{-1}) at 25± 2 °C on an orbital shaker (C24 incubator shaker, New Brunswick Scientific, USA) at 120 rpm in dark. Sub culturing was done in the same medium every fortnight in a ratio of 1: 5 of old suspension and fresh medium. Suspensions were further cultured in a 2 L air-lift bioreactor (Bio Stat (B. Braun Biotech. International, USA) and was run on a Bebicon (Hitachi Ltd.) oil-free compressor of 30 L airflow capacity air pump. Specific growth rates (μ) and doubling times (t_d) of the suspension cultures of somatic embryos were measured.

Extraction of organic acid rich fractions

Fresh plant materials (~ 10 g) were harvested, blotted to dry, weighed and crushed with liquid nitrogen to powder. Four extraction solvents were used for preparation of extracts i.e. (i) water, (ii) hot water (80 °C), (iii) absolute ethanol, and (iv) 80 % ethanol. Extraction was done by shaking in Erlenmeyer flasks with solvent: plant material ratio of 4:1, for 1 h with constant agitation. Post-extraction, samples were filtered using Whatman No. 1 filter paper and centrifuged at 10, 000 g for 10 mins to get rid of particulate matter. The supernatant was concentrated in vacuuo for 30 mins at 37 °C to dryness and taken up in 1 mL of ddH_2O. Spent media of cultures were freeze dried and taken up in 1 mL of ddH_2O. Aliquots were stored at -20 °C, until further analyses by spectrophotometry, TLC and LC-ESI-MS/ MS.

Colorimetric quantification of shikimic acid

Shikimic acid was quantified in the biomass extracts and spent media as described (Mossor and Schramm, 1972). Briefly, to 1 mL aqueous extract in test tube, 3 mL of concentrated sulphuric acid was added while mixing on ice, to which 1 mL of 0.2% aqueous p-hydroxybenzaldehyde was added. The test tube with the reaction mixture was transferred to a boiling water bath, heated for 60 min and allowed to cool. After 10 min, the extinction of the intense purple-violet dye was measured at 590 nm in glass cuvettes of 1 cm path length, using a double beam spectrophotometer (Spectrascan UV 2600 PC, Chemito, India) against appropriate blanks. The reaction is insensitive towards quinic acid. Quantification was done by plotting a standard curve for shikimic acid (Sigma-Aldrich, USA) under similar conditions in a range of 25-125 ng mL^{-1} concentration range.

Thin-layer chromatography (TLC) of shikimates

Thin-layer chromatography was performed on 10 x 10 cm silica gel 60_{254} TLC plates (Merck, Germany) sprayed with 0.1 M B(OH)3. Plates were developed in ethyl acetate: methanol: formic acid: water (8:1:1:1, v/v/v/v) or n-butanol: acetone: water (4:5:1, v/v/v) to the top and dried at 110 °C for 10 min before use. All standards and samples were manually applied using a capillary. Dried plates were spray derivatized with 60 % (v/v)

Serial No.	Culture Volume (L)	Culture Vessel Type	Inoculum (g)	Specific Growth Rate (μ^1, day^{-1})	Doubling Time (^2t_d, days)	Duration (days)	Biomass Yield (g ^{3}FW)	Shikimic Acid Content (%, w/w ^{4}DW)
1	0.05	Erlenmeyer Flask	1	0.047	11.5	14	5.5 ± 0.8	0.07±0.01
2	2	Air-lift Bioreactor	35	0.067	10.2	21	155±17	0.085±0.02

Table 1. Biomass yield and shikimic acid production in different culture volumes and vessel types. Notes: $^1\mu$: Specific Growth Rate expressed in day^{-1}; ^2t_d: Doubling Time expressed in days; FW: fresh weight, DW: dry weight

aqueous sulphuric acid or a sequential spray of (a) saturated aqueous KIO4, (b) aqueous NaHSO3 and (c) 1 % methanolic aniline, followed by heating at 130 °C.

LC-ESI-MS/ MS analyses of shikimate, quinate and their derivatives

Sandalwood tissues extracted in four conditions, were further rechromatographed on LH-20 columns and various fractions were collected for identification and characterization. Fractions were analyzed by "on-line" LC-ESI-MS/ MS using a Waters, 2695 separation module and QuattroMicroTM API HPLC coupled with a Micromass ion-trap model. The solvent flow rate was 0.250 mL min^{-1} and the injection volume was 10 µL. The temperature was set to 25 °C and the column backpressure was 490 bars. Sample solutions were injected into a reversed phase column XTerra™ MS C_{18}, 5 µm (particle size) and 2.1 (i.d.) x 100 mm (l) (Waters, Milford, USA), which was maintained at 20°C. The mobile phase consisted of the following 9.5 min sequence of linear gradients and isocratic flows of solvent B (AcCN) balanced with aqueous 7.5 mM HCOOH (solvent A) at a flow rate of 250 µL min−1: 5 % B for 0.8 min, 5-10 % B over 0.4 min, isocratic 10 % B for 0.7 min, 10-15 % B over 0.5 min, isocratic 15 % B for 1.3 min, 15-21 % over 0.3 min, isocratic 21 % B for 1.2 min, 21-27 % B over 0.5 min, 27-50 % B over 2.3 min, 50-100 % B over 1 min, and finally 100-5 % B over 0.5 min. At the end of this sequence, the column was equilibrated under initial conditions for 2.5 min (Gruz et al., 2008). The pressure ranged from 410-500 psi during the chromatographic run. The effluent was introduced into a PDA detector (scanning range of 190-370 nm, resolution 1.2 nm) and subsequently into an electrospray source (source block temperature 130 °C, desolvation temperature 300°C, capillary voltage 3 kV, cone voltage 28 V). Argon was used as both collision (collision energy 2 eV) and desolvation gas (650 L h^{-1}). The flow generated by chromatographic separation was directly injected into the ESI source. The spectrometer was operated in both negative and positive modes and the detected mass range was set to 50-900 m/z. For fragmentation pattern study, two scan events were prescribed to run simultaneously in the LCQ mass spectrometer. The first event was a full scan MS to acquire data on ions in the range (scan mode) of 50-900 m/z and the second event was a MS/ MS product scan event on selected mass ions molecule specific m/z, respectively. The data were processed using the Mass Lynx™ 4.1 software.

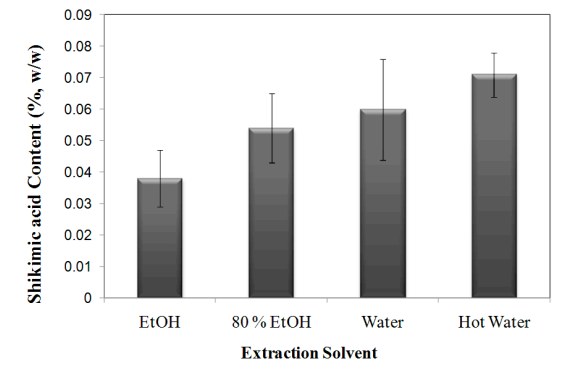

Figure 1. Effect of different extraction solvents on shikimic acid yield from in vitro embryogenic suspension cultures. Results are values from interday assays and are expressed as mean ± S.D. (n= 6). EtOH: ethanol.

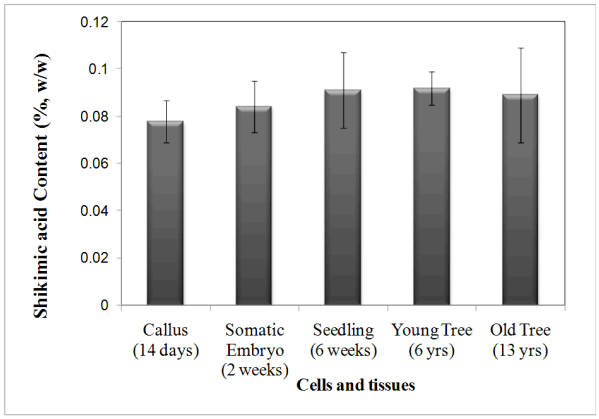

Figure 2. Yields of shikimic acid from different sandalwood tissues. Results are values from interday assays and are expressed as mean ± S.D. (n= 6).

Data analysis

All experiments were conducted in triplicates. The data represents mean ± SE (n=6). Statistical analyses were done by SPSS statistical package (version 16) and graphical representations were done with Microsoft Excel program (Windows 2007).

Results And Discussion

Previous reports (Amrhein et al., 1983, Binarová et al., 1984, Smith et al., 1986) indicate that plant cells in culture do not accumulate shikimic acid as such, but tend to do so under glyphosate (commercially known as Roundup™) mediated inhibition of the enzyme EPSP synthase. Earlier studies report that calli of *Corydalis sempervirens*, accumulated 0.03 % (w/w) shikimic acid (Amrhein et al., 1983) while tomato cell lines secreted 0.008 % (w/w) shikimic acid (Smith et al., 1986) upon treatment with 10 mM glyphosate. However, we were interested to monitor the levels of shikimic acid in sandalwood suspension cultures without any exogenous treatment under in vitro conditions. Data provided in Table 1 reveal that the specific growth rates (μ) (0.047-0.067 d^{-1}) and doubling time (t_d) (10.2-12.6 d) of cultured biomass in shake flask and air-lift bioreactor were similar. Shikimic acid yield from both the pools of biomass were also comparable (0.07-0.08 %, w/w DW). Furthermore, alfalfa cultures are known not to respond to glyphosate treatment at all (Binarová et al., 1984).

Efficiency, yield and ease of extraction are considered important parameters for industrial success of plant based natural products as downstream processes. Thus we compared the extract yields for two each solvents based on ethanol and water. Results (Figure 1) indicate that, hot water extracts yielded higher amounts of shikimic acid (0.071 ± 0.003 %, w/w) as compared to aqueous extracts and ethanol-based solvents (0.038-0.06 %, w/w). In fact, recently it was shown that hot water extraction of plant materials at temperatures greater than 100 °C but less than 200 °C yielded 100 % recoveries of shikimic acid from plant materials (Ohira et al., 2009). Evidently, alcohol additives and mild temperatures (~55 °C) have been used successfully to extract shikimic

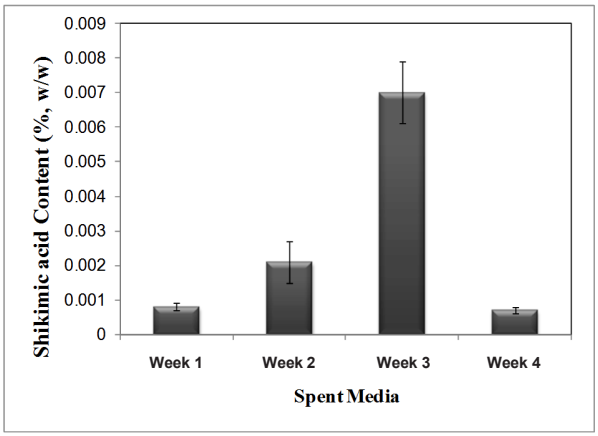

Figure 3. Yields of shikimic acid from spent media of in vitro embryogenic suspension cultures. Results are values from interday assays and are expressed as mean ± S.D. (where, n= 6).

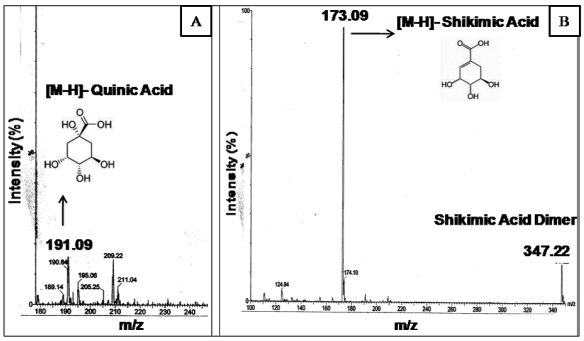

Figure 4. LC-ESI-MS/ MS based detection of TLC-eluted A. quinic acid and B. shikimic acid.

acid (Miles et al., 1994).

Metabolites are synthesized and stored differentially in different plant parts and tissues, depending on the tree, physiology and environmental constraints. Thus, we investigated the quantitative variation of extractable shikimic acid across the five developmental stages in the tree's life cycle to identify the potential material from which the highest yield could be harnessed. Results indicate that the sandalwood at various developmental stages in vitro and in vivo yielded more or less similar amounts of shikimic acid (0.078-0.089 %, w/w) (Figure 2). Besides, woody dicots are known to accumulate more shikimic acid than herbaceous species and monocots (Raghavendra et al., 2009; Yoshida et al., 1975). Apparently, comparable quantities of shikimic acid could be obtained from callus, in a matter of 14 d of growth, as compared to other older stages ranging from several weeks (i.e. for seedlings) to years (i.e. for trees), in case of sandalwood tree life cycle.

The spent media provides clear advantages than the plant suspension cultures themselves, as it is are free from other cellular contaminants and thus have potential in reducing the downstream processing costs, and enhancing the extractability and ease. It was observed that the spent media accumulated shikimic acid from 1-4 weeks, with maximum yields (0.007 %, w/w) during the third week of growth, with reduction or loss owing to degradation with further culture (Figure 3).

TLC analyses revealed, red and charred spots of shikimic acid upon derivatization (not shown). The chromatographic behaviors for shikimic acid (R_f=0.24) and quinic acid (R_f=0.62) present in the extracts were distinct. Upon quantification of TLC -elutes, it was revealed that the ratio of shikimic acid to quinic acid was roughly 1:3 for the tissues of sandalwood. Photometric quantification of such extracts gave purple-blue dye complex (λ_{max}, 590 nm), which is characteristic of shikimic acid. Equation obtained using commercially available shikimic acid, i.e. y= 2.643 x + 0.235 (R^2 = 0.993) was used for quantification of extracts obtained from tissues.

Recently, analytical methods have been developed for detection of shikimic acid by modified

spectrophotometric and HPLC (Zelaya et al., 2011) and LC-ESI-MS/ MS based methods (Avula et al., 2011). Thus, to confirm the identity of the molecules under study, we proceeded for LC-MS/ MS based validation of TLC-eluted samples.

Analyses by LC-ESI-MS/ MS (Figure 4), allowed identification of shikimic acid (MW. 174 Da) in the deprotonated state as (M-H)-ions with m/z 173.0455. Moreover, the dimeric form of shikimic acid appeared as m/z 347.22 (Table 2.). Similarly, quinic acid (MW. 192 Da) was identified in the deprotonated form as (M-H)-ions at m/z 191.09. In addition, ammonium adduct of quinic acid was detected as (M+NH4)+ ions for m/z 210. Additional shikimate and quinate derivatives obtained from the callus suspension culture extracts were, identified mostly as (M-H)-; i.e. feruloyl quinic acid (m/z 365), dicaffeoylferuloylquinic acid (m/z 497), caffeoylquinic acid-hexoside (m/z 539, as (M+Na)+, positive ionization), tricaffeoylquinic acid (m/z 677) and caffeoylquinic acid dimers (m/z 707), predominantly identified in negative ionization modes (data not presented). As a matter of fact, several plants are known to contain shikimic acid derivatives, i.e. *Quercus* (Ishimaru et al., 1987), *Senecio* (Barrero et al., 1988) and *Cyathostemma* (Mahmood et al., 1991).

Our data revealed shikimic acid producing ability of biomass from in vitro cultures in 14 days as compared to 6 years of maturity required for star anise plants to yield seeded pods. In terms of biomass yields, 1 kg of pods is obtainable post-six years of growth in star anise, whereas 0.95 kg of callus is obtainable in seven parallel batches of growth in a 2 L air-lift bioreactor, within 3 weeks of culture induction. A biomass of 30 kg would yield 1 kg of shikimic acid from Chinese star anise (Farina and Brown, 2006), whereas the same quantity of biomass would yield 210 g of shikimic acid (this study). About, 100 g of suspension cultured biomass of sandalwood would yield 700 mg of shikimic acid, a quantity enough for synthesis of 5 oseltamivir capsules (~75 mg each); with the consideration of 35 % conversion efficiency of shikimic acid into oseltamivir at industrial scale. Nevertheless, attempts to obtain shikimic acid by glyphosate treatment remain an attractive target (Tong et al., 2010), keeping in mind the immediate global needs of global shikimic acid and the epidemic nature of flu.

The findings described here, have significant potential for commercial application as substitute for Chinese star anise, towards in vitro production of shikimic acid. Moreover, the sandalwood trees are mainly distributed on the Deccan Plateau spread over 9,000 km^2 of which 91% of the coverage in the states of Karnataka and Tamil Nadu (Ral, 1990). This natural population could provide a huge bio-resource for global production and supply of shikimic acid. We are further investigating in our laboratory to enhance yield by biological elicitors and facilitation of release of shikimic acid from cells to media, so that down-stream processing becomes easier in future.

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