Immobilization mediated enhancement of phyllanthin and hypophyllanthin from *Phyllanthus amarus*

J. S. Thakur*, R. K. Agarwal, M. D. Kharya

**Plant Biotechnology Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour Central University, Sagar (M.P.) 470003, India**

Available online May 2012

**[ABSTRACT]** *Phyllanthus amarus* plant is used in the traditional system of medicine as a hepatoprotective drug for which the major lignans phyllanthin and hypophyllanthin are responsible. So far, no significant work has been done on the culture of this plant. Realizing the hepatoprotective potential, the present investigation was undertaken. A cost effective process was developed for enhancing phyllanthin and hypophyllanthin utilizing the immobilization technique. HPTLC was used to compare the phyllanthin and hypophyllanthin contents in calcium alginate immobilized cells obtained from fresh grown plants and MS medium was supplemented with different abiotic elicitors, under aseptic conditions for the treatment with chitosan, copper sulphate, phenylalanine and silver nitrate solution to make the whole process commercially viable. It was revealed that silver nitrate and phenylalanine at low concentration enhances phyllanthin and hypophyllanthin yield as compared to control immobilized cell culture. The study revealed that an increase in the content of phyllanthin and hypophyllanthin was elicitor concentration dependent and silver nitrate treatment gave a maximum yield of hepatoprotective bioactives as compared to the other abiotic elicitors used.

**[KEY WORDS]** Immobilization; Elicitors; Lignans; Hepatoprotective; HPTLC

**[CLC Number]** R917  **[Document code]** A  **[Article ID]** 1672-3651(2012)03-0207-06

1 Introduction

Biotechnology is an applied science in which living organisms are exploited for their byproducts for commercial purposes. It integrates knowledge and information from many fields. Keeping in view the worldwide demand of therapeutically useful bioactives, large scale *in vitro* production is necessary for the future production of herbal drugs and their bioactives to meet their future global requirement [1].

The aim of plant biotechnology research is to optimize the concentration of precursors, abiotic and biotic elicitors with duration of their contact for maximum response to the production of targeted secondary metabolite which is very much desired. Liver, one of the biggest body organs, performs many vital functions including digestion. Another important role is to detoxify the harmful and unwanted substances in the body. During this detoxification process, the liver suffers from challenges affecting the hepatic architecture and hepatocytes. In general, the liver suffers from hepatotoxicity which damages it. Antihepatotoxic herbs restore the bile flow and reduce total bilirubin, biliverdin, triglycerides, cholesterol and total lipids in liver [2].

To overcome these complications, a large number of herbal drugs are prescribed. Few such drugs are (1) *Phyllanthus niruri*, (2) *Ocimum sanctum*, (3) *Tephrosia purpurea*, (4) *Andrographis paniculata*, (5) *Eclipta alba* and (6) *Terminalia chebula* [3]. They contain polyphenols, tannins, lignans and alkaloids which possess serum bilirubin, reducing the effect on the hepatic tissues without any adverse effect. These constituents act as excellent hepatoprotectives in the treatment of jaundice and liver cirrhosis [4].

*Phyllanthus amarus* is an important hepatoprotective drug being used since ancient time. The hepatoprotective activity has been reported from phyllanthin and hypophyllanthin present in *P. amarus*. Although it is highly valuable as hepatoprotective agent, *P. amarus* suffers from the problem of short supply due to its low herbage, availability in limited duration and stringent requirement of climatic condition.

*Phyllanthus amarus* (Euphorbiaceae) (commonly called Bhui amla), though common to central and south India, is indigenous to the rain forests of Amazon and other tropical
areas of the world. Due to its hepatoprotective property, it is in great demand. However, scanty growth, short life span (July to October) and requirement of damp weather for growth are the factors for the short supply of *P. amarus*. These factors make this plant a suitable candidate for exploitation through biotechnology for the production of its hepatoprotective bioactives [5].

To overcome these problems, biotechnology has been used to produce the bioactives - phyllanthin and hypophyllanthin using plant tissue culture technology utilizing *Phyllanthus amarus*.

To meet the increasing demand of plant based drugs, immobilization of plant cells is a viable alternative method for the production of therapeutic bioactives and enhancing their yield through the treatment with different abiotic and biotic elicitors, precursors and phytohormones. Review of literature reveals that although large numbers of plants possess hepatoprotective activity, the *Phyllanthus amarus* has proved its hepatoprotective potential [6].

Enhancement of secondary metabolites i.e. phyllanthin and hypophyllanthin in *P. amarus* plant cells in vitro was done by media manipulation, phytohormone regulation, precursor feeding in immobilized and suspension cultures. MS medium was selected for the establishment of its in vitro immobilized cell cultures utilizing *P. amarus* leaves, under aseptic conditions.

### 2 Materials and Methods

Fresh leaves of *P. amarus* collected from the medicinal plant garden of Department of Pharmaceutical Sciences, Dr. H. S. Gour Central University, Sagar (M.P.) India, were authenticated (Herbarium No.QDS/3/99/09) from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow (U.P.) India.

From literature it was revealed that chitosan and phenylalanine play an important role in enhancing the secondary metabolite production in medicinal plants. It was also observed that copper sulphate and silver nitrate also exert a noticeable role in the enhancement of bioactives by immobilization [7].

These abiotic elicitors are therefore selected for studying their role in the production of important bioactives from *P. amarus* through immobilized system.

#### 2.1 Sterilization of material and preparation of alginate beads for immobilization

MS Media is plant growth media used in laboratories for cultivation of plant cell culture. It was invented by plant scientists Toshio Murashige and Folke. K. Skoog during Murashige’s search for a new plant growth regulator. MS Media was purchased from Hi media Laboratories Pvt. Ltd., and formulated for study purpose. (product code : PT810).

Dehydrated MS Media 34.10 g was dissolved in 1000 mL of sterilized distilled water and the medium pH was adjusted to 5.6. The freshly prepared MS medium was autoclaved and stored in refrigerator for further use as per requirement.

The collected fresh leaves (25 g) of *P. amarus* were washed with running tap water, followed by 2% tween solution, re-washed thoroughly with distilled water, and then sterilized with 70% ethanol. The leaves were subsequently surface sterilized with 0.1% mercuric chloride solution and were washed thoroughly with sterilized water in aseptic condition. Then the leaves were crushed finely using sterilized pestle mortar to get cell homogenate of *P. amarus* leaves [8].

Over-night stored sodium alginate solution 20 mL (5%) was mixed thoroughly with the cell homogenate for 30 min to eliminate air bubbles and to enhance viscosity. The beads, from cell homogenate were prepared using 25 mL injection syringe and the prepared beads were suspended in (2% W/V) calcium chloride solution. The alginate beads were then washed with 0.9% sterilized saline solution and transferred into (100 mL) sterilized conical flask containing 50 mL MS medium. These immobilized cell cultures of *P. amarus* leaves were used in experimentation for obtaining maximum yield of phyllanthin and hypophyllanthin [9].

#### 2.2 Effect of chitosan treatment

It is a linear polysaccharide produced commercially by deacetylation of chitin, from exoskeleton of crabs, shrimp etc. and cell wall of fungi.

Chitosan A.R (1 g) was dissolved in 100 mL of distilled water by heating at 60 °C for 15 minutes with 2 mL of glacial acetic acid to make 1% (W/V) chitosan solution. The pH of the solution (100 mL) was adjusted to 5.5 with 1 mol·L⁻¹ sodium hydroxide solution and autoclaved at 120 °C (15 lbs /sq.inch) for 20 minutes and was filter sterilized before addition to the immobilized cell culture. Four sets (in triplicate) of sterilized conical flasks were taken with 50 mL MS medium and labelled as AC [control] CT1, CT2 and CT3 containing 25 g alginate beads of *P. amarus*. Into CT1, CT2 and CT3 flasks, 5, 10 and 20 mL of 1% chitosan solution was added and incubated for 14 days in incubator cum shaker at 25 ± 2 °C between 80 to 100 rpm⁻¹. Immobilized cell culture (20 mL) from each flask was withdrawn and HPTLC analysis was done for the content of phyllanthin and hypophyllanthin in control and treated flasks.

From the experimental data it was observed that as the concentration of chitosan solution in immobilized cell culture increased from 5 to 20 mL, the bioactives phyllanthin and hypophyllanthin enhanced from 0.261% to 0.405%, which was higher than the control 0.120% (without chitosan treatment). The maximum increase in the yield of bioactives (0.405%) with 20 mL of 1% chitosan solution was to the tune of 238% (Table 1, Fig. 2).

#### 2.3 Effect of copper sulphate treatment

MS medium was supplemented with 1% copper sulphate solution and the stock solution 100 mL (conc. 1%) was filtered using sterilized 0.2 μm microfilter into a sterile container.
Following the process given earlier, the alginate beads were prepared from 25 g of fresh leaf cell homogenate of \textit{P. amarus} aseptically. To four sets of flasks (in triplicates) labelled as C1, C2, C3 and C4, 50 mL MS medium was taken in each sterilized flask and 2, 4, 6 and 8 mL of 1% copper sulphate solution was added from stock solution and kept in incubator cum shaker. After 14 days, 20 mL immobilized cell samples from each flask were collected and analyzed for bioactive contents using HPTLC.

Addition of different concentrations of copper sulphate solution to MS media in immobilized cell system increased the content of phyllanthin and hypophyllanthin compared to control (0.120%) and maximum enhancement of 0.560% was found with 8 mL which was nearly 4 times the enhancement as compared to control (Table 2, Fig. 3).

### Table 2 HPTLC analysis of immobilized \textit{P. amarus} cell system for phyllanthin and hypophyllanthin content with copper sulphate \((n = 3, \bar{x} \pm s)\)

<table>
<thead>
<tr>
<th>Flask</th>
<th>Copper sulphate (1%) mL</th>
<th>Phyllanthin and hypophyllanthin (W/W)</th>
<th>% increase compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Control</td>
<td>0.120 ± 0.001</td>
<td>0</td>
</tr>
<tr>
<td>C1</td>
<td>2</td>
<td>0.305 ± 0.002</td>
<td>154</td>
</tr>
<tr>
<td>C2</td>
<td>4</td>
<td>0.420 ± 0.001</td>
<td>250</td>
</tr>
<tr>
<td>C3</td>
<td>6</td>
<td>0.525 ± 0.002</td>
<td>337</td>
</tr>
<tr>
<td>C4</td>
<td>8</td>
<td>0.560 ± 0.001</td>
<td>367</td>
</tr>
</tbody>
</table>

When media supplementation was done by adding 2, 4, 6 and 8 mL of phenylalanine the content of phyllanthin and hypophyllanthin was found to be 0.560% and 0.585% to 0.590% and 0.615% in immobilized \textit{P. amarus} cell system, showing 5 times enhancement in bioactives respectively as compared to control (0.120%) (Table 3, Fig. 4).

### Table 3 HPTLC analysis of immobilized \textit{P. amarus} cell system for phyllanthin and hypophyllanthin content with phenylalanine \((n = 3, \bar{x} \pm s)\)

<table>
<thead>
<tr>
<th>Flask</th>
<th>Phenylalanine (1%) mL</th>
<th>Phyllanthin and hypophyllanthin (W/W)</th>
<th>% increase compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Control</td>
<td>0.120 ± 0.001</td>
<td>0</td>
</tr>
<tr>
<td>P1</td>
<td>2</td>
<td>0.560 ± 0.002</td>
<td>367</td>
</tr>
<tr>
<td>P2</td>
<td>4</td>
<td>0.585 ± 0.001</td>
<td>387</td>
</tr>
<tr>
<td>P3</td>
<td>6</td>
<td>0.590 ± 0.002</td>
<td>392</td>
</tr>
<tr>
<td>P4</td>
<td>8</td>
<td>0.615 ± 0.001</td>
<td>413</td>
</tr>
</tbody>
</table>
containing 50 mL of MS medium alongwith alginate beads of P. amarus cells, 2, 4, 6 and 8 mL of silver nitrate solution was added and after 14 days of incubation, 20 mL of sample was withdrawn from each flask and analyzed for bioactives.

When the immobilized cell system was supplemented with silver nitrate solution by adding 2, 4, 6 and 8 mL in MS medium, the yield of the phyllanthin and hypophyllanthin was found to be 0.690%, 0.765%, 0.770% and 0.905%, respectively, indicating maximum enhancement of 654% with 8 mL silver nitrate solution as compared to control 0.120% (Table 4, Fig. 5).

**Table 4** HPTLC analysis of immobilized P. amarus cell system for phyllanthin and hypophyllanthin content with silver nitrate 

<table>
<thead>
<tr>
<th>Flask</th>
<th>Silver nitrate (1%)/mL</th>
<th>Phyllanthin and hypophyllanthin (W/W)*</th>
<th>% increase compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Control</td>
<td>0.120 ± 0.001</td>
<td>0</td>
</tr>
<tr>
<td>S1</td>
<td>2</td>
<td>0.690 ± 0.002</td>
<td>475</td>
</tr>
<tr>
<td>S2</td>
<td>4</td>
<td>0.765 ± 0.001</td>
<td>537</td>
</tr>
<tr>
<td>S3</td>
<td>6</td>
<td>0.770 ± 0.002</td>
<td>541</td>
</tr>
<tr>
<td>S4</td>
<td>8</td>
<td>0.905 ± 0.001</td>
<td>654</td>
</tr>
</tbody>
</table>

*Readings are average mean of 4 set of flasks

In the HPTLC analysis, the base line separation of the retention times of standard phyllanthin and hypophysylanthin was found to be 0.28 and 0.37 minutes. Standard curves of phyllanthin and hypophyllanthin were constructed using simple linear regression at wavelength of 280 nm. The calibration curves were plotted showing the peak area of phyllanthin and hypophyllanthin, which were recorded separately and the percentage W/W was reported for separate treatments.

For quantification of phyllanthin and hypophyllanthin in P. amarus immobilized cells of 14 day samples from MS medium in incubator cum shaker were taken. Stock solutions of phyllanthin and hypophyllanthin were prepared by HPTLC grade methanol, to obtain concentration of 200 µg and calibration curves were plotted, using HPTLC-integration by CAMAG TLC evaluation software. HPTLC precoated plates at 60 °F 254 (Merck) and automatic sample III (CAMAG) were used and integrated with CATS V4.06, S/N : 051A011/Sc3 V1.14, S/N: 041123.

HPTLC Plates (20 cm x 20 cm) were developed using hexane: ethyl acetate solvent system (2 : 1) (Application mode CAMAG Automatic TLC Sampler III, Development mode CAMAG Twin Trough Chamber) [11].

Calibration curves were plotted showing the peak height and distance travelled by the peak after calculating the factor x area divided by amount of sample applied. The percentage (W/W) of phyllanthin and hypophyllanthin was calculated in different treated and control samples as per their concentration and was reported [12]. In the chromatogram the first peak seen was of chlorophyll and the second peak of phyllanthin, 4th peak of hypophyllanthin, while the other peaks which are seen are of other unidentified lignans, terpenes etc. present in P. amarus, apart from phyllanthin and hypophyllanthin.
3 Results and Discussion

After the immobilization of cell culture of *P. amarus* in calcium alginate beads in MS medium, studies were carried out to find out the impact of supplementation with chitosan, copper sulphate, phenylalanine and silver nitrate under aseptic conditions after immobilization on the increased accumulation of secondary metabolites to enhance the production of phyllanthin and hypophyllanthin as compared to control.

It was reported that an optimal concentration of a suitable sterilizing agent with ideal exposure period depends on the nature of plant for successful surface sterilization of the leaves. Earlier reports suggested that 0.01% W/V mercuric chloride was found to be suitable for surface sterilization of leaves.

MS medium was modified by adding chitosan, copper sulphate, phenyl alanine and silver nitrate solution, [biotic elicitors] for enhancing of *P. amarus* bioactives in immobilized cells.

3.1 Chitosan treatment

Immobilized cell system of *P. amarus* in MS medium showed maximum enhancement in the yield of phyllanthin and hypophyllanthin with 20 mL of 1% solution of chitosan. It was 238% when compared to control immobilized cell cultures after HPTLC analysis. The entrapment efficiency of chitosan depends on elicitor specificity, cell line of elicitor used, presence of growth regulators, composition of culture medium and the environmental conditions.

Elicitation of immobilized *plumbago rosea* cells with chitosan proved highly effective by using an extracellular site for the product accumulation where plumoligin production was increased about 21 times by collective use of immobilization, elicitation and two phase culture [13].

3.2 Copper sulphate treatment

With immobilized cell system of *P. amarus* in MS medium when the addition of 2, 4, 6 and 8 mL of 1% copper sulphate solution was done, the enhancement in the content of phyllanthin and hypophyllanthin was 0.305, 0.420, 0.525 and 0.560% W/W, respectively. The maximum enhancement was 367% with 8 mL of copper sulphate solution elicitation when compared to control immobilized cultures after HPTLC analysis.

There are several reports indicating enhanced production of secondary metabolites from suspension cultures of higher plants with addition of copper sulphate. It was reported that copper ions were suitable for inducing the accumulation of high levels of sesquiterpenoid phytoalexins in fruit cavities of *Datura stramonium*; in cell suspension culture, the highest levels of products were formed in response to 1 mmol·L⁻¹ copper ions [14].

3.3 Phenylalanine treatment

Immobilized cell system of *P. amarus* in MS medium elicitation with 1% solution of phenylalanine 2, 4, 6 and 8 mL showed enhancement in 0.560%, 0.585%, 0.590% and 0.615% W/W, respectively in yield of bioactives. The maximum enhancement of phyllanthin and hypophyllanthin was 413% in 8 mL of phenylalanine when compared to control immobilized cultures.

DiCosmo F and Misawa M reported that the addition of phenylalanine into the agar medium of *Taxus cuspidata* cells was found to stimulate the biosynthesis of taxol. These results are in agreement with the earlier reports of enhanced production of secondary metabolites with phenylalanine on the cell cultures of *Capsium annuum*, *Cephaelis ipecucuanha*, *Taxas wallichtana*, *T. cuspidata* etc. [16]. Ballica et al. reported that tropane alkaloids yield was five times higher in *Datura stramonium* cell cultures supplemented with L-phenylalanine than in the control cultures.

3.4 Silver nitrate treatment

Immobilized cell system of *P. amarus* in MS medium elicitation with 1% solution of silver nitrate 2, 4, 6 and 8 mL showed enhancement in 0.690%, 0.765%, 0.770% and 0.905% W/W, respectively in the yield of bioactives. The maximum enhancement of phyllanthin and hypophyllanthin was 654% in 8 mL of silver nitrate when compared to control (0.120%) immobilized cultures.

4 Conclusion

Immobilized cell cultures of *P. amarus* with different treatments in MS media, the content of phyllanthin and hypophyllanthin enhanced maximum with 1% solution of silver nitrate 654% followed by phenylalanine 413%, copper sulphate 367% and chitosan 238% when compared to control sample 0.120% (Table 5, Fig. 6). A graph was plotted to show the percentage enhancement of phyllanthin and hypophyllanthin in immobilized cell system with different treatments (X ± s).

Table 5 Comparative enhancement of phyllanthin and hypophyllanthin in immobilized cell system with different treatments (X ± s)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment</th>
<th>Phyllanthin and Hypophyllanthin (W/W)</th>
<th>Percentage enhancement/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0.120 ± 0.001</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Chitosan</td>
<td>0.405 ± 0.001</td>
<td>238</td>
</tr>
<tr>
<td>3</td>
<td>Copper sulphate</td>
<td>0.560 ± 0.001</td>
<td>367</td>
</tr>
<tr>
<td>4</td>
<td>Phenyl alanine</td>
<td>0.615 ± 0.001</td>
<td>413</td>
</tr>
<tr>
<td>5</td>
<td>Silver nitrate</td>
<td>0.905 ± 0.001</td>
<td>654</td>
</tr>
</tbody>
</table>

Fig. 6 Comparative percentage enhancement of phyllanthin and hypophyllanthin in immobilized cell system of *P. amarus*
show the percentage increase with different treatments in immobilized cell system of *P. amarus* and compared with control in which the percentage enhancement of phyllanthin and hypophyllanthin was reported.

Cell viability studies were also done using fluorescence diacetate stain method for *P. amarus* in MS medium of each supplementation and elicitation, by UV. fluorescence microscope and it was found that 66% cells were living cells in the immobilized cell cultures.[18]

On the basis of the HPTLC analysis of control as well as the treated samples of *P. amarus* immobilized cell system,[19] it was found that there was maximum increase in phyllanthin and hypophyllanthin yield using silver nitrate followed by phenylalanine, copper sulphate, chitosan, as elicitors when compared to control (0.120%).

The study revealed that the addition of copper sulphate, phenylalanine, chitosan and silver nitrate as biotic elicitors in the MS medium enhanced maximum production of phyllanthin and hypophyllanthin in immobilized *P. amarus* cells as compared to control sample. The production of hepatoprotective bioactives, phyllanthin and hypophyllanthin in *P. amarus* enhanced by immobilization cell system in MS medium by supplementing with different abiotic elicitors reported after HPTLC analysis.

**Acknowledgement**

The author is thankful to Mr. Sudhakar Agarwal, director and Dr. Jaydeep, Incharge Research and Development, Indian Herbs and Research Supply Co. Saharanpur (U.P.) for their help in the analytical work. The author is also thankful to AICTE for providing fellowship under its QIP program.

**References**


