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# Immobilization mediated enhancement of Phyllanthin and Hypophyllanthin from *Phyllanthus amarus*

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**Abstract.** Phyllanthus amarus plant is used in traditional system of medicine as hepatoprotective drug for which the major lignans phyllanthin and hypophyllanthin are responsible. As so far no significant work has been done on culture aspect of this plant, hence realizing the hepatoprotective potential, the present investigation was undertaken.

A cost effective process was developed for enhancing phyllanthin and hypophyllanthin utilizing immobilization technique. HPTLC was used to compare phyllanthin and hypophyllanthin contents in calcium alginate immobilized cells obtained from fresh grown plants and M.S. medium was supplemented with different abiotic elicitors, for treatment with chitosan, copper sulphate, phenylalanine and silver nitrate solution to make whole process commercially viable. It was revealed that silver nitrate and phenylalanine in low concentration enhances phyllanthin and hypophyllanthin yield as compared to control.

The study revealed that increase in the content of phyllanthin and hypophyllanthin was elicitor concentration dependent and silver nitrate treatment give maximum yield of hepatoprotective bioactives as compared to other abiotic elicitors used.

**Keywords**: Phyllanthus amarus, Immobilization, Elicitors, Lignans, Hepatoprotective, HPTLC.

## 1. Introduction

The aim of plant biotechnology research is to optimize the concentrations of precursors, abiotic and biotic elicitors with duration of their contact for maximum response to the production of targeted secondary metabolite is very much desired.

*Phyllanthus amarus* is an important hepatoprotective drug being used since time immemorial. 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 world. Due to its hepatoprotective property, it is in great demand. However, scanty growth and short life span (July to October) and requirement of damp weather for growth are the factors responsible for the short supply of *P. amarus*. These factors make this plant a suitable candidature for exploitation through biotechnology for production of its hepatoprotective bioactives.

To overcome these problems it was thought to use biotechnology for producing the bioactives - phyllanthin and hypophyllanthin using plant tissue culture technology utilizing *Phyllanthus amarus*.

## 2. Experimental

Fresh leaves of *P. amarus* collected from 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 noticeable role in the enhancement of bioactives by immobilization.

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

## 3. Sterilization of material and preparation of alginate beads for immobilization

The collected fresh leaves (25 gm) of *P. amarus* were washed with running tap water, followed by 2% tween solution, rewashed 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 by using sterilized pestle mortar to get cell homogenate of *P. amarus* leaves.

Over-night stored sodium alginate solution 20 ml (5%) was mixed thoroughly with cell homogenate for 30 min to eliminate air bubbles and to enhance viscosity. The beads, from cell homogenate were prepared using 25ml injection syringe and 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. This immobilized cell cultures of *P. amarus* leaves was used in experimentation for obtaining maximum yield of bioactives phyllanthin and hypophyllanthin.

### 3.1. 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 (1gm) was dissolved in 100ml of distilled water by heating at 60ºC for 15 minutes with 2 ml of glacial acetic acid to make 1% (w/v) chitosan solution. The pH of the solution (100ml) was adjusted to 5.5 with 1N sodium hydroxide solution, and autoclaved at 120ºC (15 lbs/sq.inch) for 20 minutes and was filter sterilized before addition to the immobilized cell culture. Four set (in triplicate) sterilized conical flasks were taken with 50ml MS medium and labelled as AC [ control] CT1, CT2 and CT3. containing 25gm 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 r.p.m. Immobilized cell culture (20ml) from each flask was withdrawn and HPTLC analysis was done for content of phyllanthin and hypophyllanthin in control and treated flasks.

Table 1.1 HPTLC analysis of immobilized P. amarus cell system for phyllanthin and hypophyllanthin content with Chitosan

| Flask | Chitosan (1%) ml | Phyllanthin and hypophyllanthin (w/w)* | % increase compared to control |
|---|---|---|---|
| AC | Control | 0.120±0.001 | 0 |
| CT1 | 5 | 0.261±0.001 | 117 |
| CT2 | 10 | 0.305±0.002 | 154 |
| CT3 | 20 | 0.405±0.001 | 238 |

*Readings are average mean of 3 set of flasks

From the experimental data it was observed that as the concentration of chitosan in immobilized cell culture increased from 5 to 20ml, the bioactives phyllanthin and hypophyllanthin enhanced from 0.261% to

0.405% which was higher than the control 0.120%. The maximum increase in the yield of bioactives (0.405%) with 20ml of 1% chitosan solution was to the tune of 238% **(Table 1.1)**.

## 3.2. Effect of Copper sulphate treatment

MS medium was supplemented with 1% copper sulphate solution and the stock solution 100ml (conc.1%) was filtered using sterilized 0.2 ˛ microfilter into a sterile container.

Following the process given earlier the alginate beads were prepared from 25gm of fresh leaf cell homogenate of *P. amarus* aseptically. To four set 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, 20ml immobilized cell samples from each flask was collected and analysed for bioactive contents using HPTLC.

Table1.2HPTLC analysis of immobilized P.amarus cell system for phyllanthin and hypophyllanthin content with copper sulphate.

| Flask | Copper sulphate (1%) ml | Phyllanthin and hypophyllanthin (w/w)* | % increase compared to control |
|---|---|---|---|
| AC | Control | 0.120 ± 0.001 | 0 |
| C1 | 2 | 0.305 ± 0.002 | 154 |
| C2 | 4 | 0.420 ± 0.001 | 250 |
| C3 | 6 | 0.525 ± 0.002 | 337 |
| C4 | 8 | 0.560 ± 0.001 | 367 |

*Readings are average mean of 3 set of flasks

Addition of different concentrations of copper sulphate to MS medium 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 enhancement compared to control **(Table 1.2)**.

## 3.3. Effect of Phenylalanine treatment

Phenylalanine was used as an elicitor for which 1% stock solution was prepared and sterilized with 0.2 filter, in a sterile container. In four sets (in triplicate) of flasks labelled as P1, P2, P3 and P4, 50 ml MS medium was added in each sterilized flask containing alginate beads prepared from 25gm leaf homogenate of *P. amarus* by adding 2, 4, 6 and 8 ml of 1% phenylalanine solution in each flask of respective sets. After 14 days of incubation 20 ml sample was withdrawn from each flask and analysed for bioactives by HPTLC.

Table 1.3 HPTLC analysis of immobilized P. amarus cell system for phyllanthin and hypophyllanthin content with phenylalanine.

| Flask | Phenylalanine (1%) ml | Phyllanthin and hypophyllanthin (w/w)* | % increase compared to control |
|---|---|---|---|
| AC | Control | 0.120 ± 0.001 | 0 |
| P1 | 2 | 0.560 ± 0.002 | 367 |
| P2 | 4 | 0.585 ± 0.001 | 387 |
| P3 | 6 | 0.590 ± 0.002 | 392 |
| P4 | 8 | 0.615 ± 0.001 | 413 |

*Readings are average mean of 3 set of flasks

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 *P. amarus* cell system showing 5 times enhancement in bioactives respectively as compared to control (0.120%) **(Table 1.3)**.

## 3.4. Effect of Silver nitrate treatment

Supplementation of medium was done with 1% stock solution of silver nitrate and sterilized. Four sterilized flasks in triplicates labelled as S1, S2, S3 and S4 each 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 analysed for bioactives.

Table 1.4 HPTLC analysis of immobilized P. amarus cell system for phyllanthin and hypophyllanthin content with silver nitrate

| Flask | Silvernitrate (1%) ml | Phyllanthin and hypophyllanthin (w/w)* | % increase compared to control |
|---|---|---|---|
| AC | Control | 0.120±0.001 | 0 |
| S1 | 2 | 0.690±0.002 | 475 |
| S2 | 4 | 0.765±0.001 | 537 |
| S3 | 6 | 0.770±0.002 | 541 |
| S4 | 8 | 0.905±0.001 | 654 |

*Readings are average mean of 4 set of flasks

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 8ml silver nitrate solution as compared to control 0.120% **(Table 1.4)**.

## 3.5. Estimation of phyllanthin and hypophyllanthin

For quantification of phyllanthin and hypophyllanthin in *P. amaru*s suspension and immobilized cells of 14 day samples from MS medium in incubator cum shaker, stock solutions of phyllanthin and hypophyllanthin were prepared by HPTLC grade methanol, to obtain concentration of 200µg and calibration curves were plotted, using HPTLC-integration by CAMAG TLC evaluation software. HPTLC precoated plates at 60ºF 254 (Merck), automatic sample III (CAMAG) were used and integrated with CATS V4.06, S/N : 0511A011/Sc3 V1.14, S/N: 041123. HPTLC Plates (20 x 20cm) were developed using Hexane: Ethyl acetate solvent system (2:1) (Application mode CAMAG Automatic TLC Sampler III, Development mode CAMAG Twin Trough Chamber) (**Sharma *et al.*, 1993**).

Calibration curves were plotted showing peak height and distance travelled by peak after calculating the factor × area divided by amount of sample applied and the percentage (w/w) of phyllanthin and hypophyllanthin was calculated in different treated and control samples as their concentration as reported (**Rajpal, 2002**). In the chromatogram the 1st peak seen was of chlorophyll and 2nd 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.

## 3.6. Results and discussion:

After 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 is reported that an optimal concentration of suitable sterilizing agent with ideal exposure period depends on the nature of plant for successful surface sterilization of the leaves. The earlier reports suggested that 0.01% w/v mercuric chloride was found 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.

## 3.7. Chitosan treatment

Immobilized cell system of *P.amarus* in MS medium showed maximum enhancement in the yield of phyllanthin and hypophyllanthin with 20ml of 1% solution of chitosan. It was 238% when compared to control immobilized cell cultures after HPTLC analysis **(Table 1.1)**. 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 plumbogin production was increased about 21 times by collective use of immobilization, elicitation and two phase culture **(Komariah, 2003).**

## 3.8. Copper sulphate treatment:

With immobilized cell system of P.*amarus* in M.S. medium when addition @ 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. **(Table 1.2)** 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 1mM Copper ions **(Whitehead *et al.*, 1990).**

## 3.9. Phenylalanine treatment

Immobilized cell system of P.*amarus* in MS medium elicitation with 1% solution of phenylalanine @ 2, 4, 6 and 8ml 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 8ml of phenylalanine when compared to control immobilized cultures **(Table 1.3). Dicosmo and Misawa (1995)** 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 ipecacuanha, Taxas wallichtana, T. cuspidata* etc. **(Veeresham *et al.*, 2003). Ballica *et al.*, (1993)** reported tropane alkaloids yield was five times higher in *Datura stramonium* cell cultures supplemented with L-phenylalanine then in the control cultures.

## 3.10. Silver nitrate treatment

Immobilized cell system of *P.amarus* in MS medium elicitation with 1% solution of silver nitrate @ 2, 4, 6 and 8ml showed enhancement in 0.690, 0.765, 0.770 and 0.905% w/w respectively in yield of bioactives. The maximum enhancement of phyllanthin and hypophyllanthin was 654% in 8ml of silver nitrate when compared to control (0.120%) immobilized cultures **(Table 1.4).**

Table1.5 Comparative enhancement of phyllanthin and hypophyllanthin in immobilized cell system with different treatments

| S. No. | Treatment | Phyllanthin and Hypophyllanthin (w/w) | Percentage enhancement |
|---|---|---|---|
| 1 | Control | 0.120 | 0 |
| 2 | Chitosan | 0.405 | 238 |
| 3 | Copper sulphate | 0.560 | 367 |
| 4 | Phenyl alanine | 0.615 | 413 |
| 5 | Silver nitrate | 0.905 | 654 |

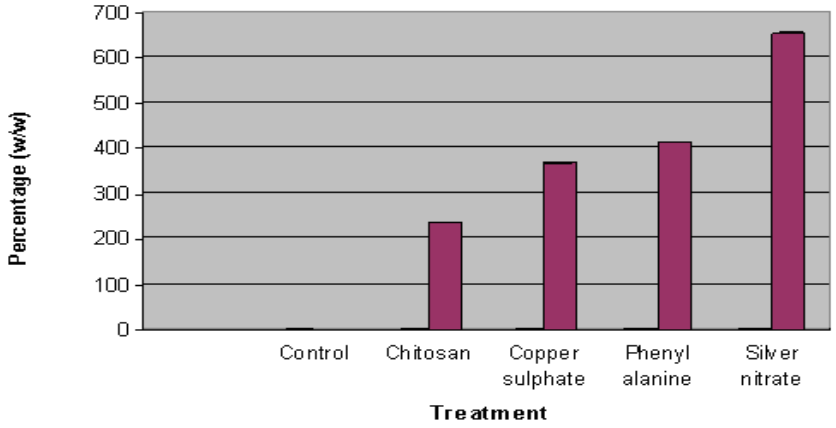


Fig1.1 Comparative percentage enhancement of phyllanthin and hypophyllanthin in immobilized cell system of P.amarus

## 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 1.5 Fig.1.1)**. A graph was plotted to 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 for *P. amarus* in MS medium in each supplementation and elicitation, by U.V. florescence microscope and it was found that 66% cells were living cells in the immobilized cell cultures.

On the basis of HPTLC analysis of control as well as treated samples of *P. amarus* immobilized cell system, 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 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.

## 5. Acknowledgement

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