PLANT SCIENCES
Study of the effect of UV-B for short duration on the callus culture of Bacopa monnieri by its qualitative and quantitative analysis using Thin layer chromatography and High-performance liquid chromatography

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ABSTRACT
In this study an attempt was made to study the effect of UV-B for short duration on the callus culture of Bacopa monnieri by its qualitative and quantitative analysis using Thin layer chromatography and High performance liquid chromatography. Bacopa monnieri L.Pennell is an important medicinal plant which belongs to family Plantaginaceae. It is rich in innumerable bioactive secondary metabolites especially which are used as neuro tonic and treatment for depression, mental health, and to enhance cognitive performance.

Five different callus culture groups were made on the basis of different concentrations of PGRs and each group had both non-treated and UV treated sets in both the sets MS media was supplemented with different concentrations of PGRs. The non-treated groups were maintained for 8 weeks without UV-B exposure but in the treated groups the cultures were maintained for 7 weeks and in the 8th week UV-B radiation were given for a period of 2 hours/day. Among all the 5 groups the best response was found in the group in which MS media was supplemented with 0.1 mg/L of 2,4-D and BAP so these sets were taken for phytochemical studies.

According to the present studies it was found that tissue culture and UV-B supplementation for short duration enhanced the secondary metabolite Bacoside which is a triterpenoid saponin and is responsible for the nootropic activity of the plant. When both the groups were compared the UV treated groups showed increased number of peaks as well as area in HPLC analysis and high range of saponins were detected in TLC analysis of UV treated callus extracts.

Keywords: Bacopa monnieri, HPLC, TLC, UV-B

INTRODUCTION
Bacopa monnieri L. Pennell is an important medicinal plant which belongs to family Plantaginaceae. It is rich in innumerable bioactive secondary metabolites especially which reduces many health issues. Bacopa is generally used as a neuro tonic and treatment for depression, mental health, and cognitive performance. Brahmi is known for its anti hepatotoxic anti inflammatory and antioxidant properties. There is a huge demand for raw materials particularly for the extraction of bioactive molecules. The conventional method of production of bacopa monnieri is not able to meet the demand therefore to overcome this, many biotechnological approaches such as plant tissue culture have been established through various tissue culture techniques such as callus culture, organ culture and cell suspension culture. Water hyssop or Brahmi is one of the most important semi aquatic plant which is generally used as a memory enhancer since ancient time in Indian subcontinent. It is a reputed nootropic herb which is native to India and Australia and also grows in warm wetlands and rice fields but the demand is largely met from wild populations which leads to depletion of the herb therefore it has been listed as an threatened species by the International Union for the conservation of natural and national resources and prioritised in list of 32 medicinal plants for cultivation and conservation by national medicinal plant board of India (2004).

Bacopa monnieri contains alkaloids bramine, nicotinine and herpestine. The main component of bacopa which is essential for its nootropic activity is bacoside A and bacoside B. Triterpenoid saponin A, B, C and pseudo jujubogenin was also found. D-mannitol, betulinic acid and stigmastanol was also found.

Bacopa is bitter, stringent which have cooling properties and is reported to improve the intellect it is used in the treatment of hoarseness, diabetes, cardiac disorders, anemia, insanity and epilepsy. It is also used in boils as a blood purifier, used in cataract complaints.

Plant cell and organ cultures have emerged as potential sources of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colouring agents, biopesticides, and food additives. In recent years, various strategies have been developed to assess biomass accumulation and synthesis of secondary compounds in cultures. Biomass accumulation and metabolite biosynthesis are two stage events, and the parameters that control the growth and multiplication of cultured cells/organs and biomass accumulation are controlled in the first stage. Parameters that assist with the biosynthesis of metabolites are controlled in the second stage. The selection of high-producing cells or organ clones; optimization of medium parameters such as suitable medium, salt, sugar, nitrogen, phosphate, and plant growth regulator levels; and physical factors such as temperature, illumination, light quality, medium pH, agitation, aeration, and environmental gas (e.g., oxygen, carbon dioxide, and ethylene) are
controlled in the first stage of the culture process. Elicitation, replenishment of nutrient and precursor feeding, permeabilization, and immobilization strategies assist with the accumulation of metabolites and can be applied in the second stage of the culture process. By following stage-specific strategies, it is possible to produce large amounts of biomass with an increase in the accumulation of secondary compounds.

2. MATERIALS AND METHODS

Different phytochemical tests were conducted for the qualitative and quantitative analysis of secondary metabolites (SMs). Analysis was done using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) methods.

A. Plant materials

Five different callus culture groups were made on the basis of different concentrations of PGRs and each group had both non-treated and UV treated sets in both the sets MS media was supplemented with different concentrations of PGRs. The non-treated groups were maintained for 8 weeks without UV-B exposure but in the treated groups the cultures were maintained for 7 weeks and in the 8th week UV-B radiation were given for a period of 2 hours/day. Among all the 5 groups the best response were found in the group in which MS media was supplemented with 0.1 mg/L of 2,4-D and BAP so these sets were taken for phytochemical studies.

B. Preparation of callus extracts (sample)

The separation of bioactive substances from plant tissues through the use of a specific solvent is known as extraction. A solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been employed. The main idea is to grind the plant material (dry or wet) finer, which improves the surface area for extraction and hence enhances the rate of extraction (Tiwari P. et al., 2011). A callus that was eight weeks old was moved to a separate volumetric flask of 10 ml and then dissolved in methanol. For the measurement of secondary metabolites, the filtrate was utilised in TLC and HPLC after the solution was ground for 20 minutes and filtered using Whatman filter paper no. 41.

C. Thin layer chromatography

1) Principle

A chromatographic method called TLC is used to separate mixtures of chemicals. Based on differential adsorption and analytical partitioning between the liquid stationary phase and mobile solvent phase, chemicals are separated.

2) Mobile phase

Solvent system: ethyl acetate: methanol: toluene: water (4:1:1:0.5) used for separation of saponins in Bacopa monnieri

3) Stationary phase

Stationary phase was prepared using TLC plate coated with silica gel, which are commercially available 60 F254 (Merck silica gel 60 F254 plate).

4) Procedure

Each solvent extract was subjected to thin layer chromatography (TLC) as per conventional one-dimensional ascending method using silica gel 60F254, 7X6 cm (Merck) were cut with ordinary household scissors. Plate markings were made with soft pencil. Glass capillaries were used to spot the sample for TLC applied sample volume 1- micro litre by using capillary at distance of 1 cm at 5 tracks. In the twin trough chamber with different solvent system ethyl acetate: methanol: toluene: water (4:1:1:0.5) used for Bacopa monnieri. After pre-saturation with mobile phase for 20 min for development were used. The movement of the active compound was expressed by its retention factor (Rf), values were calculated for different samples. The developed thin layer chromatographic plates were visualized in normal light, short UV light (254nm), and long UV light (365nm) using TLC cabinet (Electronic India).

Detection and Calculation of Rf Value

Once the chromatogram was developed the Rf Value of the spot was calculated using the formula:

\[ R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}} \]

D. High performance liquid chromatography

1) Basic principle

HPLC stands for High performance liquid chromatography (sometimes also referred to as High Pressure Liquid Chromatography). HPLC is a chromatographic technique used in analytical chemistry and analytical recent biochemistry to separate a mixture of compounds for the purpose to identify, quantify and purify the individual specific components of the complex mixture. Separation is based on the polar (hydrophilic) or non-polar (hydrophobic) tendency of analytic between two liquid phases.

2) HPLC instrumentation

Chromatographic system

The HPLC system (Waters) consisted of a pump (515), a U.V. Visible detector, a Thermo C 18 (250 X 4.6 mm, 5μm) column, a Data Ace software.

Chromatographic conditions

Mobile phase: 0.05 M sodium sulfate buffer pH 2.3 and acetonitrile (68.5:31.5, v/v).

Flow rate: 1 ml/min
UV – detection at $\lambda_{\text{max}}$ - 205 nm
Volume for injection: - 20 µl.

**Preparation of sample**

10 mg of extract was transferred to 10 ml volumetric flask containing methanol. The solution was sonicated for 25 min and the final volume was made with methanol. The mixture was then filtered through a 0.45 µm filter. The stock solution was further diluted sufficiently with methanol to get sample solution of drug concentration of 10µg/mL. Sample volume of 20µl was injected to determine the retention time of Bacoside A

**Selection of separation variable**

**Table 1: Separation Variable**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td></td>
</tr>
<tr>
<td>Dimension</td>
<td>250 mm x 4.60 mm</td>
</tr>
<tr>
<td>Particle Size</td>
<td>5 µµ</td>
</tr>
<tr>
<td>Bonded Phase</td>
<td>Octadecylsilane (C_{18})</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td></td>
</tr>
<tr>
<td>0.05 M sodium sulfate buffer pH 2.3</td>
<td>68.5</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>31.5</td>
</tr>
<tr>
<td>Diluent</td>
<td>Methanol</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Sample Size</td>
<td>20 µl</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>205 nm</td>
</tr>
<tr>
<td>Retention time</td>
<td>2.353 min</td>
</tr>
</tbody>
</table>

**3. RESULTS AND DISCUSSION**

A. Qualitative estimation of phenolic compounds by TLC

TLC of methanolic callus BM-C extract (non treated) showed Rf values of 0.72 and BM-UV (UV treated callus) showed 3 fractions with Rf values ranging from 0.70 to 0.92 at long UV exposure (Table 1)Rf values of all samples were identified of saponins bacoside A and B which indicated the presence of saponins.(fig1)
Normal Light  Short UV  Long UV
Figure 1. TLC of Bacopa monnieri

Table 2: TLC of Bacopa monnieri Control

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Mobile phase</th>
<th>Spot Distance</th>
<th>R_f value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Distance Travelled by Solvent = 5.0 cm&lt;br&gt;Etanol acetate: Methanol: Toluene: Water (4:1:1:0.5)&lt;br&gt;No. of bands at Long UV = 1&lt;br&gt;No. of bands at Short UV = 0&lt;br&gt;No. of bands at Normal Light = 0</td>
<td>= 3.6</td>
<td>= 0.72</td>
</tr>
</tbody>
</table>

Table 3: TLC of Bacopa monnieri UV Treated

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Mobile phase</th>
<th>Spot Distance</th>
<th>R_f value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Distance Travelled by Solvent = 5.0 cm&lt;br&gt;Etanol acetate: Methanol: Toluene: Water (4:1:1:0.5)&lt;br&gt;No. of bands at Long UV = 3&lt;br&gt;No. of bands at Short UV = 0&lt;br&gt;No. of bands at Normal Light = 0</td>
<td>= 3.5, 4.1, 4.6</td>
<td>= 0.70, 0.82, 0.92</td>
</tr>
</tbody>
</table>

B. Quantitative estimation of bacosides by HPLC

In the present study a methods was developed by using HPLC for quantitative estimation of saponins in Methanolic extracts of Bacopa monnieri callus using Mobile phase of 0.05 M sodium sulfate buffer pH 2.3 and acetonitrile (68.5:31.5, v/v). It was observed that there was a significant increase in the peak numbers and area of UV-B treated Bacopa callus as discussed in table 4. A representative chromatogram of Bacoside A has been given in Fig. 2.

<table>
<thead>
<tr>
<th>Non-treated callus extract</th>
<th>Treated callus extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Number</td>
<td>Rt. (min)</td>
</tr>
<tr>
<td>1.</td>
<td>2.353</td>
</tr>
<tr>
<td>2.</td>
<td>3.129</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4-Results for HPLC analysis of Bacopa monnieri non-treated and treated callus extract
The UV-treated samples of *Bacopa monnieri* were compared with control samples showed that the number of peaks of UV-treated samples were higher than control samples. It means UV-B light (for short period) can enhance the productivity of compounds in the plants. UV-B induction of these secondary biosynthetic pathways and the UV-B induction regulatory genes are likely to be used in combination with new high-throughput strategies to identify the most preferred route correlating with desired metabolite biosynthesis in different plants under UV-B exposure experiments. Kumari Rima et al., (2013), Eichholz I. et al., (2011), Johnson C. B. et al., (1999)

**Figure 2: Chromatogram of Control**

**Figure 3: Chromatogram of UV Treated**

### 4. CONCLUSION

Secondary metabolites (SMs) have a significant economic role in a variety of ecological processes, including pollination, ecological consequences, plant defence, and plant breeding. Due to their toxicity and ability to repel herbivores and microbes, plants produce a wide variety of natural products (SMs), some of which are also crucial for plant communication with other organisms and some of which serve a specific purpose in defence against abiotic stress (such as UV exposure). These SMs have little to do with plant growth and development. Plant SMs have biological processes that are vital to human existence. Plant SMs have advantages, and they are used in perfumes, cosmetics, food industry, traditional and contemporary medicine. When compared to the Mobile phase SS-I, the *Bacopa monnieri* callus MeOH extract demonstrated the highest fractions with Rf values. In comparison to the untreated callus extract, the largest proportion of Rf values were found in the SS-II phase in the UV-treated callus extract. These investigations show that the SS-II phase and UV treated callus extract provided the best results for the study of saponins. In comparison to untreated callus extract, the UV treated callus extract identified more peaks. The UV-treated sample showed that, as compared to the untreated sample, exposure to UV-B light (for a brief period of time) might increase the number of peaks.

These findings demonstrated the potential for economic and medical exploitation of secondary metabolites in PTC with UV supplementation. Increased UV-B radiation appeared to have a favourable, neutral, or negative impact on plant growth, chlorophyll content, cell size, growth index, and callus induction rate, according to earlier research. Short-term exposure to UV-B light stresses the callus more, which prompts it to create SMs as a defensive biochemical. Therefore, UV-B therapy may be advantageous for the growth of SMs; however, prolonged exposure to UV-B radiation may be toxic for calluses and result in cell death.
5. ACKNOWLEDGEMENTS
We appreciate the Principal and my mentor, Dr. Pinky Dwivedi of the Govt. Madhav Science PG College in Ujjain, for approving the experimental efforts.

REFERENCES
2. Arya Deepika and Vidyadhar Patni (2013), Comparative analysis of total flavonoids and quercetin content in vivo and in vitro and enhancement of quercetin via precursor feeding inpluchea lanceolata oliver & hiern, 5, 617-621