FLAVONOIDS – CHEMICAL CONSTITUENTS OF BOERHAAVIA DIFFUSA L. THROUGH TLC AND HPTLC
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ABSTRACT:
Boerhaavia diffusa L. (Satodi) is important medicinal plant. Plant tissue culture is very useful to increase the production of secondary metabolites. Callus was obtained on MS supplemented with 1.5 mg/l NAA and 1.5 mg/l KIN. Chromatography is the second important technique for analysis and isolation of these herbal drugs. Qualitative and quantitative analysis was done through TLC and HPTLC. TLC of in vitro suggested quercetin, kaempferol, 8 unidentified free flavonoids and 5 unidentified bound flavonoids were obtained in in vitro materials. HPTLC of in vitro materials was done and scanned at different wavelengths (200 nm, 254 nm, 366 nm). Calibration spectrum, Rf values and amount of particular spot were recorded at particular wavelength through scanning. Total 13 flavonoids at 200 nm, 9 at 254 nm, 10 at 366 nm were scanned from in vitro (8 week old callus) B. diffusa. Absorption spectra of total 8 flavonoids at 190 nm were obtained from B. diffusa. Some of these are confirmed while others are unidentified.

KEY WORD: Flavonoids, Boerhaavia diffusa, TLC, HPTLC.

INTRODUCTION:
Plants are very important commercial source of chemical compounds including primary and secondary metabolites. Ayurveda practitioner employ these plant to cure swelling, poultes lesions, tubercular ulcers, scabies, ophthalmia, muscular pain, dropsy, rheumatism, diabetes and even cancer. These properties are mainly because of its primary and secondary metabolites of drugs. This drug is naturally present in the parts like leaf, stem, root, seed or some times in the whole plant. These parts can be artificially cultured on the media and can get maximum amount of drugs. For it, Murashige and Skoog’s, 1962 (MS) medium is used as a basal nutrient medium. Different types of growth promoters like 2, 4-D,
IAA, IBA, NAA, Kinetin, BAP are used to prepare hormonal medium. Even it can be prepared with alone auxin or cytokinin or in combination of both (Vasil, 1984). Amount of drugs, which are artificially produced in 8 week old callus can be compared with naturally occur in plant (whole plant). Plant tissue culture approach has been found to be advantageous as it provides a continuous and reliable source of artificial product year around without the destruction of entire plant. With the help of tissue culture, high quantities of desired compounds can be obtained.

Flavonoids have 15-carbon in their basic skeleton. They are widely spread, soluble phenolic derivatives that are variously coloured. They are restricted to the vacuole though some are found in chloroplast and chromoplast. Flavonoids are glycosides and their glycones vary depending upon the flavan structure, which comprises two aromatic rings joined in a chroman structure by a 3-carbon unit (C_6-C_3-C_6). Free glycones are found in dead woody tissue and formed from free flavonoids through hydrolysis and are variously classified depending upon the state of oxidation of the C_3 unit in the molecule. The flavonoids, chalcones etc. are chief classes of flavonoids, which contribute to the colour scheme of the plants. On the contrary, flavonols and flavones do not contribute to the colour of various plant organs. Bioflavonoids have as many as C_{30} atoms in their basic skeleton and are obtained from dimerisation of flavones by C-C linkage. They are found in the gymnosperms. Flavonoids directly form the C_6-C_3 portion of phenylpropanoids. Flavonoids are distinguished by various increasing state of the C_3 chain. Glycosides involving fine aglycones were isolated from parsley cell suspension culture. The flavones (apigenin, luteolin, chrysoniol) occurred either as 7-0-glycoside or 7-0-apiglycosides while the flavonols (quercetin, isorhamnetin) are 3-0-monoglycoside or 3-7-diglycosides. β-glycosidase enzyme for degradation of flavonol-3-glycoside but each cell has not this enzyme so degradation of flavonoid is not so common. Based on the number of carbon atoms in their skeleton, phenolics are divided into several major groups (Hess, 1975) (1) Simple phenolics (2) Phenol carboxylic acid (3) Flavan derivatives. Hence, the present investigation was taken up for qualitative and quantitative analysis of in vitro produced flavonoids through TLC and HPTLC.

**PLANT MATERIAL:**

**Scientific name** : *Boerhaavia diffusa* L.

**Family** : Nyctaginaceae

**Common names** : Satodi, Punarnava (Nadkarni, 1954)

**Chemical constituents:**

Alkaloids : Punarnavine-1, Punarnavine-2

Steroids : β-Sitosterol, β-Sitosterol-D-glucoside, Sitosteryl oleate, Sitosteryl palmitate
Flavonoids: C-methyl flavone, Kaempferol, Quercetin
Rotenoids: Boerhaavinone A, Boerhaavinone B, Boerhaavinone C, Boerhaavinone D, Boerhaavinone E, Boerhaavinone F
Lignans (Irridoids): Liriodendrin, Stringarsionol, Mono-D-glucoside, β-D-glucoside

**Medicinal properties and uses:** Whole plant is diuretic, laxative, expectorant, stomachic, diaphoretic, emetic, anthelmintic, febrifuge, purgative, cardio-tonic, saporific, refrigerant. It has anti-biliary, anti-pyretic, anti-inflammatory and anti-dote to spider and snake bites activities. It is used in dropsy, jaundice, gonorrhoea, asthma, anaemia, oedima, ascites, calculus, cough, colic, haemorrhage, heart diseases, insomnia, abdomen tumour, malaria, hysteria, convulsion, gastritis, enteritis, dysmenorrhoea and leprosy.

**METHODOLOGY:**

(A) **TISUE CULTURE**

1. Preparation of nutrient medium
   - Preparation of stock solutions
   - Mix the solutions and stir it
   - Dissolve sugar and add agar-agar
   - Heat the above solution till agar-agar dissolve
   - Add stock solutions of PGRs and make up the volume
   - Adjust pH with HCl or NaOH to 6.8
   - Pour the medium into culture vials
   - Plug the vials with non-absorbent cotton
   - Autoclave for 15 minutes at 120° C and cool it
2. Inoculation and Culturing of the explants
   - Explants should be washed in soap water, tap water and finally rinsed with distilled water
   - Under aseptic condition treatment with 0.1% HgCl₂ is given for few minutes and finally rinsed with DW
   - Explants are introduced into the vial over a flame to avoid microbial contamination
   - Immediately plug the vial
3. Maintaining the culture vials under controlled light and temperature conditions
4. Periodic observations of the cultured explants
5. Maintaining the culture through subculture
6. Conclude and make inferences about the experiment conducted / field trial of *in vitro* raised plantlets

**(B) THIN LAYER CHROMATOGRAPHY (TLC)**

The operation performed in TLC is essentially the same as in paper chromatography. This technique involves several steps:

1. Preparing thin layer (e.g. silica gel G.)
2. Choice of solid for support (e.g. glass plate)
3. Sample application

Extraction of flavonoids: (Subramanian and Nagaraja, 1969)

**Procedure:** 100 mg dried, powdered callus was extracted with methanol in soxhlet apparatus. Petroleum ether was mixed with methanolic extracts. Both the layer was separated with the help of separating funnel. Methanolic fraction was several times washed with petroleum ether. Petroleum ether fraction (fraction I) was discarded due to the presence of fatty substances. Solvent ether was added to the first methanolic part in the ratio of 1:1. This fraction (fraction-2) was further analysed for free flavonoids. Ethyl acetate was added to the methanolic part in the ratio of 1:1. This fraction (fraction-3) was further analysed for bound flavonoids.

Sample extract: Fraction-1 was discarded.

Fraction-2 was redissolved in solvent ether

Fraction-3 was redissolved in ethyl acetate

4. Choice of solvent (mobile phase)

Benzene : Acetic Acid (125:25) for flavonoids

5. Development of chromatograph

6. Detecting or spraying reagent

7. Identification and calculation of Rf value

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

**(C) HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)** (Sethi, 1996)

- **Formulation**: Herbal drugs
- **Classification**: Alkaloids / Steroids / Flavonoids
- **Dosage form**: Liquid
Sample preparation

**Extraction of flavonoids:** (Subramanian and Nagarajan, 1969)

**Procedure:** 100 mg dried, powdered callus was extracted with methanol in soxhlet apparatus. Petroleum ether was mixed with methanolic extracts. Both the layer was separated with the help of separating funnel. Methanolic fraction was several times washed with petroleum ether. Petroleum ether fraction (fraction I) was discarded due to the presence of fatty substances. Solvent ether was added to the first methanolic part in the ratio of 1:1. This fraction (fraction-2) was further analysed for free flavonoids. Ethyl acetate was added to the methanolic part in the ratio of 1:1. This fraction (fraction-3) was further analysed for bound flavonoids.

Sample extract: Fraction-1 was discarded. Fraction-2 was redissolved in solvent ether Fraction-3 was redissolved in ethyl acetate

**Mobile phase:** Benzene : Acetic Acid (125:25) for flavonoids

**Conditions of Chromatography:**

1. Test plate : HPTLC precoated plate, silica gel 60 F<sub>254</sub> – aluminium (Merk)
2. Format : 10 X 10 cm<sup>2</sup>
3. Thickness : 250 µm
4. Spotting volume : 2 µl, 4 µl, 6 µl, 8 µl (Linomat IV)
5. Separation technique : Ascending
6. Development chamber : Twin-trough glass chamber (10 X 10 cm<sup>2</sup>) (Camag) (saturate for 10 minutes prior to development)
7. Mobile phase : Benzene : Acetic Acid (125:25) for flavonoids
8. Spraying reagent : 0.1 % AlCl₃ in alcohol
9. Relative Humidity : 52%
10. Temperature : 24º C
11. Migration distance : 80 mm
12. Migration time : (30 minutes for flavonoids)
13. Detection : UV

**Densitometric Scanning (Camag Scanner III)**

1. Wavelength and Mode
   a. 200 / 254 nm - Absorbance / Reflectance
   b. 366 nm – Fluorescence / Reflectance
2. Slit Dimension : 6 X 0.45 mm.
RESULTS AND DISCUSSION:

Table (1): Tissue culture results of *Boerhaavia diffusa*

<table>
<thead>
<tr>
<th>Explants</th>
<th>Level of Auxins</th>
<th>Level of Cytokinins</th>
<th>Callus initiation (In week)</th>
<th>Characters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>Concentration (mg/l)</td>
<td>Name</td>
<td>Concentration (mg/l)</td>
</tr>
<tr>
<td>Stem and Buds</td>
<td>NAA</td>
<td>0.5</td>
<td>KIN</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf</td>
<td>2.4-D</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.4-D</td>
<td>2</td>
<td>BAP</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (2): TLC results of Free & Bound flavonoids of 8 week old callus of *Boerhaavia diffusa*

<table>
<thead>
<tr>
<th>Materials</th>
<th>Compounds</th>
<th>Spray treatment</th>
<th>Iodine treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rf Colour</td>
<td>Rf Colour</td>
</tr>
<tr>
<td>Free flavonoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callus</td>
<td>Unidentified 1</td>
<td>- -</td>
<td>0.41 Dark brown</td>
</tr>
<tr>
<td></td>
<td>Unidentified 2</td>
<td>0.58 Brown</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 3</td>
<td>0.66 Green yellow</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 4</td>
<td>0.70 Green yellow</td>
<td>0.71 Brown</td>
</tr>
<tr>
<td></td>
<td>Unidentified 5</td>
<td>0.73 Green yellow</td>
<td>0.73 Brown</td>
</tr>
<tr>
<td></td>
<td>Unidentified 6</td>
<td>0.76 Green yellow</td>
<td>0.76 Brown</td>
</tr>
<tr>
<td></td>
<td>Unidentified 7</td>
<td>0.81 Green yellow</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 8</td>
<td>0.84 Green yellow</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>0.86 Yellow</td>
<td>0.86 Brown</td>
</tr>
<tr>
<td></td>
<td>Unidentified 9</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Bound flavonoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callus</td>
<td>Unidentified 1</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 2</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 3</td>
<td>0.62 Yellow green</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 4</td>
<td>0.66 Yellow green</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 5</td>
<td>0.69 Yellow green</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 6</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Unidentified 7</td>
<td>0.73 Yellow</td>
<td>0.73 Brown</td>
</tr>
</tbody>
</table>
Table (3): HPTLC results of Flavonoids of 8 week old callus of *Boerhaavia diffusa*

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Spot number</th>
<th>Scanning at different wavelengths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>200 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rf</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unidentified 8</td>
<td>0.75</td>
<td>Yellow</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.78</td>
<td>Yellow</td>
</tr>
<tr>
<td>Unidentified 9</td>
<td>0.88</td>
<td>Green brown</td>
</tr>
<tr>
<td>Unidentified 10</td>
<td>0.91</td>
<td>Brown</td>
</tr>
<tr>
<td>Unidentified 11</td>
<td>0.94</td>
<td>Brown</td>
</tr>
</tbody>
</table>

**REFERENCES:**


**Figure 1: quantitative estimation at 200 nm through HPTLC**
Figure 2: quantitative estimation at 254 nm through HPTLC

Figure 3: quantitative estimation at 366 nm through HPTLC
Photo 1: HPTLC photo under fluorescent light  Photo 2: HPTLC photo under UV light without stain

Photo 3: HPTLC photo under UV light with stain  Photo 4: HPTLC photo under visible light