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# ALKALOIDS – 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 total 6 unidentified alkaloids were analysed from *B. diffusa.* 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 9 alkaloids at 200 nm were scanned from *in vitro* (8 week old callus) *B. diffusa.* Absorption spectra of total 8 alkaloids at 190 nm were obtained from *B. diffusa.* Some of these are confirmed while others are unidentified.

***KEY WORD:*** *Alkaloids, 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, poultices 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.

The term alkaloid was proposed by Meissner (1918) for the basic, nitrogen containing compounds of plant origin and defined as "natural plant compounds that have a basic character and contain at least one nitrogen atom in a heterocyclic ring" or "basic nitrogenous plant products mostly optically active and possessing nitrogen heterocycles as their structural units with a pronounced physiological action". Pelletier (1982) proposed a definition as "an alkaloid is cyclic organic compound contains nitrogen in a negative oxidation state which is of limited distribution among living organisms". The nitrogen may exist as a primary amine ($RNH_2$), secondary amine ($R_2NH$) and tertiary amine ($R_3N$). Such compounds are basic but the degree of basicity varies greatly depending upon the structure of the molecule, the presence and location of other functional groups. The free alkaloids are usually soluble in ether or chloroform or other relatively nonpolar immiscible solvents in which however alkaloid salts are insoluble. The most common amino acids that act as precursors in alkaloid biosynthesis are ornithine, lysine, methionine, phenylalanine, tyrosine and tryptophan. Hence, the present investigation was taken up for qualitative and quantitative analysis of *in vitro* produced alkaloids 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, Boerhaavinon 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.

## *MATERIALS AND METHODS:*

### (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 $120^0$ 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% $HgCl_2$ 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 alkaloids:** (Constabel et al., 1981)

**Procedure**: 100 mg dried, powdered callus mass was suspended in 50 ml methanol, stirred for 5 minutes and filtered. The callus was further washed with 50 ml methanol and filterate was collected. This step was done for three times at the interval of 24 hours. Methanolic filterates were pooled together and concentrated *in vacuo*. The dried content was partitioned between 50 ml ethyl acetate and 50 ml 1N HCl (8.24 ml concentrated HCl was diluted up to 100 ml DW.) with the help of separating funnel. The aqueous portion (acedic) was removed and organic fraction (ethyl acetate) was repeatedly washed with 1N HCl till colourless aqueous portion was obtained. The combined aqueous portion (acedic) was neutralized with sodium bicarbonate and pH was adjusted at 10 with 10N NaOH and then partitioned with 50 ml ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness. The dry residue was triturated with ethyl acetate or dichloromethane and filtered or decanted away from the insoluble materials. Evaporation of the filterate was used for indole alkaloids. Residue was redissolved in 2-5 ml ethyl acetate for the analysis of alkaloids.

**Sample extract:** Residue was redissolved in ethyl acetate

4. Choice of solvent (mobile phase)

Methanol : Ethyl acetate (1:9) for alkaloids

5. Development of chromatograph

6. Detecting or spraying reagent

1% Cerric ammonium sulphate (1g Cerric ammonium sulphate was dissolved in 100 ml hot ortho phosphoric acid) (Fransworth et al., 1964) for alkaloids

7. Identification and calculation of Rf value

$$Rf = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by solvent}}$$

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

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

  **Extraction of alkaloids:** (Constabel et al., 1981)

  **Procedure**: 100 mg dried, powdered callus mass was suspended in 50 ml methanol, stirred for 5 minutes and filtered. The callus was further washed with 50 ml methanol and filterate was

collected. This step was done for three times at the interval of 24 hours. Methanolic filterates were pooled together and concentrated *in vacuo*. The dried content was partitioned between 50 ml ethyl acetate and 50 ml 1N HCl (8.24 ml concentrated HCl was diluted up to 100 ml DW.) with the help of separating funnel. The aqueous portion (acedic) was removed and organic fraction (ethyl acetate) was repeatedly washed with 1N HCl till colourless aqueous portion was obtained. The combined aqueous portion (acedic) was neutralized with sodium bicarbonate and pH was adjusted at 10 with 10N NaOH and then partitioned with 50 ml ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness. The dry residue was triturated with ethyl acetate or dichloromethane and filtered or decanted away from the insoluble materials. Evaporation of the filterate was used for indole alkaloids. Residue was redissolved in 2-5 ml ethyl acetate for the analysis of alkaloids.

**Sample extract:** Residue was redissolved in ethyl acetate

- Conditions of Chromatography:

1. Test plate : HPTLC precoated plate, silica gel 60 $F_{254}$ – aluminium (Merk)
2. Format : 10 X 10 $cm^2$
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 $cm^2$) (Camag) (saturate for 10 minutes prior to development)
7. Mobile phase : Methanol : Ethyl acetate (1:9) for alkaloids
8. Spraying reagent : 1% Cerric ammonium sulphate (1g Cerric ammonium sulphate was dissolved in 100 ml hot ortho phosphoric acid) (Fransworth et al., 1964)
9. Relative Humidity : 52%
10. Temperature : 24° C
11. Migration distance : 80 mm
12. Migration time : (30 minutes)
13. Detection : UV

- Densitometric Scanning (Camag Scanner III)

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

.

## *RESULTS AND DISCUSSION:*

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

| Explants | Level of Auxins | | Level of Cytokinins | | Callus initiation (In week) | | | | *Characters* |
|---|---|---|---|---|---|---|---|---|---|
| | Name | Concentration (mg/l ) | Name | Concentration (mg/l ) | 1 | 2 | 3 | 4 | |
| Stem and Buds | NAA | 0.5 | KIN | 0.5 | - | - | - | + | Brownish white coloured and friable callus |
| | | 1 | | 1 | - | - | + | + | |
| | | 1.5 | | 1.5 | + | ++ | +++ | +++ | |
| | | 2 | | 2 | - | ++ | ++ | ++ | |
| | | 3 | | 3 | - | - | + | ++ | |
| Leaf | 2,4-D | 0.5 | - | - | - | - | - | + | White coloured and much friable callus |
| | | 1 | | - | - | - | - | + | |
| | | 1.5 | | - | - | - | ++ | ++ | |
| | | 2.0 | | - | - | ++ | ++ | ++ | |
| | 2,4-D | 1 | BAP | 1 | - | - | - | + | Less growth of callus |
| | | 2 | | 2 | - | - | - | + | |
| | | 3 | | 3 | - | - | ++ | ++ | |
| | | 4 | | 4 | - | ++ | ++ | +++ | |

**Table (2): TLC results of Alkaloids of 8 week old callus of *Boerhaavia diffusa***

| Materials | Compounds | Spray treatment | | Iodine treatment | |
|---|---|---|---|---|---|
| | | Rf | Colour | Rf | Colour |
| Callus | Unidentified 1 | - | - | 0.08 | Brown |
| | Unidentified 2 | 0.11 | Brown | - | - |
| | Unidentified 3 | 0.50 | Brown | 0.50 | Brown |
| | Unidentified 4 | 0.77 | Bluish green | - | - |
| | Unidentified 5 | - | - | - | - |
| | Unidentified 6 | 0.91 | Greenish brown | 0.90 | Brown |

**Table (3): HPTLC results of Alkaloids of 8 week old callus of *Boerhaavia diffusa***

| Secondary metabolite | Spot number | Scanning at different wavelengths | | | | | |
|---|---|---|---|---|---|---|---|
| | | 200 nm | | 254 nm | | 366 nm | |
| | | Rf | Area % | Rf | Area % | Rf | Area % |
| Alkaloids | Spot 1 | 0.01 | 58.13 | 0.01 | 60.81 | - | - |
| | Spot 2 | - | - | - | - | 0.03 | 3.98 |
| | Spot 3 | - | - | - | - | 0.07 | 13.73 |
| | Spot 4 | 0.18 | 17.46 | 0.18 | 16.91 | 0.18 | 18.01 |
| | Spot 5 | - | - | - | - | 0.23 | 4.60 |
| | Spot 6 | 0.28 | 3.81 | 0.28 | 6.16 | 0.28 | 18.20 |
| | Spot 7 | 0.35 | 1.05 | - | - | - | - |
| | Spot 8 | - | - | - | - | 0.38 | 8.50 |
| | Spot 9 | 0.51 | 3.86 | 0.51 | 5.35 | 0.52 | 19.22 |
| | Spot 10 | 0.54 | 1.45 | - | - | - | - |
| | Spot 11 | 0.58 | 1.10 | - | - | - | - |
| | Spot 12 | - | - | 0.60 | 0.55 | 0.61 | 5.01 |
| | Spot 13 | - | - | - | - | 0.68 | 2.45 |
| | Spot 14 | 0.73 | 2.23 | 0.74 | 6.23 | 0.74 | 4.00 |
| | Spot 15 | - | - | 0.80 | 0.10 | - | - |
| | Spot 16 | 0.86 | 10.93 | 0.85 | 3.29 | - | - |
| | Spot 17 | - | - | 0.88 | 0.59 | 0.88 | 2.30 |
| **Total** | | **9** | | **9** | | **11** | |

Plant tissue culture or the aseptic culture of cells, tissues and organs is an important tool in both basic and applied studies. Callus was obtain on MS medium supplemented with combinations of 2,4-D + BAP and NAA + KIN in *B. diffusa*. The satisfactory result was obtained on MS + 1.5mg/l NAA + 1.5mg/l KIN **(Table-1)**. TLC results suggested that total 6 unidentified alkaloids were analysed from *B. diffusa.* Some of secondary metabolites were identified with iodine treatment only **(Table-2)**. HPTLC results suggested that total 9 alkaloids at 200 nm, 9 at 254 nm, 11 at 366 nm were scanned from *in vitro* (8 week old callus) *B. diffusa.* Absorption spectra of total 8 alkaloids at 190 nm were obtained from *B. diffusa* **(Table-3, Figure-1, 2, 3, Photo-1, 2, 3).** Seth et al., 1986; Jain and Khanna, 1989; Srivastava and Padhya, 1995 have estimated punarnavosides – as anti-fibrinolytic agent from *B. diffusa*. Gupta and Ahmed, 1984 identified flavonoids, Srivastava and Shukla, 1998 identified unsaturated acids from *B. diffusa.* Saxena et al (2012) had deeply studied few medicinal plants and its chemical constituents with the help of TLC and HPTLC at Botany department, Gujarat University, Ahmedabad, Gujarat.

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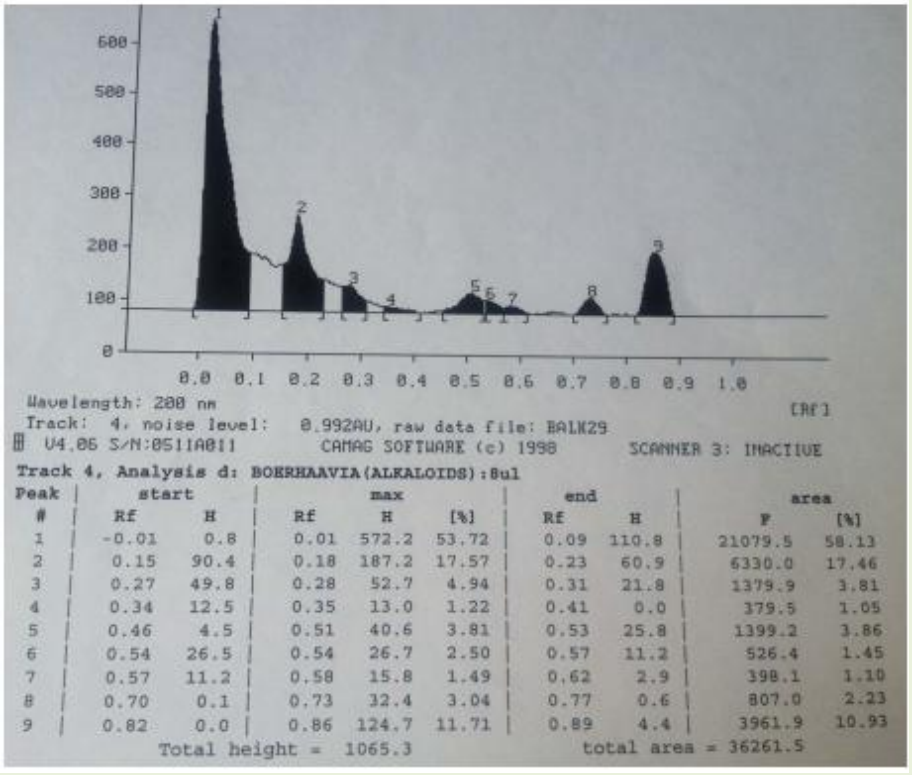
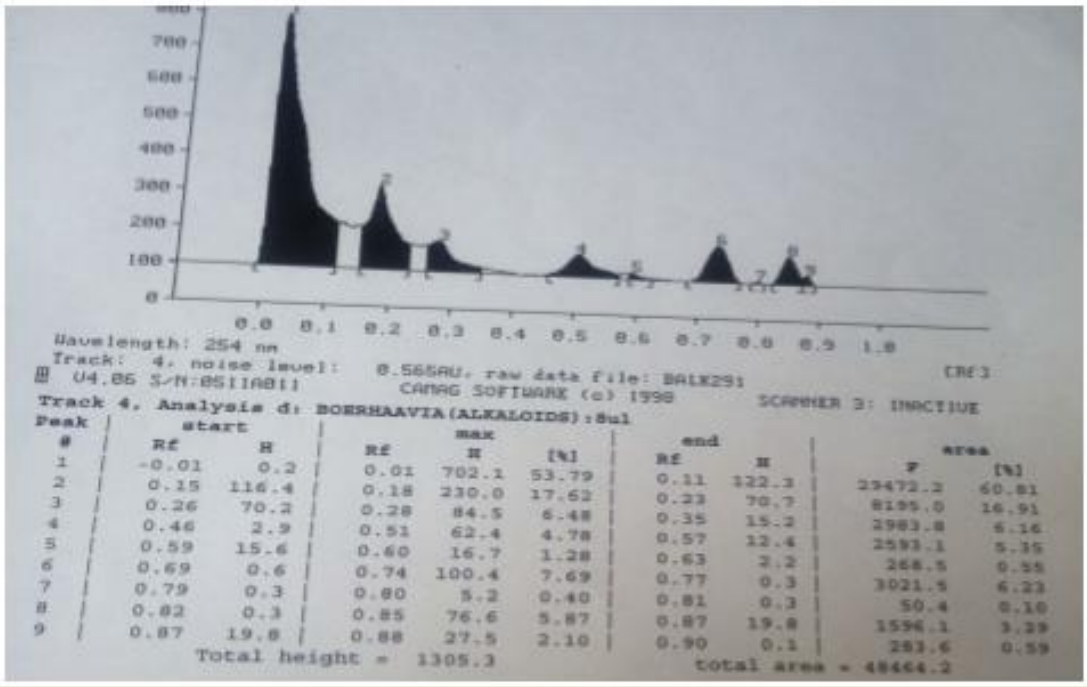
**Figure 1: quantitative estimation at 200 nm through HPTLC**



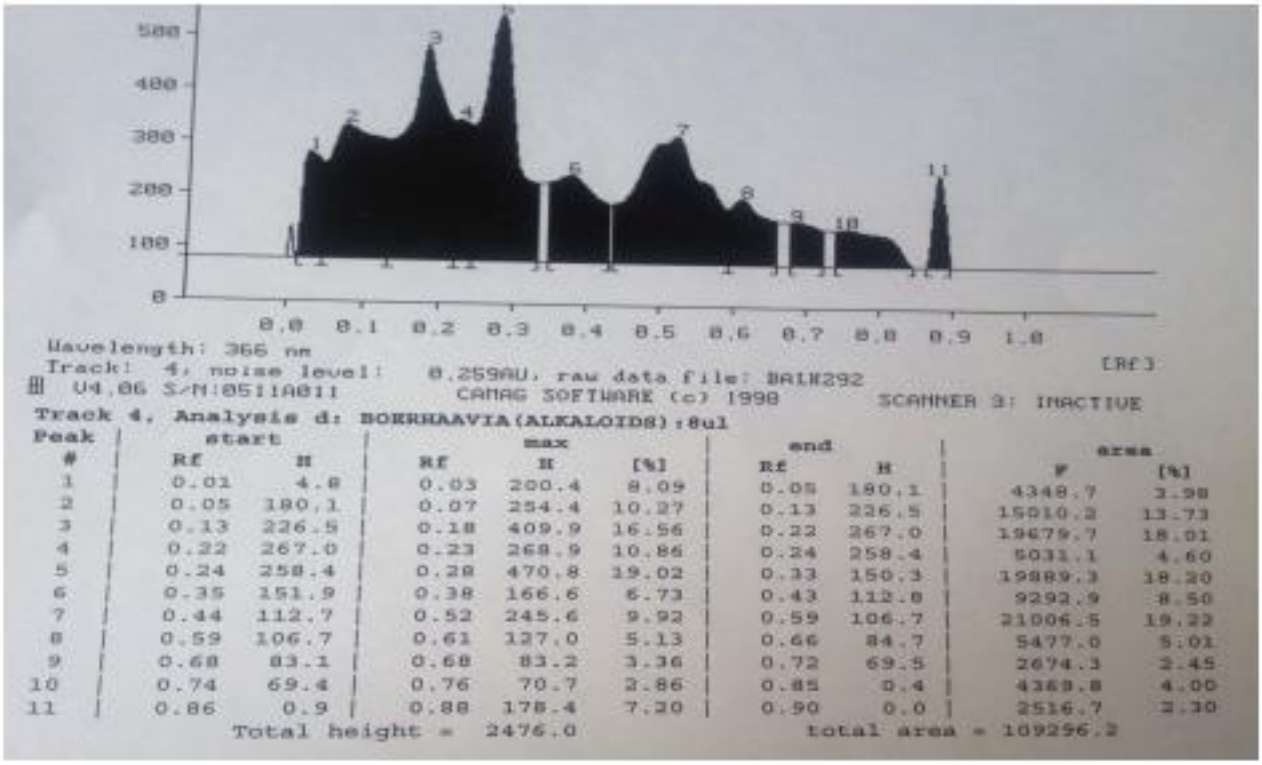

| Peak # | start Rf | start H | max Rf | max H | max [%] | end Rf | end H | area F | area [%] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | -0.01 | 0.8 | 0.01 | 572.2 | 53.72 | 0.09 | 110.8 | 21079.5 | 58.13 |
| 2 | 0.15 | 90.4 | 0.18 | 187.2 | 17.57 | 0.23 | 60.9 | 6330.0 | 17.46 |
| 3 | 0.27 | 49.8 | 0.28 | 52.7 | 4.94 | 0.31 | 21.8 | 1379.9 | 3.81 |
| 4 | 0.34 | 12.5 | 0.35 | 13.0 | 1.22 | 0.41 | 0.0 | 379.5 | 1.05 |
| 5 | 0.46 | 4.5 | 0.51 | 40.6 | 3.81 | 0.53 | 25.8 | 1399.2 | 3.86 |
| 6 | 0.54 | 26.5 | 0.54 | 26.7 | 2.50 | 0.57 | 11.2 | 526.4 | 1.45 |
| 7 | 0.57 | 11.2 | 0.58 | 15.8 | 1.49 | 0.62 | 2.9 | 398.1 | 1.10 |
| 8 | 0.70 | 0.1 | 0.73 | 32.4 | 3.04 | 0.77 | 0.6 | 807.0 | 2.23 |
| 9 | 0.82 | 0.0 | 0.86 | 124.7 | 11.71 | 0.89 | 4.4 | 3961.9 | 10.93 |

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




| Peak # | start Rf | start H | max Rf | max H | max [%] | end Rf | end H | area F | area [%] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | -0.01 | 0.2 | 0.01 | 702.1 | 53.79 | 0.11 | 122.3 | 29472.2 | 60.81 |
| 2 | 0.15 | 116.4 | 0.18 | 230.0 | 17.62 | 0.23 | 70.7 | 8195.0 | 16.91 |
| 3 | 0.26 | 70.2 | 0.28 | 84.5 | 6.48 | 0.35 | 15.2 | 2983.8 | 6.16 |
| 4 | 0.46 | 2.9 | 0.51 | 62.4 | 4.78 | 0.57 | 12.4 | 2593.1 | 5.35 |
| 5 | 0.59 | 15.6 | 0.60 | 16.7 | 1.28 | 0.63 | 2.2 | 268.5 | 0.55 |
| 6 | 0.69 | 0.6 | 0.74 | 100.4 | 7.69 | 0.77 | 0.3 | 3021.5 | 6.23 |
| 7 | 0.79 | 0.3 | 0.80 | 5.2 | 0.40 | 0.81 | 0.3 | 50.4 | 0.10 |
| 8 | 0.82 | 0.3 | 0.85 | 76.6 | 5.87 | 0.87 | 19.8 | 1596.1 | 3.29 |
| 9 | 0.87 | 19.8 | 0.88 | 27.5 | 2.10 | 0.90 | 0.1 | 283.6 | 0.59 |

**Figure 3: quantitative estimation at 366 nm through HPTLC**




| Peak # | start Rf | start H | max Rf | max H | max [%] | end Rf | end H | area F | area [%] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.01 | 4.8 | 0.03 | 200.4 | 8.09 | 0.05 | 180.1 | 4348.7 | 3.98 |
| 2 | 0.05 | 180.1 | 0.07 | 254.4 | 10.27 | 0.13 | 226.5 | 15010.2 | 13.73 |
| 3 | 0.13 | 226.5 | 0.18 | 409.9 | 16.56 | 0.22 | 267.0 | 19679.7 | 18.01 |
| 4 | 0.22 | 267.0 | 0.23 | 268.9 | 10.86 | 0.24 | 258.4 | 5031.1 | 4.60 |
| 5 | 0.24 | 258.4 | 0.28 | 470.8 | 19.02 | 0.33 | 150.3 | 19889.3 | 18.20 |
| 6 | 0.35 | 151.9 | 0.38 | 166.6 | 6.73 | 0.43 | 112.8 | 9292.9 | 8.50 |
| 7 | 0.44 | 112.7 | 0.52 | 245.6 | 9.92 | 0.59 | 106.7 | 21006.5 | 19.22 |
| 8 | 0.59 | 106.7 | 0.61 | 127.0 | 5.13 | 0.66 | 84.7 | 5477.0 | 5.01 |
| 9 | 0.68 | 83.1 | 0.68 | 83.2 | 3.36 | 0.72 | 69.5 | 2674.3 | 2.45 |
| 10 | 0.74 | 69.4 | 0.76 | 70.7 | 2.86 | 0.85 | 0.4 | 4369.8 | 4.00 |
| 11 | 0.86 | 0.9 | 0.88 | 178.4 | 7.20 | 0.90 | 0.0 | 2516.7 | 2.30 |

Total height = 2476.0 total area = 109296.2

**Photo 1: HPTLC photo under fluorescent light**

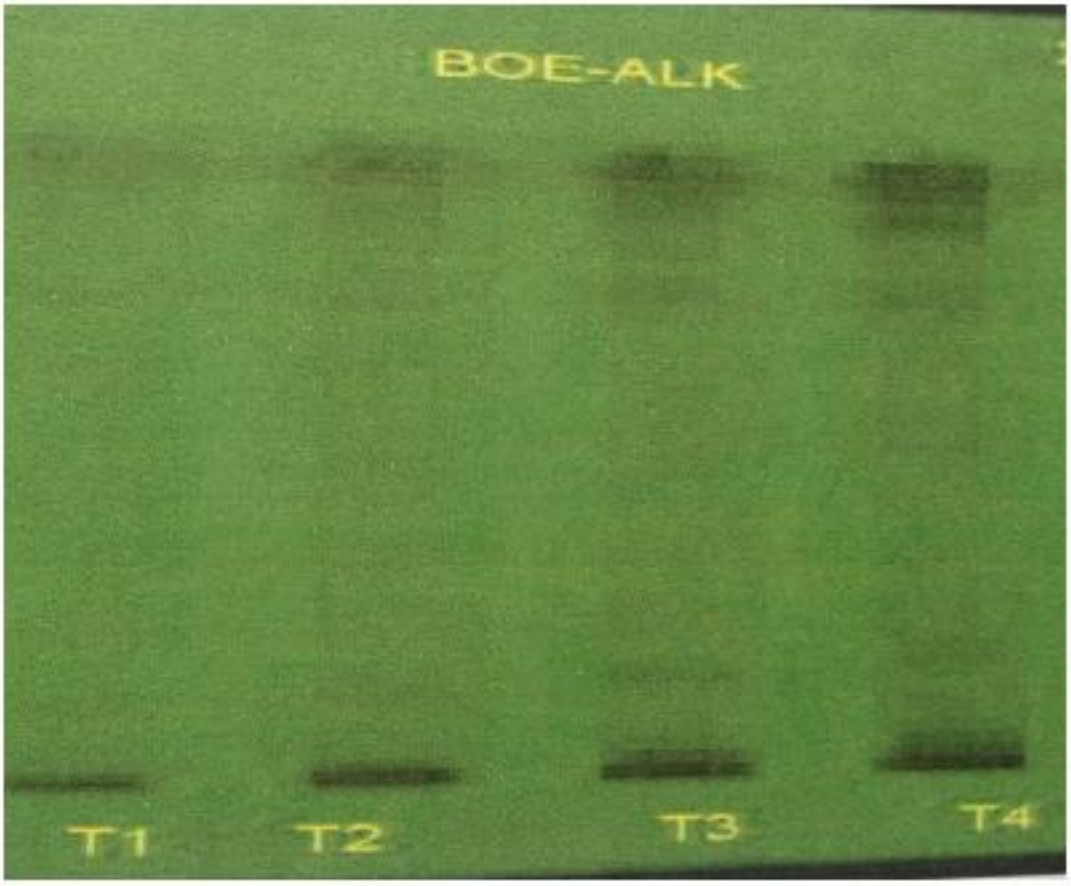


**Photo 2: HPTLC photo under UV light without stain**

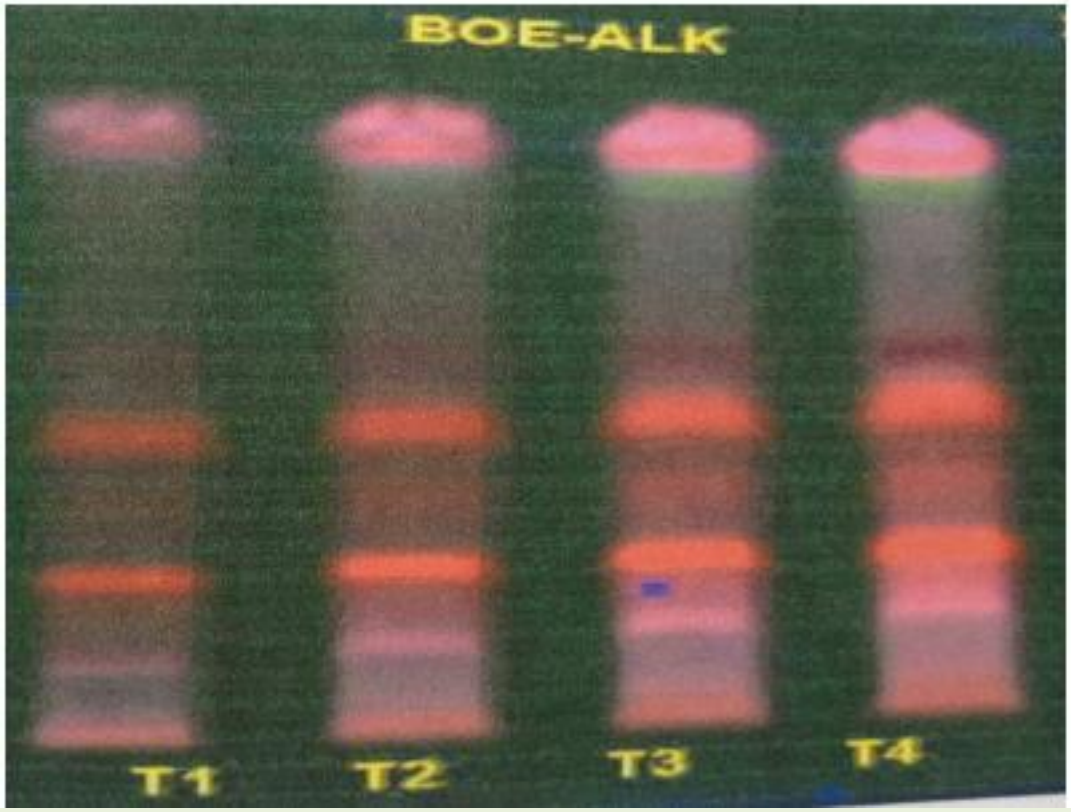



**Photo 3: HPTLC photo under visible light**