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# CHROMATOGRAPHIC METHODS OF FRACTIONATING SEVERAL CHEMICAL CONSTITUENTS IN *PHYLA NODIFLORA*

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**ABSTRACT:**

*Phyla nodiflora* Linn., Synonym- *Lippia nodiflora* Rich also known as *Jala-pippali* is an important herb which can be used medicinally. The aim of the present study is to fractionate several chemical constituents in *Phyla nodiflora*. Chromatography was used for designing of multi component solvent system for different extracts where, for methanolic extract Hexane: Toluene: Ethyl acetate (2: 1.5: 0.5); for chloroform and petroleum ether Hexane: Ethyl acetate (3:1) were selected. Five different phenolic components were extracted and comparison was done through HPTLC and it was found that in phenolic BuOH extract maximum active constituents were separated.

**INTRODUCTION:**

*Jala pippali* is an aquatic plant of Verbenaceae family[1, 2] having fruits like those of pippali[3]. The plant is botanically *Phyla nodiflora* Linn., syn- *Lippia nodiflora* Rich. Lippia is name in honour of Augustus Lippi, a French traveler of seventeenth century. It is a creeping prostate perennial herb, rooting at nodes[1, 4, 5, 6] and mostly found in tropical and sub tropical regions. Throughout India in wet places along bunds of irrigation channels, canal edges and riverbanks and also ascending up to 900 m[7, 8, 9]. It is astringent, cooling, appetizing, stomachic, constipating, anthelmintic, digestive, aphrodisiac, opthelmic, diuretic and febrifuge. It is useful in vitiated conditions of pitta, burning sensation, anorexia, flatulence, colic, dyspepsia, diarrhoea, ulcers, strangury, asthma, bronchitis, knee joint pain, gonorrhea, irritation of internal haemorrhoids, cardiopathy, erysipelas and fever. Tender stalks and leaves are slightly bitter and prescribed in form of infusion of children suffering from indigestion and to women after delivery[4, 8, 10].

This plant contains glycosides- nodiflorin A ($C_{28}H_{34}O_{12}$, m pt - 186ºC), nodiflorin B, lippiflorin A (3', 4', 5,6 - tetrahydroxy -7-0-L - arabinosyl flavone ) and lippiflorin B (4' -O-L - rhamnoside of Lippiflorin A). A new flavone also detected is nodifloretin - chracterised as 4', 5, 6, 7 - tetrahydroxy -3-methoxyflavone. The leaves also contain tannin (8%), fat (9%), rutin, a waxy ester and β-sitosterol[7, 11, 12, 13]. The aim of the work is to fractionate these chemical constituents. Therefore, the present work is designed as follows: 1. Chromatography: Multi component mobile phase designing for different extracts. 2. Extraction of three different phenolic components and comparison of methods through HPTLC.

**MATERIALS AND METHODS:**

**Identification of the Plant**:

*Jala-pippali* was identified by referring its taxonomical and morphological characters mentioned in different Materia medica and Floras like Flora of Presidency of Bombay, Flora Simlensis etc. The whole plant required for the present study was collected personally from Botanical Garden, Sassoyi, G.A.U., Jamnagar. The material was washed and then dried under sun and shade.

**Chemicals:** All the chemicals used in experiment are mainly CDH, Qualligen and Ranbaxy.

**Chromatography:** Multi Component Mobile Phase Designing For Different Extracts. Here TLC was used for designing of multi-component solvent. Three extract, methanolic, petroleum ether and chloroform extract were prepared[14].

**a) Mobile phase designing for methanolic extract for T.LC. by Triangle Series:**

Multi component solvent system was designed using triangle series[15]. L.R. Synder founded these series. He classified the solvents

according to their solvent strength in nine groups. From these groups, any solvent was taken and mixed with Hexane in different ratios and the TLC was run. Here Hexane was taken as a diluent as its strength in zero.

Total solvent strength is $S = \Sigma^n \psi_i s_i$

Where, i=1, $\psi$ = Volume fraction, s = Strength of solvent and it can be defined as the sum of the strength of individual solvent multiplied by its volume fraction[3, 4].

For 1st TLC plate, solvent system used was Hexane : Toluene (3:1) and in 2nd TLC plate, same solvent in the ratio 2:2, followed by changing the ratio to 1:3 in 3rd TLC plate. $R_f$ values were noted down.

Now another solvent system, Hexane: Ethyl acetate was used in same pattern as described above. $R_f$ values were again noted down. From these $R_f$ values $\Delta hR_f$ values were obtained. All the $R_f$ values are multiplied by 100 and were termed as $\Delta hR_f$ values. They are regarded only as guide values for the migration distances even when all experimental data are accurately measured.

Strength of all the solvent systems, which were used, was calculated from this and the strength of those solvent systems, which give good separation were noted down. Now the system was changed from doublet to triplet. These solvents, which give good separation, were mixed in definite proportion, so that their strength is equal to the strength of that solvent system, which gives good separation. By this method, TLC for methanolic extract was designed and finally the solvent system used was Hexane: Toluene: Ethyl acetate in the ratio 2:15:0.5 and the strength was 1. 45.

**b) Mobile phase designing for Petroleum ether extract for TLC Screening Series:**

Here Mobile phase was designed using screening series. In 1st series, TLC was run using neat solvents like methanol, acetic acid, ethyl acetate, toluene and chloroform. This system gives an idea that which solvent picks the spot and separates the components. The solvents were selected and we shifted to 2nd series. Selected solvents are combined with Hexane in different proportions and their TLC was run. Their $R_f$ values and $\Delta hR_f$ values were also noted down.

Finally the solvent system used was Hexane: Ethyl acetate in the ratio 3:1 and strength 1:1.

**c) Mobile phase designing for Chloroform extract for TLC:**

Mobile phase was designed as per the procedure for petroleum ether extract and the solvent system finally selected was Hexane: Ethyl acetate in the ratio 3:1 and having strength 1.1.

Extraction of Phenolic Components and Comparison through HPTLC:

To the sample, add 2M HCl and hydrolyze it on water-bath for 30 minutes. In a test-tube take acetic acid, water and hydrochloric acid in the ratio (30:10:3). This solution in the test-tube was added in the hydrolyzed sample. Boiled for 10 minutes and

then cooled. If not red, stop the work. If red color appeared, then centrifuge or filter it. From the centrifuge collect the upper layer and if filtered, collect the filtrate. Then go for ethyl acetate partition. Ethyl acetate layer was separated, dried, then methanol was added and this phenolic methanol extract was filled in the vial. To the rest portion other than ethyl acetate, amyl alcohol or butanol was added. This butanol layer was divided into 2 parts. One part of this butanol layer was used directly as phenolic butanol and to another part, methonolic HCL was added. This was used as phenolic methanolic HCl[16].

These three samples obtained were subjected for HPTLC separation with other two samples that were fresh methanolic extract and methanolic extract prepared three months before. For HPTLC separation pre-coated silica gel TLC plate (10 x 10 cm) of uniform thickness of 0.2 mm was used. These five spots were spotted on the plate in the sequence given below:

| | |
|---|---|
| MeOH | - Fresh |
| MeOH | - Dec |
| Phenolic | - BuOH |
| Phenolic | - MeOH |
| Phenolic | - MeOH - HCl |

The solvent system used was Hexane: Toluene: Ethyl acetate in the ratio 2:1.5:0.5. The plate was developed in twin trough chamber and was scanned under different wavelengths starting from 200 nm to 450 nm. The $R_f$ values and color of resolved bands were noted down and were presented in the charts.

**RESULTS AND DISCUSSIONS:**

**Chromatography:** Multi component mobile phase designing of three different extracts: T.L.C. was used for designing of multi component solvent system of three extracts, petroleum ether, chloroform and methanolic extract. Two methods, Triangle series and Screening series, were used for designing purpose and results obtained were tabulated and shown in Table 1 and Table 2.

The solvent system finally selected for methanolic extract was Hexane: Toluene: Ethyl acetate in the ratio (2: 1.5: 0.5) because the number of spots were more which indicate that number of components separated were more. The distance between two spots as indicated by $\Delta$ $hR_f$ values were also appropriate. $\sum \Delta$ $hR_f$ and $\Pi \Delta$ $hR_f$ values were also higher in comparison to other tested solvent mixtures. The solvent system found to be best for separation of Petroleum ether extract was Hexane: Ethyl acetate (3:1) but number of spots were more in Hexane: Chloroform. But Hexane : Chloroform was not selected because its $\Delta hR_f$ values were very close to each other indicating that components were not separated properly. Hexane: Ethyl acetate showed good separation and its $\sum \Delta$ $hR_f$ and $\Pi$ $hR_f$ values were also higher when compared to other tested solvent mixtures.

**Table 1: Triangle Series**

| Sr. No. | Mobile Phase | Volume Fraction | Strength | No. of Spots | $R_f$ | $\Delta h R_f$ | $\Sigma \Delta h R_f$ | $\pi \Delta h R_f$ |
|---|---|---|---|---|---|---|---|---|
| | | | | **Methanolic Extract** | | | | |
| 1 | Hexane : Tolune | 0.75<br>0.25 | 0.6 | 1 | 0.06 | - | - | - |
| 2 | Hexane : Tolune | 0.5<br>0.5 | 1.2 | 1 | 0.14 | - | - | - |
| 3 | Hexane : Tolune | 0.25<br>0.75 | 1.8 | 2 | 0.07, 0.12 | 5 | 5 | 5 |
| 4 | Tolune | 1 | 2.4 | 4 | 0.05, 0.11, 0.15, 0.19 | 6,4,4 | 14 | 96 |
| 5 | Hexane : Ethyl Acetate | 0.75<br>0.25 | 1.1 | 3 | 0.31, 0.40, 0.48 | 9,8 | 17 | 72 |
| 6 | Hexane : Ethyl Acetate | 0.5<br>0.5 | 2.2 | 2 | 0.68, 0.74 | 6 | 6 | 6 |
| 7 | Hexane : Acetic Acid | 0.75<br>0.25 | 1.5 | 3 | 0.16, 0.31, 0.38 | 15,7 | 22 | 105 |
| 8 | Hexane : Toluene : Ethyl Acetate | 0.5<br>0.375<br>0.125 | 1.45 | 7 | 0.07, 0.29, 0.58, 0.68, 0.78, 0.85, 0.90 | 22, 29, 10, 10, 7, 5 | 83 | 2233000 |
| | | | | **Chloroform Extract** | | | | |
| 1 | Hexane : Ethyl acetate | 0.75<br>0.25 | 1.1 | 8 | 0.03, 0.07, 0.19, 0.35, 0.58, 0.70, 0.93, 0.99 | 4, 12, 16, 23, 12, 13, 6 | 96 | 29251584 |

**Table 2: Screening Series**

| Petroleum Ether Extract | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sr. No.** | **Mobile Phase** | **Volume Fraction** | **Strength** | **No. of Spots** | **$R_f$** | **$\Delta h\ R_f$** | **$\Sigma\Delta hR_f$** | **$\pi\ \Delta h\ R_f$** |
| 1 | Methanol | 1 | 5.1 | Spot drag | - | - | - | - |
| 2 | Acetic Acid | 1 | 6.0 | Spot drag | - | - | - | - |
| 3 | Ethyl Acetate | 1 | 4.4 | 3 | 0.03, 0.52, 0.77 | 49, 25 | 74 | 1225 |
| 4 | Chloroform | 1 | 4.3 | 4 | 0.66, 0.15, 0.22, 0.79 | 9, 7, 57 | 73 | 3591 |
| 5 | Toluene | 1 | 2.4 | 4 | 0.03, 0.05, 0.11, 0.80 | 2, 6, 69 | 77 | 828 |
| 6 | Hexane : Chloroform | 0.25<br>0.75 | 3.225 | 7 | 0.03, 0.05, 0.08, 0.09, 0.13, 0.19, 0.80 | 2, 3, 1, 4, 6, 61 | 77 | 8784 |
| 7 | Hexane : Chloroform | 0.50<br>0.50 | 2.15 | 3 | 0.03, 0.05, 0.08 | 2, 3 | 5 | 6 |
| 8 | Hexane : Ethyl acetate | 0.25<br>0.75 | 3.3 | 3 | 0.01, 0.70, 0.81 | 69, 11 | 80 | 759 |
| 9 | Hexane : Ethyl acetate | 0.50<br>0.50 | 2.2 | 2 | 0.80, 0.87 | 79 | 79 | 79 |
| 10 | Hexane : Ethyl acetate | 0.75<br>0.25 | 1.1 | 4 | 0.15, 0.63, 0.77, 0.94 | 48, 14, 17 | 79 | 11424 |

The solvent system found to be best for separation of Petroleum ether extract was Hexane: Ethyl acetate (3:1) but number of spots were more in Hexane: Chloroform. But Hexane : Chloroform was not selected because its $\Delta hR_f$ values were very close to each other indicating that components were not separated properly. Hexane: Ethyl acetate showed good separation and its $\sum \Delta hR_f$ and $\Pi hR_f$ values were also higher when compared to other tested solvent mixtures.

**Extraction of three different phenolic components and comparison of methods through HPTLC**

For HPTLC separation, five spots were spotted on different tracks on the pre-coated silica gel T.L.C. plate (Fig 1) in the sequence given below:

Track 1 = MeOH - Fresh

Track 2 = MeOH - Dec

Track 3 = Phenolic - BuOH

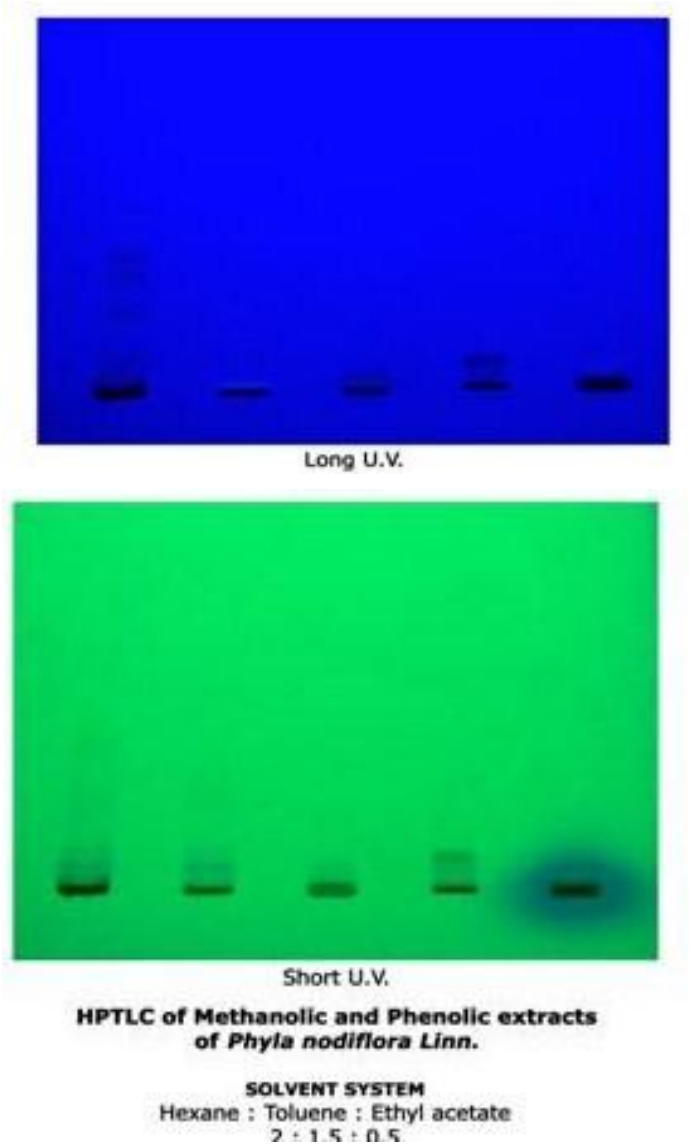
Track 4 = Phenolic - MeOH

Track 5 = Phenolic - MeOH - HCl

The results obtained are given in the Table 3:

From the above table, values of Track 1 and Track 2 were compared, as both are methanolic extracts, one fresh prepared and another extract prepared three months before. Number of spots was more in track 1 as compared to track 2. More spots were observed when visualized in the wavelength 200 to 300 nm. But only three $R_f$ values (0.38, 0.49, 0.99) were common in both the tracks.

**Fig 1:**




When the spectra of these three $R_f$ values were compared, then it was found that the spectra of 0.38 and 0.99 $R_f$ values were identical which proved that the component detected on both tracks were same. The spectra of 0.49 $R_f$ values of both tracks were not identical, so both the components were different. When observed under wavelength 300 nm to 450 nm, the components were separated only on track 1.

**Table 3: Extraction of phenolic components and comparison of methods through HPTLC**

| Solvent System | Visuliz-ation | Track 1 | | Track 2 | | Track 3 | | Track 4 | | Track 5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. of Topics | Rf value | No. of Topics | Rf value | No. of Topics | Rf value | No. of Topics | Rf value | No. of Topics | Rf value |
| Hexane : Toluene: Ethyl acetate 2 : 1.5 : 0.5 | 200 nm | 14 | 0.01, 0.04, 0.07,0.14, 0.22, 0.28, 0.38,0.44, 0.49, 0.60, 0.64, 0.70, 0.76, 0.99 | 11 | 0.05, 0.18, 0.24, 0.38, 0.49, 0.61, 0.66, 0.79, 0.81, 0.84, 0.99 | 18 | 0.01, 0.05, 0.08, 0.17, 0.19, 0.21, 0.23, 0.30, 0.37, 0.47, 0.54, 0.66, 0.67, 0.70, 0.75, 0.76, 0.84, 0.99 | 15 | 0.05, 0.18, 0.23, 0.27, 0.34, 0.51, 0.68, 0.70, 0.74, 0.76, 0.78, 0.82, 0.85, 0.89, 0.99 | 11 | 0.01, 0.16, 0.51, 0.67, 0.70, 0.74, 0.81, 0.88, 0.89, 0.99 |
| | 250 nm | 12 | 0.00, 0.04, 0.08, 0.16, 0.28, 0.38, 0.44, .0.47, 0.60, 0.70, 0.76, 0.99 | 4 | 0.24, 0.49, 0.62, 0.67 | 13 | 0.00, 0.05, 0.08, 0.17, 0.21, 0.28, 0.29, 0.38, 0.48, 0.66, 0.67, 0.70, 0.99 | 12 | 0.18, 0.27, 0.50, 0.68, 0.70, 0.74, 0.76, 0.78, 0.82, 0.86, 0.90, 0.99 | 6 | 0.01, 0.42, 0.88, 0.89, 0.92, 0.99 |
| | 300 nm | 8 | 0.00, 0.05, 0.08, 0.15, 0.28, 0.44, 0.54, 0.60 | 3 | 0.25, 0.62, 0.67 | 10 | 0.00, 0.05, 0.08, 0.17, 0.27, 0.29, 0.39, 0.65, 0.67, 0.88 | 9 | 0.18, 0.27 0.68, 0.70 0.74, 0.82, 0.86, 0.99 | 8 | 0.01, 0.14, 0.29, 0.35, 0.42, 0.88, 0.89, 0.92, |
| | 350 nm | 7 | 0.00, 0.07, 0.16, 0.18, 0.28, 0.32, 0.44 | - | - | 7 | 0.00, 0.08, 0.17, 0.21, 0.30, 0.65, 0.67 | 6 | 0.18, 0.27, 0.50, 0.68, 0.82, 0.86 | 7 | 0.00, 0.01, 0.14, 0.29, 0.35, 0.42, 0.88 |
| | 400 nm | 8 | 0.00, 0.07, 0.10, 0.15 0.18, 0.28, 0.32, 0.44 | - | - | 5 | 0.00, 0.08, 0.17, 0.29, 0.65 | 1 | 0.86 | 4 | 0.00, 0.01, 0.14, 0.42 |
| | 450 nm | 7 | 0.00, 0.05, 0.07, 0.18, 0.28, 0.32, 0.44 | - | - | 3 | 0.01, 0.08 0.21 | - | - | 4 | 0.00, 0.01, 0.14, 0.42 |

This proved that the fresh methanolic extract was better than the extract prepared three months before.

Similarly the values of phenolic components on track 3, 4 and 5 were also compared. Number of spots was found to be more on track 3 when visualized in wavelength 200 to 300 nm. The $R_f$ values (0.70 and 0.99) of two spots on three different tracks were same. When the spectra of these three $R_f$ values were compared, the spectra of $R_f$ 0.99 were identical showing same component on all three tracks. The spectra of the $R_f$ values, which were almost, same on the three tracks i.e. 0.75 on track 3, 0.74 on

track 4 and again 0.74 on track 5, was also identical. When the spectra of $R_f$ value 0.66 on track 3, 0.68 on track 4 and 0.67 on track 5 were compared then $R_f$ 0.66 and 0.67 on track 3 and 5 respectively, were same but $R_f$ 0.68 on track 4 was different. This showed that on track 3 and 5, the components were same and component on track 4 was different. When visualized in wavelength 300 nm to 450 nm the total number of spots on all three tracks declined.

The above results showed that track 3 i.e. Phenolic BuOH extract is better than other extracts. All the five tracks were compared and the common components in each track are given below:

Track 1 = 19

Track 2 = 11

Track 3 = 21

Track 4 = 15

Track 5 = 16

The percentage of common components in track 1 is 23.17%, track 2 is 13.41% and track 3 is 25.61% track 4 is 18.29% and track 5 in 19.51%. One $R_f$ value 0.99 is common in all the five tracks and when the spectra were compared, it was identical. In all the five tracks, there is one common component. As the plant *"Jala-pippali"* contains chemical constituent-nodifloretin, so this common component can be nodifloretin. From the above data, more number of common components separated was in track 3 i.e. Phenolic BuOH extract. So this method of extraction of phenolic components can be considered as the best.

**CONCLUSION:**

Multi component mobile phase designing of different extracts were carried out so that more number of components are separated out.

Extraction of three different phenolic components was done and the methods were compared through HPTLC. The best method was of extraction with butanol as maximum number of common components was separated out by this method.

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