HPTLC Fingerprints of Alcoholic Extracts of Phyllanthus species collected from Various Parts of Eastern Ghats in Andhra Pradesh

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Abstract: The genus Phyllanthus L. consists of 750 to 850 species distributed in tropical and subtropical around the world while 51 species in India. Most of them have been used in traditional medicine since ancient times. Phyllanthus spp., like P. amarus, P. debilis, P. fraternus, P. urinaria and P. virgatus are called bhumyamalaki, used in Ayurveda. More than 514 compounds have been isolated from Phyllanthus, the majority of which are lignins, triterpenoids, flavonoids, and tannins. Lignins like phyllanthin and hypophyllanthin have been shown hepatoprotective activity and tannins exhibit various biological activities. Corilagin, geranin, and gallic acid are another three most prevalent compounds in this genus. P.amarus shows high concentration of Phyllanthin than P. fraternus, P. virgatus, P. maderaspatensis, P. urinaria, and P. debilis. Our aim is to establish physical constants and fingerprint profile of Phyllanthus species collected from various parts of Eastern Ghats in Andhra Pradesh using high performance thin layer chromatography (HPTLC) technique. It is one of the fast growing techniques, used to analyze the phytochemical constituents. Leaves of thirteen Phyllanthus species were used to prepare methanolic extracts. Extracted samples used for carrying out HPTLC. Sample was spotted on TLC plate as triplicate and developed the plate with the mobile phase as Toluene: Ethyl acetate: Methanol (7: 2: 1). Dried TLC plate was then scanned under the UV at 366nm. All the Phyllanthus spp. exhibits significant fractions of phyllanthin, niranthin, quercetin and phenolic compounds in alcoholic extracts. It can be used as a diagnostic tool for the correct identification of potential Phyllanthus spp. and it is useful as a phytochemical marker and also a good estimator of genetic variability in the plant populations.

Keywords: Phyllanthus genus, HPTLC, Phytochemicals and secondary metabolites

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1. INTRODUCTION

Phyllanthus L. is the largest genus in the family of Phyllanthaceae. Phyllanthus has been used for treatment of diabetes, intestinal parasites and liver disorder, kidney and bladder problems. The Phyllanthus spp. contain therapeutically important organic compounds such as lignans, alkaloids, flavonoids, polyphenols, ellagitannins and triterpenoids. More than 514 compounds have been isolated from Phyllanthus, the majority of which are lignins, triterpenoids, flavonoids, and tannins. Crude extracts of Phyllanthus spp. exhibit inhibitory effects on the hepatitis B virus (HBV). Previous reviews depict the biological activities of Phyllanthus species, mostly from P. amarus, P. emblica L. and P. niruri L. Primarily contains lignin (e.g., phyllanthin and hypophyllanthin) and geraniin and 5 flavonoids (quercetin, astralin, quercetin, isoquercitrin and rutin). Due to the presence of potential polyphenolic compounds in Phyllanthus, the present study was focused on the quantitative screening of thirteen Phyllanthus species from different geographical regions in Andhra Pradesh. Natural products of plant origin are widely recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities. The subject of phytochemistry is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with structures, biosynthesis, turnover, metabolism, natural distribution, and biological functions of these substances. Epidemiological and experimental studies suggest that medicinal herbs have great potential in the management of different types of cancers including lung, breast, colon, liver, prostate, skin, and ovarian carcinomas. In this connection, medicinal plant extracts, and their purified compounds have significant growth inhibitory potential against various types of cancerous cells in vitro as well as in vivo. Flavonoids have various pharmacological activities including anticancer, anti-inflammatory, antioxidant, anti-diabetic, and antiviral activities through various cell-signaling pathways. Most of the flavonoids reported from Phyllanthus were flavonol and glycoside forms. From the ethanolic extract of P. urinaria, two new acetylated flavonoid glycosides, along with the known isolates, quercetin and quercetin 3-O-alpha-L-rhamnopyranoside have been isolated. A new flavone sulfonic acid was isolated from the methanolic extract of P. urinaria. The isolated flavonoids from P. urinaria showed antioxidant, anti-inflammatory, antitumor, and anti-H. Pylori activities. Phenolic compounds are the major group of phytochemicals that include at least one aromatic ring, with one or more hydroxyl groups attached. Phytochemical investigation of ethanolic extract from whole plants of P. urinaria resulted in the isolation of nine compounds including trimethyl-3, 4-dehydrochelubate, methylgallate, and methyl brevifolin carboxylate. High performance thin layer chromatography is rational for expansion of chromatographic fingerprints to determine major active constituents of medicinal plants. The separation and resolution are much better, and the results are much more reliable and reproducible than TLC. Combined with digital scanning profiling, it has the main advantage of in situ qualitative and quantitative measurements by scanning densitometry. Besides, the colorful pictorial HPTLC image provides extra, intuitive visible colour and/or fluorescence parameters for parallel assessment on the same plate. It also revealed a better separation of individual secondary metabolites. As a mega biodiversity nation, India is endowed with high species richness of medicinal plants. Lignins and tannins exhibit various activities and are considered to be the biological active compounds of this genus. Corilagin, geraniin, and gallic acid are three most prevalent compounds in this genus, and the pharmacological researches mainly focus on phyllanthin, niranthin, and geraniin. Several analytical procedures involving HPLC have been described. Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity. So, in this study we opted HPTLC for analysis of photochemical of Phyllanthus species.

2. MATERIALS AND METHODS

2.1 Materials

Toluene, Ethyl acetate, Methanol (HPLC grade) and prepared aluminum TLC plates were purchased from (Merck KGaA, Germany).

2.2 Study Area

The study area is primarily tropical deciduous vegetation found in Orissa, Telangana, Andhra Pradesh, Tamilnadu and some parts of the Karnatakta States in isolated hill ranges of the Eastern Ghats in peninsular India. Eastern Ghats of Andhra Pradesh (the link between 13°30’19.07"N; 77°28’84.45"E) covers the hilly terrain of coastal Andhra with nine districts and three districts in the Telangana State. The intensive field studies were conducted in the forests of the Eastern Ghats in Andhra Pradesh yielded 13 Phyllanthus spp. among them seven herbaceous, two shrubs and four trees.

2.3 Plant material

Phyllanthus spp. was collected from different geographical regions in Eastern Ghats of Andhra Pradesh. Collected specimens were identified with the help of floras and preserved at Sri Krishnadevaraya University, Anantapur. All the thirteen Phyllanthus species with their respective voucher numbers, habit, Latitude & Longitude and area of collection of specimen were listed in Table-I. Plant specimens were washed under the tap water and allowed them to shade dry for two weeks. 10g of each dried leaves with 200ml of alcohol used for the hot extraction process with the help of Soxhlet apparatus. Phyllanthus leaves were chosen for the evaluation of the phytochemical diversity. Methanolic extract of dried and powdered samples were subjected to HPTLC fingerprints.

2.4 Preparation of samples for HPTLC fingerprints

The powdered samples of leaves (1 gm each) of Phyllanthus were employed for the extraction of total photochemical in reflux condenser using 25 ml alcohol for 4 hours at 80°C. The alcohol extracts were evaporated to dryness in rotary vacuum evaporator and the residues obtained were re-dissolved in methanol (10 ml each), which were used for the application on HPTLC plate for the development of fingerprints.
<table>
<thead>
<tr>
<th>Plant extract code</th>
<th>Name of Test species/code</th>
<th>Voucher specimen</th>
<th>Habit</th>
<th>Latitude &amp; Longitude</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>P. amarus</em> Schum &amp; Thonn. (Pa)</td>
<td>SKU 50217</td>
<td>Herb</td>
<td>14°28′32.32″N; 78°43′08.32″E</td>
<td>Yvu garden (KDP Dist.)</td>
</tr>
<tr>
<td>2.</td>
<td><em>P. emblica</em> L. (Pe)</td>
<td>SKU 50219</td>
<td>Tree</td>
<td>14°28′26.75″N; 78°43′06.56″E</td>
<td>Yvu garden (KDP Dist.)</td>
</tr>
<tr>
<td>3.</td>
<td><em>P. indofesheri Bennet</em> (Pi)</td>
<td>SKU 50246</td>
<td>Tree</td>
<td>14°36′43″N; 77°38′42″E</td>
<td>S.K.U Campus (ATP Dist.)</td>
</tr>
<tr>
<td>4.</td>
<td><em>P. maderaspatensis</em> L. (Pm)</td>
<td>SKU 50221</td>
<td>Herb</td>
<td>14°36′43″N; 77°38′42″E</td>
<td>S.K.U Campus (ATP Dist.)</td>
</tr>
<tr>
<td>5.</td>
<td><em>P. narayanaswami</em> Gamble (Pn)</td>
<td>SKU 50206</td>
<td>Herb</td>
<td>18°16′27″N; 82°19′26″E</td>
<td>Araku (VSKP Dist.)</td>
</tr>
<tr>
<td>6.</td>
<td><em>P. pinnatus</em> (Wt.) Webster (Ppi)</td>
<td>SKU 50201</td>
<td>Shrub</td>
<td>15°00′38.82″N; 78°01′25.53″E</td>
<td>Bhugga (ATP Dist.)</td>
</tr>
<tr>
<td>7.</td>
<td><em>P. polyphyllus</em> Willd. (Ppo)</td>
<td>SKU 50222</td>
<td>Tree</td>
<td>13°41′57″N; 79°20′21″E</td>
<td>Tirumala (CTR Dist.)</td>
</tr>
<tr>
<td>8.</td>
<td><em>P. reticulatus</em> Poir. (Pre)</td>
<td>SKU 50223</td>
<td>Shrub</td>
<td>14°12′21.72″N; 78°07′43.35″E</td>
<td>Kalasamudram (ATP Dist.)</td>
</tr>
<tr>
<td>9.</td>
<td><em>P. rheedei</em> Wt. (Prh)</td>
<td>SKU 50204</td>
<td>Herb</td>
<td>15°53′07.39″N; 78°49′34.22″E</td>
<td>Rollapenta (KNL District)</td>
</tr>
<tr>
<td>10.</td>
<td><em>P. rotundifolius</em> Kl. Ex Wildl. (Pro)</td>
<td>SKU 50224</td>
<td>Herb</td>
<td>17°43′21″N; 83°19′29″E</td>
<td>A.U. Campus (VSKP Dist.)</td>
</tr>
<tr>
<td>11.</td>
<td><em>P. tenellus</em> Roxb. (Pt)</td>
<td>SKU 50225</td>
<td>Herb</td>
<td>13°42′46″N; 79°20′31″E</td>
<td>Tirumala (CTR Dist.)</td>
</tr>
<tr>
<td>12.</td>
<td><em>P. urinaria</em> L. (Pu)</td>
<td>SKU 50207</td>
<td>Herb</td>
<td>15°07′46.70″N; 78°40′41.63″E</td>
<td>Ahobilam (KNL Dist.)</td>
</tr>
<tr>
<td>13.</td>
<td><em>P. virgatus</em> Forst. (Pv)</td>
<td>SKU 50202</td>
<td>Herb</td>
<td>14°14′39.70″N; 78°09′48.36″E</td>
<td>Kalasamudram (ATP Dist.)</td>
</tr>
</tbody>
</table>
2.5 HPTLC instrumentation and general conditions for fingerprints

All the samples were spotted in the form of bands (width 4 mm) with a Camag Microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm × 10 cm with 0.2 mm thickness, E. Merck, Germany) using a Camag Linomat V (Switzerland). A constant application rate of 150 nl/s was employed and space between two bands was 7.7 mm. The mobile phase consisted of Toluene: Ethyl acetate: Methanol (7: 2: 1) Linear ascending development was carried out in twin trough glass chambers, saturated with mobile phase. The optimized chamber saturation time for mobile phase was 15 min at room temperature. The chromatogram was developed up to 80 mm.

3. STATISTICAL ANALYSIS

All the instruments were operated by winCATS software (v. 1.4.3 CAMAG) resident in the system. All the data obtained was subjected to one way analysis of variance ANOVA. The significant difference among the means was compared by DUNCAN’s multiple range tests.

4. RESULTS AND DISCUSSION

Authentication of \( \textit{Phyllanthus} \) species at chemical and genetic level plays a crucial role in both research and commercial purpose. The HPTLC method reported here is suitable for the rapid screening of germplasm of \( \textit{Phyllanthus} \) spp. for the determination of chemical profiles and quantification of the quercetin, phyllanthin, niranthin, and phenolic compounds. Earlier \( \textit{Phyllanthus} \) species like \( \textit{P.urinaria}, \textit{P.fraternus}, \textit{P.maderaspatensis}, \textit{P.amarus}, \textit{P.debilis} \) and \( \textit{P.virgatus} \) extracts were used for quantification of phyllanthin and niranthin using HPLC technique. \( \textit{P. amarus} \) is highly valued in the treatment for liver ailments. Some phytochemical compounds were unique to specific plants and its parts In \( \textit{Phyllanthus} \), lignans like phyllanthin, niranthin and little amount of phenolic compounds. Methanolic extracts of thirteen plants were spotted on silica gel “G” plate as shown in TLC plate was developed using toluene: ethyl acetate: methanol (7: 2: 1) mobile phase shows various spots under UV (366nm). In this study we mainly focused on comparative quantification of quercetin (fig-3), phyllanthin (fig-4), Niranthin (fig-5) and phenolic composition (fig-6), of thirteen methanolic leaf extracts of \( \textit{Phyllanthus} \) spp. in which phyllanthin generally showed \( R_f \) value at 0.31, quercetin generally showed \( R_f \) value at 0.02 and 0.65 phenolic compounds \( R_f \) 0.97. Densitograms of thirteen plant extracts were shown (fig-7). When we cross verified them, we obtained different peak heights with approximately similar percent of peak areas at RF value 0.02 and 0.65. In figure-3 we clearly given quercetin percentage of area in densitogrames with their respective plant extract code. Among those \( \textit{P. maderaspatensis} \) (Pm) Shows high percentage area of quercetin (84%) fallowed by \( \textit{P.Polyphyllus}, \textit{P.rheedei}, \textit{P.tenellus}, \textit{P.urinaria} \) and \( \textit{P.virgatus} \) exhibits 63.7%.
Plant extracts exhibit another common peak with Rf value at 0.31. This peak indicates phyllanthin in all thirteen species. P. virgatus shows with highest percentage of peak area 17.9% (fig-4). P. reticulatus shows with lowest percentage of peak area 0.2% followed by P. polypellus and P. rheedei showed least percent of peak area 0.7 and 0.8 respectively. Plant extracts exhibit another common peak with Rf value at 0.42. As we know that niranthin generally showed Rf value at 0.42. P. pinnatus (Wt.) shows with highest percentage of peak area 17.2%(fig-5).P. amarus (Pa) shows peak area 11.8%. P. rotundifolius (Pro) showed least percentage of peak area 0.1%. In the same way percentage of peak areas remaining plant extracts were reported (fig-5) and phenolic compounds showed their peaks with Rf values 0.97. Their distribution in extracts of Phyllanthus species codes 1-13 was shown respectively (fig-6). The extracts of P. rheedei, P. maderaspatensis, and P. narayanaswamii exhibited significant amount of phenolic compound composition i.e., 11.2%, 11.0% and 10.1% respectively. The extract of P. Polyphyllus Willd. (Ppo) exhibit least amount of phenol composition 4.9%. Flavonoids are naturally occurring compounds widely distributed as secondary metabolites in the plant kingdom. They are recognized for having beneficial clinical properties, such as antiinflammatory, cardioprotective, antiviral, antibacterial, and anticancer activities. Quercetin is one of the flavonoids found in the fruits of medicinal plants, (P. emblica) as a traditional medicine for diabetes. P. emblica is referenced in “Rasayana,” a branch of 5000-year-old Indian medical system “Ayurveda,” which focuses on enhancing good health, preventing diseases by boosting the immune system, as well as rejuvenating and revitalizing the body and mind. It is still used extensively not only in India, but also in Iran, Iraq, Thailand, China, Italy, Germany, and other countries as a laxative, diuretic, astringent, and antiemetic. It is also used to treat other ailments, including anemia, jaundice, and tumors. Methanolic extracts of present work are good source of quercetin, so these extracts may useful as phyto medicine Phyllanthin is one of the active principle compound present in Phyllanthus spp. Hence it is used as marker compound in herbal drug industry to identify Phyllanthus species. Several analytical procedures involving quantitative and qualitative determination of Phyllanthus species by HPLC and HPTLC. The present study, Phyllanthin and niranthin were detected in alcoholic leaf extracts of thirteen Phyllanthus species. Earlier phyllanthin found in only P. amarus. For the first time we are reporting phyllanthin in P. narayanaswamii, P. rheedei, P. polypellus, P. pinnatus and P. maderaspatensis. from Eastern Ghats of Anhra Pradesh.

![Fig 3: Distribution of percentage area of quercetin in thirteen Phyllanthus spp. extracts in densitograms (codes:1-13)](image-url)
Fig 4: Distribution of percentage area of phyllanthin in thirteen *Phyllanthus* spp. extracts in densitogram (codes: 1-13)

Fig 5: Distribution of percentage area of niranthin in thirteen *Phyllanthus* spp. Extracts in densitogram (codes: 1-13)

Fig 6: Distribution of percentage area of phenolic compounds in thirteen *Phyllanthus* spp. extracts in densitograms (codes: 1-13)
5. CONCLUSION

In the present study, phytochemicals in Phyllanthus spp., collected from various parts of Eastern Ghats of Andhra Pradesh were analyzed by HPTLC clearly explained the diversity of phytochemicals like, quercetin, phyllanthin, niranthin and phenolic compounds in Phyllanthus spp.. Methanolic extracts of all these plants were a good source of quercetin. phyllanthin was reported in P.narayanaswamii, P.rheedei, P.rotundifolius and P.maderaspatensis first time. Niranthin and phenolic phytochemicals are found in least amount. So, we hope the results of this study may be useful for development of Phytomedicine against various diseases.

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8. AUTHORS CONTRIBUTION STATEMENT

Mr. Akkulanna. S conceptualized and gathered the data for this work. Mr. P. Malleswarareddy was analysed the data and necessary inputs were given towards the designing of manuscript. All authors contributed to discussion of methodology and results to the final manuscript.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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