Chapter 4

Estimation of relative concentration of phyllanthin in herbaceous *Phyllanthus* species
4. Estimation of relative concentration of phyllanthin in herbaceous

*Phyllanthus* species

4.1 Introduction

The genus *Phyllanthus* L. is the largest genus of the family Phyllanthaceae (GRIN Database, 2011). The genus *Phyllanthus* has a long history of use in the treatment of diabetes, Intestinal parasites and liver, kidney and bladder problems (Kirtikar and Basu, 1935; Nadkarni, 1954). They are particularly useful in the treatment of pain, jaundice, gonorrhea, chronic dysentery, skin ulcers, and hepatitis B (Calixto et al., 1998; Duke’s Phytochemical and Ethnobotanical Databases, 2011). Owing to the reported medicinal properties of the *Phyllanthus* species, several phytochemical and bioactivity studies have been performed.

The major lignans of the genus, namely, phyllanthin (Fig. 4.1) and hypophyllanthin (Fig. 4.2), have been shown to be antihepatotoxic against carbon tetrachloride and galactosamine induced hepatotoxicity in primary cultured rat hepatocytes (Syamsunder et al., 1985). However, study by Khatoon et al. (2006) showed these compounds might not be exclusively responsible for hepatoprotective activity as these are present only in *Phyllanthus amarus* while *P. fraternus* and *P. maderaspatensis* also possess significant hepatoprotective activity. Moreover, quantitative and qualitative determination of phyllanthin was carried out only in few *Phyllanthus* species like *P. amarus*, *P. fraternus*, *P. maderaspatensis* and *P. urinaria*. Therefore, to know the relative concentration of phyllanthin in different *Phyllanthus* species, the present work has been taken up.

![Fig. 4.1. Phyllanthin](image1)

![Fig. 4.2. Hypophyllanthin](image2)
4.2 Materials and methods

4.2.1 Chemicals

Analytical grade solvents, methanol and formic acid were obtained from Merck Ltd. (Mumbai, India). Standard phyllanthin was purchased from Sigma chemicals (Bangalore). Polytetrafluoroethylene (PTFE) membrane syringe filter (0.45 µm) was obtained from phenomenex, USA.

4.2.2 Preparation of working standard

Stock solution was prepared by suspending 10 mg of phyllanthin in 20 ml of analytical grade methanol in a 100 ml calibrated volumetric flask and the volume was made up to 100 ml. The solution was filtered through PTFE membrane syringe filter (0.45 µm) before injection.

4.2.3 Plant materials and preparation of extracts

Seven herbaceous Phyllanthus species namely, *P. amarus*, *P. debilis*, *P. maderaspatensis*, *P. virgatus*, *P. urinaria*, *P. scabrifolius* and *P. tenellus* were collected from different parts of Karnataka. Aerial parts were separated, washed and shade-dried for a day and then dried completely in a hot air oven at 38 °C. The plant material was powdered using rotary grinder and stored in airtight containers. 100 g of each Plant powder was extracted three times in a soxhlet apparatus with methanol (300 ml) until the plant material became colorless. The combined methanolic extract was concentrated to dryness using rotary evaporator. The resultant residue was used for further chromatographic analyses.

4.2.4 Preparation of sample

The sample solutions were prepared by dissolving 10 mg extract of each Phyllanthus species in 20 ml of analytical grade methanol in a 100 ml calibrated volumetric flask, sonicated and the volume made up with methanol and filtered through PTFE membrane syringe filter (0.45 µm) before injection in to HPLC. Each sample was injected in duplicates.

4.2.5 HPLC analysis

The HPLC (high-performance liquid chromatography) analysis was performed using LC 20 AD Class LC-solution software, connected to SPD-20A UV/VIS
detector, binary pump and temperature controlled column oven. Spherisorb S10 ODS$_2$
(250 × 4.6 mm, 5 μm) reversed phase column was used for all analysis. The column
oven temperature was set at 35 °C and sample and working standards were eluted
with an isocratic mode at a UV wavelength of 220 nm and 20 μl injection volume.
Methanol and 0.2% formic acid in water in the ratio of 65:35 were used as mobile
phase at a flow rate of 1 ml/min.

4.2.6 Estimation of relative concentration of phyllanthin

Concentration of phyllanthin in different *Phyllanthus* species was calculated
relative to the standard based on the comparison of peak area in HPLC profiles of
samples and standard phyllanthin.

4.3 Results

HPLC profiles of phyllanthin (standard) and herbaceous *Phyllanthus* species
are given in figs. 4.3 and 4.4. Standard phyllanthin showed the retention time ($R_t$) at
24.144 min. $R_t$ of phyllanthin in *P. amarus*, *P. debilis*, *P. maderaspatensis*, *P.
virgatus*, *P. urinaria*, *P. scabrifolius* and *P. tenellus* were 24.954, 24.866, 24.893,
24.875, 24.911, 24.897 and 24.897 min respectively. *P. amarus* showed highest
phyllanthin concentration (0.58%) compared to other *Phyllanthus* species. In *P.
debilis*, *P. maderaspatensis*, *P. virgatus*, *P. urinaria*, *P. scabrifolius* and *P. tenellus*
phyllanthin was present in very minute concentrations (Table 4.1).
Fig. 4.3. HPLC profiles of Phyllanthin in herbaceous *Phyllanthus* species. A - Phyllanthin standard (*R*, 24.144 min), B - *P. amarus* (*R*, 24.954 min), C - *P. debilis* (*R*, 24.866 min) and D - *P. maderaspatensis* (*R*, 24.893 min)
Fig. 4.4. HPLC profiles of Phyllanthin in herbaceous *Phyllanthus* species. A - *P. virgatus* (R, 24.875 min), B - *P. urinaria* (R, 24.911 min), C - *P. scabrifolius* (R, 24.897 min) and D - *P. tenellus* (R, 24.897 min)

Table 4.1. Relative concentration of phyllanthin in herbaceous *Phyllanthus* species

<table>
<thead>
<tr>
<th>Phyllanthus species</th>
<th>Relative concentration of phyllanthin (%)</th>
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</thead>
<tbody>
<tr>
<td><em>P. amarus</em></td>
<td>0.58</td>
</tr>
<tr>
<td><em>P. debilis</em></td>
<td>0.02</td>
</tr>
<tr>
<td><em>P. maderaspatensis</em></td>
<td>0.01</td>
</tr>
<tr>
<td><em>P. virgatus</em></td>
<td>0.03</td>
</tr>
<tr>
<td><em>P. urinaria</em></td>
<td>0.01</td>
</tr>
<tr>
<td><em>P. scabrifolius</em></td>
<td>0.01</td>
</tr>
<tr>
<td><em>P. tenellus</em></td>
<td>0.02</td>
</tr>
</tbody>
</table>
4.4 Discussion

Phyllanthin and hypophyllanthin are the active principles present in *Phyllanthus* species and hence they are used as marker compounds in herbal drug industry to identify *Phyllanthus* species. Several analytical procedures involving quantitative and qualitative determination of phyllanthin from *Phyllanthus* species by HPLC (Sharma et al., 1993; Murali et al., 2001; Wang and Lee, 2005) and HPTLC (high-performance thin layer chromatography) (Deb and Mandal, 1996; Pothier, 1996; Gupta et al., 1996, 1998, 1999a, b; Gupta and Verma, 1996; Sane et al., 1997; Saxena et al., 2000; Srivastava et al., 2000) were carried out. HPLC analysis produced better resolution and increased peaks than HPTLC (Annamalai and Lakshmi, 2009). In the present study, phyllanthin was detected in aerial parts of all seven herbaceous *Phyllanthus* species. However, only *P. amarus* showed high phyllanthin concentration (0.58%). Tripathi et al. (2006) reported 0.53% phyllanthin in *P. amarus* by HPLC analysis. Khatoon et al. (2006) reported the presence of phyllanthin only in *P. amarus* and could not detect it in *P. fraternus* and *P. maderaspatensis*. These results may be due to very minute concentration of phyllanthin and its poor resolution in HPTLC.

*Phyllanthus debilis*, *P. maderaspatensis*, *P. virgatus*, *P. urinaria*, *P. scabrifolius* and *P. tenellus* showed very minute concentrations of phyllanthin in the present work. Tripathi et al. (2006) also reported very minute concentration of phyllanthin in *P. maderaspatensis* and *P. virgatus*. Present results validate their observation. However, they could not able to detect phyllanthin in *P. debilis* and *P. urinaria*. For the first time phyllanthin was reported in *P. scabrifolius* and *P. tenellus* in the present study.

Although, phyllanthin was detected in all seven *Phyllanthus* species in the present analysis, only *P. amarus* showed higher phyllanthin concentration. The higher phyllanthin concentration might be the reason for much-reported bioactive potentials of *P. amarus* than other *Phyllanthus* species. HPLC method developed here is easy, precise and showed better resolution of phyllanthin in different *Phyllanthus* species. Therefore, this method can be used for standardization of *Phyllanthus* species in herbal drug industry.