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Research Article

Botany for Medicinal science

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 toestablish 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 Phyllantaceae. *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[1]. More than 514 compounds have been isolated from *Phyllanthus*, the majority of which are lignins, triterpenoids, flavonoids, and tannins[2]. Crude extracts of *Phyllanthus spp*..exhibits inhibitory effects on the hepatitis B virus (HBV). Previous reviews depicts the biological activities of *Phyllanthus* species, mostly from *P. amarus, P. emblica* L. and *P. niruri* L.[3,4,5,6]Primarily contains lignin (e.g., phyllanthin and hypophyllanthin)[7,8] geranin and 5 flavonoids (quercetin, astralgin, quercitrin, isoquercitrin and rutin). Due to the presence of potential poly phenolic 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[9,10]. The subject of phyto chemistry 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[11,12]. 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[13]. 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*[14].Flavonoids have various pharmacological activities including anticancer, anti-inflammatory, antioxidant, anti-diabetic, and antiviral activities through various cell-signaling pathways[15]. Most of the flavonoids reported from *Phyllanthus* were flavonol and glycoside forms[16]. From the ethanolic extract of *P. urinaria*, two new acetylated flavonoid glycosides, along with the known isolates, quercetin and quercetin 3-O-α-L-rhamnopyranoside have been isolated[17]. A new flavone sulfonic acid was isolated from the methanolic extract of *P. urinaria*[18].The isolated flavonoids from *P. urinaria* showed antioxidant, anti-inflammatory, anticancer, and anti-*H. Pylori* etc. activities[19] 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-dehydrochebulate, methylgallate, and methyl brevifolincarboxylate[20]. 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[21]. 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[22,23,24].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 [25,26,27,28]. 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 Karnataka 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 1: List of *Phyllanthus*species code and geographical regions

Plant extract code	Name of Test species/code	Voucher specimen	Habit	Latitude & Longitude	Area
1.	*P.amarus* Schum & Thonn. (Pa)	SKU 50217	Herb	14˚28'32.32"N; 78˚43'08.32"E	Yvu garden (KDP Dist.)
2.	*P.emblica* L. (Pe)	SKU 50219	Tree	14˚28'26.75"N; 78˚43'06.56"E	Yvu garden (KDP Dist.)
3.	*P. indofesheri Bennet* (Pi)	SKU 50246	Tree	14˚36'43"N;77˚38'42"E	S.K.U Campus (ATP Dist.)
4.	*P.maderaspatensis* L. (Pm)	SKU 50221	Herb	14˚36'43"N; 77˚38'42"E	S.K.U Campus (ATP Dist.)
5.	*P.narayanaswamii* Gamble (Pn)	SKU 50206	Herb	18˚16'27"N; 82˚19'26"E	Araku (VSKP Dist.)
6.	*P.pinnatus* (Wt.) Webster (Ppi)	SKU 50201	Shrub	15˚00'38.82"N; 78˚01'25.53"E	Bhugga (ATP Dist.)
7.	*P. polyphyllus* Willd. (Ppo)	SKU 50222	Tree	13˚41'57"N; 79˚20'21"E	Tirumala (CTR Dist.)
8.	*P.reticulatus* Poir. (Pre)	SKU 50223	Shrub	14˚12'21.72"N; 78˚07'43.35"E	Kalasamudram (ATP Dist.)
9.	*P. rheedei* Wt. (Prh)	SKU 50204	Herb	15˚53'07.39"N; 78˚49'34.22"E	Rollapenta (KNL District)
10.	*P.rotundifolius* Kl. Ex Willd. (Pro)	SKU 50224	Herb	17˚43'21"N; 83˚19'29"E	A.U. Campus (VSKP Dist.)
11.	*P.tenellus* Roxb. (Pt)	SKU 50225	Herb	13˚42'46"N; 79˚20'31"E	Tirumala (CTR Dist.)
12.	*P.urinaria* L. (Pu)	SKU 50207	Herb	15˚07'46.70"N; 78˚40'41.63"E	Ahobilam (KNL Dist.)
13.	*P.virgatus* Forst (Pv)	SKU 50202	Herb	14˚14'39.70"N; 78˚09'48.36"E	Kalasamudram (ATP Dist.)

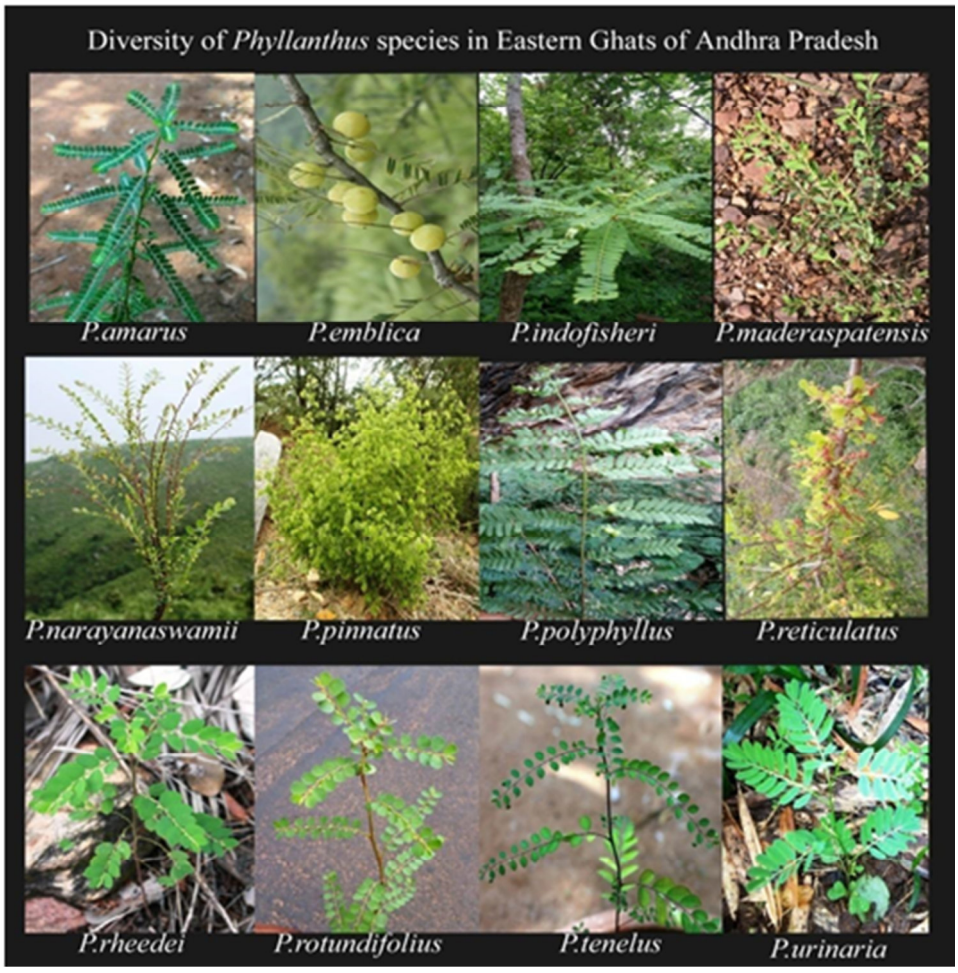


Fig 1: Selected thirteen *Phyllanthus* spp. with their geographical structures

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 x 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[29]. 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

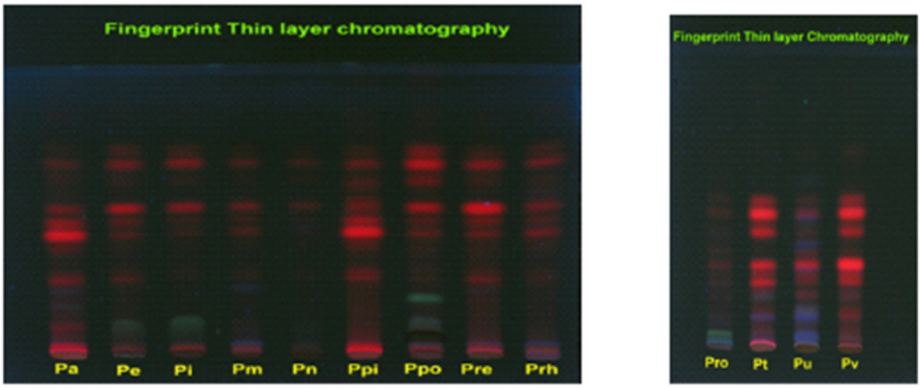


Fig 2. HPTLC Chromatograms of *Phyllanthus amarus* Schum & Thonn. (Pa), *P.emblica* L. (Pe), *P. indofesheri Bennet* (Pi), *P.maderaspatensis* L. (Pm), *P.narayanaswamii* Gamble (Pn), *P.pinnatus* (Wt.) Webster (Ppi), *P. polyphyllus* Willd. (Ppo), *P.reticulatus* Poir. (Pre), *P.rheedei* Wt. (Prh), *P.rotundifolius* Kl. ex Willd. (Pro), *P.tenellus* Roxb. (Pt), *P.urinaria* L. (Pu), *P.virgatus* Forst (Pv) alcohol extracts at UV 366 nm.

Authentication of *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 *Phyllanthus* spp. for the determination of chemical profiles and quantification of the quercetin, phyllanthin, niranthin, and phenolic compounds. Earlier *Phyllanthus* species like *P.urinaria, P.fraternus, P.maderaspatensis, P.amarus, P.debilis* and *P.virgatus* extracts were used for quantification of phyllanthin and niranthin using HPLC technique. *P. amarus* is highly valued in the treatment for liver ailments. Some phytochemical compounds were unique to specific plants and its parts In *Phyllanthus*, lignans like phyllanthin and niranthin are potential therapeutic compounds which serve as hepatoprotective agents. Synthesis of these compounds varies in geographical regions with high concentration in high altitude while less in low altitude. An earlier report revealed that high concentration of Phyllanthin and niranthin were found in leaves than that of fruits, branches and roots of *P.niruri* and also found in methanolic extract of *P.amarus,* but was not found in *P.maderaspatensis, P.fraternus* and *P.virgatus*[30]. HPTLC studies have shown that it is more resourceful than ordinary TLC methods. HPTLC chromatograms (fig-2) of all extracts of 13 *Phyllanthus* spp. were found to be a significant amount of quercetin (flavonoid). 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 *Phyllanthus spp*.. in which phyllanthin generally showed R_f value at 0.31[31], quercetin generally showed R_f value at 0.02 and 0.65[32] phenolic compounds R_f 0.97[33]. 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 *P. maderaspatensis L.* (Pm) Shows high percentage area of quercetin (84%) fallowed by .*P.Polyphyllus*, *P.rheedei*, *P.tenellus P.urinaria* and *P.virgatus* exhibits 63.7%,

82.9%, 72.4%, 82.9% and 66.2% respectively. Plant extracts exhibit another common peak with R_f 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% fallowed by *P. polyphyllus*and *P. rheedei* showed least percent of peak area 0.7 and 0.8 respectively. Plant extracts exhibit another common peak with R_f value at 0.42. As we know that niranthin generally showed R_f value at 0.42[34].*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 R_f 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.narayanswamii* 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[35], cardioprotective[36], antiviral, antibacterial, and anticancer activities[37]. 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 [38]. 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[39]. 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[40] 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.polyphyllus, P.Pinnatus* and *P.maderaspatensis.* from Eastern Ghats of Anhra Pradesh.

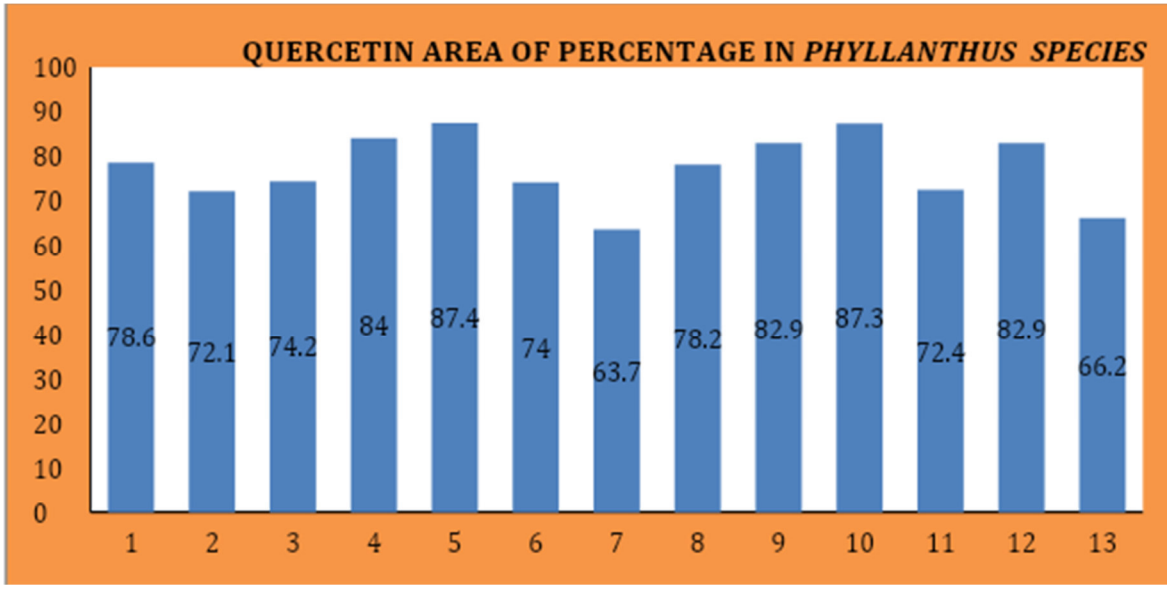


Fig 3: Distribution of percentage area of quercetin in thirteen *Phyllanthus spp.*. extracts in densitograms (codes:1-13)

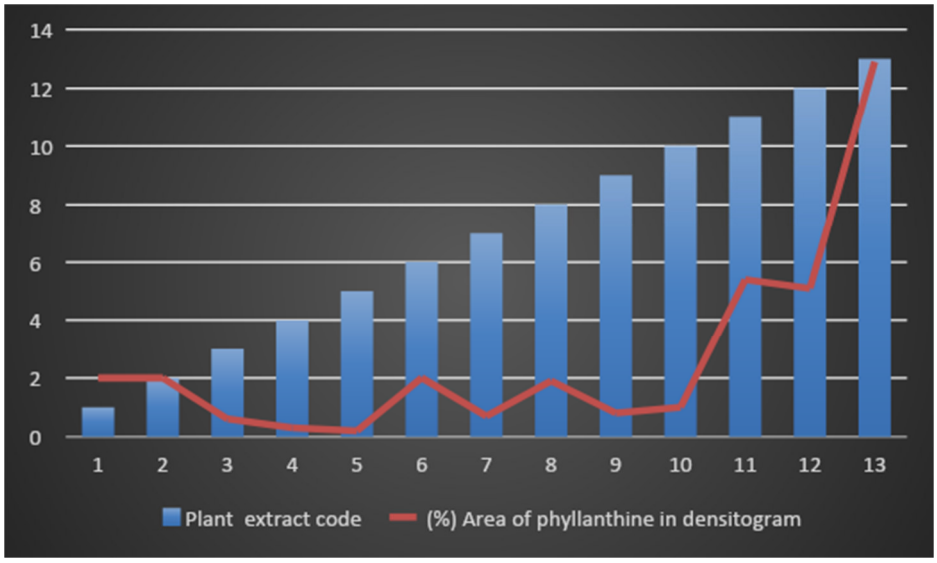


Fig 4: Distribution of percentage area of phyllanthin in thirteen *Phyllanthus spp.*. extracts in densitogram (codes: 1-13)

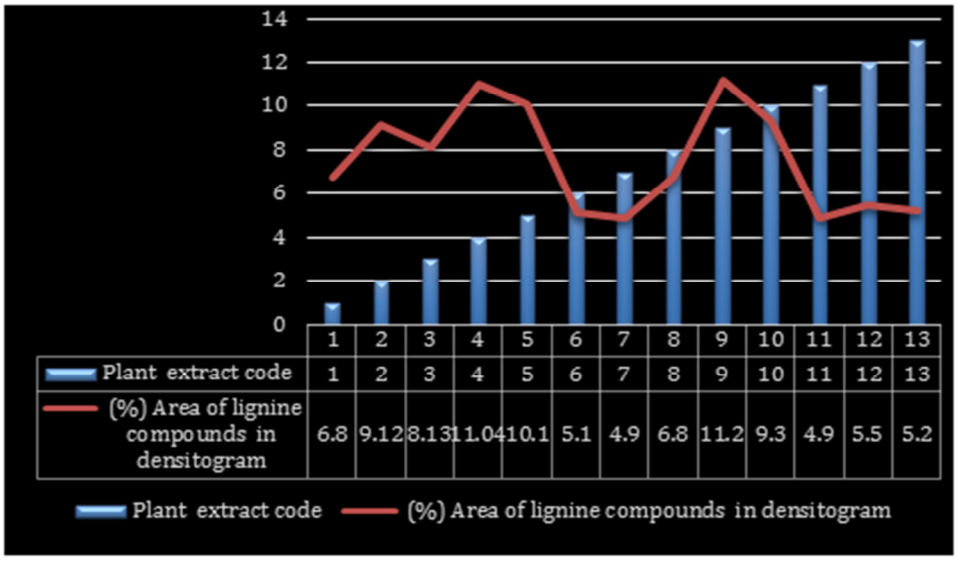


Fig 5: Distribution of percentage area of niranthin in thirteen *Phyllanthus spp.*. Extracts in densitogram (codes:1-13)

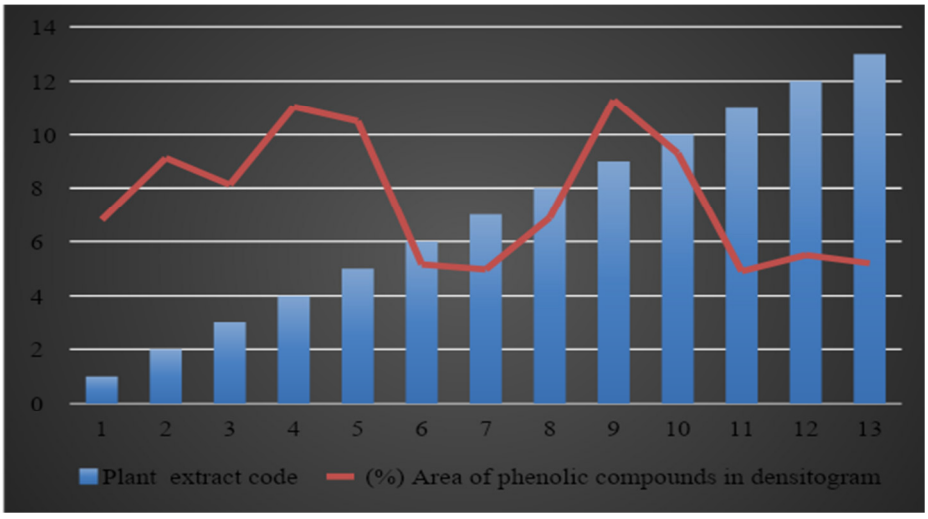
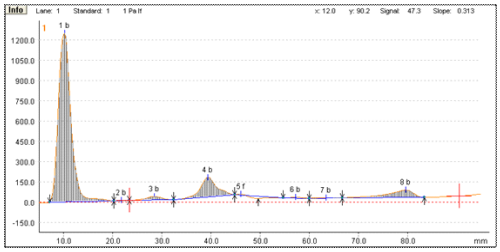
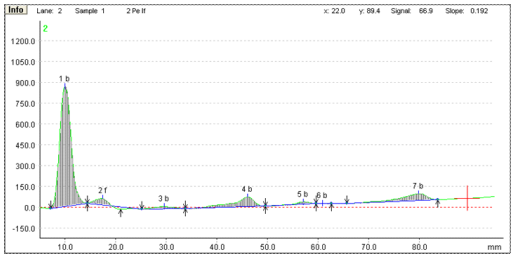


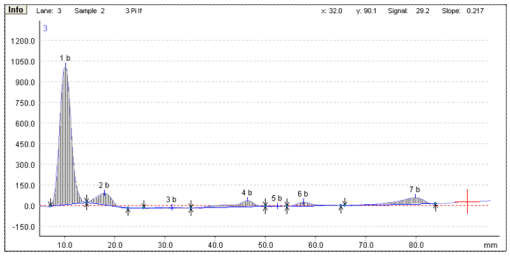
Fig 6: Distribution of percentage area of phenolic compounds in thirteen *Phyllanthus spp.*. extracts in densitograms (codes:1-13)



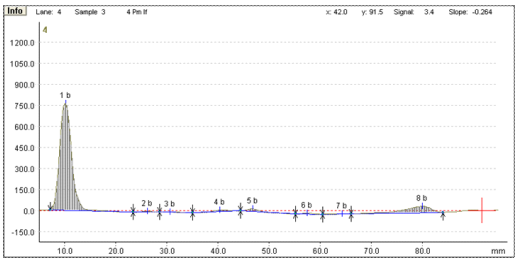
Densitogram of sample extract-I



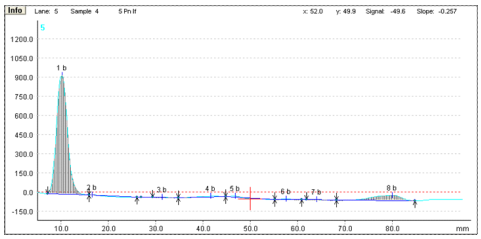
Densitogram of sample extract-II



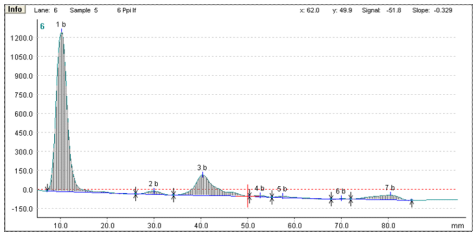
Densitogram of sample extract-III



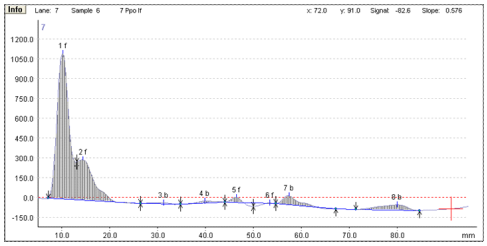
Densitogram of sample extract-IV



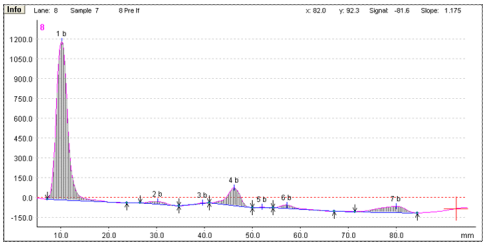
Densitogram of sample extract-V



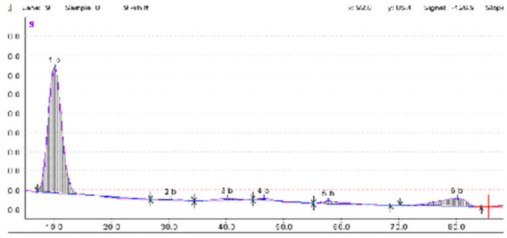
Densitogram of sample extract-VI



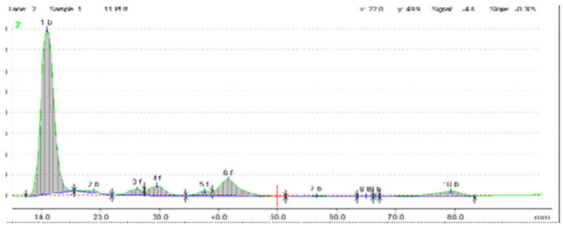
Densitogram of sample extract-VII



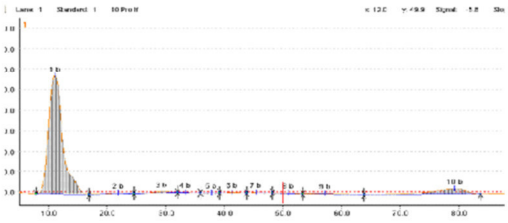
Densitogram of sample extract-VIII



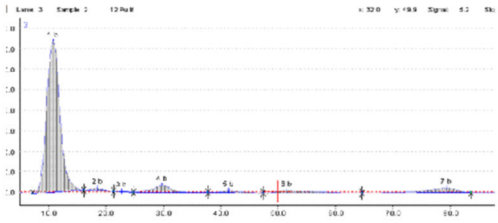
Densitogram of extract - IX



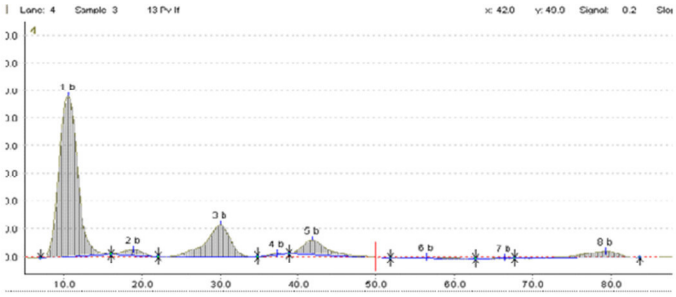
Densitogram of extract - XI



Densitogram of extract -X



Densitogram of extract - XII



Densitogram of extract - XIII

Fig 7.Densitograms of methanolic extracts of *Phyllanthus* species with 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 and phenolic compounds in *Phyllanthus spp*.. Methanolic extracts of all these plants were a good source 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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