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Assessment of Phytochemical Diversity in *Phyllanthus amarus* Using HPTLC Fingerprints

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Abstract— *Phyllanthus amarus* is an important medicinal plant and immensely used in the Indian System of Medicine to cure human diseases. Different types of secondary metabolites present in various organs of it, helped in evaluation of phytochemical diversity in different geographical conditions. We studied on 19 populations of this species collected from different geographical regions of India. All collected populations were differed to each other based on morphological as well as phytochemical markers. The population P_{18} showed more phytochemical diversity as compared to P_3 and P_6 which was evaluated by NTSYS-pc program. The phytochemical diversity was found more in leaves followed by seeds, stem and roots from one geographical region to another region.

Keywords : HPTLC, Hepatoprotective, Similarity Index, Secondary Metabolites.

INTRODUCTION

Phyllanthus amarus (family: Euphorbiaceae) is widely distributed in all tropical and subtropical regions of the planet (Edeoga et al., 2006) and Paleobotanical studies have not found the exact geographic origin of this plant. This plant may be indigenous to the tropical Americas (Cabieses, 1993; Morton, 1981; Tirimana, 1987) and the Philippines or India (Cabieses, 1993, Chevallier, 2000). It is a common pantropical weed that grows well in moist, shady and sunny places (Cabieses, 1993). It is commonly known as "Bhuiamlki" in India and has long been used for the treatment of liver, kidney and bladder problems. In a number of countries, the aerial part of *P. amarus* is highly valued in traditional medicine for its healing properties (Foo and Wong, 1992). Phyllanthin and hypophyllanthin present in this plant are reported as therapeutically active constituents and served as a hepato-protective agent (Padma and Setty, 1999). This species is also used in the most popular Ayurvedic formulations, Chyawanprash, which is consumed at large scale, not only in India but also throughout the world (Ratna Bhushan, 2003)**.** Interest in this plant has been heightened by reports of anti-viral activities and its potential as a remedy for hepatitis B-virus infection (Eldeen et al., 2010). Different plant parts are also ethnobotanically reported to have various therapeutic activities, e.g., leaves as expectorant, diaphoretic and the seeds as carminative, laxative, tonic to the liver, diuretic, diaphoretic, useful in bronchitis, earache, griping, opthalmia and ascites (Kirtikar and Basu, 2001). The fresh roots and leaves have been reported to be potent remedy for the jaundice (Kirtikar and Basu, 2001). A variety of natural products have been found to inhibit activities of unique enzymes and proteins crucial to the life cycle of HIV including efficient intervention with the reverse transcription process, but also virus binding, the integrase or protease (De Clercq, 2000; Jung et al., 2000; Cos et al., 2004; Eldeen et al., 2010**).** *P. amarus* extract also has good antioxidant properties which help in removal of free redicals from the human body (Mhaskar et al., 2000; Raphael and Kuttan, 2003; Londhe et al., 2009; Harikumar et al., 2009; Rai et al., 2007). The phenolic constituents of *P. amarus* mitigated the effect of anti-mycin A-induced mitochondrial apoptotic cascade (Guha et al., 2010).

In India, *Phyllanthus* species constituents, one of the most important groups of species traded as raw herbal drugs. These species are also exported in the powder form for the extraction of a number of phytochemicals or for use in the preparation of traditional formulations in the treatment of liver disorder (Kamble et al., 2008). Medicinal plants are capable of synthesizing an overwhelming variety of low-molecular-weight organic compounds called secondary metabolites, usually with unique and complex structures. Presently, 100,000 such compounds have been isolated from higher plants (Verpoorte and Memelink, 2002). The biosynthesis of secondary metabolites varies among plants, even in different organs of plants, and their biosynthesis depends on the environmental factors in which they grow **(**Khan et al., 2010). Intra-specific variation in phyto-constituents has been documented extensively among the plants (Chew and Rodman, 1979). *P. amarus* has various groups of compounds such as alkaloids, flavanoids, hydrolysable tannins, major lignans and polyphenols. Among these groups of compounds, many has been isolated, characterized and their medicinal

properties were investigated *in vivo*. These compounds are amarin, 1-galloyl-2,3-dehydrohexahydroxydiphenyl (DHHDP)-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, rutin, quercetin 3-O-glucoside, gallic acid, ellagic acid, phenazine, phyllanthin, hypophyllanthin, amariinic acid, amarulone, catechin, gallocatechin, securinine, norsecurinine, epibubbialine, astragalin and epigallocatechin and have complex structure and diverse medicinal properties.

Genetic diversity plays an important role in plant conservation and their survival in adverse conditions. Many environmental factors such as precipitation, mean temperature, soil, wind speed, low and high temperature extremes, duration of snow-cover, length of the vegetation period, and the intensity of radiation under clear sky conditions have been reported to differ between low and high altitude sites (Korner, 1999). Moreover, study on phytochemicals of wild populations of plants at different altitudes were performed, and it is not conclusive whether the observed variations are a response of individual plants to environmental factors related to altitude or a genetic adaptation of the populations growing at different altitudes to their specific environment (Ruhland and Day, 2000; Zidorn and Stuppner, 2001a; Zidorn et al., 2005b). The work on genetic diversity in *Phyllanthus amarus* has been work out through RAPD fingerprint by collecting plant from different geographical regions of India (Jain et al., 2003) but there is no literature found on phytochemicals based diversity in this species. Therefore, in present study, phytochemical diversity was assessed based on total secondary metabolites extracted from various populations of *P. amarus* collected from different geographical regions of India.

MATERIALS AND METHODS

Plant materials

P. amarus accessions (19) collected from different geographical regions of India and its various parts such as roots, leaves, stem and seeds were chosen for the evaluation of the phytochemical diversity. HPTLC fingerprints of methanolic extract of dried and powdered samples were developed.

Preparation of samples for HPTLC fingerprints

The powdered samples of leaves, stem, roots and seeds (1 gm each) of *P. amarus* were employed for the extraction of total phytochemicals in reflux condenser using 25 ml each methanol for 4 hours at 80°C. The methanolic extracts were evaporated to dryness in rotatory vacuum evaporator and the residues obtained were redissolved in methanol (10 ml each), which were used for the application on HPTLC plate for the development of fingerprints.

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 (2: 1, v/v). Linear ascending development was carried out in twin trough glass chamber, 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

and TLC plates were dried with air current provided by an airdryer. The Developed chromatogram was scanned at different wavelengths in multi-wavelength scanning mode to select best suitable wavelength. The slit dimension was kept at 5 mm × 0.1 mm, and 20 mm/s scanning speed was employed. Finally, 254 nm wavelength was selected for densitometric scanning using Camag TLC scanner IV at absorbance mode. The same chromatographic plate was sprayed with sulphuric acid reagent (5% sulphuric acid in methanol) and dried at 110 °C for 10 min to develop complete chromatographic profile after derivatization. Again the plate was scanned at different wavelengths in visible region, from which wavelength of 615 nm was selected. Deuterium and tungsten lamps were used as radiation sources.

Table 1 ***Morphmetric traits of Phyllanthus amarus populations***

Populations	Locations	Altitude (ft)	Plant Height (cm)	Leaflet per compound leaf	Compound leaf per plant	Length of compound leaf	No of seed per compound leaf
P_1	Gowahti (Assam)	170	29.651 ± 4.52	15.33 ± 1.24	19.33 ± 2.62	4.93 ± 0.12	25.66 ± 1.24
P_2	Goalpara (Assam)	114	27.371 ± 3.72	13.33 ± 0.47	16.33 ± 1.69	4.55 ± 0.42	24.33 ± 1.24
P_3	Pantnagar (Uttaranchal)	793	30.377 ± 1.68	14.0 ± 1.63	19.66 ± 1.69	5.05 ± 0.12	25.33 ± 1.24
P_4	Dehradun (Uttaranchal)	2155	32.518 ± 1.61	14.33 ± 2.35	20.33 ± 0.47	5.22 ± 1.68	27.66 ± 0.09
P_5	Kathua (J&K)	1007	29.963 ± 3.81	17.33 ± 3.29	20.00 ± 0.81	5.14 ± 0.25	27.00 ± 2.16
P_6	Bhadarwah (J&K)	5295	31.926 ± 2.56	17.66 ± 0.35	21.00 ± 0.81	4.86 ± 0.26	27.66 ± 1.69
P_7	Jammu (J&K)	1072	32.89 ± 2.00	17.33 ± 1.24	21.66 ± 1.24	5.49 ± 0.4	27.00 ± 0.81
P_8	Udhampur (J&K)	2480	34.25 ± 3.27	17.33 ± 0.47	17.00 ± 2.16	5.53 ± 0.39	25.66 ± 1.24
P_9	Hyderabad (A.P)	1607	46.904 ± 2.58	23.66 ± 1.69	30.00 ± 1.63	6.52 ± 1.71	38.33 ± 1.24
P_{10}	Karimnagar (A.P)	869	40.636 ± 3.71	20.00 ± 0.81	23.33 ± 2.05	6.43 ± 0.8	34.66 ± 5.24
P_{11}	Agra (U.P)	561	37.733 ± 3.72	19.00 ± 0.81	20.66 ± 0.94	6.22 ± 1.68	32.33 ± 4.02
P_{12}	Gorakhpur (U.P)	229	35.56 ± 4.45	19.66 ± 2.05	16.33 ± 1.69	10.3 ± 2.89	28.66 ± 2.35
P_{13}	Basti (U.P)	255	35.584 ± 6.08	18.33 ± 1.24	16.33 ± 1.24	6.47 ± 1.43	29.66 ± 3.09
P_{14}	Aligarh (U.P)	295	35.516 ± 3.08	19.33 ± 0.62	20.66 ± 1.69	5.93 ± 2.16	31.00 ± 4.96
P_{15}	Kota (Rajsthan)	889	42.545 ± 6.18	20.00 ± 0.00	23.33 ± 1.69	6.78 ± 0.48	36.33 ± 4.02
P_{16}	Jaipur (Rajsthan)	1417	43.711 ± 5.78	22.33 ± 1.24	23.66 ± 1.24	6.94 ± 0.37	39.00 ± 4.32
P_{17}	Indore (M.P)	1791	51.933 ± 6.4	27.00 ± 2.16	31.00 ± 1.41	10.33 ± 1.64	41.33 ± 3.29
P_{18}	Sagar (M.P)	1689	48.188 ± 6.63	24.66 ± 0.94	29.33 ± 1.41	11.47 ± 1.3	40.66 ± 4.49
P_{19}	Betul (M.P)	2181	57.004 ± 6.47	27.66 ± 1.69	31.66 ± 2.05	10.79 ± 0.45	44.00 ± 4.32

Values are mean ± SD for three replicate in each group

RESULTS

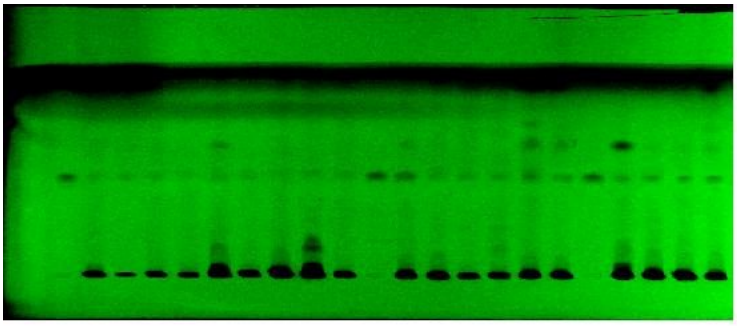
All 19 accessions were compared to each other based on Rf values of the phytochemical markers obtained on scanning of the chromatographic plate at 254 and 615 nm wavelengths. Based on the presence and absence of the compound, the dendrogram was constructed for the correlation among the accessions. In all accessions, phyllanthin compound was found and its content was varied from one geographical region to another region.

Phytochemical diversity evaluation at wavelength 254 nm

The chromatographic plate was scanned under UV wavelength and all compounds were noted for all accessions for the comparison. Phytochemical diversity was analyzed among organs, viz., roots stem, leaves and seeds from developed HPTLC fingerprints (Fig. 1, 2, 3 and 4). The polymorphism was detected 55.56 %, 68.75 %, 86.95 % and 82.35 % in roots, stem, leaves and seeds respectively (Table 2). The average polymorphism in whole plant was found 73.40 % under different environmental conditions.

Table 2. ***Secondary metabolites in different organs of Phyllanthus amarus at wavelength 254 nm***

S. No	Parameter	Root	Stem	Leaf	Seed
1	**Total No of compounds**	74	87	158	84
2	**Total No of loci for compounds**	18	16	23	17
5	**Polymorphic loci**	10	11	20	14
6	**Monomorphic loci**	1	1	1	1
7	**Unique loci**	7	4	2	2
8	**Polymorphism %**	55.56	68.75	86.95	82.35
9	**Average % polymorphism**	73.40			



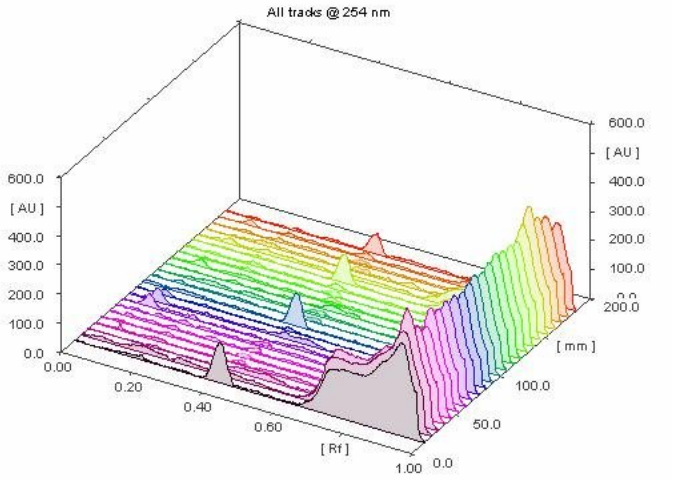
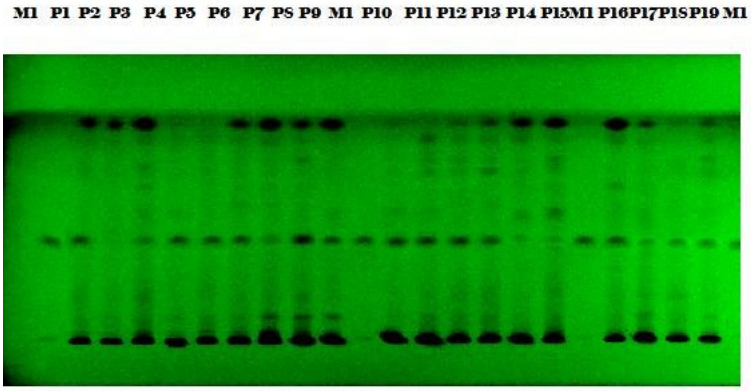


Fig 1. Phytochemical fingerprint and 3d-spectra for roots methanolic extract taken at 254 nm wavelength.



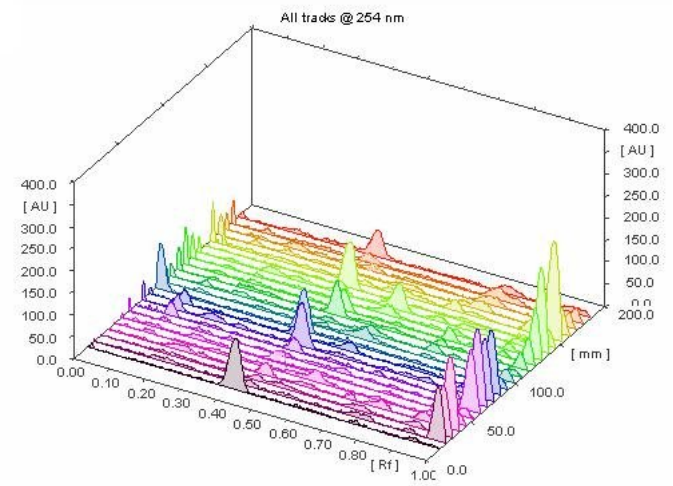


Fig 2. Phytochemical fingerprint and 3d-spectra for stem methanolic extract taken at 254 nm wavelength.

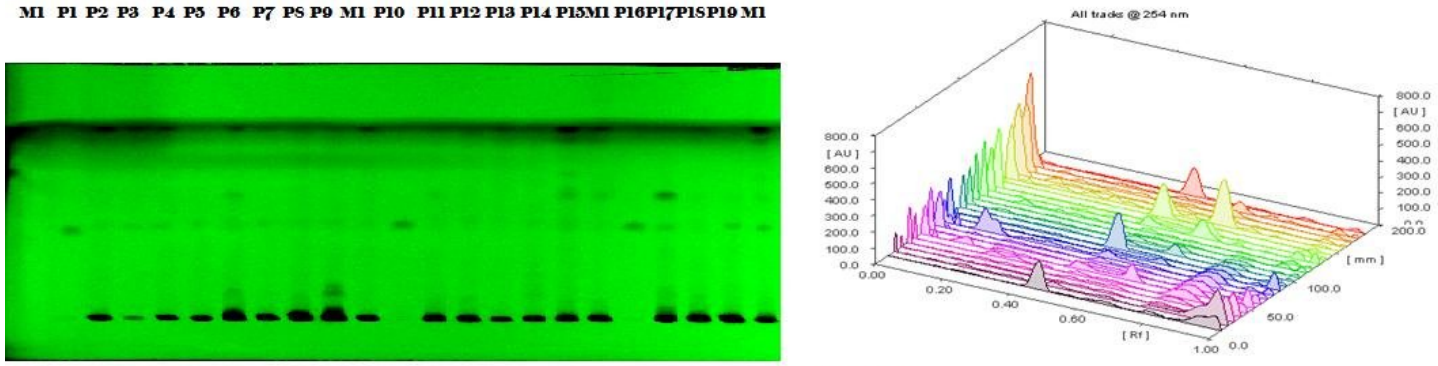


Fig 3. Phytochemical fingerprint and 3d-spectra for seeds methanolic extract taken at 254 nm wavelength.

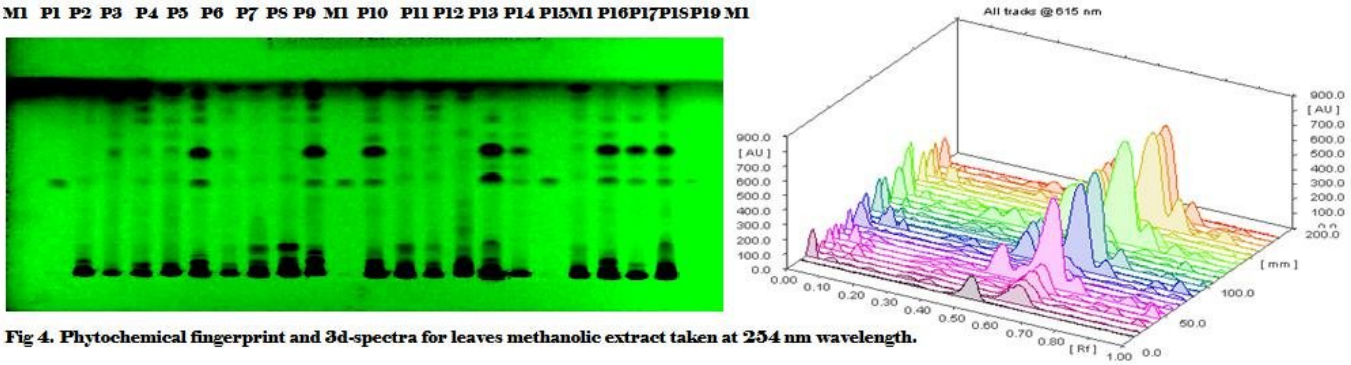


Fig 4. Phytochemical fingerprint and 3d-spectra for leaves methanolic extract taken at 254 nm wavelength.

Phytochemical diversity evaluation at wavelength 615 nm

After spray of the same chromatographic plate with sulphuric acid reagent (5% sulphuric acid in methanol) and dried at 110 °C for 10 min to develop complete chromatographic profile after derivatization. Again the plate was scanned at different wavelengths in visible region, from which wavelength of 615 nm was selected. Phytochemical diversity was also evaluated at 615 nm wavelength from HPTLC fingerprints (Fig. 5, 6, 7 and 8). The polymorphism in different organs, viz., roots, stem, leaves and seeds was 65.0%, 78.57%, 83.34% and 73.33%, respectively and average polymorphism in whole plant was 75.06% (Table 3).

Table 3: ***Comparison of secondary metabolites in different organs of Phyllanthus amarus at wavelength 615 nm***

S. No	Parameter	Roots	Stem	Leaves	Seeds
1	**Total No of compounds**	80	128	165	84
2	**Total No of loci for compounds**	20	28	30	15
3	**Polymorphic loci**	13	22	25	11
4	**Monomorphic loci**	1	1	1	1

5	**Unique loci**	6	5	4	3
6	**Polymorphism %**	65	78.57	83.34	73.33
7	**Average % polymorphism**	75.06			

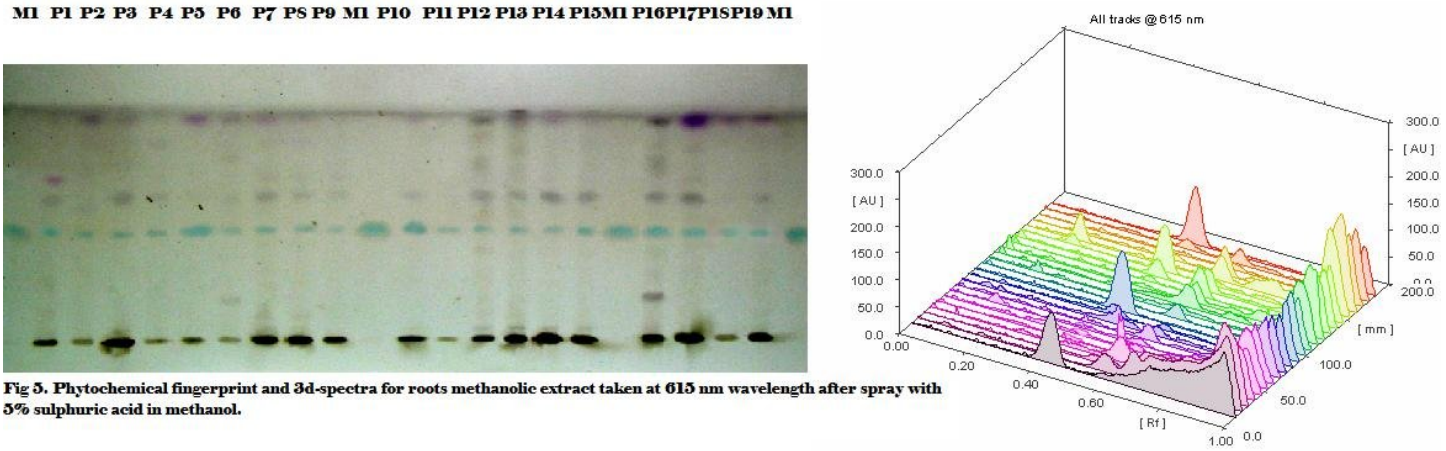


Fig 5. Phytochemical fingerprint and 3d-spectra for roots methanolic extract taken at 615 nm wavelength after spray with 5% sulphuric acid in methanol.

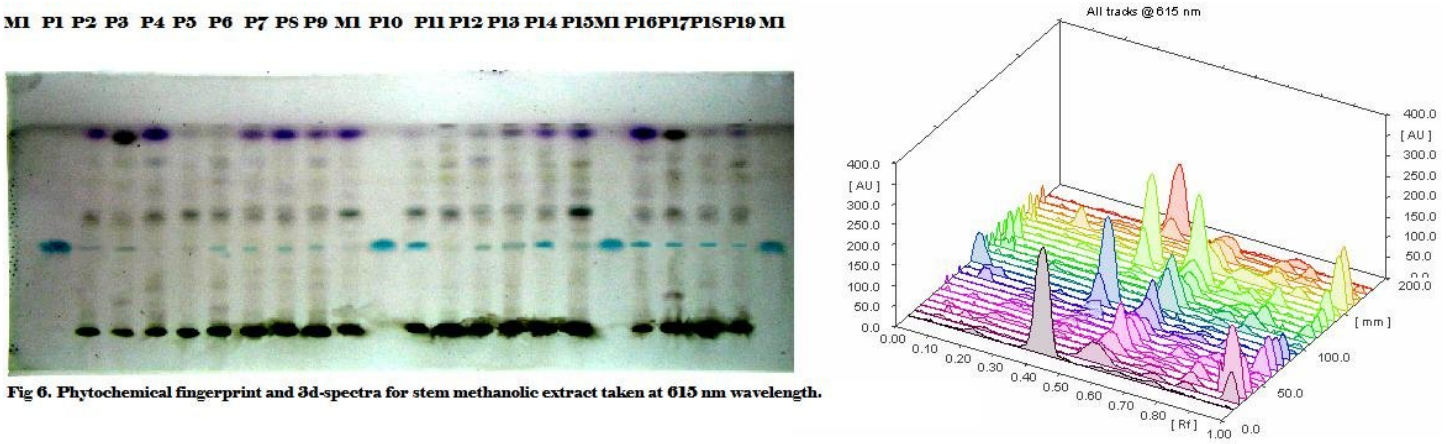


Fig 6. Phytochemical fingerprint and 3d-spectra for stem methanolic extract taken at 615 nm wavelength.

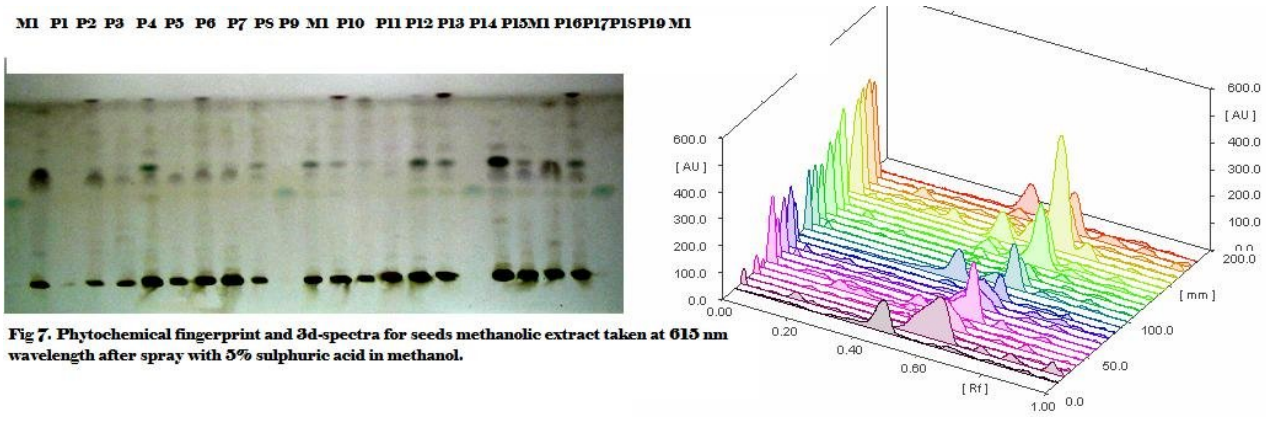


Fig 7. Phytochemical fingerprint and 3d-spectra for seeds methanolic extract taken at 615 nm wavelength after spray with 5% sulphuric acid in methanol.

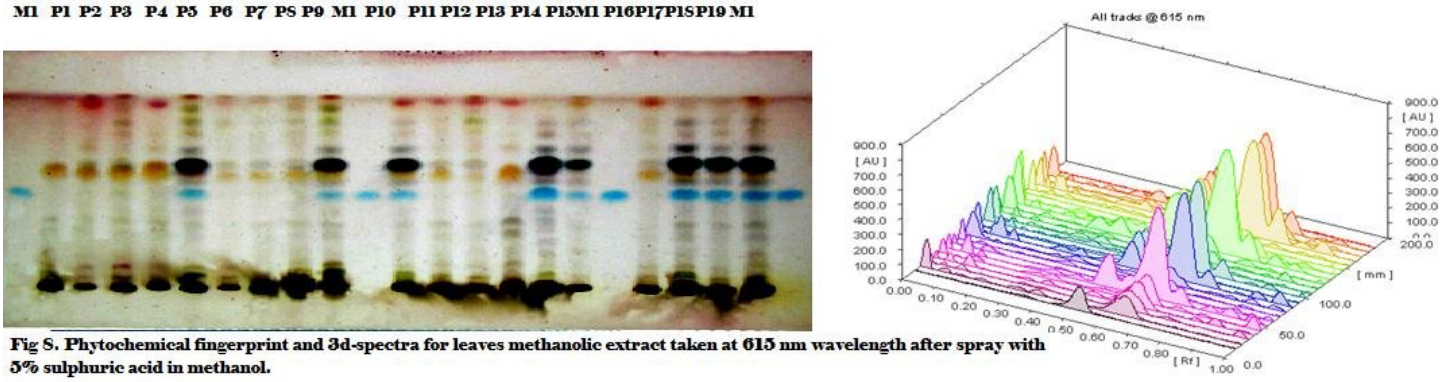


Fig 8. Phytochemical fingerprint and 3d-spectra for leaves methanolic extract taken at 615 nm wavelength after spray with 5% sulphuric acid in methanol.

Evaluation of phytochemical diversity among organs

The organ diversity among accessions was checked at 254 nm and 615 nm wavelengths. There were numerous unique compounds detected in these organs and varied among the accessions. At 254 nm the monomorphic, polymorphic and unique loci were 1, 17 and 32, respectively (Table 4) whereas at 615 nm, these were 3, 41 and 17, respectively.

Table 4: ***Comparative polymorphism in organs of Phyllanthus amarus***

S. No	Parameter	Loci No at 254	Loci at 615nm
1	**Monomorphic**	1	3
2	**Unique loci**	32	41
3	**Polymorphic loci**	17	20
4	**% Polymorphism**	34	31.25

Cluster analysis based on phytochemicals

Based on presence and absence of the phytochemicals in the various organs of the *Phyllanthus amarus*, a dendrogram was constructed for the correlation among the accessions collected from different geographical region of India.

Clustering

The unweighted pair group method with arithmetic average (UPGMA) cluster analysis was used for the construction of the dendrogram for the correlation among the collected accessions. 19 accessions of *P. amarus* were divided into 6 clusters at 55.0% similarity level (Fig. 9). The cluster I had the accessions (P_1, P_2 and P_7); cluster II (P_3, P_6, P_5 and P_4); cluster III (P_8, P_9, P_{10}, P_{11}, P_{12}, P_{16} and P_{13}), cluster IV (P_{14} and P_{15}); cluster V (P_{17} and P_{19}) and cluster VI (P_{18}). The three clusters (I, II and III) further had sub clusters.

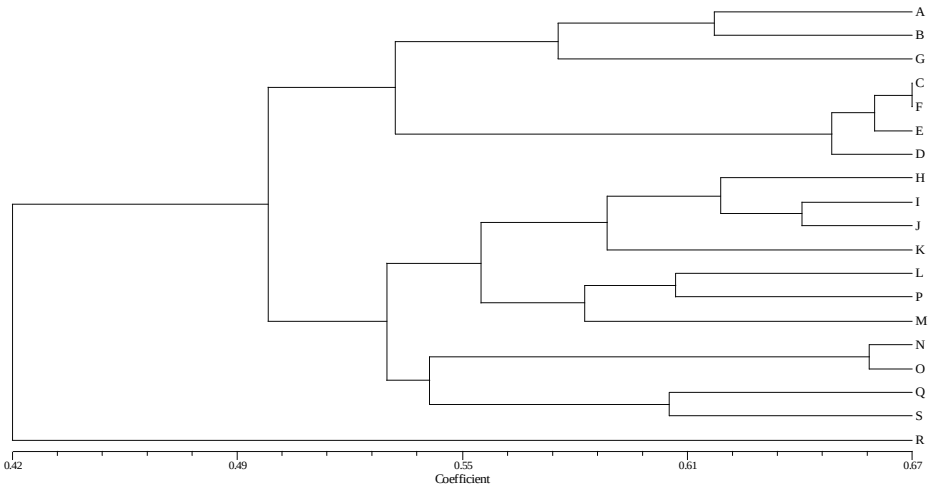


Figure 9 ***UPGMA dendrogram showing clustering of 19 accessions of Phyllanthus amarus.***

Similarity coefficient

Jaccard's similarity coefficients were estimated for all accessions using NTSYS pc program. Its range was found 31.3 % between accession P_2 and P_{18} and 66.7% between P_3 and P_6 (Table 5). Thus, results revealed closeness between P_3 and P_6 and high diversity between P_2 and P_{18} accessions.

Table 5: **Jaccard's coefficient of similarity matrix for phytochemicals data for 19 accessions of *Phyllanthus amarus***

	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	P_{16}	P_{17}	P_{18}	P_{19}
00																		
13	1.000																	
38	0.583	1.000																
26	0.511	0.631	1.000															
76	0.521	0.652	0.652	1.000														
19	0.523	**0.667**	0.653	0.662	1.000													
89	0.574	0.516	0.516	0.519	0.586	1.000												
04	0.500	0.550	0.508	0.529	0.582	0.557	1.000											
93	0.464	0.525	0.569	0.527	0.529	0.516	0.624	1.000										
58	0.418	0.471	0.527	0.507	0.473	0.504	0.607	0.637	1.000									
96	0.437	0.541	0.519	0.469	0.547	0.484	0.582	0.564	0.608	1.000								
55	0.419	0.500	0.478	0.483	0.551	0.512	0.524	0.568	0.500	0.551	1.000							
40	0.465	0.493	0.515	0.486	0.508	0.492	0.541	0.611	0.528	0.582	0.561	1.000						
40	0.394	0.464	0.496	0.500	0.477	0.460	0.450	0.519	0.484	0.444	0.492	0.508	1.000					
73	0.433	0.464	0.542	0.555	0.523	0.496	0.532	0.653	0.570	0.500	0.540	0.570	0.655	1.000				
08	0.466	0.507	0.496	0.532	0.573	0.508	0.557	0.565	0.557	0.523	0.603	0.597	0.574	0.613	1.000			
15	0.467	0.493	0.462	0.467	0.496	0.481	0.515	0.547	0.538	0.541	0.511	0.563	0.530	0.485	0.542	1.000		
38	**0.313**	0.366	0.430	0.437	0.397	0.328	0.437	0.431	0.500	0.443	0.471	0.462	0.418	0.442	0.430	0.464	1.000	
85	0.396	0.444	0.486	0.479	0.478	0.438	0.462	0.496	0.485	0.511	0.492	0.508	0.573	0.560	0.548	0.602	0.544	1.000

DISCUSSION

The phenotypic traits of collected populations of *Phyllanthus amarus* were highly varied from one geographical region to another region of India (Khan et al., 2010). These diverse phenotypic traits are important for the conservation and further may be used high production of potential compounds *in vitro* conditions through biotechnological approaches. *Phyllanthus* species has recently become a

focal point of several studies due to their broad therapeutic use in folk medicine and their wide distribution as well as their diverse secondary metabolite entities (Kalida and Mohan, 2009). Morphological marker(s) have been routinely used to identify genetic diversity but major disadvantages associated with these markers are, the limited number of morphological characters available for analysis further, and these characters also influenced by environmental factors.

Phenotypic variation is especially pronounced in the physiological responses of a plant under growth conditions. The morphometric traits of collected populations, viz., plant height, leaflet per compound leaf, compound leaf per plant, length of compound leaf and number of fruits had negative correlation with increased altitudes. The traits including plant height (27.371 ± 3.72 cm), leaflet per compound leaf (13.33 ± 0.47), length of compound leaf (4.55 ± 0.42 cm) and number of fruits per compound leaf (24.33 ± 1.24) were more reduced in population (P_2) at low altitude 114 ft as compared to high altitude (Table 1). Some populations P_2, P_{12} and P_{13} had similar compound leaf per plant at various altitudes including 114 ft, 229 ft and 255 ft, and it was 16.33 approximately. The number of leaflet and fruits per compound leaf were highest in population P_{19} at altitude 2181 ft, and these were 27.66 ± 1.69 and 44.00 ± 4.32 as compared to the populations collected at low altitude. At elevated altitude, some populations P_4 (2155 ft) and P_6 (5295 ft) showed slight variation in plant height to each other, and it was 32.518 ± 1.61 (cm) and 31.926 ± 2.56 (cm), even these populations had wide differences in altitude. The reason behind this wide variation in morphometric traits may be other various environmental factors present at various locations along with altitudes. Similarly, the most of the morphological data such as length of petiole in sago palm was highly variable according to the environmental conditions (Kjaer et al., 2004).

In dynamic environments, plants can respond to the changing conditions through altered production of secondary metabolites. Secondary metabolites based phytochemical diversity in *P. amarus* was evaluated at wavelength 254 nm and 615 nm. At wavelength 254 nm, the Phytochemical diversity among organs was 55% (root), 81.25% (stem) and 88.46% (leaf), whereas at wavelength 615 nm, the Phytochemical diversity among organs was 65% (roots), 83.33% (stem), 86.66% (leaves) and 80% (seeds) respectively. The average phytochemical diversity in *P. amarus* at wavelength 254 nm and 615nm was 71.67% and 75.66% respectively. The 19 accessions were clustered into 6 clusters and accession P_{18} collected from Sagar (M.P.) showed more Phytochemical diversity as compared to other accessions. The accessions P_3 and P_6 showed more closeness to the other accessions. However, these accessions had different altitudes along with different environmental conditions. Their morphological markers were also varied from one geographical region to another region (Table 1). Phyllanthin compound was found in all accessions but its content was varied (data not shown). Such differences in biosynthesis of secondary metabolites may be due to variations in genetic and environmental factors. Thus, the phytochemical diversity assessed among accessions of *P. amarus* based on secondary metabolites was higher than the DNA based markers as reported by researchers in the literature. This higher phytochemical diversity would help to the future researchers who may exploit this important medicinal herb for various research aspects.

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