Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 5, 2015

Original Article

IMPACT OF CERTAIN BIOTIC AND ABIOTIC FACTORS ON PHYLLANTHIN AND HYPOPHYLLANTHIN CONTENT OF *PHYLLANTHUS AMARUS* SCHUM.& THONN. FROM THREE DIFFERENT HABITATS

DHANDAYUTHAPANI KANDAVEL[1], SOUNDARAPANDIAN SEKAR[2]

[1]Department of Botany, Periyar EVR College (Autonomous), Tiruchirappalli 620023, Tamilnadu, India, [2]Department of Industrial Biotechnology, Bharathidasan University, Tiruchirappalli 620024, Tamilnadu, India
Email: kandavel1976@gmail.com

Received: 06 Feb 2015 Revised and Accepted: 05 Mar 2015

ABSTRACT

Objective: *Phyllanthus amarus* is an important medicinal plant used for its hepatoprotective and other medicinal benefits. The bioactive potential of this plant is due to the presence of two lignans, Phyllanthin and Hypophyllanthin. Overharvest and overexploitation of this plant is reducing its natural population. To develop a proper agrotechnique for cultivation, a wide analysis of habitat ecology and the influence of some factors crucial in the content of these two is essential, which has been attempted**.** Three different study areas differing in soil profile and color where chosen and used as study areas for comparison.

Methods: Phytosociological associations of plants, morphometric and anatomical studies, soil physico-chemical properties, rhizospheric microbes, endophytic microbes, genetic relatedness through RAPD and HPLC profile of Phyllanthin and Hypophyllanthin content was analyzed between the study areas.

Results: This plant has a natural preference to grow with *Cynodondactylon* and in wild conditions the content of the two lignans does not fluctuate drastically and are not very significantly correlated to any biotic or abiotic factor in particular to enhance or decrease them.

Conclusion: Agrotechnique for this plant can be developed keeping different geographic locations as cultivation areas since this plant has good adaptive skills and maintains the balanced biochemical potential.

Keywords: *Phyllanthus amarus*, Phyllanthin, Hypophyllanthin, Hepatoprotective, Agrotechnique, Phytosociology, HPLC, RAPD, Rhizospheric microbes, Endophytes.

INTRODUCTION

*Phyllanthus amarus*Schum.&Thonn. (Phyllanthaceae) is spread throughout the tropical and subtropical countries and commonly used in the Indian Ayurvedic system in problems of stomach, genitourinary system, liver, kidney and spleen[1]. Phyllanthin (a bitter constituent) and Hypophyllanthin (a non-bitter constituent) [2,3] are the active principles responsible for the hepatoprotective role [4] and are present in combination only in *Phyllanthus amarus* and not in the other related species [5]. The highest amounts of phyllanthin (0.7% w/w) and hypophyllanthin (0.3% w/w) have been reported in leaves, whereas; in the stem these are in minor quantities [6]. Around 80% of the global trade in medicinal plant species relies on harvest from the wild. Many species are in danger of over-exploitation and even extinction through over-collection and habitat loss. Reasons for the rarity of medicinal plant species include an array of factors such as habitat specificity, narrow range of distribution, introduction of exotic, habitat alterations, climatic changes, heavy livestock grazing, explosion of human population, fragmentation and degradation of population and genetic drift [7].

Cultivation of medicinal plants is a viable alternative. Many manufacturers prefer wild medicinal plants over cultivated ones as there is a general feeling that wild plant species contain better chemical contents [7]. In Botswana, traditional medicinal practitioners avoid cultivated material as they consider them devoid of the power of the material collected from wild [8]. Moreover, information on the propagation of medicinal plants is available for less than 10% and agro technology is available for 1% of the total known plants globally [9,10]. In India, only 82 medicinal plants have recommended agro practices, by National Medicinal Plants Board (NMPB), New Delhi [11]. In this light, it is imperative to check whether habitat ecology has any role in the phytochemical content before arriving at a proper agrotechnique method for cultivation of P. amarus. In this study, three different study areas differing in soil color and profile were chosen and phytosociological analyses, morphometric, anatomical, soil physico-chemical assessment, rhizospheric microbes, endophytes, genetic diversity and phytochemical content were assessed.

MATERIALS AND METHODS

Materials

Analytical reagent grade chemicals of Sigma–Aldrich for laboratory chemicals and Himedia for microbial media was used for the experiments. The place of work is Tiruchirappalli district and is centrally located in the state of Tamilnadu, India with a total geographic extent of 4404.12 sq. Km. It lies between 78° 10' to 79°5' east longitude and 10°15' and 11 °2' north latitude. It lies at an altitude of 78 m above sea level. The annual mean maximum temperature is 37.7 °C and the annual mean minimum temperature are 18.9 °C. The annual total rainfall is 778 mm. Three Revenue Villages were chosen as study sites representing the major soil types; red (Koppu North), brown (Sathamangalam) and grey (Sikkathambur).

Phytosociological, morphometric and anatomical analyses

Phytosociological studies were carried out following quadrat sampling technique [12]. Since herbaceous species were studied, sampling size of 1 X 1 m^2 was followed [13]. For each study area, ten quadrats were laid and the plants present were counted, recorded and identified using standard identification manuals[14-22]. Primary data thus obtained was analyzed for density, frequency, relative density, relative frequency. Further Shannon-Wiener diversity index (H΄), Simpson diversity index (λ) and Species Importance Value (SIV) [23-25] was calculated. So renson Index (Cs) was calculated to assess the similarity coefficient of the different study areas [26]. The plants were also tested for any morphometric variation. The following characters were recorded for each plant collected from these quadrats and the mean value was drawn for each quadrat: plant height (cm), number of compound leaves per

plant, number of leaflets per compound leaf, length of branchlets (cm), number of fruits per plant, primary root length (cm) and number of secondary roots. Correlation matrix was developed based on this data. Stem and branchlet samples from all the study areas were collected. The plant samples (stem, branchlets and roots) were cross sectioned and compared for any significant changes in the tissue composition, distribution and variation.

$$\text{Species Importance Value} = \text{Relative frequency} + \text{Relative density}$$

$$\text{Shannon-Weiner Diversity Index } (H') = -\Sigma pi \log pi$$

$$\text{Simpson Diversity Index } (\lambda) = \Sigma\, pi^2$$

$$\text{Where, } Pi = \frac{\text{Number of individual of a species}}{\text{Total number of individuals}}$$

So renson index (Cs) = 2j/(a+b)

Where,

j = the number of species found in both sites

a = the number of species found in site A

b = the number of species found in site B

Soil sampling

Rhizosphere soil samples were collected from the study areas following zigzag pattern across the sampling field to ensure homogeneity. All the samples thus collected in a field area are thoroughly mixed,labeled and stored at 4 °C until further analysis.

Soil physico-chemical properties

Soil samples were analyzed for the following parameters: pH and Electrical Conductivity [27]; Available Nitrogen [28]; Organic Carbon [29]; Available Phosphorus [30]; Available Potassium [31]; Micronutrients [32].

Rhizospheric microbes

Rhizosphere soil samples were collected from the plant roots in each study area. 1 gram of soil was taken and mixed with 9 ml of sterile distilled water, vortexed, serially diluted and plated. For bacterial isolation, Nutrient Agar plates were used. The plates were incubated at 37oC for 1-2 day (s) and bacterial, colonies were counted and expressed as CFU/gram dry weight of soil. For fungi, Dichloran Rose Bengal Agar with Chloramphenicol (100 µg/ml) was used.

The plates were incubated at 28 °C for 3-4 day(s) and the number of fungal colonies was counted and expressed as CFU/gram dry weight of soil. For Actinomycetes, Actinomycete Isolation Agar with Nalidixic Acid (25 µg/ml) and Cycloheximide (50 µg/ml) was used. The plates were incubated at 30oC for 7–10 days and the actinomycetes colonies were counted and expressed as CFU/gram dry weight of soil. The dilutions with countable colonies (30 to 300) were looked upon and considered.

Endophytes isolation

The seedlings from the study areas were uprooted, kept in sterile polyethylene bags and brought to the laboratory. Root sections of 2-3 cm were excised using a sterile scalpel from ten plants. Root sections were taken just below the soil line. The samples were surface disinfected with sodium hypochlorite (1.05%) and washed in four changes of 0.02 M phosphate buffer (pH 7.0) solution. Measured quantity of 0.1 ml aliquot from the final buffer wash was removed and transferred in 9.9 ml nutrient agar to serve as sterile check. Samples were discarded, if growth was detected in the sterile check within 48 h [33].

Selected samples were triturated in 9.9 ml of buffer in sterile pestle and mortar. The triturate was serially diluted in potassium phosphate buffer solution and plated on Trypticase Soy Agar (TSA). The medium was supplemented with penicillin G 100 units per ml and streptomycin 100 µg/ml concentrations. The isolates were counted after 48 hrs.

Genetic diversity among *P. amarus* in the study areas using RAPD analysis

Genomic DNA from the accessions was extracted [34] and twenty different primers used in this study were synthesized by Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore. The primers given in table 1 are designated as MAP01 to MAP20 [35] and the sequences were screened for the accessions. The PCR reaction mix (25 µl)used in the experiment is: DNA template-1.72 µl (25 ng); dNTP mix-0.5 µl (200 µM each); Taq DNA polymerase-0.5 µl (0.2 unit); PCR buffer-2.5 µl; Primer-1 µl (10 pmol); Sterile distilled. H_2O-18.78 µl. The amplifications were carried out using a DNA engine thermocycler (Eppendorf Pro-S gradient cyler) and the PCR conditions under which the amplification was carried out is: Initial Denaturation–94°C/5 min; 40 cycles of Denaturation, Annealing and extension at 94°C/1 min,38°C/1 min and 72 °C/2 min respectively; Final Extension–72°C/5 min. The amplified products were separated on 1.8% agarose gel containing 0.5 µg/ml of ethidium bromide and photographed with Alpha InnotechAlphaimager (USA). The profile was analyzed using the Alphaimager systems (version 1.2.0.1). Using the software the molecular weight of each band (by comparing with the marker bands) and their respective Rf values were obtained. Presence and absence of a particular molecular weight band in all the samples for a particular primer were recorded as a binary scoring matrix with 1 in the presence of a band and 0 in the absence of a band. Using RAPDistance Package (Version 1.04) Distance matrix was calculated and dendrogram plotted to assess the genetic relatedness among the accessions.

Table 1: Sequences of primers used in RAPD analysis of *P. amarus* genome

Oligo name	5'<-----Sequence----->3'
MAP01	AAATCGGAGC
MAP02	GTCCTACTCG
MAP03	GTCCTTAGCG
MAP04	TGCGCGATCG
MAP05	AACGTACGCG
MAP06	GCACGCCGGA
MAP07	CACCCTGCGC
MAP08	CTATCGCCGC
MAP09	CGGGATCCGC
MAP10	GCGAATTCCG
MAP11	CCCTGCAGGC
MAP12	CCAAGCTTGC
MAP13	GTGCAATGAG
MAP14	AGGATACGTG
MAP15	AAGATAGCGG
MAP16	GGATCTGAAC
MAP17	TTGTCTCAGG
MAP18	CATCCCGAAC
MAP19	GGACTCCACG
MAP20	AGCCTGACGC

HPLC quantification of Phyllanthin and Hypophyllanthin from leaf samples of P. amarus in the study areas

Seven grams of fresh leaf material were taken and mixed with 2.1 g of Na_2CO_3 dissolved in 30 ml of Distilled Water. The material is kept for maceration at room temperature for 18 h. The macerate was then taken in a Soxhlet apparatus (Extractor–100 ml) and boiled with 200 ml of methanol containing 3% potassium hydroxide for 1 h [36]. The refluxed material was filtered and the residue was again refluxed with the same volume of methanol containing 3% potassium hydroxide for 1 h. The filtrate was collected and combined with the earlier filtrate. The combined volume was noted and 10 µl from this sample was injected into Shimadzu High Performance Liquid Chromatographic system equipped with LC10A pump & SPD-M 10A*vp* Photo diode Array Detector in combination with Class-VP software and LC 2010HT integrated system equipped with Quaternary gradient, auto injector in combination with Lab solution software. 8 mg of Phyllanthin and hypophyllanthin reference standards was weighed in a 50 ml volumetric flask, dissolved in 25 ml of methanol, sonicated for 5 minutes, warmed on

a water bath for 5 minutes, cooled and made up to 50 ml with methanol. Further diluted, to get 16 µg and 1.6 µg each. The mobile phase used in this isocratic elution was acetonitrile: phosphate buffer (pH-2.8) run in a C18, 2.5µ, 100 x 3.0 mm Phenomenex column at a flow rate of 0.4 ml/min detected at 230 nm.

Statistical analyses

Wherever required appropriate statistical validations like Correlation matrix and Pearson's Correlation Coefficient were made with the help of SPSS predictive analysis software, version 18.

Calculation for quantifying phyllanthin (%)

$$\frac{\text{Area of Phyllanthin in sample}}{\text{Area of the standard Phyllanthin}} \text{x} \frac{\text{Weight of Phyllanthin in mg}}{\text{Standard dilution}} \text{x} \frac{\text{Sample dilution}}{\text{Sample weight in mg}} \text{x} \frac{\text{Purity of Phyllanthin x 100}}{100}$$

Calculation for quantifying Hypophyllanthin (%)

$$\frac{\text{Area of Hypophyllanthin in sample}}{\text{Area of the standard Hypophyllanthin}} \text{x} \frac{\text{Weight of Hypophyllanthin in mg}}{\text{Standard dilution}} \text{x} \frac{\text{Sample dilution}}{\text{Sample weight in mg}} \text{x} \frac{\text{Purity of Hypophyllanthin x 100}}{100}$$

RESULTS AND DISCUSSION

The quadrat analysis revealed the ecological preferences of *P. amarus*. The enumeration of plants of the quadrats in all the study areas indicated the richness of *P. amarus* population in places where they grow, whereas, other species of *Phyllanthus* do not show this population richness. From the recorded plants, fifteen species were found exclusively in Sathamangalam; fourteen species were found exclusively in Sikkathambur and 23 species were found exclusively in Koppu.

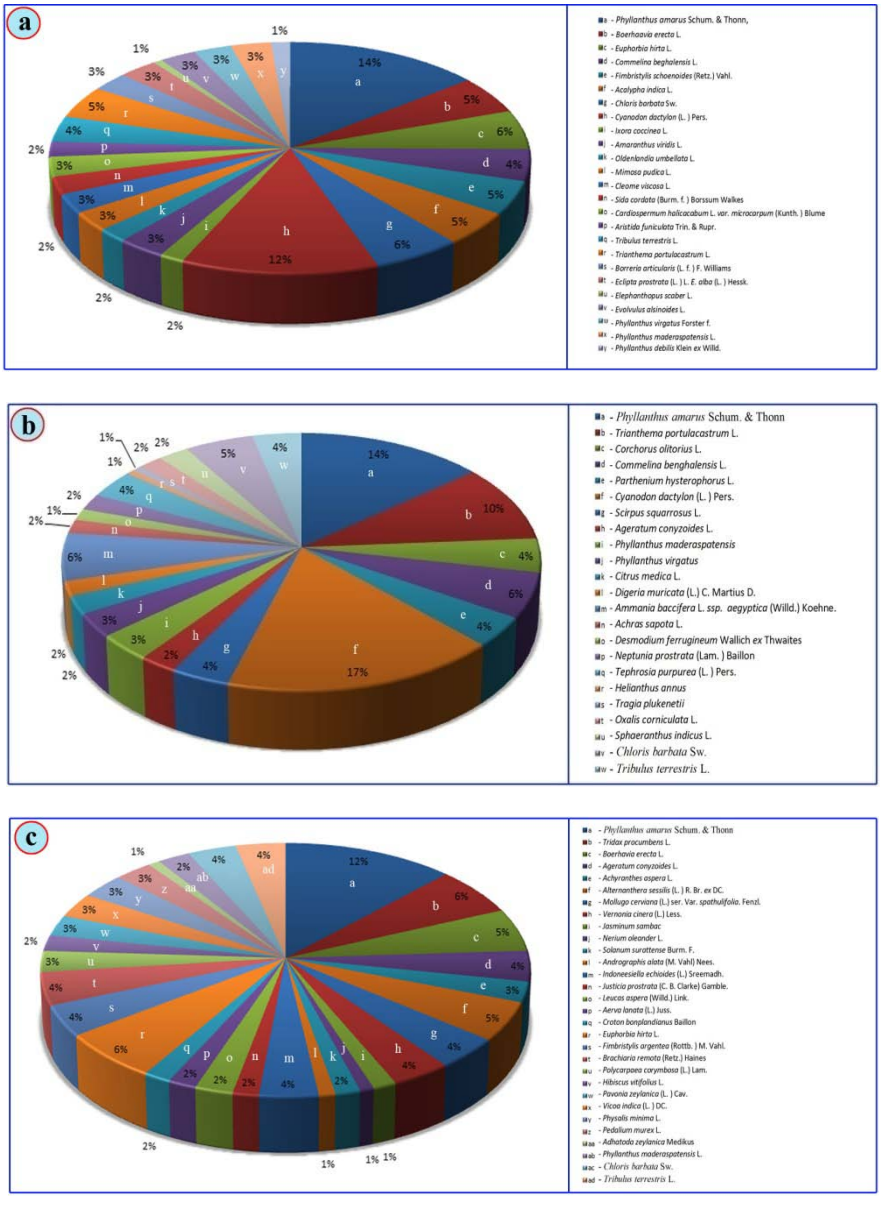

Fig. 1: Species Importance value (%) for the study areas (a) Sathamangalam(b) Sikkathambur (c) Koppu

This reveals the inclination of *P. amarus* to be more associated with *C. dactylon* in the study areas. Simpson Diversity Index (λ) given in fig. 2 was lowest in Koppu revealing high diversity and the Shannon-Wiener Diversity Index (H^1) given in fig. 2 was highest in Koppu justifying its richness.

This indicates the adaptability of the plant and reflects its capability to grow along with diverse plant species in different habitats. The species Importance value presented in fig. 1 in Sathamangalam revealed *P. amarus* with highest presence of 14.3% (SIV = 28.63) followed by*Cynodondactylon* with 12.29% presence(SIV = 24.58); Sikkathambur revealed the highest presence of 17% for *Cynodondactylon*(SIV=33.77) and with 14.04% *P. amarus* (SIV=28. 08) followed and in Koppu the higher of 12.39% presence was by *P. amarus* (SIV=24.77) followed by *Tridaxprocumbens* and *Euphorbia hirta* with 6% presence (SIV = 12.36 and 12.35 respectively).

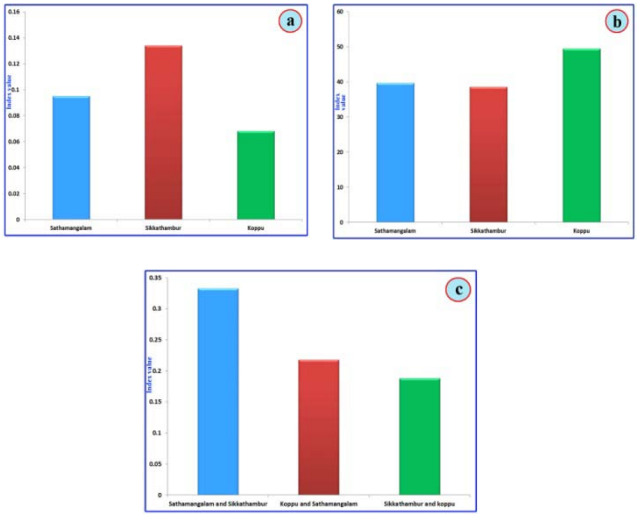

Fig. 2: Comparative analyses of various phytosociological parameters from the three study areas: (a) Simpson Diversity Index (λ)(b) Shannon-Wiener Diversity (H^1)(c) Sorenson Index (Cs)

In Sikkathambur, λ value was highest and a median value was found in Sathamangalam. The H^1 value was more or less equal in Sathamangalam and Sikkathambur. As Shannon-Weiner diversity index is not affected by sample size the results can be taken as a generalized pattern followed by *P. amarus* [37].

Sorenson Index given in fig. 2 implies a similarity in the coefficient between Sathamangalam and Sikkathambur in the diversity whereas Sikkathambur and Koppu have a different diversity pattern.

These analyses disclose certain crucial leads like the distribution pattern, species associations and geospatial variations unique to this species which helped to understand the propagation template it follows in its natural ecosystem, which provides vital clues when agrotechniques were developed.

The correlation matrix of morphometric observations given in table 2 resonates the fact that changes in the values of parameters observed do not significantly affect the overall phenotypic expression in different ecological niches, as, significant positive relationship exists.

Variation in morphometric characters was linked in earlier studies to changes in flavonoids and other production dynamics [38,39] but as the correlation is positive here the variations, if any, in phytochemical constituents between the study areas cannot be attributed to morphometric parameters of this plant in different study areas. Anatomical comparisons of stem, root and branchlets did not show any major variation among the study areas.

Table 2: Correlation matrix for morphometric observations in the study areas

	Sathamangalam	Koppu	Sikkathambur
Sathamangalam	1		
Koppu	0.825**	1	
Sikkathambur	0.793**	0.899**	1

**. Correlation is significant at the 0.01 level (2-tailed).

Correlation coefficient given in table 3 between Sathamangalam and Koppu (r=0.958, p<0.01), between Sathamangalam and Sikkathambur (r= 0.809, p<0.01) and between Koppu and Sikkathambur (r = 0.732, p<0.01) resonate the fact that changes in the values of parameters observed do not significantly affect the overall soil physico-chemical properties in different ecological niches as significant positive relationship exists.

Table 3: Correlation matrix for soil physicochemical parameters in the study areas

	Sathamangalam	Koppu	Sikkathambur
Sathamangalam	1		
Koppu	0.958**	1	
Sikkathambur	0.809**	0.732**	1

**. Correlation is significant at the 0.01 level (2-tailed).

Phenolic content was said to be influenced by soil type in some plants [40] and influence of calcareous oil in influencing linalool, linalyl acetate and trans-myrtanol acetate was detected in *Myrtuscommunis* [41]. As the correlation matrix show positive correlation, any change, in phytochemical profile among the study areas cannot be directly related to soil physico-chemical variables, as the variations are negligible based on statistical analysis of the data. Plant-Soil Feedback studies conducted have suggested that factors like plant abundance, root exudate, etc., can change the soil nature [42] and the positive correlation of the data here may also be due to the influence of *P. amarus* over the soil in which it grows.

The role of soil parameters in influencing the richness of Rhizospheric microbes and endophytic microbes was assessed by employing Pearson's Correlation Coefficient. In Sathamangalam, rhizobacteria and rhizospheric actinomycetes were significantly and negatively correlated with phosphorus and copper at 0.05 and 0.01 levels of significance, respectively. Rhizospheric actinomycetes were positively correlated with rhizobacteria at 0.01 significance level as seen from table 4. In Sikkathambur, positive correlation at 0.01 significance exists between endophytic actinomycetes and soil pH and endophytic bacteria and nitrogen.

Similarly, strong negative correlation exists between endophytic actinomycetes and organic carbon percentage at 0.01 significance level and Iron, Manganese and endophytic actinomycetes at 0.05 significance level as seen from table 5. In Koppu, rhizospheric fungi were strongly and positively correlative with endophytic bacteria at 0.01 significance level. Endophytic fungi were strongly and positively correlative with iron at 0.01 significance level and strongly negatively correlative with zinc at 0.01 significance level. Negative correlation also exists between endophytic fungi and nitrogen and potassium at 0.05 level of significance as evident from table 6. Microorganisms in soil were said to be not only inhabitants, but also active participants in the formation and reorganization and hence the stage of soil formation may have an influence over the diversity found [43]. Generally less than 3,000 bp long amplifications result from RAPD PCR [44] but in our case 4000 bp fragments were produced. Of twenty primers used (MAP01 to MAP20) other than MAP07 all other primers responded to all the accessions. The banding patterns revealed polymorphism among different primers and were similar to a large extent among the accessions.

Using RAPDistance Package (Version 1.04) Distance matrix was calculated and dendrogram plotted and given in fig. 6 to assess the genetic relatedness among the accessions.

Table 4: Pearson's Correlation coefficient between soil physico-chemical parameters and viable microbial population in Sathamangalam

	pH	EC (dSm^{-1})	N (Kg/ac)	P (Kg/ac)	K (Kg/ac)	O. C (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)	R_ Bact	Endo Bact	R_Act	Endo Act	R_Fungi	Endo Fungi
pH	1															
EC (dSm^{-1})	.945	1														
N (Kg/ac)	.877	.672	1													
P (Kg/ac)	-.683	-.885	-.249	1												
K (Kg/ac)	.992	.979	.809	-.771	1											
O. C (%)	-.987	-.985	-.788	.792	-.999*	1										
Fe (ppm)	.047	-.283	.521	.697	-.081	.115	1									
Mn (ppm)	.705	.434	.959	.037	.608	-.581	.742	1								
Zn (ppm)	-.959	-.814	-.977	.449	-.915	.901	-.327	-.876	1							
Cu (ppm)	-.655	-.866	-.212	.999*	-.746	.768	.724	.075	.415	1						
R_ Bact	.655	.866	.212	-.999*	.746	-.768	-.724	-.075	-.415	-1.000**	1					
Endo_Bact	-.866	-.982	-.520	.957	-.923	.935	.459	-.255	.690	.945	-.945	1				
R_Act	.655	.866	.212	-.999*	.746	-.768	-.724	-.075	-.415	-1.000**	1.000**	-.945	1			
Endo_Act	-.929	-.756	-.993	.363	-.873	.857	-.414	-.918	.996	.327	-.327	.619	-.327	1		
R_Fungi	.327	.000	.741	.466	.203	-.171	.959	.901	-.581	.500	-.500	.189	-.500	-.655	1	
Endo_Fung	-.786	-.945	-.392	.989	-.859	.875	.581	-.115	.579	.982	-.982	.990	-.982	.500	.327	1

* Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed)

EC-Electrical Conductivity; O. C-Organic Carbon; R_Bact-Rhizospheric bacteria; Endo Bact-Endophytic bacteria; R_Act: Rhizospheric Actinomycetes; Endo Act-Endophytic Actinomycetes; R_Fungi-Rhizospheric Fungi; Endo Fungi-Endophytic Fungi.

Table 5: Pearson's Correlation coefficient between soil physico-chemical parameters and viable microbial population in Sikkathambur

	pH	EC (dSm^{-1})	N (Kg/ac)	P (Kg/ac)	K (Kg/ac)	O. C (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)	R_ Bact	Endo_Bact	R_Act	Endo_Act	R_Fungi	Endo_Fungi
pH	1															
EC (dSm^{-1})	.866	1														
N (Kg/ac)	.866	.500	1													
P (Kg/ac)	-.971	-.721	-.961	1												
K (Kg/ac)	.693	.240	.961	-.846	1											
O. C (%)	-1.000**	-.866	-.866	.971	-.693	1										
Fe (ppm)	-.999*	-.888	-.843	.959	-.660	.999*	1									
Mn (ppm)	-.997*	-.825	-.902	.986	-.747	.997*	.992	1								
Zn (ppm)	-.500	-.866	.000	.277	.277	.500	.539	.432	1							
Cu (ppm)	.693	.240	.961	-.846	1.000**	-.693	-.660	-.747	.277	1						
R_ Bact	-.866	-1.000**	-.500	.721	-.240	.866	.888	.825	.866	-.240	1					
Endo_Bact	.866	.500	1.000**	-.961	.961	-.866	-.843	-.902	.000	.961	-.500	1				
R_Act	-.770	-.986	-.348	.594	-.074	.770	.798	.718	.938	-.074	.986	-.348	1			
Endo_Act	1.000**	.866	.866	-.971	.693	-1.000**	-.999*	-.997*	-.500	.693	-.866	.866	-.770	1		
R_Fungi	-.500	.000	-.866	.693	-.971	.500	.460	.565	-.500	-.971	.000	-.866	-.167	-.500	1	
Endo_Fungi	.000	.500	-.500	.240	-.721	.000	-.045	.077	-.866	-.721	-.500	-.500	-.638	.000	.866	1

* Correlation is significant at the 0.05 level (2-tailed), ** Correlation is significant at the 0.01 level (2-tailed)

EC-Electrical Conductivity; O. C-Organic Carbon; R_Bact-Rhizospheric bacteria; Endo Bact-Endophytic bacteria; R_Act: Rhizospheric Actinomycetes; Endo Act-Endophytic Actinomycetes; R_Fungi-Rhizospheric Fungi; Endo Fungi-Endophytic Fungi.

Distance matrix ranged from 0.118 to 0.212 and classified the accessions into two clusters. Cluster I included plants from Sathamangalam and Koppu whereas Cluster II comprised of plants from Sikkathambur. Even though the accessions are from geographically distinct study areas, the accessions did not show long genetic distances and a coherent genetic pattern is exhibited among the study areas which is similar to an earlier work [45]. Simultaneous quantification of phyllanthin and hypophyllanthin from the study areas give in fig. 7 revealed the higher percentage of the duo in Sikkathambur followed by Sathamangalam and Koppu respectively.

Table 6: Pearson's Correlation coefficient between soil physico-chemical parameters and viable microbial population in Koppu

	pH	EC (dSm^{-1})	N (Kg/ac)	P (Kg/ac)	K (Kg/ac)	O. C(%)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)	R_ Bact	Endo Bact	R_Act	Endo Act	R_Fungi	Endo Fungi
pH	1															
EC (dSm^{-1})	.982	1														
N (Kg/ac)	.899	.799	1													
P (Kg/ac)	-.397	-.564	.046	1												
K (Kg/ac)	.891	.789	**1.000***	.062	1											
O. C (%)	.000	.189	-.439	-.918	-.454	1										
Fe (ppm)	-.866	-.756	**-.998***	-.115	**-.999***	.500	1									
Mn (ppm)	.792	.663	.980	.245	.983	-.610	-.991	1								
Zn (ppm)	.866	.756	**.998***	.115	**.999***	-.500	**-1.000****	.991	1							
Cu (ppm)	-.381	-.200	-.748	-.697	-.759	.924	.792	-.866	-.792	1						
R_ Bact	-.936	-.853	-.995	.049	-.994	.352	.987	-.956	-.987	.682	1					
Endo Bact	.655	.500	.920	.434	.926	-.756	-.945	.980	.945	-.948	-.879	1				
R_Act	.596	.737	.183	-.974	.167	.803	-.115	-.017	.115	.515	-.276	-.217	1			
Endo_Act	.982	.929	.965	-.217	.961	-.189	-.945	.893	.945	-.549	-.986	.786	.434	1		
R_Fungi	.655	.500	.920	.434	.926	-.756	-.945	.980	.945	-.948	-.879	**1.000****	-.217	.786	1	
Endo_Fun	-.866	-.756	**-.998***	-.115	**-.999***	.500	**1.000****	-.991	**-1.000****	.792	.987	-.945	-.115	-.945	-.945	1

*. Correlation is significant at the 0.05 level (2-tailed), **. Correlation is significant at the 0.01 level (2-tailed).

EC-Electrical Conductivity; O. C-Organic Carbon; R_Bact-Rhizospheric bacteria; Endo Bact-Endophytic bacteria; R_Act: Rhizospheric Actinomycetes; Endo Act-Endophytic Actinomycetes; R_Fungi-Rhizospheric Fungi; Endo Fungi-Endophytic Fungi. Molecular marker analyses among accessions from the three study areas given in fig.3, 4, 5 revealed bands ranging from fifty bp to 4000bp in size.

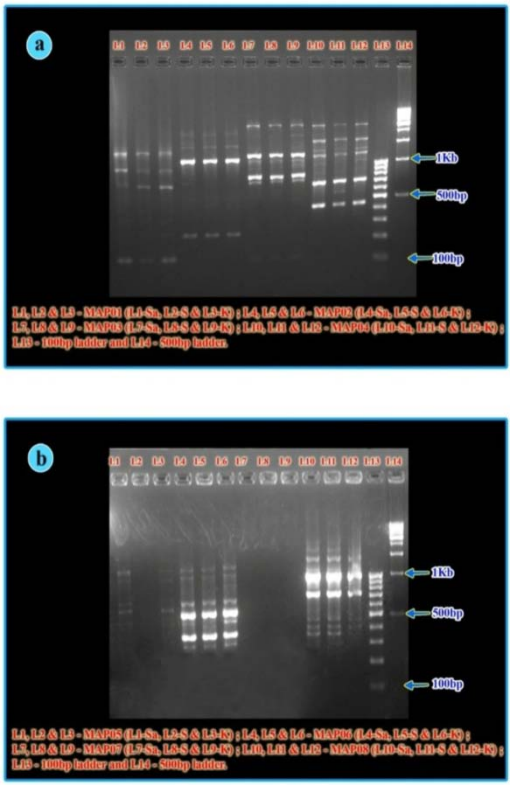

Fig. 3: Submarine Agar gel electrophoresis of RAPD based on PCR products of *P. amarus* genome from the study areas using MAP01 to MAP08 primers: MAP01, MAP02, MAP03 and MAP04MAP05, MAP06, MAP07 and MAP08; Sa–Sathamangalam; S–Sikkathambur; K–Koppu

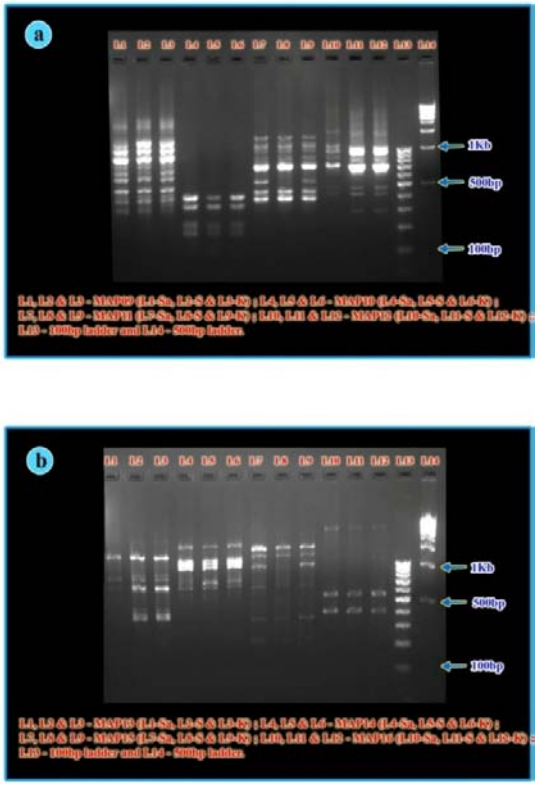

Fig. 4: Submarine Agar gel electrophoresis of RAPD based on PCR products of *P. amarus* genome from the study areas using MAP09 to MAP16 primers: (a) MAP09, MAP10, MAP11 and MAP12 (b) MAP13, MAP14, MAP15 and MAP16; Sa–Sathamangalam S–Sikkathambur K–Koppu

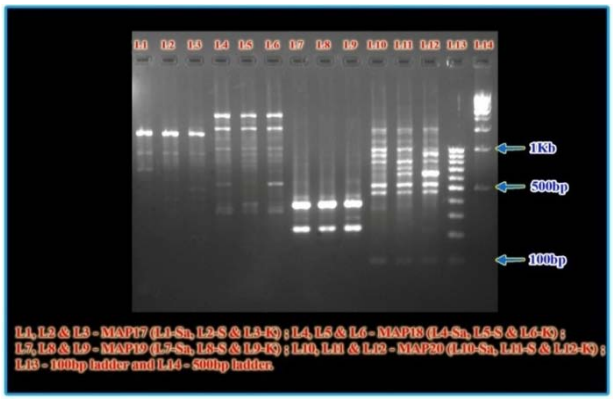

Fig. 5: Submarine Agar gel electrophoresis of RAPD based on PCR products of *P. amarus* genome from the study areas using MAP17 to MAP20 primers: Sa–Sathamangalam S–Sikkathambur K–Koppu

Recovery of phyllanthin and hypophyllanthin were at 99% for both, revealing the effectiveness of the method, better than, earlier reports of 98.7 and 97.3% recovery for the phyllanthin and hypophyllanthin [46]. The ratios of phyllanthin and hypophyllanthin for Koppu, Sathamangalam and Sikkathambur were, 0.58:0.9, 0.68:1.04 and 1.04:1.36 respectively, indicating higher levels of hypophyllanthin than phyllanthin, compared to higher levels of phyllanthin than hypophyllanthin reported (1.4:0.6) in some studies [36].

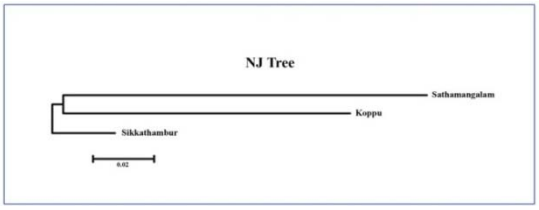

Fig. 6: Distance matrix Dendrogram showing diversity of the *Phyllanthus amaraus* schum.& Thonn. from the study areas

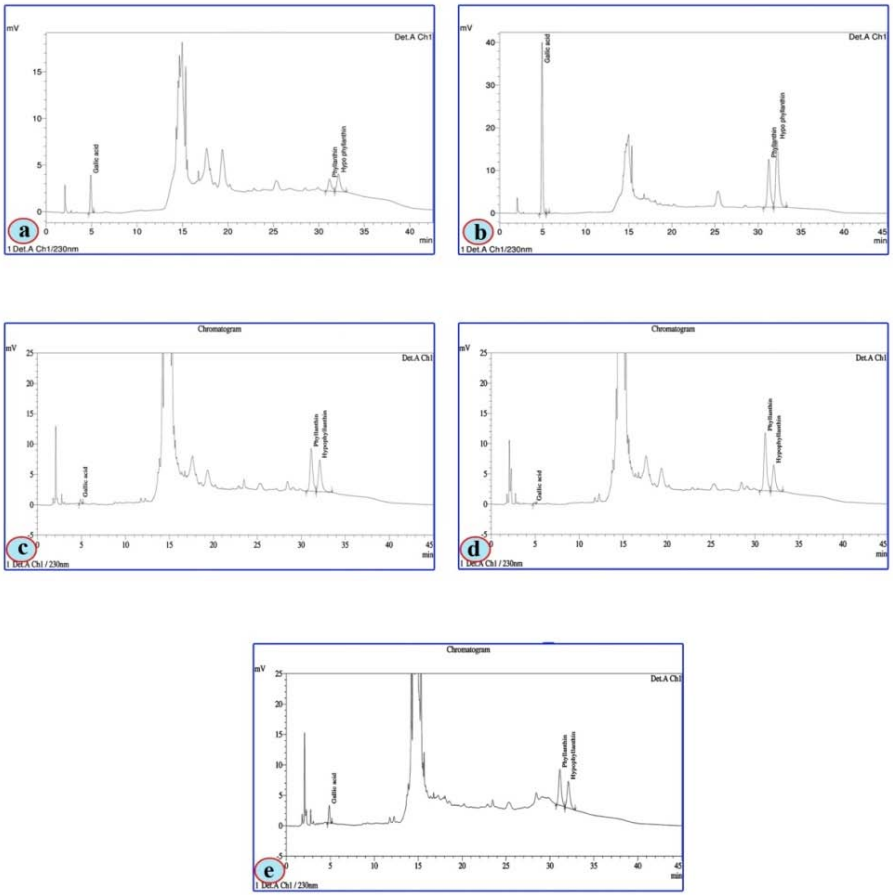

Fig.7: HPLC quantification of Phyllanthin and Hypophyllanthin in *Phyllanthus amaraus* schum.& Thonn. from the study areas: (a) Mixed standards of Gallic acid, Phyllanthin and Hyphophyllanthin standard (1.6 µg each) (b) Mixed standards of Gallic acid, Phyllanthin and Hyphophyllanthin standard (16 µg each) (c) Phyllanthin and Hyphophyllanthin from *P. amaraus*of Sathamangalam(d) Phyllanthin and Hyphophyllanthin from *P. amaraus*of Sikkathambur(e) Phyllanthin and Hyphophyllanthi*n* fro*m P. amaraus*of Koppu

CONCLUSION

Phytosociological analysis by quadrats in the three sites revealed the association of P. amarus with other plants. P. amarus was found associated more closely with the grass, Cynodondactylon. Comparison of morphometric, anatomical and soil physico-chemical features in the three areas showed no momentous variations. Microbial diversity in the three study areas showed significant variations. The content of phyllanthin and hypophyllanthin as well as genetic diversity of P. amarus showed slight variations in the study areas. It is thus possible to cultivate P. amarus in different geographical locations without compromising its medicinal value.

ACKNOWLEDGEMENTS

The authors thank University Grants Commission, Govt. of India, New Delhi for the award of Teacher fellowship (FDP) to Kandavel.

CONFLICT OF INTERESTS

We declare that we have no conflict of interest

REFERENCES

1. Patel JR, Tripathi P, Sharma V, Chauhan NS, Dixit VK. *Phyllanthus amarus*: ethnomedicinal uses, phytochemistry and pharmacology: a review. J Ethnopharmacol 2011;138:286-13.
2. Row LR, Satyanarayana P, Subba Rao GSR. Crystalline constituents of Euphorbeaceae–the synthesis and absolute configuration of phyllanthin. Tetrahedron 1967;23:1915-8.
3. Row LR, Satyanarayana P, Srinivasulu C. Revised structure of hypophyllanthin from *Phyllanthus niruri* Linn. Tetrahedron 1970;26:3051-7.
4. Shyamsundar KV, Singh B, Thakur R, Hussain A, KisoY, Hikino H. Antihepatotoxic principles of *Phyllanthus niruri* herbs. J Ethnopharmacol 1985;14:41-4.
5. Khatoon S, Rai V, Rawat AKS, Mehrotra S. Comparative pharmacognostic studies of three *Phyllanthus* species. J Ethnopharmacol 2006;104:79–86.
6. Sharma A, Singh RT, Handa SS. Estimation of phyllanthin and hypophyllanthin by high performance liquid chromatography in *Phyllanthus amarus*. Phytochem Anal 1993;4:226-9.
7. Kala CP, Dhyani PP, Sajwan BS. Developing the medicinal plants sector in northern India: challenges and opportuinites. J Ethnobiol Ethnomed 2006;2:32.
8. Cunningham AB. Management of medicinal plant resources. In: Seyani JH, Chikuni, Chikuni AC. Editors. Proceedings of the 13th Plenary Meeting of AETFAT, Zomba, Malawi 1991;1:173–89.
9. Lozoya. Ethnobotany and the search of new drugs. UK: John Wiley and Sons; 1994.
10. Khan IA, Khanum A. Role of biotechnology in medicinal and aromatic plants. Hyderabad: Ukaaz Publications; 2000.
11. NMPB. Agrotechniques of Selected medicinal Plants 2012. Available from: http://www.nmpb-mpdb.nic.in.
12. Cottam G, Curtis JT. The use of distance measures in phytosociological sampling. Ecol 1956;37:451-60.
13. McIntosh R. P Pattern in a forest community. Ecol 1962;43:25-33.
14. Matthew KM. Illustrations on the flora of the tamil nadu carnatic. The Rapinat Herbarium. Tiruchirappalli, India: St. Joseph's College; 1982.
15. Matthew KM. The flora of the tamil nadu carnatic. Part I. Polypetalae. The Rapinat Herbarium Tiruchirappalli, India: St. Joseph's College; 1983a.
16. Matthew KM. The flora of the tamil nadu carnatic. Part II. Gamopetalae & Monochlamydeae. The Rapinat Herbarium. Tiruchirappalli, India: St. Joseph's College; 1983b.
17. Matthew KM. The flora of the tamil nadu carnatic. Part III. Monocotyledones. The Rapinat Herbarium. Tiruchirappalli, India: St. Joseph's College; 1983c.
18. Matthew KM. Further Illustrations on the flora of the tamil nadu carnatic. The Rapinat Herbarium. Tiruchirappalli, India: St. Joseph's College; 1988.
19. Gamble JS, Fischer CEC. The flora of the presidency of madras. XI Parts. London: Adlard Son, Ltd; 1915-1936.
20. Nair NC, Henry AN. Flora of Tamil Nadu. Vol. I. Coimbatore, India: Botanical Survey of India, Department of Environment; 1983.
21. Henry AN, Kumari GR, Chitra V. Flora of Tamil Nadu. Vol. II. Coimbatore, India: Botanical Survey of India, Department of Environment; 1983.
22. Henry AN, Chitra V, Balakrishnan NP. Flora of Tamil Nadu. Vol III. Coimbatore, India: Botanical Survey of India, Department of Environment; 1989.
23. Boom BM. A forest inventory in Amazonian Balivia. Biotropica 1986;18:287-94.
24. Magurran AE. Ecological diversity and its measurement. 451, Williams Street, Princeton, New Jersey: Princeton University Press; 1988.
25. Michael P. Ecological methods for field and laboratory investigation. New Delhi: Tata McGraw Hill; 1990.
26. Janson S, Vegelius J. Measures of ecological association. Oecologia 1982;49:371-6.
27. Peters JB. Recommended methods of manure analysis. University of Wisconsin, Madison, Cooperative Extension Publication A3769;2003. Available from: http://uwlab.soils.wisc.edu/pubs/A3769.pdf
28. Subbiah BB, Asija GL. A rapid procedure for estimation of available N in soils. Curr Sci 1956;25:259–62.
29. Walkley A, Black IA. An examination of the Degtareff method for determining soil organic matter and a proposed modification of the chromic acid titration. Soil Sci 1934;34:29-38.
30. Olsen S, Cole C, Watanabe F, Dean L. Estimation of available phosphorus in soils by extraction with sodium bicarbonate., Washington, DC: USDA Circular Nr 939, US Gov. Print. Of fiœ; 1954.
31. Amerine MA, Kishaba TT. Use of the flame photometer for determining the sodium, potassium and calcium content of wine. Am J Enol Vitic 1952;3:77-86.
32. Clesceri LS, Greenberg AE, Eaton AD. Standard methods for the Examination of water and waste water. Washington DC: American Public Health Association; 1998.
33. Mathiyazhagan S, Kavitha K, Nakkeeran S, Chandrasekar G, Manian K, Renukadevi P, *et al.* PGPR mediated management of stem blight of *Phyllanthus amarus* (Schum. and Thonn.)caused by *Corynesporacassiicola* (Berk and Curt) Wei. Arch Phytopathol Plant Prot 2004;37:183–99.
34. Moller EM, Bahnweg G, Sandermann H, Geiger HH. A simple and efficient protocol for isolation of high molecular weight DNA from filament fungi, fruit bodies and infected plant tissue. Nucl Acids Res 1992;20:6115-6.
35. Jain N, Shasany AK, Sundaresan V, Rajkumar S, Darokar MP, Bagchi GD, *et al.* Molecular dieversity in *Phyllanthus amarus* assessed through RAPD analysis. Curr Sci 2003;85:1454-8.
36. Sharma A, Singh RT, Handa SS. Estimation of phyllanthin and hypophyllanthin by high performance liquid chromatography in *Phyllanthus amarus*. Phytochem Anal 1993;4:226-9.
37. Spellerberg IF. Shannon-Wiener Index. In: Jorgensen SE, Fath B. editors. Encyclopedia of Ecology. Elsevier; 2008. p. 3249-52.
38. Horwath AB, Grayer RJ, Keith-Lucas MD, Simmonds MSJ. Chemical characterisation of wild populations of *Thymus* from different climatic regions in southeast Spain. Biochem Syst Ecol 2008;36:117-33.
39. Boström C, Roos C, Rönnberg O. Shoot morphometry and production dynamics of eelgrass in the northern Baltic Sea. Aquat Bot 2004;79:145–61.
40. Vilanova M, Santalla M, Masa A. Environmental and genetic variation of phenolic compounds in grapes (*Vitisvinifera*) from northwest Spain. J Agric Sci 2009;147:683-97.
41. Flamini G, Cioni PL, Morelli I, Maccioni S, Baldini R. Phytochemical typologies in some populations of *Myrtuscommunis* L. on Caprione Promontory (East Liguria, Italy). Food Chem 2004;85:599–04.
42. Kulmatiski A, Beard KH, Stevens JR, Cobbold SM. Plant–soil feedbacks: a meta-analytical review. Ecol Lett 2008;11:980–92.
43. Pace NR. A molecular view of microbial diversity and the biosphere. Sci 1997;276:734–40.
44. Semagn K, Bjørnstad A, Ndjiondjop MN. An overview of molecular marker methods for plants. Afr J Biotechnol 2006;5:2540-68.
45. Darokar MP, Suman PSK, Shasany AK, Kumar S. Low levels of genetic diversity detected by RAPD analysis in geographically distinct accessions of Bacopamonnieri. Genet Resour Crop Evol 2001;48:555–8.
46. Tripathi AK, Verma RK, Gupta AK, Gupta MM, Khanuja SPS. Quantitative determination of phyllanthin and hypophyllanthin in *Phyllanthus* species by high-performance thin layer chromatography. Phytocheml Anal 2006;17:394–7.