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ASSESSMENT OF ALIZARIN, PURPURIN AND GENETIC FIDELITY OF *RUBIA CORDIFOLIA* L. FROM EASTERN GHATS OF TAMIL NADU, INDIA

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ABSTRACT

Rubia cordifolia L. is a widely used medicinal plant belongs to the family Rubiaceae. The evaluation of the alizarin, purpurin and genetic fidelity of *Rubia cordifolia* L. is crucial for the identification of this economically and ecologically valuable climber. In this study, we quantify the phytochemicals (alizarin and purpurin) using RP-HPLC analysis and Genetic fidelity studies carried out using RAPD markers. Phytochemical studies showed gradual variation among the samples. The maximum amount of alizarin and purpurin was found in the plant samples of Kalrayan hills (GBR3- 32.4% and 33.4%) followed by Kolli Hills (GBR1- 27.0% and 16.5%), Pachamalai (GBR6- 23.8% and 20.7%), Shervaroy hills (GBR2 16.6% and 9.4%), Jawadhu hills (GBR5- 13.4% and 11.7%), Chitteri hills (GBR4- 12.9% and 0.59%) and Yelagiri hills (GBR7 8.7% and 0.87%) respectively. 15 RAPD primers were used to distinguish the genetic fidelity among *Rubia cordifolia* L. collected from Eastern Ghats of Tamil Nadu. 15 RAPD primers amplified 246 polymorphic bands. Each primer classified the species under investigation into clear, completely separated clusters, although maximum conformity was achieved with respect to species relationships when the RAPD method was employed. Overall, results indicated that RP-HPLC represents an effective tool for phytochemical studies and RAPD represents an efficient molecular marker system for the assessment of genetic fidelity and diversity.

KEYWORDS: ***Rubia cordifolia*** **, RP-HPLC, alizarin, purpurin, RAPD, genetic fidelity, Eastern Ghats.**

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INTRODUCTION

Rubia cordifolia Linn. is a flowering plant species belong to the family Rubiaceae. It is commonly known as Manjistha and in English Indian madder. Roots and stems are an active part of the plant. Plant has many pharmacological actions like blood purifier activity, anticancer, astringent, antidysentric, antiseptic, deobstruent properties and antirheumatic, hepatoprotective[1]. Hepatoprotective action is mainly shown by rubiadin[2]. The plant contains various chemical constituents like anthraquinones[3], iridoids[4], hexapeptides, rubiprasins, quinones, and triterpenoids[5]. The plant contains substantial amounts of anthraquinone especially in the roots, the coloring matter present in the root is a mixture of purpurin (trihydroxyanthraquinone) and munjisthin (xanthopurpurin-2-carboxylic acid) having antitumor properties. It also hasradioprotective[6], anticancer and antioxidant[7], antihyperglycemic[8], antistress, antimicrobial [9], anti-inflamatory[10], astringent and antidysentric properties. The roots are very effective in purifying blood and used as laxative, analgesic, lactogogue, emmanogogue, diuretic and are used in eye sores, paralysis, lethargy, enlargement of spleen, pains in the joints, rheumatism and uterine pains. The stem is described as a cure for snake bite and scorpion sting [11]. Apart from its medicinal value, this plant has also been used as natural food colorants and as a natural hair dye. Various chemical constituents like iridoid glycoside, naphthoic acid esters, bocyclic-hexapeptide and novel anti-tumour bicyclic-hexapeptide-RA-XVII[12] along with anthraquinones have been isolated and identified from *R. cordifolia*. In the past few years, vital constituents have been studied by many researchers using HPLC and HPTLC. Moreover, chloroquine, primaquine and bulaquine[13], tizanidine and rofecoxib[14], 2-azaanthraquinone[15] and rubiadin[16] were analyzed using HPLC. Taking into account the evidence provided by morphology, molecular markers might be a useful tool for evaluation of genetic differentiation and similarity among populations of *R.cordifolia* L. Genetic diversity patterns can provide insights into evolutionary and demographic history of a taxon[17]. The understanding of processes that structured genetic diversity within and among populations (namely inbreeding, gene flow, genetic drift, and selection) might avoid future risk of diversity erosion[18].The study on genetic diversity of plant species in natural forests has practical applications for breeding, conservation and the clarification of the mechanisms of species differentiation. Genetic diversity studies followed on the heels of the development of molecular genetic techniques[19,20].The genetic diversity of plants was previously analyzed by either isozyme or restriction fragment length polymorphism (RFLP) analysis. However, random amplified polymorphic DNA (RAPD) markers also used[20]. Compared with RFLP assays, RAPD renders some loss of information because RAPD markers are dominant rather than codominant[21]. The main advantages of RAPD technology are its simplicity, reduced running time and lower cost. Moreover, it does not use radioactive probes and requires only small amounts of DNA (15-25 ng)[22] and has been applied to differentiate strains belonging to the same species[20]. RAPD results are also easy to interpret, and prominent polymorphisms can be reliably reproduced[23]. Random amplified polymorphic DNA (RAPD) markers have found the widest application in analyses of genetic variation below the species level, particularly in investigations of population structure and differentiation, including estimation of FST analogues and genetic variation within populations[24]. Confirming this, comparison of the use of different nuclear DNA markers for estimating intraspecific genetic diversity in plants, demonstrated that STMS (sequence tagged microsatellite sites)-based among-population diversity estimates are similar to those derived by the dominantly inherited markers[25]. Such analysis are crucial for conservation genetics, and the rapidity with which RAPD markers can be generated is an advantage, because they can deliver crucial information within the time constraints frequently demanded by urgent conservation decisions[26]. Furthermore, some papers demonstrate that new statistical approaches, particularly Bayesian methods, for estimating population genetic data significantly alleviate the bias related to their dominant nature [27, 28]. This technique has been used to reveal

genetic diversity among species level[29-31]. In the present study RP-HPLC was used for phytochemical quantification and RAPD markers were used for genetic fidelity studies. There are no earlier reports on phytochemical constituents and RAPD analysis in *R.cordifolia* L. from the Eastern Ghats of Tamil Nadu. Therefore, the present work is a first attempt to understand the genetic fidelity of this medicinal plant.

MATERIALS AND METHODS

Plant material

Rubia cordifolia L. collected from 7 accessions of Eastern Ghats. Table 1 summarizes that the details of study area. Root samples of *Rubia cordifolia* Linn. was directly collected from various hilly (Table 1 and Figure 1) regions of Eastern Ghats, Tamil Nadu. The plant was identified using Tamil Nadu carnatic flora[32].

Table 1
Details of study area

S.No	Name of the study area	Accession Code	Latitude	Longitude
1	Kolli Hills	GBR1	11°24'N	78°33'E
2	Shervaroy Hills	GBR2	11°77'N	78°20'E
3	Kalrayan Hills	GBR3	11°85'N	78°63'E
4	Chitteri Hills	GBR4	11°83'N	78°4'E
5	Jawadhu Hills	GBR5	12°69'N	78°73'E
6	Pachamalai	GBR6	11°36'N	78°60'E
7	Yelagiri Hills	GBR7	12°57'N	78°63'E

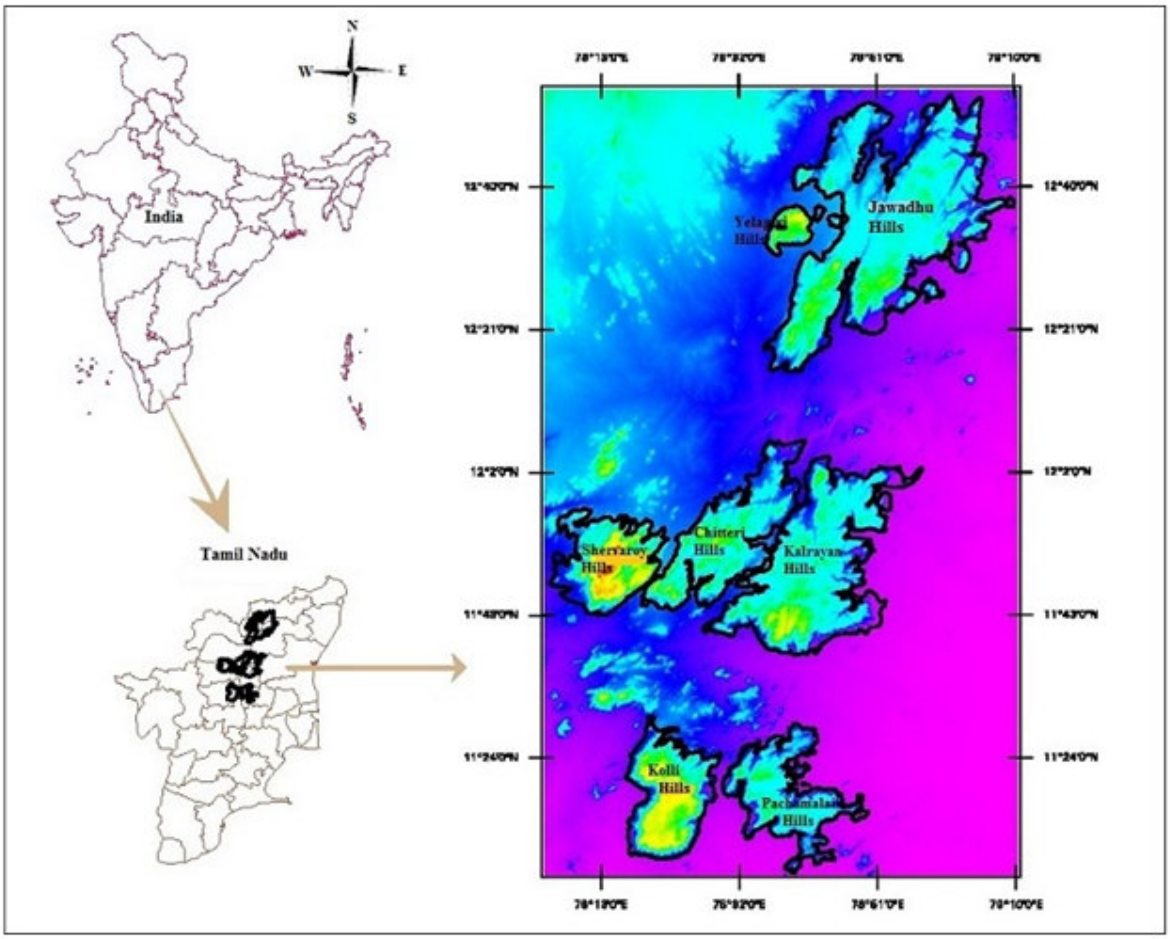

Figure 1
Map summarizes that the study area at Eastern Ghats.

Chemicals and Instruments

Chemicals used for the experimentation were HPLC grade and analytical reagent (AR). Methanol (Himedia) and Water used as solvent. Instruments used during research work were HPLC (Make Data Ace Company Pvt. Ltd.) with UV detector, UVspectrophotometer (Make *JASCO*), Rotary Evaporator, Electronic Balance (Make *SHIMADZU* Model *AY-120*). RP-HPLC Chromatographic method was developed using column Hi-Qsil C18 (250×4.6mm) using UV detector.

RP-HPLC analysis

Preparation of standard stock solution for RP-HPLC analysis

Standard alizarin (Himedia) and purpurin (Sigma Aldrich) were used for confirmation.

Standard stock solution

10mg of alizarin and purpurin was accurately weighed and dissolved in 10 ml of methanol to obtain stock solution (1000µg/ml).

Working standard solution

1ml of standard stock solution was diluted to 10ml with methanol.

Selection of Detection Wavelength

The standard solution was scanned over range 200nm to 400nm. From the UV spectrum of Marker, it was found that marker shows good absorbance at 300nm; hence 300nm was selected as detection wavelength.

Method development

Selection of Mobile phase and chromatographic studies were carried on working standard solution using C18 column. Mobile phase consisting of different proportions of methanol and distilled water. After several trials optimum mobile phase was found to be methanol and water in the ratio of 80:20. This mobile phase gave peak with acceptable retention time 10min and acceptable peak parameters.

Summary of chromatographic parameters selected

a) Column: HiQ-SiL C18 (250×4.6mm) column
b) Mobile phase: Methanol: water (80:20 %v/v)
c) Flow rate: 1.00 ml/min
d) Detection Wavelength: 300 nm
e) Sample injection volume: 20 µl loop
f) Temperature: ambient

Preparation of Sample solution

Extract was prepared as per the procedure given in[33] as follows, 2 gm root powder of *Rubia cordifolia* L. was accurately weighed and dispersed in 20 ml of ethyl acetate. This solution was kept aside overnight at ambient temperature. The solution was filtered through Whatmann filter paper No. 41 (Himedia) and filtrate was evaporated on a water bath to obtain a solid mass of extract (Approximately 500 mg dissolved in 2 ml of methanol). The extract was dissolved in methanol and diluted appropriately by the method of Khodke et al. All the experiments and samples accessed thrice for confirmation of results.

DNA isolation

The study was conducted on seven accessions belonging to various hill regions of Eastern Ghats, Tamil Nadu, India which are listed in Table 1. All the plant materials were collected from their natural habitat of the study area. Total genomic DNA was isolated from the leaf tissue of the mother plants using the CTAB method[34] with minor modifications. Two grams of leaf tissue were ground in liquid nitrogen and suspended in three volumes of CTAB extraction buffer (2% cetyltrimethylammonium bromide (CTAB - Himedia), 100 mMTris±HCl (pH 8.0 - Himedia), 20 mM EDTA (Himedia), and 1% b-mercaptoethanol (Himedia). The suspension was incubated at 60°C for 25 min, extracted with an equal volume of chloroform (Sigma Aldrich): isoamyl alcohol (Aldrich) (24:1) and centrifuged at 5000 rpm for 10 min. The DNA was precipitated from the aqueous phase with a two-third volume of isopropanol (Himedia) at 220°C for 1 h. The pellet, recovered by centrifugation at 10000 rpm for 10 min, was dissolved in TE buffer (10 mM Tris±HCl, 1 mM EDTA, pH 8.0) and treated with RNAse (Genei) at 37°C for 1 h. The DNA was purified by phenol/ chloroform extraction and ethanol precipitation in the presence of 0.3 M sodium acetate (Aldrich) (pH 5.2). The pellet was dissolved in TE buffer and the DNA concentration was estimated in 1% agarose gel (Merck).

RAPD analysis

RAPD analysis of genomic DNA was carried out using 15 decamer random oligonucleotide primers (OPA 01 to 15) obtained from Operon Tech. (Alamanda, USA) (Table 2).

Table 2
Details of RAPD Primers and sequence used for this study

Primer name	Sequence	Number of bands	Number of polymorphic bands	Percentage of polymorphism(%P)
OPA-01	5′-CAGGCCCTTC-3′	32	20	62.5
OPA-02	5′-TGCCGAGCTG-3′	69	55	79.7
OPA-03	5′-AGTCAGCCAC-3′	33	23	69.6
OPA-04	5′-AATCGGGCTG-3′	35	27	77.1
OPA-05	5′-AGGGGTCTTG-3′	19	08	42.1
OPA-06	5′-GGTCCCTGAC-3′	16	10	62.5
OPA-07	5′-GAAACGGGTG-3′	33	23	69.6
OPA-08	5′-GTGACGTAGG-3′	16	11	68.7
OPA-09	5′-GGGTAACGCC-3′	13	05	38.4
OPA-10	5′-GTGATCGCAG-3′	24	12	50.0
OPA-11	5′-CAATCGCCGT-3′	22	14	63.6
OPA-12	5′-TCGGCGATAG-3′	11	4	36.3
OPA-13	5′-CAGCACCCAC-3′	18	14	77.8
OPA-14	5′-TCTGTGCTGG-3′	8	6	75.0
OPA-15	5′-TTCCGAACCC-3′	23	14	60.8
	Total number of bands	372	246	66.1

The polymerase chain reaction (PCR) was carried out in a volume of 25 ml containing 20 ng of genomic DNA, 2.5 ml of 10s% assay buffer, 2 ml of 5 mM MgCl2, 0.5 ml of 5 mM dNTPs (Pharmacia), 15 ng of primer and 1 unit of Taq DNA Polymerase (Sigma). Amplification was carried out in a DNA thermal cycler (Corbett Research thermal cycler Model No: CG1-96, Australia) programmed for 45 cycles. Each cycle comprised 1 min at 94°C (first cycle was for 3 min at 94°C), 1 min at 37°C and 2 min at 72°C. An additional cycle of 15 min at 72°C was used for primer extension. The amplified samples were electrophoresed in 1.5% agarose gel in 0:5 TBE (Tris±borate±EDTA) buffer. Amplification with each primer was repeated twice to confirm the reproducibility of the results. Cluster analysis performed using UPGMA (Unweighed pair group means average) by STATISTICA Ver. 5.0.

RESULTS

RP-HPLC of alizarin and purpurin:

Method development for the separation of alizarin and purpurin

Different compositions of mobile phase for RP-HPLC analysis was tested in order to obtain high resolution, symmetrical and reproducible peaks for alizarin and purpurin. The desired resolution of compound was achieved by using Methanol: water (80:20 %v/v) as the mobile phase. On this system, separation was good and peaks of alizarin and purpurin were well defined. The detection wavelength of 300 nm was found to be optimal high sensitivity to alizarin and purpurin spots.

Estimation of alizarin and purpurin from different accessions

Estimation of the alizarin and purpurin was carried out by comparing the peak area of the sample with the standard and linear equation and chromatograms were shown in Figure 2.

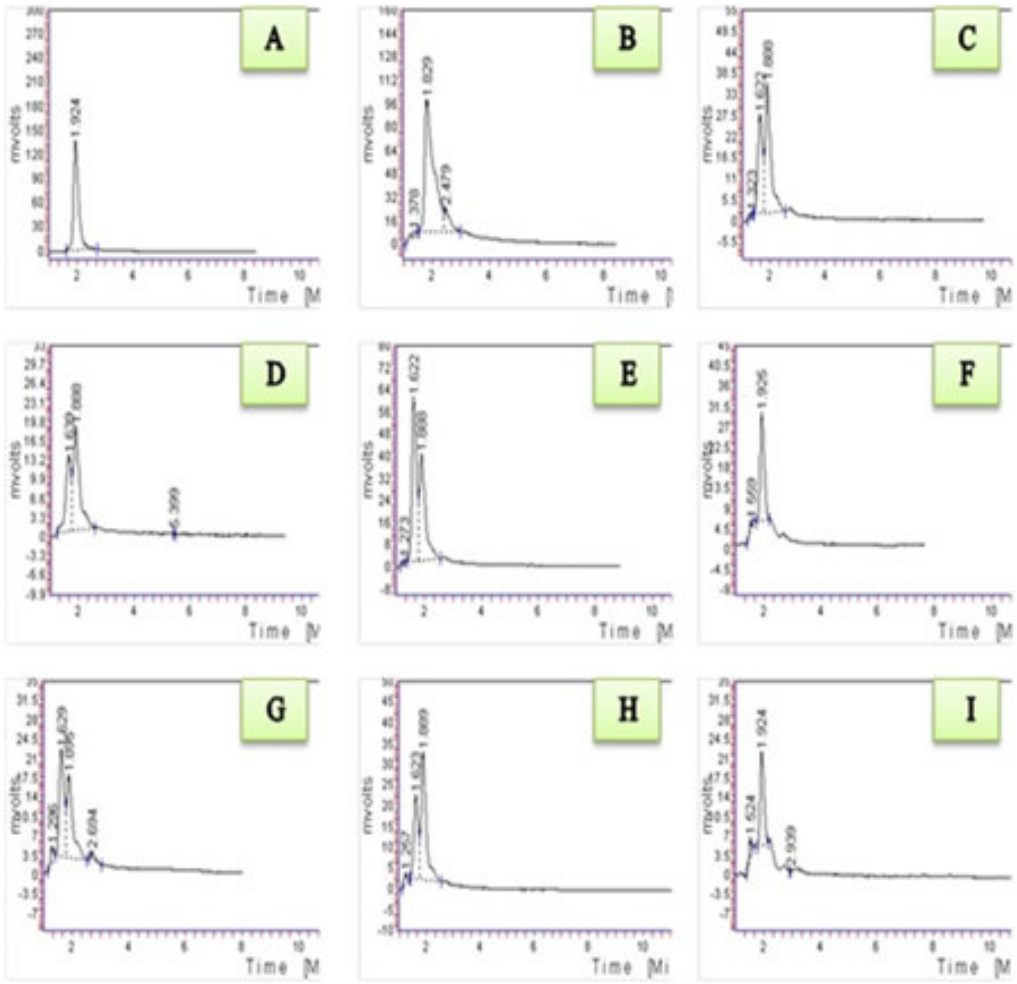

Figure 2

RP – HPLC chromatogram of Rubia cordifolia L. root samples; A – Alizarin Standard; B – Purpurin Standard; C – GBR1 (Kolli Hills); D- GBR2 (Shervaroy Hills); E- GBR3 (Kalrayan Hills); F-GBR4 (Chitteri Hills); G-GBR5 (Jawadhu Hills); H-GBR6 (Pachamalai); I- GBR7 (Yelagiri Hills)

Root portion was subjected to RP- HPLC quantification and the results obtained were shown in Figure 3. alizarin and purpurin content was quantified from roots of *Rubia cordifolia* L. and the values in percentage (mg) was found to be maximum in plants of Kalrayan hills (GBR3- 32.4% and 33.4%) followed by Kolli Hills (GBR1- 27.0% and 16.5%), Pachamalai (GBR6- 23.8% and 20.7%), Shervaroy hills (GBR2 - 16.6% and 9.4%), Jawadhu hills (GBR5- 13.4% and 11.7%), Chitteri hills (GBR4- 12.9% and 0.59%) and Yelagiri hills (GBR7 - 8.7% and 0.87%) respectively.

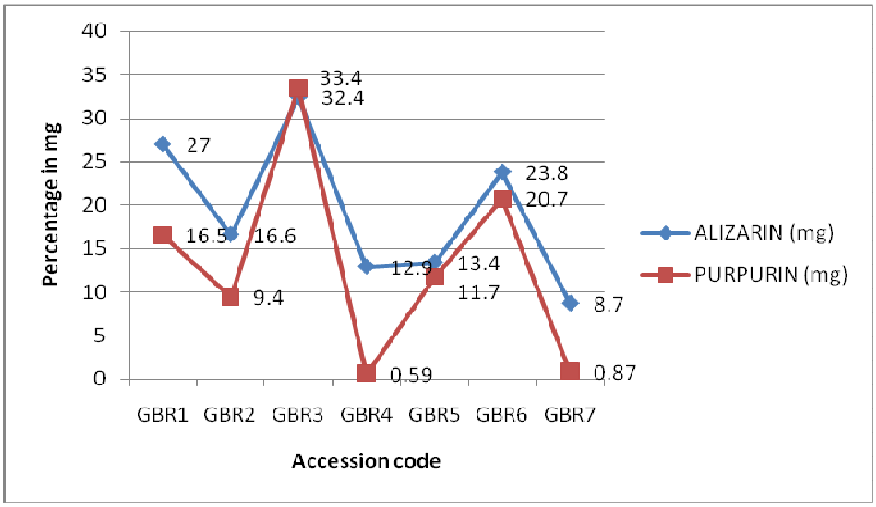

Figure 3

Graph summarizes that percentage of Alizarin and Purpurin present in root samples of Rubia cordifolia L. analyzed by RP-HPLC method collected from the 7 accessions of Eastern Ghats.

An interesting feature was observed from the perusal of the data which revealed that the plant was collected during the month of February in all the study area but alizarin and purpurin level showed gradual variations among plant samples. This may lead to the finding of genetic fidelity of this plant using RAPD analysis.

RAPD analysis

Marker analysis

Table 2 summarizes the sequence details of the total number of bands amplified, number of polymorphic bands and percentage of polymorphism detected for different marker systems in *Rubia cordifolia* L. For RAPD, the 15 primers produced a total of 246 polymorphic bands. The findings exposed that the highest levels in terms of polymorphic band number and polymorphism percentages (79.7%) were obtained with primer OPA-02 (55 fragments) Figure 4.

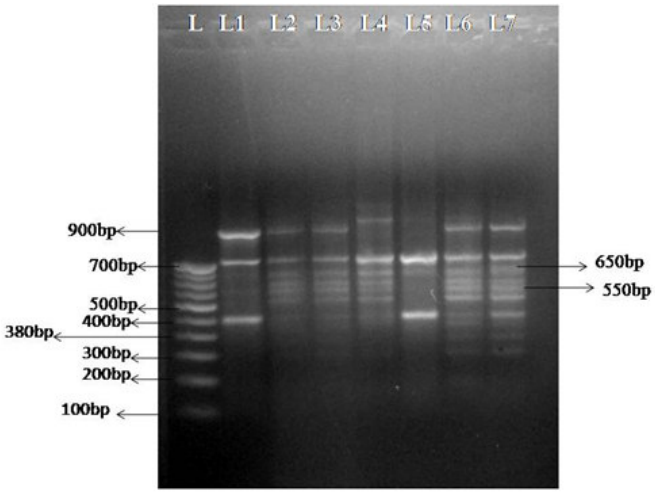

Figure 4

Primer OPA 02 - RAPD amplified products visualized in 1.0% agarose gel stained with ethidium bromide. L-100bp Ladder(Fermentas); L1-Kolli hills (GBR1);L2- Shervaroy Hills (GBR2); L3-Kalrayan hills (GBR3); L4-Chitteri hills (GBR4); L5- Jawadhu hills (GBR5); L6- Pachamalai hills (GBR6); L7- Yelagiri hills (GBR7)

Whereas the lowest rates of bands were attained with primers OPA-14 and OPA-12 (6 and 4 fragments respectively). An average of 7.83 bands per primer ranging from about 800 bp to 1000 bp was produced.

Cluster analysis

The clustering patterns obtained by the UPGMA cluster analysis of RAPD dendrogram are given in Figure – 5

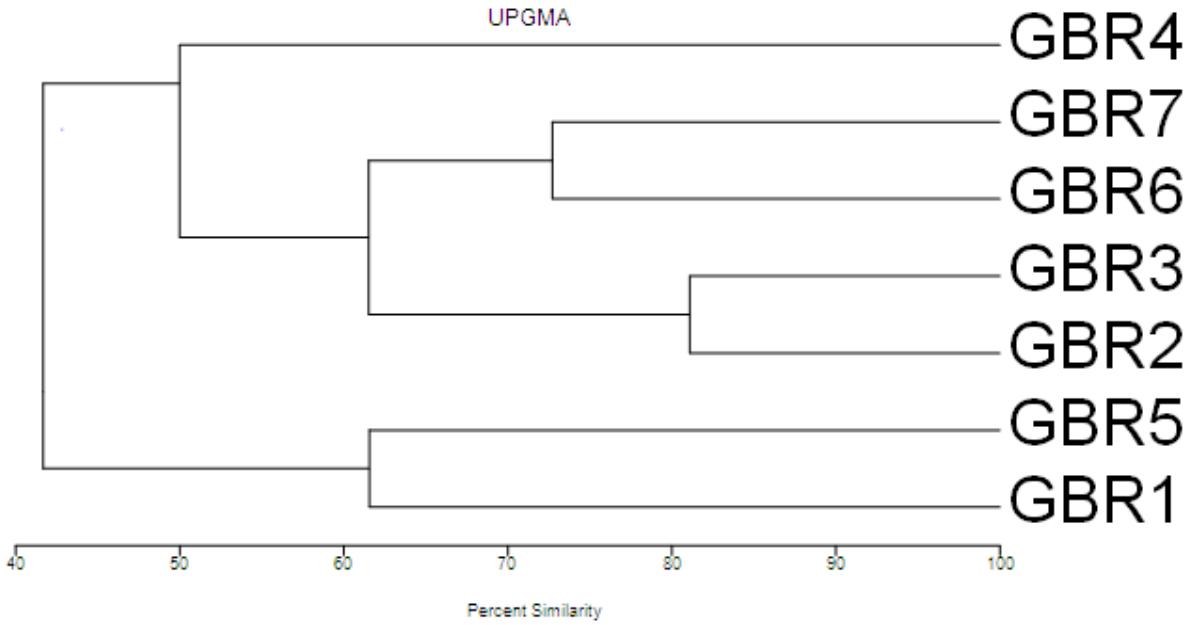

Figure 5

RAPD dendrogram based on UPGMA summarizes that genetic fidelity of Rubia cordifolia L. collected from 7 accessions of Eastern Ghats, Tamil Nadu, India. GBR1- Kolli hills; GBR2- Shervaroy hills; GBR3- Kalrayan hills; GBR4 – Chitteri hills; GBR5- Jawadhu hills; GBR6- Pachamalai; GBR7- Yelagiri hills.

The analysis of the RAPD data exposed a clear separation of the accessions into three groups by cutting the dendrogram at a genetic similarity (GS) value 0.27, with the greatest separation of GBR1 and GBR5 accessions. The results also showed that within-group variance components were low for RAPD which indicates that the genetic background attributable to the geographical origin contributes to genetic diversity.

DISCUSSION

RP-HPLC Analysis

The RP- HPLC method is rapid, simple and accurate for quantitative monitoring of compounds in *R. cordifolia.* In the present study, we developed RP-HPLC method for determination of alizarin and purpurin from the roots of *Rubia cordifolia* L. collected from different places of Eastern Ghats, Tamil Nadu. The data revealed variation in the alizarin and purpurin content which may be due to the environmental factors and biogeographical impact influence on the species and also due to genetic makeup of species leads to this variation in the phyto constituents. The present work is similar to the report of [16] who developed and validated isocratic HPLC method for determination of rubiadin in roots of R. *cordifolia.* L. RP-HPLC method for rubiadin was developed using mobile phase methanol: water in the ratio of 80: 20. Stationary phase use of his method was Hi-Qsil C18 (250×4.6 mm). Specific studies have been carried out for the estimation of alizarin in *Rubia* species[35] HPLC method for the quantitative analysis of alizarin in field grown roots and tissue cultures of *R. tinctorum* and *R. peregrina.* Alizarin was quantified by HPLC in the presence of trichlorophenol as internal standard using an RP C-18 Nova-pak® column eluted isocratically with methanol: 10% aqueous acetic acid (60:40). The minimum detection limit of alizarin was found to be 10.5 ng. The field grown roots and callus

of *Rubia tinctorum* contained 939.6 (±17.4) and 335.2 (±4.9) µg/g dry weight total alizarin respectively, while similar tissues of *Rubia peregrina* contained 147.9 (±10.0) and 216.8 (µ0.4) µg/g dry weight total alizarin. Similar work was done by[36] who developed a HPLC method to determine the non-glycosidic anthraquinones alizarin (1,2-dihydroxy-9,10-anthracenedione), emodin (1,3,8-trihydroxy-6-methyl-9,10 anthracene-dione) and anthraquinone (9,10-anthracenedione). The anthraquinones were separated by isocratic elution on a 125x4.6 mm I.D. column containing ODS Hypersil 5 reversed-phase material using methanol-5% acetic acid (pH 3.0) (70:30) as the mobile phase.

RAPD Analysis

The application of molecular marker systems has revolutionized the pace and precision of plant genetic analysis and helped to develop efficient plant conservation strategies. Although several molecular marker systems are currently available in the literature, most of the studies so far performed have employed only one marker system for the analysis and characterization of cultivated chestnuts. Recently, comparison between molecular markers method on genetic diversity in chestnut is becoming common[34, 37]. The dominant markers (RAPD, AFLP and ISSR) used in this study provide a large number of polymorphic loci and were in general agreement with other studies in genetic diversity measurements[38].RAPD markers have been successively used for fingerprinting and germplasm collections in many plant species. The use of Operon Tech (OP series) primers is an apt molecular tool to study genetic variations in Rubiaceae plants which is evident by the work of[39] who studied genetic relations and phylogeny of subtribe Coffeeae by RAPD markers in the genus *viz.*, *Coffea* and *Psilanthus*. In the present study analysis of plant relationship in *Rubia cordifolia* L. using RAPD marker indicated close similarity of the genomes distributed in same geological area. However in the present study *Rubia cordifolia* L. collected from Kolli hills (GBR1) showed the close relationship with Jawadhu hills (GBR5). In the meantime Shervaroy (GBR2) with Kalrayan (GBR3) and Pachamalai (GBR6) with Yelagiri (GBR7) showed close relationship. This result also supported by the phylogenetic theory based on biogeography of the study areas of the plants which delimits the speciation of the species[40].

CONCLUSION

In conclusion, results of this study designated that RP-HPLC analysis is a useful tool for the quantification and variations of vital Phyto-constituents in plants and RAPD dendrogram study reliable tool for genetic variation and similarity index within the populations of plants. Further analysis together with data from other methods can be used to make a more accurate reconstruction of the genus *Rubia cordifolia* L. Additionally; the large number of unique bands obtained that signify the power of RAPD markers in similarity and diversity analysis in the taxonomic level. Finally, this study concludes that the phytochemical variations occurred due to natural parameters like climate, soil (Texture, type, temperature, physico - chemical properties), humidity, range of rainfall and seasonal variations of the area. These variations may not influence the species genetically.

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COMPETING INTERESTS

We have no competing interests.

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