

Detection of Molecular and Phytochemical Variations in Tamilnadu Landraces of *Gymnema sylvestre* Genotype: An Important Anti-Diabetic Plant

U. Jinu¹, M. Thiagarajan¹, S. Javed², A. A. Alatar², and P. Venkatachalam^{1,3*}

ABSTRACT

Gymnema sylvestre, a potential anti-diabetic medicinal plant, is used in many herbal drug formulations. The medicinal properties have been credited to the presence of gymnemic acid, which is effective in controlling blood glucose and lipid levels in the body. Molecular and biochemical variations of seven populations of *G. sylvestre* were investigated using DNA markers and phytochemical fingerprints of gymnemic acid content. Results obtained through these techniques were used in a comparative analysis and revealed a correlation between genetic variations and biochemical content analysis. Thirteen primers were used for RAPD-PCR analysis revealing 112 bands, of which 62 were found to be polymorphic. The percentage of polymorphic bands detected ranged from 11.1 to 84.6% with an average of 54.2%. The average number of amplified bands per primer was 8.62 while the mean number of polymorphic bands per primer was 5.42. A dendrogram of the genetic similarities among the populations was constructed using the genetic distance coefficients and the seven accessions were clustered into four major groups. The total gymnemic acid content level varied significantly among these groups from 3.84 to 7.12 mg g⁻¹ DW. A principle coordinate analysis confirmed the results of clustering. Maximum yield of gymnemic acid content (7.12 mg g⁻¹ DW) was recorded with L7 accession. Results suggest that there might be a positive correlation between genetic makeup and gymnemic acid content; however, this has to be further investigated by using other molecular approaches.

Keywords: Cluster analysis, Genetic diversity, Gymnemic acid, Random amplified polymorphic DNA.

INTRODUCTION

India is endowed with enormous natural wealth in terms of ecosystems, species richness, and genetic diversity, enlisting it in the 12 mega biodiversity hotspots of the world. Large numbers of plant species are being used for medicinal purposes in various traditional as well as modern medical systems. Herbal drug and pharmaceutical industry is one of the fastest growing

industries for herbal-based medicine production. Currently, there is an increasing realization of health hazards associated with indiscriminate use of modern medicines. However, lack of scientific inputs in this area has also limited its potential uses and benefits. Moreover, unscientific wild harvesting of important medicinal plants have resulted in over exploitation of natural population and a threat to important gene pools.

¹ Plant Genetic Engineering and Molecular Biology Lab, Department of Biotechnology, Periyar University, Periyar Palkalai Nagar, Salem-636 011, TN, India.

² Department of Botany and Microbiology, College of Science, King Saud University, P. O. Box: 2455, Riyadh 11451, Kingdom of Saudi Arabia.

³ Department of Biology, Western Kentucky University, Bowling Green, KY 42101, U. S. A.

* Corresponding author: e-mail: pvenkat67@yahoo.com



Gymnema sylvestre R. Br. (Apocynaceae), a woody shrub, found in the tropical and subtropical regions of Indian subcontinent, possesses great medicinal potential. It is also known as “Gur-Mar” in Hindi, literally meaning the “sugar killer” because of its effectiveness in the treatment of diabetes in the traditional medical systems of the Indian subcontinent. Recent scientific studies have confirmed its effectiveness in diabetes mellitus because of its hypoglycemic, anti-hyperlipidemic and anti-oxidant properties (Tiwari *et al.*, 2014; Jain and Devi, 2016). These activities have been credited to gymnemic acid, a group of complex triterpenic glycosides. Gymnemagenin, a common aglycone of gymnemic acids produced after acidic and basic hydrolysis, inhibits glucose absorption and is commonly used by several workers as an analytical marker to determine the quality of *Gymnema* plant materials (Puratchamani and Jha, 2004).

Molecular and biochemical markers have opened a new avenue to investigate the genetic diversity. The identified markers could serve as basis for selection of elite genotypes and future improvement (Karaca and Ince, 2008). Also, Ince and Karaca (2015) have been successfully applied used the touchdown direct amplification of minisatellite-region DNA polymerase chain reaction (Td-DAMD-PCR) technique for identification of genetic diversity in *Dianthus*. Correlations between the metabolic contents and DNA fingerprints among geographically distinct plant populations have been reported in several species, *Fructus xanthii* (Han *et al.*, 2008), *Ocimum gratissimum* (Vieira *et al.*, 2001), *Tanacetum vulgare* (Keskitalo *et al.*, 2001). However, no report is available on combined diversity analysis using molecular and biochemical methods in *Gymnema* spp. *G. sylvestre* shows high level of variability in bioactive compounds levels among the accessions collected from different locations (Pandey and Yadav, 2010). Variation in gymnemic acid content may affect the quality of pharmaceutical products and herbal medicine preparation. Although independent studies of either genetic or phytochemical

profile analysis of various medicinal plants including *Gymnema* have been reported (Tripathi *et al.*, 2012; Shilpha *et al.*, 2013; Mahar *et al.*, 2013), to the best of our knowledge, correlation studies for genetic and biochemical markers have not been done. Therefore, it is one of the prerequisites to conduct genetic diversity study for the analysis of gymnemic acid contents level among the different *Gymnema* accessions growing from various agroclimatic conditions. The genetic diversity study is highly essential for the selection of the high gymnemic acid yielding varieties for industrial applications in the future. Earlier reports suggest that a positive correlation exists between genetic diversity and the level of bioactive molecules, as recorded in various medicinal plants (Hu *et al.*, 2007; Han *et al.*, 2008; Azizi *et al.*, 2012).

It is hypothesized that: (i) Identification of potential genotype for higher yield of gymnemic acid content may be beneficial for pharmaceutical industry, and (ii) This will be highly useful for selective cultivation of best genotypes for sustainable production. The present study aimed at establishing the correlation between genetic diversity with the variability in gymnemic acid yield among different accessions of *G. sylvestre*, so as to identify the potential accession with increased accumulation of bioactive molecules by estimation of genetic variability using molecular as well as phytochemical fingerprinting analysis.

MATERIALS AND METHODS

Plant Materials

Seven accessions of *G. sylvestre* were collected from various locations in Tamilnadu, India (Table 1). Young leaves were collected and used for DNA extraction and HPLC analysis. The accessions were selected to represent a wide range of geographical as well as agro-climatic conditions, based on distribution of *G. sylvestre* within Tamilnadu, India.

Table 1. Accession and collection details of *G. sylvestre* plants with longitude and latitude of the study area.

Accessions ID	Area of the study	Latitude	Longitude
L1	Horticulture Research Station, TNAU, Yercaud, Salem Dt, TN	11° 77' N	78° 20' E
L2	ABS Botanical garden, Kaaripatti, Salem (Dt), TN	11° 65' N	78° 28' E
L3	Centre for Siddha medicinal garden, Mettur, Salem Dt. TN	11° 47' N	77° 48' E
L4	TAMCOL Medicinal Garden, Kolli Hills, Namakkal Dt. TN	11° 14' N	78° 20' E
L5	Forest Research Centre, Kanjamalai, Salem, Dt. TN	11° 37' N	78° 30' E
L6	Yelgri Hills, Thirupattur, Vellore Dt. TN,	12° 34' N	78° 38' E
L7	Morappur, Salem Dt. TN	11° 11' N	77° 19' E

Preparation of Hydro-Methanolic Extracts

The coarse powder (25 g) was extracted with 500 mL of hydro-methanol (20:80) using a cold extraction method. The extract was filtrated using Whatman filter paper (No. 1) and then concentrated at 40°C using a rotary evaporator. The residue was preserved in ultra-deep freezer (-80°C) until further biochemical analysis.

Acid Base Hydrolysis Method

The leaf extract (50 mg) was weighed accurately and dissolved in 50% (v/v) methanol to make 50 mL. To 10 mL of this solution, 2 mL of 12% (w/v) potassium hydroxide (KOH) was added and heated on a boiling water bath for 1 h. After cooling, 5.5 mL of 4N HydroChloric acid (HCl) was added and heated on a boiling water bath for 1 h. Thereafter, on cooling, pH was adjusted to 8.0 using 12% (w/v) KOH, volume was maintained to 100 mL with 50% (w/v) methanol, filtered and subjected to HPLC quantification of biochemical compound.

High Performance Liquid Chromatography (HPLC) Analysis

Quantification analysis was performed using HPLC (Shimadzu, Tokyo, Japan, model LC10A) instrument equipped with

Shimadzu ApD-M 10 Avp Photodiode Array Detector (PDA) in order to determine peak purity and similarity test of gymnemagenin. HPLC grade solvents (Sigma Alrich, USA) were pre-filtered using a Millipore (Billerica, MA, USA) system and analysis was performed on a water column (Milford, MA, USA) C18 Spherisorb S10 ODS2 (250×4.6 mm, id 10 µm). The mobile phase was methanol:water (0.1% TFA) (50:50) at a flow rate of 0.6 mL min⁻¹. The detection wavelength was 210 nm, which was close to the absorption maxima for compound. The injection volume for sample was 20 µL and column temperature was 26°C.

DNA Isolation

Total DNA was extracted from leaves using a modified CTAB method based on the protocol of Doyle and Doyle (1990). DNA quality as well as integrity was analyzed on 0.8% (w/v) agarose gel electrophoresis. The DNA band was visualized under UV transilluminator and the image was captured by using gel documentation system (Alpha Inotech Gel Imaging System, USA). For each DNA sample, a series of assays were carried out to estimate the optimum DNA concentrations (15-25 ng µL⁻¹) for PCR amplifications. This was accomplished by comparing the dilutions to a known DNA standard after separation on 0.8% (w/v) agarose gel and stained with ethidium bromide. The DNA was diluted with sterile TE buffer to 10-15 ng µL⁻¹ and used for PCR amplification.



DNA Amplification by Polymerase Chain Reaction (PCR)

Random oligonucleotide primers (Operon Inc, USA) were used for RAPD analysis (Williams *et al.*, 1990). The reaction was carried out in a volume of 20 μL consisted of 2 μL , 10X PCR buffer (10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2), 2 μL , 1.5 mM dNTPs (dATP, dGTP, dCTP and dTTP), 250 nM random decamer primers (1.0 μL), 0.5 units of Taq DNA polymerase, 2 μL of genomic DNA (15 ng) and finally added 13 μL of sterile water. DNA amplifications were performed in a PCR thermal cycler (Cyber lab, USA) under the amplification profile of initial denaturation at 94°C for 4 minutes, followed by 40 cycles consisting of denaturation at 94°C for 1 minute, annealing at 37°C for 1.30 minutes, extension at 72°C for 2 minutes with a final extension at 72°C for 7 minutes. After PCR cycles, loading buffer was added to the amplified products and they were analyzed on 1.5% (w/v) agarose gel electrophoresis containing 0.5 $\mu\text{g mL}^{-1}$ ethidium bromide using 1X TAE buffer. Electrophoresis was performed at 60V for 2 hours until the bromophenol blue dye front migrated to the bottom of the gel. The molecular standard used was the lambda DNA double digested by *EcoRI/Hind III*. The gels were visualized under UV transilluminator and photographed with Alpha Inotech gel documentation system (USA). In order to optimize the DNA amplification rate, different concentrations of Taq DNA polymerase (0.1–0.5 units) and MgCl_2 (1.5 to 2.5 mM) were used for PCR amplification reactions. Among the random oligo nucleotide primers, those that exhibited clear banding pattern after PCR amplification were selected for further RAPD analysis (Venkatachalam *et al.*, 2008).

DNA Fingerprinting Data Scoring and Analysis

RAPD banding patterns were analyzed by biostatic type scoring. The amplified DNA

band was scored '+' as presence and '-' as absence for all prominent bands within a fingerprint profile (Collard and Mackill, 2009). Therefore, a sequence of '1' and '0' was generated for each primer/species to form a data matrix. DNA fragment sizes were estimated from the agarose gel by comparison with PCR molecular weight marker. RAPD-PCR amplification with each primer was performed with 5 replicates and repeated three times to obtain consistent banding pattern. A dendrogram was generated by cluster analysis using Unweighted Paired Group Method with Arithmetic average (UPGMA) based on Jaccard's similarity co-efficient analysis. The fit of dendrogram obtained was checked by bootstrapping.

Phylogenetic Tree Construction

For evaluation purposes, the genetic relationship between different locations of *Gymnema* plant species and DNA bands from seven accessions produced by different random primers were scored and used for construction of phylogenetic tree. To identify the amplified DNA fragment size, double standard DNA marker (lambda DNA double digested by *EcoRI/Hind III*) was used to determine the size of each amplified DNA fragment. RAPD fragments were assigned a DNA length and recorded as a binary matrix for each individual as presence (1) or absence (0) of a given band. Phylogenetic relationship of *G. sylvestre* plant species was generated using Hierarchical clustering of DARwin 5.0 software based on UPGMA method.

RESULTS AND DISCUSSION

Gymnema sylvestre R.Br. is considered as a potential herb for diabetes treatment, because of the presence of important blood glucose and lipid regulating substances, specially gymnemic acid. However, *G. sylvestre* is a highly cross-pollinated species,

showing high levels of genetic variation among populations. The wild harvesting is thus less effective. It is also essential to conserve its gene pool from rapid disappearance caused by excess collection from the natural habitat. Therefore, identification of elite and potential genotypes is essential for sustainable industrial application. Genetic diversity analysis and its correlation to phytochemical yield would be one of the prerequisites for selection of potential and high yield germplasm accession and implementation of effective commercial crop cultivation for extraction of large-scale secondary metabolites. Although various molecular techniques are being used for genetic diversity analysis, the Randomly Amplified Polymorphic DNA (RAPD) analysis is one of the most effective PCR based methods as it is rapid, simple, cost effective tool, and reveals high levels of polymorphism among populations.

Assessment of Genetic Diversity by RAPD-PCR Analysis

Genomic DNA was isolated from healthy leaf tissue samples using modified CTAB method and used for RAPD-PCR analysis. PCR amplification yielded prominent bands and clear lanes with 10-15 ng DNA in the reaction mixture. PCR assay was carried out to obtain the optimum Taq DNA polymerase concentration, among the various concentrations tested, the intensity of DNA amplicons at 0.5 unit was found to be the best concentration for amplification of most stable/reproducible bands. The yield of RAPD products declined if the $MgCl_2$ concentration was increased from 1.5 mM to 2.5 mM in the reaction mixture and smeared lanes appeared in the electrophoresis profile pattern. One of the reasons may be due to the inhibition of Taq DNA polymerase activity in the presence of excess Mg^{2+} in the reaction mixture. In the present study, $MgCl_2$ at 1.5 mM concentration was found to be the best for

PCR amplification using RAPD-PCR markers in DNA samples of *G. sylvestre* (Figure 1).

The presence and absence of the DNA bands in the RAPD profile for six random primers is presented in Figure 1. RAPD profile generated using selected random primers was found to be clear and reproducible banding patterns and consistent for all the 7 accessions. For preliminary screening, about one hundred oligo-nucleotide primers were tested for amplification of the genomic DNA from the seven accessions of *G. sylvestre* plant species (L1 to L7). The RAPD primer sequence, total DNA bands, and polymorphic bands frequency details are represented in Table 2. Among the hundred primers screened, 13 random oligonucleotide primers (OPA04, OPA10, OPA13, OPB15, OPB17, OPB18, OPC06, OPC09, OPC16, OPC20, OPD03, OPD06 and OPD08) produced clear and reproducible DNA fingerprints for all the seven accessions of *G. sylvestre*. All the primers revealed DNA polymorphism among 7 populations and 62 amplicons were found to be polymorphic (Table 2). It is interesting to note that the average of DNA polymorphism noticed was 54.2% among the 7 accessions collected from different regions of Tamilnadu (20 to 280 km distribution, Table 2). The highest percent of polymorphic bands (84.6%) was noticed with OPA04 primer followed by OPC16 (81.8%) (Figure 1), while the lowest percentage polymorphism obtained was 11.1% with OPA13 (Figure 1). Anew the number of polymorphic bands ranged from 1 to 11 with a mean average of 4.78 per primer. RAPD has been applied for determination of genetic diversity in *Withania somnifera* accessions collected from various geographical locations (Dharmar and Britto, 2011). Similarly, an attempt was made to determine the genetic variability among 17 accessions of *G. sylvestre* using 20 primers by RAPD-PCR technique and about 50% polymorphism



Table 2. Primers selected for RAPD analysis, the total number of bands amplified by each primer and polymorphism among 7 *G. sylvestre* accessions collected from different geographical locations of Tamilnadu.

Primers code	Nucleotide Sequence 5' to 3'	Total No of bands	No of polymorphic bands	Polymorphism (%)	DNA bands size (in bp)
OPA04	AATCGGGCTG	13	11	84.6	1509-200
OPA10	GTGATCGCAG	13	7	53.8	895-200
OPA13	CAGCACCCAC	9	1	11.1	1520-150
OPB15	GGAGGGTGTT	6	3	50.0	698-350
OPB17	AGGGAACGAG	11	7	63.6	1980-400
OPB18	CCACAGCAGT	9	4	44.4	1894-450
OPC06	GAACGGACTC	6	2	33.3	1680-250
OPC09	CTCACCGTCC	10	6	60.0	1606-250
OPC16	CACACTCCAG	11	9	81.8	1872-200
OPC20	ACTTCGCCAC	3	2	66.7	876-400
OPD03	GTCGCCGTCA	7	3	42.8	1350-350
OPD06	ACCTGAACGG	3	2	66.7	980-450
OPD08	GTGTGCCCCA	11	5	45.4	1980-400
Total		112	62		
Mean		8.62	4.78	54.2%	

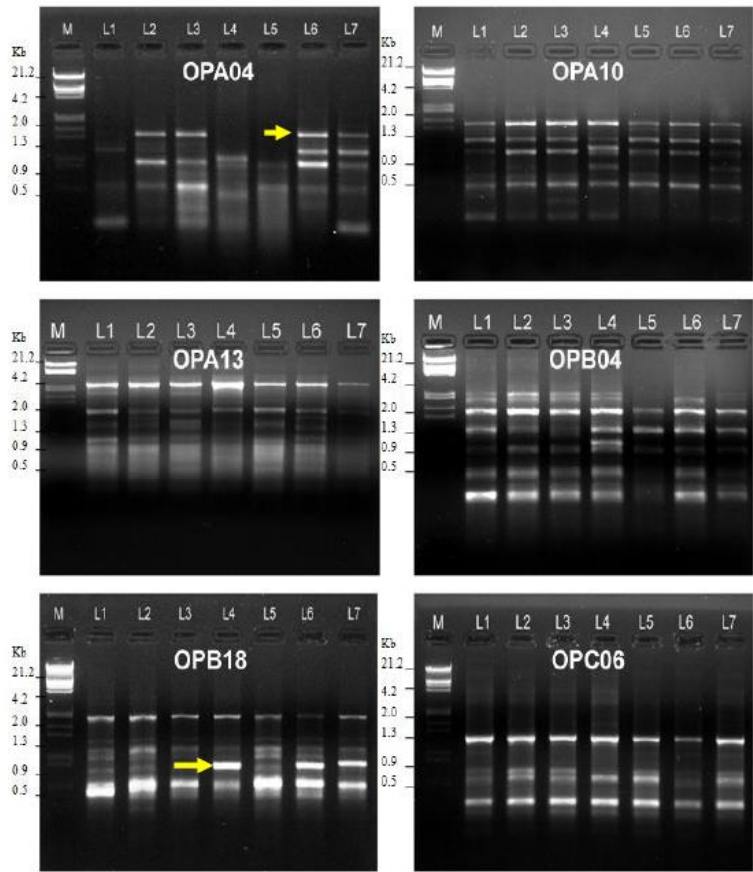


Figure 1. RAPD pattern generated using various selected oligo-nucleotide primers. (Accession ID: L1- Yercaud Hills; L2- Kaaripatti; L3- Mettur; L4- Kollihills; L5- Kanjamalai Hills; L6- Yelgri Hills; L7- Morappur). Arrow (➡) indicates the presence of DNA band.

was reported by Balamuralikrishna *et al.* (2012). Earlier, Nair and Keshavachandran (2006) reported that the percent of DNA polymorphism was 73.2% recorded by using 15 RAPD primers. Lal *et al.* (2013) reported that RAPD fingerprinting profile generated by using 5 random primers revealed 100% polymorphism among 12 *G. Sylvestre* accessions collected from various locations of Gujarat. Recently, Mouna *et al.* (2014) reported considerable genetic diversity within the *G. sylvestre* populations collected from Karnataka region and about 85% of polymorphism was noticed with 12 accessions examined by ISSR marker analysis.

Genomic DNA from the seven different populations was amplified using random primers to detect the polymorphic DNA as molecular markers (Table 2). PCR amplification using OPA10, OPB15, OPB17, OPB18, OPC06, OPC09, OPC20, OPD03, OPD06 and OPD08 random primers produced different major DNA bands within 7 accessions. Figure 1 shows that populations L2, L3, L6 and L7 have a specific 1.6 kb DNA band amplified by

OPA04 primer, while this band was absent in L1, L4, and L5 populations. In the case of OPC18 primer, a specific DNA fragment with the size of 1.2 kb was amplified in L4, L6, and L7 populations and it was not amplified in L1, L2, L3 and L5 populations (Figure 1).

For Principal Coordinates Analysis (PCoA), dissimilarity matrix values were calculated using a binary data matrix with Jaccard simple co-efficient analysis to ordinate 7 accessions from different locations on a scattered plot. The genetic distance values were used for hierarchical cluster analysis using UPGMA agglomeration method using Darwin 5.0 software. According to PCoA results, the codes of the accessions from each location were plotted on the four components corresponding to different regions, where the 7 populations were collected from Tamilnadu. The PCoA displayed the separation of populations (L2, L1), (L4, L3), (L6, L5) and (L7) according to genetic distances and based on dissimilarity matrix values on the scattered plot graph (Figure 2). It is noteworthy to mention that PCoA

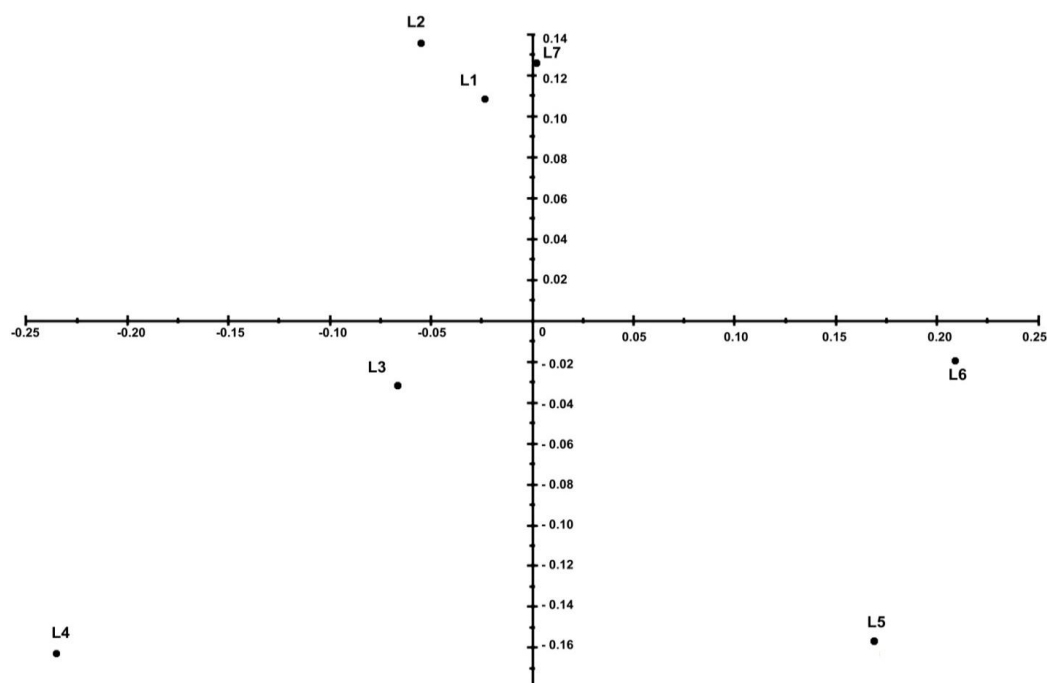


Figure 2. Principal coordinates analysis of RAPD markers from seven accessions of *G. sylvestre* (Accession ID: As defined in Figure 1).



values migrated from negative to positive side on the scattered plot graph, suggesting the increase of means in high degree of similarity. Similar result was also reported recently in *Rauvolfia serpentina* (Nair et al. 2014).

The ratios of the common DNA markers and genetic distance coefficients among the populations were calculated using Jaccard co-efficient. The genetic distance coefficients among the populations were distributed between 0.22 and 0.51 (Table 3). A dendrogram generated from Jaccard similarity matrix and the UPGMA method revealed clear genetic relationships among the *Gymnema* accessions. A dendrogram of genetic similarities among the populations was constructed using the genetic distance coefficients shown in Table 3. Based on the

clustering analyses, the populations were classified into four major distinct clusters: group I (populations L4 and L3), group II (populations L2 and L1), group III (populations L6 and L5) and group IV (population L7) (Figure 3). It is important to mention that accessions from different geographical origins were formed in the same clusters, for instance the accession L5 (Kanjamalai Hills) with L6 -Yelgri Hills and L3-Mettur with L4-Kolli Hills in the UPGMA dendrogram together notwithstanding the distance of 285 and 135 km between them. Interestingly, two geographically very near to accessions viz., L1-Yercaud Hills and L2-Kaaripatti were clustered into the same group and, on the contrary, the L7-Morappur population was very close to L1-Yercaud Hills but formed

Table 3. Similarity index matrix based on Jaccard’s simple coefficient analysis revealed by RAPD markers.

Accession ID ^a	L1	L2	L3	L4	L5	L6	L7
L1	0.000						
L2	0.222	0.000					
L3	0.267	0.280	0.000				
L4	0.400	0.384	0.319	0.000			
L5	0.360	0.387	0.346	0.428	0.000		
L6	0.350	0.358	0.337	0.475	0.246	0.000	
L7	0.306	0.272	0.337	0.397	0.510	0.507	0.000

^a L1- Yercaud Hills; L2- Kaaripatti; L3- Mettur; L4- Kolli Hills; L5– Kanjamalai Hills; L6- Yelgri Hills, L7- Morappur.

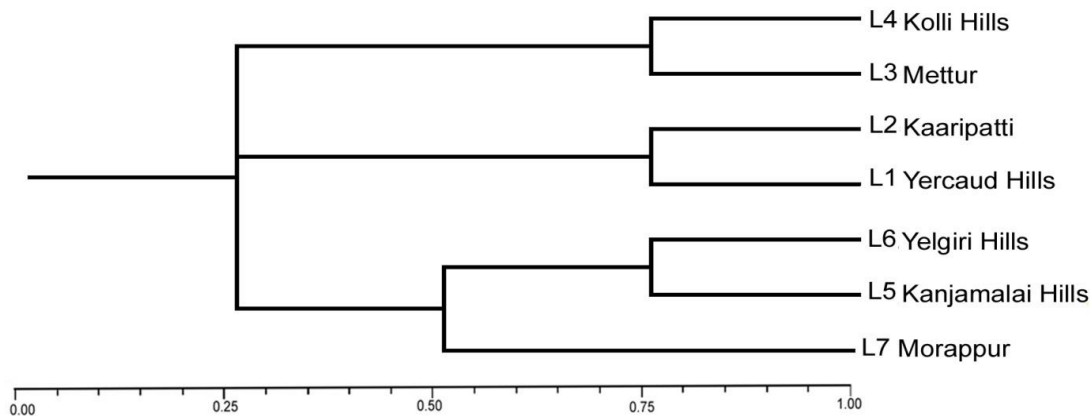


Figure 3. Un-weighted Pair Group Method with Arithmetic average (UPGMA) tree showing the genetic relationships between seven accessions of *G. sylvestre* as determined by RAPD markers.

separate cluster in the dendrogram and showed more genetic distance. Similar results were also reported earlier in *Rauvolfia serpentina* (Nair *et al.*, 2014).

Based on the phytochemical analysis, the L7 accession remained having higher level of variation in the content rate than other accessions. The present results showed the existence of genetic variations among the 7 *G. sylvestre* accessions and the maximum distance recorded was 0.51 for L7 accession. Therefore, this population could be utilized for the production of high quality herbal formulations and also may be selected for large scale cultivation and supply of quality materials to pharmaceutical industry. Balamuralikrishna *et al.* (2012) reported that there was considerable association between molecular and biochemical diversity with respect to gymnemic acid content in *G. sylvestre*. Shahnawaz *et al.* (2012) studied the genetic diversity of *G. sylvestre* using RAPD and ISSR markers and reported the genetic diversity of 22 *Gymnema* populations spread across Western Ghats of Maharashtra. Similar genetic variability was also noticed in the present study. A relationship between genetic variability and geographic distribution has been reported earlier in other medicinal plants (Grass *et al.*, 2006; Zong *et al.*, 2008; Rahimmalek *et al.*, 2009; Djabou *et al.*, 2012; Sundaresan *et al.*, 2012). In the present study, RAPD results provided useful insights to understand the existence of genetic variation through evolutionary dynamics among 7 populations of *G. sylvestre* across different geographical locations within Tamilnadu.

Determination of Phytochemical Diversity by HPLC Analysis

In identification of genetic variability using DNA fingerprints, there will not be any change irrespective of the tissue used for analysis, but the phytochemical content will largely vary depending on the tissue type used, due to the influence of physiological nature and environmental factors (Nair *et al.*, 2014). In this context, it would be more appropriate to

justify applying both DNA and phytochemical-based methods for determination of genetic diversity. In this context, it would be more appropriate to justify applying both DNA and phytochemical-based methods for determination of genetic diversity effectively. In order to find out the differences in phytochemical constituents level from *G. sylvestre* and also to provide comprehensive information on biochemical quality, the main bioactive molecule i.e., gymnemic acid was selected and used for analysis from 7 populations distributed in various locations within Tamilnadu (from 20 to 285 km distance). In the present study, the acid-base hydrolysis extraction method was used for extraction of higher yield of gymnemagenin content from the leaves of *G. sylvestre*. Gymnemagenin is a glycone of gymnemic acid and is separated after acidic and basic hydrolysis. Also, addition of water in methanol increased the yield of gymnemagenin to certain extent by improving its solubility. The phytochemical fingerprint and gymnemic acid content level of 7 accessions was determined by using HPLC at an Ultra violet wavelength of 210 nm (Figure 4). Among the populations tested, the higher level of gymnemic acid content recorded was 7.18 mg g⁻¹ DW with accession L7, followed by L1 (6.97 mg g⁻¹ DW) and L2 (6.89 mg g⁻¹ DW), whereas the low level of gymnemic acid content observed was 3.84 mg g⁻¹ DW with L5 population (Figure 5). It is very important to mention that the DNA fingerprinting profile data showed maximum genetic variation in the UPGMA dendrogram for L7 accession that had the high gymnemic acid content followed by L1 and L2 populations. Results showed a strong positive correlation between molecular and phytochemical content variability in *G. sylvestre*. Phytochemical analysis clearly showed the presence of rich biochemical diversity among the 7 populations. The present results provide the evidences for existence of phytochemical diversity among the accessions tested and the best accession could be selected based on the level of bioactive compound for bio-resource conservation and pharmaceutical utilization

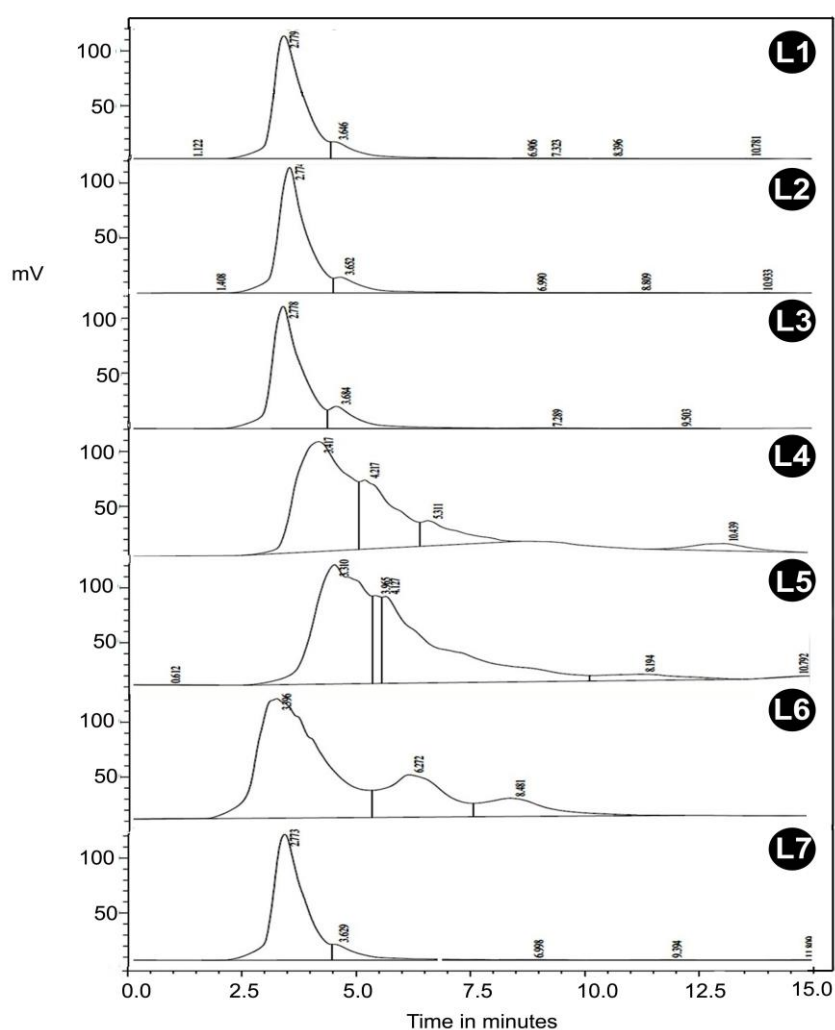


Figure 4. Chromatograms of methanolic extracts of *G. sylvestre*. HPLC chromatograms showing presence of gymnemic acid at 2.8 minutes. (Accession ID: As defined in Figure 1).

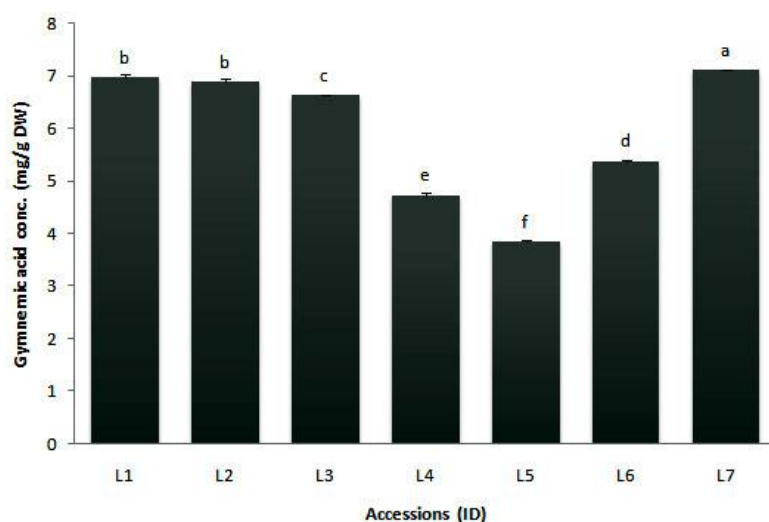


Figure 5. Distribution of gymnemic acid in *G. sylvestre* (Accession ID: As defined in Figure 1).

of this important medicinal herb. Earlier, Han *et al.* (2008) observed a correlation between genetic diversity and variations in phenolic contents of *Fructus xanthii*. Balamuralikrishna *et al.* (2012) also investigated the genetic diversity and compared with gymnemic acid content in *G. sylvestre* and observed a correlation between genetic and biochemical diversity. Recently, Nair *et al.* (2014) demonstrated that there was a considerable variation in the reserpine content levels among the populations tested and a maximum reserpine content noticed was 1.312 g 100 g⁻¹ with *R. serpentina* accession 7. It is also reported that the level of reserpine content from different accessions of this plant varied with geological conditions and the occurrence of variability might be largely due to changes in environmental conditions of the selected location (Nair *et al.* 2014).

The results of the present study reveal some relationships among genetic makeup, gymnemic acid content, and place of origin of each *G. sylvestre* population. The genetic relationships between populations from the same region were closer than those between populations from different regions. There were significant differences in the bioactive compound levels in accessions collected from the various locations of Tamilnadu (Table 1), suggests that genetic factors may also play a critical role in determining the level of bioactive compounds in the plants. Similar results were also reported earlier (Tao *et al.*, 2009; Hu *et al.*, 2007; Han *et al.*, 2008; Azizi *et al.*, 2012). Based on the phytochemical chromatograms and molecular markers, seven *G. sylvestre* populations were perfectly matched in PCoA groupings. Results strongly suggest that the level of gymnemic acid in each population was positively correlated with the data generated by genetic diversity analysis. Similarly, Ince and Karaca (2015) have successfully applied the e-microsatellite DNA markers to identify medicinal value species and quantitative detection of dry herbal products in *Salvia* species. In this context, the present findings clearly suggest the necessity of comparative analysis of both bioactive component level and genetic variability among

the populations originating from different geographical regions to obtain ideal medicinal plant accession for pharmaceutical industry applications. Taken together, the present results reveal that the L7 (Morappur) accession, which showed maximum genetic diversity in the dendrogram, is positively correlated with the highest level of bioactive compound (Gymnemaic acid) compared to other accessions. Moreover, one of the bands (1.6 kb) amplified with OPA 4 primer (Figure 1) corresponded closely with high yielding accessions. This can be further confirmed by using other molecular tools for screening high yielding genotypes.

CONCLUSIONS

In conclusion, the combined molecular and biochemical fingerprint analysis showed the existence of genetic and phytochemical diversity among 7 *Gymnema* populations originating from different geographical regions. Fingerprinting analysis showed a relationship between genetic distance and chemotype variations among the accessions tested. Dendrogram result strongly indicated that the maximum level of genetic diversity was with L7 accession, which had the highest level of gymnemic acid content (7.18 mg g⁻¹ DW) compared to other accessions. The influence of the geographic origin on genetic diversity and biochemical contents in *G. sylvestre* plants could provide an important insight to select the potential genotype for large-scale cultivation, and L7 population deserves special consideration for pharmaceutical applications due to the presence of higher gymnemic acid content. We also found a band that showed high correlation with the gymnemic acid production; this can be used as a marker. Further, this band can be sequenced to find out the genetic basis of this correlation. To the best of our knowledge, this is the first report to provide valuable information on existence of correlations between the genetic diversity and chemotype pattern in this important medicinal plant species.



ACKNOWLEDGEMENTS

Dr. P. Venkatachalam acknowledged financial assistance for this work is provided by University Grants Commission under UGC Major Project No. 37-297/2009 (SR), Govt. of India, New Delhi.

REFERENCES

1. Azizi, A., Hadian, J., Gholami, M., Friedt, W. and Honermeier, B. 2012. Correlations between Genetic, Morphological and Chemical Diversities in a Germplasm Collection of the Medicinal Plant *Origanum vulgare* L. *Chem. Biodiver.*, **9**: 2784-2801.
2. Balamuralikrishna, R., Reddy, S. R. R., Vijayalaxmi, M., Anugna, K., Divyavani, N. and Reddy, K. J. 2012. Molecular Characterization of 17 Accessions of *Gymnema sylvestre* R. Br. using RAPD Markers. *Int. J. Pharma. Bio. Sci.*, **3**: 126-135.
3. Baum, B. R., Mechanda, S. and Livesey, J. F. 2001. Predicting Quantitative Phytochemical Markers in Single *Echinacea* Plants or Clones from Their DNA Fingerprints. *Phytochemistry*, **56**: 543-549.
4. Chodiseti, B., Rao, K. and Giri, A. 2013. Phytochemical Analysis of *Gymnema sylvestre* and Evaluation of Its Antimicrobial Activity. *Nat. Prod. Res.*, **27**: 583-587.
5. Collard, B. C. Y. and Mackill, D. J. 2009. Conserved DNA-Derived Polymorphism (CDDP): A Simple and Novel Method for Generating DNA Markers in Plants. *Plant Mol. Biol. Rep.*, **27**: 558-562.
6. Das, J., Thapaa, S., Pradhana, D., Thoratb, S. S. and Talukdarc, N. C. 2013. Intra-Specific Genetic Diversity, Phytochemical Analysis and Antioxidant Activities of a Potential Himalayan Swertia (*Swertia bimaculata* Hook. F. and Thomas.). *Ind. Crops Prod.*, **49**: 341-347.
7. Jain, N. and Devi, V. K. 2016. In Vitro Evaluation of Once-a-Day Chronotherapeutic Drug Delivery System of *Gymnema sylvestre*. *Ind. Crops Prod.*, **88**: 58-64.
8. Dharmar, D. K. and Britto, A. J. D. 2011. RAPD Analysis of Genetic Variability in Wild Populations of *Withania somnifera* (L.) Dunal. *Int. J. Biol. Technol.*, **2**: 21-25.
9. Djabou, N., Allali, H., Battesti, M. J., Tabti, B., Costa, J. and Varesi, A. M. L. 2012. Chemical and Genetic Differentiation of Two Mediterranean Subspecies of *Teucrium scorodonia* L. *Phytochemistry*, **74**: 123-132.
10. Doyle, J. J. and Doyle, J. L. 1990. A Rapid Total DNA Preparation Procedure for Fresh Plant Tissue. *Focus*, **12**: 13-15.
11. Grass, S., Zidorn, C., Frank, R. B., and Stuppner, H. 2006 Comparative Molecular and Phytochemical Investigation of *Leontodon autumnalis* (Asteraceae, Lactuceae) Populations from Central Europe. *Phytochemistry*, **67**: 122-131.
12. Han, T., Hu, Y., Zhou, S. Y., Li, H. L., Zhang, Q. Y., Zhang, H., Huang, B. K., Rahman, K., Zheng, H.C., And Qin, L.P. 2008. Correlation between the Genetic Diversity and Variation of Total Phenolic Acids Contents in *Fructus Xanthii* from Different Populations in China. *Biomed. Chromatogr.* **22**: 478-486.
13. Hu, Y., Zhang, Q., Xin, H., Qin, L. P., Lu, B. R., Rahman, K., and Zheng, H. 2007. Association between Chemical and Genetic Variation of *Vitex rotundifolia* Populations from Different Locations in China: Its Implication for Quality Control of Medicinal Plants. *Biomed. Chromatogr.*, **21**: 967-975.
14. Ince, A. G. and Karaca, M. 2015. E-Microsatellite Markers for Some Naturally Occurring *Salvia* Species in the Mediterranean Region. *Turk. J. Biol.*, **39**: 69-77.
15. Ince, A. G. and Karaca, M. 2015. Td-DAMD-PCR Assays for Fingerprinting of Commercial Carnations. *Turk. J. Biol.*, **39**: 290-298.
16. Kang, M., Lee, M., Choi, M., Min, K. S. and Shibamoto, T. 2012. Hypoglycemic Activity of *Gymnema sylvestre* Extracts on Oxidative Stress and Antioxidant Status Diabetic Rats. *J. Agric. Food Chem.*, **60**: 2517-2524.
17. Karaca, M. and Ince, A. G. 2008. Minisatellites as DNA Markers to Classify Bermudagrasses (*Cynodon* spp.): Confirmation of Minisatellite in Amplified Products. *J. Genet.*, **87**: 83-86.
18. Keskitalo, M., Pehu, E. and Simon, J. E. 2001. Variation in Volatile Compounds from Tansy (*Tanacetum vulgare* L.) Related to Genetic and Morphological Differences of Genotypes. *Biochem. System. Ecol.*, **29**: 267-285.
19. Kumar, A. and Yadav, P. S. 2010. Variation in Gymnemic Acid Content and Non-Destructive Harvesting of *Gymnema sylvestre* (Gudmar). *Pharmacog. Res.*, **2**: 309-312.
20. Lal, S., Mistry, K. N., Daxini, N. and Patel, S. 2013. Assessment of Genetic Diversity in Chakkarakolli (*Gymnema sylvestre* R. Br.)

- using Randomly Amplified Polymorphic DNA (RAPD) Marker from Different Locations of Gujarat, India. *Inter. J. Biomed. Res.*, **6**: 21-29.
21. Li, X. B. and Tu, P. F. **2003**. Fingerprint System of Chinese Medicinal Materials. *Chin. Tradit. Herb. Drugs*, **34**: 385-387.
 22. Mahar, K.S., Rana, T.S., Ranade, S.A., Pande, V., and Palni, L.M.S. 2013. Estimation of Genetic Variability and Population Structure in *Sapindus trifoliatus* L., using DNA Fingerprinting Methods. *Trees*, **27**: 85-96.
 23. Malik, J. K., Manvi, F. V., Alagawadi, K. R. and Noolvi, M. 2008. Evaluation of Anti-Inflammatory Activity of *Gymnema sylvestre* Leaves Extract in Rats. *Int. J. Green Pharm.*, **2**: 114-115.
 24. Mouna, H. M., Reddy, P. J. M., Rajasekharan, P. E., Shareef, I. and Sreekanth, B. 2014. Assessment of Genetic Diversity in the Medicinal Climber *Gymnema sylvestre* from Karnataka, India. *Int. J. Innov. Res. Sci. Eng. Tech.*, **3**: 10497-10501.
 25. Nair, S., and Keshavachandran, R. 2006. Genetic Variability of *Chakkarakolli* (*Gymnema sylvestre* R. Br.) in Kerala Assessed using Morphological and Biochemical Markers. *J. Trop. Agri.*, **44**: 64-67.
 26. Nair, V. D., Raj, R. P. D., Panneerselvam, R. and Gopi, R. 2014. Assessment of Diversity among Populations of *Rauvolfia serpentina* Benth. Ex. Kurtz. from Southern Western Ghats of India, Based on Chemical Profiling, Horticultural Traits and RAPD analysis. *Fitoterapia*, **92**: 46-60.
 27. Obiadalla-Ali, H. A. O., Mohamed, N. E. M., Abdelsabour, G. A. and Khaled, A. G. A. 2015. Inbreeding, Outbreeding and RAPD Markers Studies of Faba Bean (*Vicia faba* L.) Crop. *J. Adv. Res.*, **6**: 859-868.
 28. Ohmori, R., Iwamoto, T., Tago, M., Takeo, T., Unno, T. and Itakura, H. 2005. Antioxidant Activity of Various Teas against Free Radicals and LDL Oxidation. *Lipids*, **40**: 849-853.
 29. Pandey A. K., Yadav S. 2010, Variation in Gymnemic Acid Content and Non-destructive Harvesting of *Gymnema sylvestre* (Gudmar). *Pharmacognosy Res.* **2(5)**:309-12.
 30. Pasha, C., Sayeed, S., Ali, S. and Khan, Z. 2009. Anti Salmonella Activity of Selected Medicinal Plants. *Turk. J. Biol.*, **33**: 59-64.
 31. Puratchimani, V. and Jha, S. 2004. Standardization of *Gymnema sylvestre* R.Br. with Reference to Gymnemagenin by High-Performance Thin-Layer Chromatography. *Phytochem. Anal.*, **15**: 164-166.
 32. Rachh, P. R., Rachh, M. R., Soniwala, M. M., Joshi, V. D., and Suthar, A. P. 2010. Effect of *Gymnema* Leaf Extracts on UV Induced Damage on *Salmonella typhi*. *Asian J. Biol. Sci.*, **3**: 28-33.
 33. Rahimmalek, M., Bahreininejad, B., Khorrami, M. and Tabatabaei, B. E. S. 2009. Genetic Variability and Geographic Differentiation in *Thymus daenensis* subsp. *Daenensis*, An Endangered Medicinal Plant, as Revealed by Inter Simple Sequence Repeat (ISSR) Markers. *Biochem. Genet.*, **47**: 831-842.
 34. Saneja, A., Sharma, C., Aneja, K. R. and Pahwa, R. 2010. *Gymnema sylvestre* (Gurmar): A Review. *Der Pharm Lett.*, **2**: 275-284.
 35. Shanmugasundaram, E. R. B., Gopinath, K. L., Shanmugasundaram, K. R., and Rajendran. 1990. Possible Regeneration of the Islets of Langerhans in Streptozotocin-Diabetic Rats given *Gymnema sylvestre* Leaf Extracts. *J. Ethnopharmacol.*, **30**: 265-79.
 36. Shah Nawaz, M., Zanan, R. L., Wakte, K.V., Mathure, S. V., Kad, T. D., Deokule, S. S. and Nadaf, A. B. 2012. Genetic Diversity Assessment of *Gymnema sylvestre* (Retz.) R. Br. ex Sm. populations from Western Ghats of Maharashtra, India. *Genet. Resour. Crop. Evol.*, **59**: 125-134.
 37. Shilpha, J., Silambarasan, T., Pandian, S. K., and Ramesh, M. 2013. Assessment of Genetic Diversity in *Solanum trilobatum* L., An Important Medicinal Plant from South India using RAPD and ISSR Markers. *Genet. Resour. Crop Evol.*, **60**: 807-818.
 38. Srikanth, A. V., Maricar, S., Narsu, L. M., Kumar, P. R. and Reddy, B. M. 2010. Anticancer Activity of *Gymnema sylvestre* R.Br. *Inter. J. Pharm. Sci. Nanotech.*, **3**: 897-899.
 39. Sundaresan, V., Sahni, G., Verma, R. S., Padalia, R. C., Mehrotra, S. and Thul. S. T. 2012. Impact of Geographic Range on Genetic and Chemical Diversity of Indian Valerian (*Valeriana jatamansi*) from Northwestern Himalaya. *Biochem. Genet.*, **50**: 797-808.
 40. Tao, J., Luo, Z. Y., Msangi, C. I., Shu, X. S., Wen, L., Liu, S. P., Zhou, C. Q., Liu, R. X. and Hu, W. X. 2009. Relationships among Genetic Makeup, Active Ingredient Content, and Place of Origin of the Medicinal Plant *Gastrodia Tuber*. *Biochem. Genet.*, **47**: 8-18.



41. Tiwari, P., Mishra, B. N. and Sangwan, N. S. 2014. Phytochemical and Pharmacological Properties of *Gymnema sylvestre*: An Important Medicinal Plant. *BioMed. Res. Int.* ID 830285, doi.org/10.1155/2014/830285
42. Tripathi, N., Chouhan, D. S., Saini, N. and Tiwari, S. 2012. Assessment of Genetic Variations among Highly Endangered Medicinal Plant *Bacopa monnieri* (L.) from Central India using RAPD and ISSR Analysis. *3 Biotech.*, 2: 327-336.
43. Venkatachalam, P., Nair, L. B., Geetha, N. and Thulaseedharan, A. 2008 Genetic Relationship Analysis in Rubber Tree (*Hevea brasiliensis* Muell. Arg.) by DNA Fingerprinting. *Plant Cell Biotech. Mol. Biol.*, 9: 101-108.
44. Vieira, R. F., Grayer, R. J., Paton, A. and Simon, J. E. 2001. Genetic diversity of *Ocimum gratissimum* L. based on Volatile Oil Constituents, Flavonoids and RAPD Markers. *Biochem. Syst. Ecol.*, 29: 287-304.
45. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J.A., and Tingey, S.V. 1990. DNA Polymorphisms Amplified by Arbitrary Primers are Useful as Genetic Markers. *Nucl. Acids Res.*, 18: 6531-6535.
46. Zong, M., Liu, H. L., Qiu, Y. X., Yang, S. Z., Zhao, M. S. and Fu, C. X. 2008. Genetic Diversity and Geographic Differentiation in the Threatened Species *Dysosma pleiantha* in China as revealed by ISSR Analysis. *Biochem. Genet.*, 46: 180-196.

تشخیص تغییرات ملکولی و فیتوشیمیایی در نژادهای بومی Tamilnadu از ژنوتیپ *Gymnema sylvestre*: یک گیاه مهم ضد دیابتی

ی. جینو، م. تیاگارجان، س. جاود، ا. ا. العطار، و پ. ونکاتاجالام

چکیده

Gymnema sylvestre گیاهی است با پتانسیل دارویی ضد دیابت که در بسیاری از نسخه های گیاه دارویی استفاده می شود. خواص دارویی آن به حضور اسید ژیمنمیک (gymnemic acid) نسبت داده می شود که در کنترل گلوکز خون و سطح چربی بدن موثر است. در این پژوهش، تغییرات ملکولی و بیوشیمیایی در ۷ جمعیت *G. sylvestre* با استفاده از نشانگر DNA و اثرانگشت فیتوشیمیایی محتوای اسید ژیمنمیک بررسی شد. نتایج به دست آمده از این روش در یک تحلیل مقایسه ای استفاده شد و آشکار ساخت که بین تغییرات ژنتیکی و تجزیه محتوای مواد بیوشیمیایی همبستگی وجود دارد. سیزده آغازگر برای تجزیه RAPD-PCR استفاده شد و ۱۱۲ باند آشکار شد که ۶۲ تای آنها پلی مورفیک بود. درصد باندهای پلی مورفیک بین ۱۱/۱٪ تا ۸۴/۶٪ با میانگین ۵۴/۲٪ بود. میانگین تعداد باندهای افزایش یافته (amplified) در هر آغازگر ۸/۶۲ بود در حالیکه میانگین تعداد باندهای پلی مورفیک در هر آغازگر ۵/۴۲ بود. در ادامه، یک دندروگرام (dendrogram) از تشابهات ژنتیکی با استفاده از ضرایب فواصل ژنتیکی ساخته شد و ۷ نمونه ثبت شده در ۴ گروه اصلی دسته بندی شد. در میان این گروه ها، کل محتوای اسید ژیمنمیک بین ۳/۸۴mg/g DW تا ۷/۱۲mg/g DW تغییرات زیاد و معناداری میکرد. تجزیه و تحلیل مختصات اصلی نتایج دسته بندی مزبور را تایید کرد. حد بیشینه محتوای اسید ژیمنمیک (۷/۱۲mg/g DW) به نمونه ثبت شده L7 تعلق داشت. نتایج حاکی از آن است که بین ساختار ژنتیکی و محتوای اسید ژیمنمیک احتمالاً یک همبستگی مثبت وجود دارد، با این همه، این مطلب باید با استفاده از دیگر روش های ملکولی بیشتر بررسی شود.