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MOLECULAR ANALYSIS AND PHYTOCHEMICAL PROFILING TO EXPLORE THE DIVERSITY OF *GARCINIA* SPECIES COLLECTED FROM WESTERN GHATS OF INDIA

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Hydroxycitric acid made the genus *Garcinia* economically important. Genetic and chemical diversity has been studied in *Garcinia* species using molecular markers, HCA and antioxidant activity. Nine species were collected and screened for molecular diversity and six were subjected to analyse antioxidant and HCA content and its interspecies variability. A total of 129, 125 and 89 bands with polymorphism of 78.74%, 78.4% and 93.36% were obtained using ISSR, RAPD and EST-SSR, respectively. The average PIC value obtained with ISSR, RAPD and EST-SSR markers was 0.9161, 0.9440 and 0.8903, respectively. Determined HCA content by HILIC-HPLC system using 0.1% orthophosphoric acid and acetonitrile (30:70) as mobile phase in fruit powder of various *Garcinia* species was found to be significantly different. *G. gummi-gutta*, *G. indica* and *G. xanthochymus* are rich of HCA containing 12.44±1.04%, 7.92±0.83% and 6.3±0.286%, respectively. *G. morella*, *G. talbotii* and *G. celebica* contained very negligible amount of HCA, 0.023±0.012%, 0.083±0.034% and 0.34±0.013%, correspondingly. *G. talbotii* showed high antioxidant capacity (95.40±0.720). Below that *G. indica* and *G. xanthochymus* were showing significant amount of total phenols (1.23±0.015 and 1.07±0.008), flavonoids (11.17±0.075 and 12.35±0.219) and antioxidant activity (90.73±0.976 and 91.37±0.854). Correlation analysis found significant association between molecular and chemical variation indicating influence of genetic background on the observed HCA and antioxidant profiles. The conducted analysis showed the most distinct species at the genetic and chemical levels were *G. gummi-gutta*, *G. indica* and *G. xanthochymus*. This study signifies the utility of molecular and chemical fingerprints for commercial exploitation of HCA from *Garcinia* species.

Key words: diversity, *Garcinia*, hydroxycitric acid, ISSR, molecular markers, RAPD

INTRODUCTION

Garcinia L. is a pantropically distributed genus with high species richness in SE Asia, and composed of 240 species (Saleh 2006). In India the genus *Garcinia* is represented by 43 species including 9 endemic to the Western Ghats only (Shameer *et al.* 2019). For genus *Garcinia* Western, Ghats is considered as

secondary centre for origin (Abraham *et al.* 2006). It includes *G. indica* (Thouars) Choisy, *G. gummi-gutta* (L.) Roxb., *G. talbotii* Raizada ex Santapau, *G. morella* (Gaertn.) Desr. which are widely distributed. Besides them three species *G. celebica* L. (syn. *G. hombroniana* Pierre), *G. xanthochymus* Hook. f. ex T. Anderson and *G. mangostana* L. are introduced (Shameer *et al.* 2019). These indigenous and introduced species provides important resources in Indian continent. Some species are also well-known among local populations due to their various culinary purposes and medicinal properties. More recently the pharmacological properties of few *Garcinia* species have been deeply explored (Hart and Cock 2016). The genus is known for a variety of biologically active metabolites such as polyisoprenylated benzophenones (Meli Lannang *et al.* 2010), xanthones (Louh *et al.* 2008) and triterpenoids (Meli Lannang *et al.* 2008, Nguyen *et al.* 2011). Considerable efforts have been made in order to understand small and complex molecules from different parts of the various species of *Garcinia* including xanthones and their derivatives (Bennett and Lee 1989, Rama Rao *et al.* 1980) in addition to their biological and pharmacological activity. There have been extensive claims on the beneficial effects of *Garcinia* plant extracts especially based on their antioxidant properties (Hassan *et al.* 2013, Jung *et al.* 2006).

In the current scenario, the assessment of genetic and chemical diversity is the most renowned approach for identification and characterisation of elite species. Molecular markers with biochemical and phytochemical traits play an important role to depict genetic diversity (Jugran *et al.* 2012). The presence of high numbers of sympatric *Garcinia* species in almost every tropical region (Ashton 1988, Sweeney 2008) may well have led to a situation where the genus received comparatively less attention from taxonomists because of its supposed complexity. Some past studies have produced estimation of genetic diversity at genus level (Mansyah *et al.* 2010, Mohan *et al.* 2010, Qosim *et al.* 2011, Tharachand *et al.* 2015, Thatte *et al.* 2012). However, information on genetic diversity and genetic relationships of *Garcinia* is very limited (Mohanan *et al.* 2017, Saleh 2006).

Collective marker studies have been found in some species of *Garcinia*. Studies on various *Garcinia* species involving random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) markers were successfully used by various researchers throughout the world (Mansyah *et al.* 2010, Mohan *et al.* 2010, Qosim *et al.* 2011, Tharachand *et al.* 2015, Thatte *et al.* 2012). In our recent report, we demonstrated the phytogeographical variation and molecular marker study in characterisation of natural and conserved populations of *G. xanthochymus* as well (Anerao *et al.* 2016*b*). Single attempt has been made available on group of *Garcinia* species from Indian continent (Parthasarathy *et al.* 2013). However, data on molecular and chemical diversity of *Garcinia* is sparsely available.

The genus has received importance of new global economy of medicinal and pharmaceutical industries because of the presence of bioactive molecule hydroxycitric acid (HCA), knowing for its antiobesity property. HCA, found in *Garcinia* species inhibits the extra mitochondrial enzyme adenosine triphosphate-citrate (pro-3S)-lyase essential in fat production which results in reduction in the body weight (Heymsfield *et al.* 1989). Recent developments in the field of determination and quantitation of bioactive compound and its comparative analysis from economic crops have led to renewed interest in determination of HCA using a number of analytical methods. Lewis and Neelakantan (1965) had identified HCA in mid-sixties. Most studies have determined HCA content in *Garcinia* using High performance liquid chromatography (HPLC), Liquid chromatography (LC) and Reverse Phase High performance liquid chromatography (RPHPLC) (Dang *et al.* 2011, Jayaprakasha and Sakariah 1998, 2000, 2002, Jena *et al.* 2002). Comparative estimation of HCA was carried in four commonly available species (Ashish *et al.* 2008). Several studies have produced the percentage of HCA, but still there is insufficient information to conclude a general statement. Considering the high commercial value of this compound and widespread occurrence in *Garcinia* species makes HCA a useful biochemical marker for chemosystematics and evolutionary relationships. Due to lack of standard protocol for determination and inconsistent results from the previous methods, researchers are looking for new, more sensitive, reliable and fast techniques which give quick results (Dang *et al.* 2011, Jayaprakasha and Sakariah 1998, 2000, Jayaprakasha *et al.* 2003, Kumar *et al.* 2013).

Due to the lack of information available on the molecular diversity in economically imperative species of *Garcinia* and tremendous demand of HCA in market there is a need of molecular fingerprinting as well as the antioxidant and HCA profiling of *Garcinia* species. Such study helps to prevent the sale of the invariably adulterated and different unrelated drugs in the crude drug market of the world under the name of weight loss.

In spite of their numerous species and economic relevance, there is no ample information available on genetic relationship, antioxidant activity and HCA content of *Garcinia*. It was considered that quantitative measures would conveniently enhance and extend the qualitative analysis. The study was also focused on method development and quantification of HCA from six *Garcinia* species from Western Ghats using High Interaction Liquid Chromatography-High Performance Liquid Chromatography (HILIC-HPLC) and will be compared with the available market product. The HCA profile can be used to identify species, because of its distinctness to each species, and evidence of tampering can be evaluated by comparing the known species fingerprint to that of the suspected adulterated HCA powder. Objectives of the present study were molecular characterisation of the germplasm of *Garcinia*

using molecular markers and to compare the level of information provided by RAPD, ISSR, Expressed sequence tags-SSR (EST-SSR) and variability in the content of potential metabolite HCA using HILIC-HPLC analysis and antioxidant activity for the assessment of genetic similarities to find the genetic and phytochemical relationship among species.

MATERIAL AND METHODS

Fruits, seeds and samplings of nine species of *Garcinia* including *G. indica* (Thouars) Choisy, *G. xanthochymus* Hook. f. ex T. Anderson, *G. morella* (Gaertn.) Desr., *G. gummi-gutta* (L.) Roxb. (syn. *Garcinia affinis* Wight et Arn.), *G. talbotii* Raizada ex Santapau, *G. celebica* L. (syn. *G. hombroniana* Pierre), *G. madruno* (Kunth) Hammel, *G. elliptica* (Graham) Wall. ex Wight (syn. *G. acuminata* Planch. et Triana) and *G. livingstonei* T. Anderson were collected for executing the research work from various parts of the Western Ghats (Fig. 1). Botanical names of the species and their synonyms were referred from the plant list (2010). The global positioning system of collection sites was mentioned in Table 1. Dried fruit powders of *G. indica*, *G. gummi-gutta*, *G. xanthochymus*, *G. talbotii*, *G. morella* and *G. celebica* were utilised for determination of HCA and antioxidant assay.

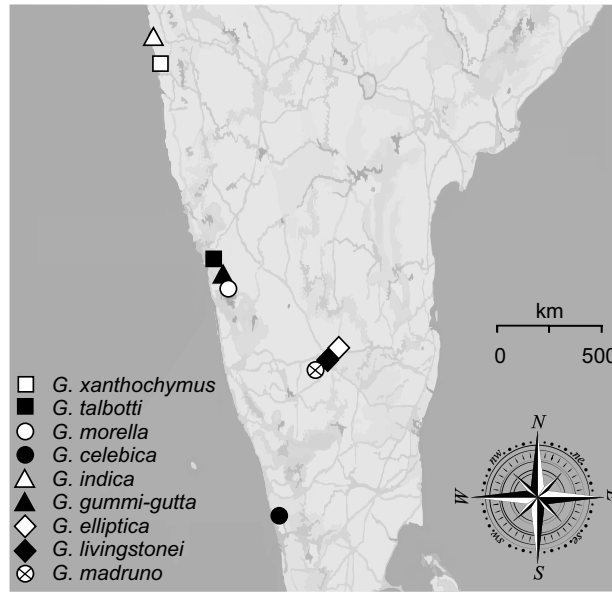

Fig. 1. Distribution map showing the *Garcinia* species collected from various locations of Western Ghats

Table 1
List of the *Garcinia* species utilised and their collection details

Garcinia species	Origin/ Location	GPS location	Alt. (m)
G. elliptica Wall. ex Wight (Sect. *Hebradendron*)	GKVK, Bangalore	13° 4′ 49.31″ N; 77° 34′ 35.90″ E	936
G. gummi-gutta (L.) Roxb. (Sect. *Brindonia*)	Kadakeri Karnataka	14° 20′ 59.66″ N; 74° 51′ 41.97″ E	594
G. celebica L. (Sect. *Garcinia*)	NBPGR, Kerala	10° 33′ 06.94″ N; 76° 16′ 28.82″ E	15
G. indica (Thouars) Choisy (Sect. *Brindonia*)	Dive-Agar, Maharashtra	18° 10′ 23.62″ N; 72° 59′ 28.64″ E	4
G. livingstonei T. Anderson (Sect. *Teracentrum*)	GKVK Bangalore	13° 04′ 49.31″ N; 77° 34′ 36.47″ E	953
G. madruno (Kunth) Hammel (Sect. *Rheediopsis*)	Lalbaugh Garden Bangalore	10° 33′ 05.17″ N; 76° 16′ 28.82″ E	906
G. morella (Gaertn.) Desr. (Sect. *Hebradendron*)	Menasi, Karnataka	14° 16′ 44.86″ N; 74° 49′ 0.33″ E	610
G. talbotii Raizada ex Santapau (Sect. *Xanthochymus*)	Mabagi, Karnataka	14° 35′ 24.73″ N; 74° 33′ 51.41″ E	308
G. xanthochymus Hook. f. ex T. Anderson (Sect. *Xanthochymus*)	Dapoli, Maharashtra	17° 45′ 13.65″ N; 73° 10′ 56.09″ E	182

Molecular analysis

The young leaf material was used for extraction of DNA. The details of each technique were mentioned in the following subsections. The chemicals were obtained from Thermo Fischer. Reactions were carried out in a total volume of 25 µl consisting of 100 ng of template DNA, 1X Taq buffer, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase, 0.2 mM dNTPs, and 0.5 µM Primer.

DNA extraction and PCR amplification: DNA was isolated from young leaves following the cetyltrimethyl ammonium bromide (CTAB) and Sodium dodecyl sulphate (SDS) protocols as mentioned in published protocol (Anerao *et al.* 2016*a*).

PCR amplification of ISSR: For the ISSR analysis, a total of 35 primers were checked and out of that 11 primers were screened down for genetic analysis. The total reaction volume was carried in 25 µl of reaction mixture containing 100 ng μl^{-1} of template DNA, 1X Taq buffer, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase, 0.2 mM dNTPs and 0.5 µM RAPD primer. The polymerase chain reaction (PCR) amplification was carried out in a thermocycler (Gold AmpGene) programmed as follows: 5 min at 95 °C for 1 cycle, followed by 1 min at 94 °C, 1 min at annealing temperature (depending on the primer used, melting temperature is mentioned in Table 2) 1 min at 72 °C for 45 cycles, and 7

Table 2
Marker parameters calculated for each primer utilised with *Garcinia* species

Sr. no.	Primer	Primer sequences	Tm (°C)	Mean PIC	MI
1	A1	AGAGAGAGAGAGAGAGT	51	0.9486	7.589
2	A 2	AGAGAGAGAGAGAGAGAA	51	0.9667	7.733
3	A3	AGAGAGAGAGAGAGAGTA	51	0.9087	9.99
4	A 4	GAGAGAGAGAGAGAGAGAA	53	0.9717	5.830
5	A 5	GAGAGAGAGAGAGAGAGAAC	50	0.9602	6.721
6	A 6	GAGAGAGAGAGAGAGAGAAT	50	0.9758	2.927
7	A 7	GACGACGACGACGAC	50	0.7409	2.222
8	A 8	CCCGGATCCGAGAGAGAGAGAGAGAGA	53	0.9153	8.237
9	A 9	GACAGACAGACAGACA	53	0.7409	2.222
10	A 10	CACACACACACACACAAT	53	0.9747	10.721
11	A 809	AGAGAGAGAGAGAG AGC	50	0.9737	11.685
12	RAPD 1	AAAGCTGCGG	32	0.9408	8.467
13	RAPD 2	AAAGCTGCGG	34	0.9369	13.116
14	RAPD 3	AAGCGACCTG	32	0.9190	11.947
15	RAPD 4	AATCGCGCTG	32	0.9549	10.503
16	RAPD 5	AATCGGGCTG	32	0.9734	9.734
17	RAPD 6	ACACACGCTG	32	0.9782	14.673
18	RAPD 7	ACATCGCCCA	32	0.9208	10.128
19	RAPD 8	ACCACCCACC	34	0.9214	11.056
20	RAPD 9	ACCGCCTATG	32	0.9593	10.552
21	RAPD 10	ACGATGAGCG	32	0.9354	11.224
22	E-SSR 1	For: CATGGAGTTGTGATACCTAC Rev: CAGAGTTAGCCATATAGAGTG	51	0.9879	2.963
23	E-SSR 2	For: ATTATTTACCCTACAGAGTGC Rev: GTATTATCGGTAATGTCTTCAT	51	0.9879	2.963
24	E-SSR 3	For: GAACGAGAAATCGGGAAC Rev: GCAGCCATTGAATACAGAG	51	0.9516	1.903
25	E-SSR 4	For: TTGATGCAACTTTCTGCC Rev: ATGTGATTGTTAGAATGAACTT	53	0.8324	6.65
26	E-SSR 5	For: GCTTTATCCACATCAATATCC Rev: TCCTACAATAACTTGCC	54	0.8458	6.76
27	E-SSR 6	For: AATTATCCTATCCCTCGTATC Rev: AGAAACATGATGTGAACC	54	0.6378	2.551

Table 2 (continued)

Sr. no.	Primer	Primer sequences	Tm (°C)	Mean PIC	MI
28	E-SSR 7	For: GTTTAGAAAGTGCTGTGTGAC	56	0.9226	16.6
		Rev: GATGTATGGGACCTAATG			
29	E-SSR 8	For: GACATACAGGAAACGGTGGAG	55	0.8599	6.019
		Rev: ATTGTAAATGACCATCAACTA			
30	E-SSR 9	For: GCTTCGATCAATCTAGCTTCCC	60	0.9105	9.105
		Rev: GCAAACTACGCCACCCCG			
31	E-SSR 10	For: CGAGGGAGGGATGAGGAGC	60	0.9672	6.770
		Rev: CACATTCATCCACCCTCCTATA			

min at 72 °C for final extension. PCR products were separated on 2% agarose gel stained with 0.1 μg ml^{-1} of ethidium bromide using 1X Tris Acetic EDTA (TAE) buffer solution, and visualised under UV light.

PCR amplification of RAPD: For the RAPD analysis, 10 RAPD primers were utilised. PCR reaction mixture and concentrations of the reagents were same as mentioned above. Only variables are primer annealing temperatures. Screened RAPD primers sequence and their melting temperatures were mentioned in Table 2. An aliquot of 10 μl of the amplified products was subjected to electrophoresis on a 2% agarose gel cast in 1X TAE. A digital image of the ethidium bromide-stained gel was captured using a Gel doc Imaging System.

PCR amplification of EST-SSR: For the EST-SSR analysis, 10 primers pairs with forward and reverse sequences (Table 2) were considered for amplification on *Garcinia* species. PCR reactions were set up in a total volume of 25 μl. 0.5 μM of each of the forward and reverse primers were utilised for amplification. Remaining procedure was similar for the RAPD and ISSR. PCR products were separated on 12% of denaturing UREA-Polyacrylamide gel electrophoresis (PAGE) Gel and run in 1X Tris Borate EDTA (TBE) buffer at 100 V. Further the visualisation of amplified DNA gel was carried out using slight modifications of the reported silver-stain protocols (Bassam and Gresshoff 2007, Kumar *et al.* 2015).

HPLC determination of hydroxycitric acid

Most of the *Garcinia* species are dioecious in nature and number of populations are very few. Because of that we could not collect sufficient fruits of *G. livingstonei* and *G. madruno. G. elliptica* was a male. Fruits of only six species of *Garcinia* were made available for phytochemical analysis. Ten fresh ripen fruits of each *Garcinia* species namely *G. gummi-gutta*, *G. indica*, *G. xanthochymus*, *G. morella*, *G. talbotii* and *G. celebica* were collected from different

Table 3
Details of chromatographic parameters

Parameters	Details
Column	ZIC®CHILIC 250 × 4.6 mm, 3 μm
Flow rate	0.75 ml/minute
Detection	UV @210 nm
Column oven temperature	30 °C
Injection volume	20 μL

locations of Maharashtra, Karnataka and Kerala in 2015–2016. Fruit rinds were chopped and then dried in the hot air oven at 60 °C. The rinds were powdered and used for extracting hydroxycitric acid. Commercial product of HCA was procured from the Isha Agro Developers PVT LTD and analytical grade standard of pure HCA lactone (synonym of *Garcinia* acid) (Article No. 27750-13-6) procured from Sigma Aldrich.

Method development: The objective of any analytical method is to obtain consistent, reliable and accurate data. To achieve better retention and resolution for HCA, HILIC was selected as a method of analysis. The separation was achieved on ZIC®CHILIC 250 × 4.6 mm, 3 μm column for the analysis of HCA. The details of chromatographic parameters are mentioned in Table 3.

The LOD and LOQ for the present HPLC method developed were measured as per guidelines described by the International Conference on Harmonization (ICH 2005). The mean values were calculated based on at least three independent replicates (n = 3) and results are presented as means ± standard deviations (SD). The percentage of HCA was calculated based on the area under curve of respective species chromatogram.

Mobile phase preparation: 0.1 v/v orthophosphoric acid (EMSVRB Grade, received from Merck Article No. 1.00573.1000) in miliQ water was used as mobile phase with acetonitrile (Gradient grade for chromatography, Article No. 6183002500) in a ratio of 30:70 (v/v).

Preparation of stock and calibration of standard solutions: Standard solution of pure HCA was prepared by dissolving 4.0 mg in 2 ml (2,000 ppm) of diluent in a vial (stock solution). Serial dilutions were made from stock solution to obtain concentrations of 50, 100, 200, 300, 500, 600 and 750 ppm. The linearity of the method for standard was established by injection of different concentrations.

Method precision: To assess the reproducibility of the method of analysis for HCA, 500 ppm standard was injected in triplicate and performed under identical chromatographic conditions. The results are reported in terms of relative standard deviation (RSD). Standard curve was plotted for the determination of limit of detection (LOD) and limit of quantification (LOQ) of HCA on HILIC-HPLC.

Sample preparation: One gram of dried, powdered fruit rind was (dissolved) extracted in 30 ml of 0.1% orthophosphoric acid and sonicated for 30 min in ultrasonic bath. After sonication, volume was made up with HPLC grade acetonitrile (70 ml). Samples were again sonicated for 15 min to extract the acid. Extracts were filtered through 0.45 μm nylon membrane filtrate (Article No. SLHN033NK) to remove fine particles and filtrate was used for HILIC-HPLC detection.

Estimation of antioxidant activity, total phenolics and total flavonoids

DPPH assay: Methanolic extracts obtained from fruits of six *Garcinia* species at a concentration of 1 mg ml^{-1} were examined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay to confirm the antioxidant activity. DPPH radical-scavenging activities were performed by making slight changes in Zhao *et al.* (2012) protocol. The fruit extracts were prepared in methanol and ascorbic acid was used as a standard. 0.002% of DPPH solution was prepared and stored at 20 °C until required. 1 ml of 0.002% of DPPH solution prepared in methanol was mixed with 1 ml of the sample solution and standard solution separately. These solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm using UV-visible Spectrophotometer (LabIndia UV-1800). Blank was prepared by adding 1 ml of 0.002% DPPH solution in 1 ml of methanol. The optical density was recorded and % inhibition was calculated using the formula given below,

$$\text{Percent (\%) inhibition of DPPH activity} = (A - B / A) \times 100$$

A = optical density of the blank and B = optical density of the sample.

FRAP assay: Second antioxidant assay ferric reducing antioxidant power (FRAP) was carried out by using a modified method (Benzie and Szeto 1999). The stock solutions were prepared using 300 mM acetate buffer and 16 ml acetic acid, pH 3.6, 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution was maintained at 37 °C. Methanolic extracts of fruits (150 μl) were react with 2,850 μl of the FRAP solution for 30 min in the dark condition. Ascorbic acid was used as standard and same procedure was used for it. The coloured product (ferrous tripyridyltriazine complex) was measured at 593 nm. Results were expressed in μg Fe (II)/g dry mass and compared with that of ascorbic acid.

Total phenolics content: Spectrophotometric method was used to determine concentration of phenolics in plant extracts. The plant sample was extracted with methanol by using cold maceration and concentration of 1 mg/ml was prepared for determining the total phenolic content. 0.5 ml of methanolic solution of plant extract, 2.5 ml of 10% (v/v) Folin-Ciocalteu's reagent and 2.5

ml 7.5% $NaHCO_3$ (w/v) was used as reaction mixture. The blank was prepared by mixing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent and 2.5 ml of 7.5% of $NaHCO_3$ (w/v). Gallic acid was used as standard. The samples were kept in room temperature for 2 hours. The absorbance was measured using spectrophotometer at λmax 765 nm. The concentration of phenolics was read (mg/ml) from the calibration curve by using the measured absorbance. The content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Total flavonoids content: The total flavonoids content in the plant extracts was determined using spectrophotometric method. The sample was prepared by adding 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ (w/v) solution dissolved in methanol. The samples were then incubated for an hour at room temperature. The absorbance was measured using spectrophotometer at λmax 415 nm. Rutin was used as standard using same process and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

Statistical data analysis

The amplified products of ISSR, RAPD and EST-SSR PCR were manually scored for band presence (1) or absence (0) for the nine species of *Garcinia* and a binary data matrix was constructed. To analyse the suitability of all three markers types, the performance of the markers was measured using two parameters; polymorphic information content (PIC) and marker index (MI). The PIC value for each locus was calculated using formula (Roldán-Ruiz *et al.* 2000); PICi = 2fi (1–fi), where PICi is the polymorphic information content of the locus, fi is the frequency of the amplified fragments and 1–fi is the frequency of nonamplified fragments. The frequency was calculated as the ratio between the number of amplified fragments at each locus and the total number of accessions (excluding missing data). The PIC of each primer was calculated using the average PIC value from all loci of each primer. Effective multiplex ratio (EMR) was calculated using formula; EMR = n_p (n_p / n) where n_p is the number of polymorphic loci and n is the total loci number. Marker index for both the markers was calculated to characterise the capacity of each primer to detect polymorphic loci among the genotypes. Marker index for each primer was calculated as a product of polymorphic information content and effective multiplex ratio (Varshney *et al.* 2007). The relationship among the population was calculated through Jaccard's coefficient and the analysis was performed using the algorithm computed by PAST version 3.12 (Paleontological Statistics) (Hammer *et al.* 2001). Dendrograms were constructed by Unweighted

pair-group method with arithmetic average (UPGMA) method. The principal component analysis (PCA) was carried out to visualise genetic diversity among population. Nei's genetic diversity, Shannon's index and percentage of polymorphic loci were calculated using POPGENE 1.31 software (Yeh *et al.* 1997). Mantel test was performed to compute matrix correlation between the distance matrices generated from each primer system to test the goodness of fit between ISSR- RAPD, ISSR- EST-SSR and RAPD-EST SSR. All data in the HCA and antioxidant assay were expressed as the mean ± standard deviation (SD) of triplicates (n = 3). Correlation coefficient between molecular markers and chemical diversity has been tested by analysis of variance (ANOVA) and $p < 0.05$ was considered statistically significant.

RESULTS

Molecular diversity analysis

Molecular markers are widely used in plant genetics, breeding, biological diversity analysis and cultivar recognition since they can directly manifest genetic differences at the DNA level. Total of 31 markers including ISSR, RAPD and EST-SSRs were utilised for evaluating allelic assortment in *Garcinia*.

ISSR polymorphism: Thirty-five primers were initially tried for amplification, out of that, analyses of eleven ISSR primers were screened for polymorphism. Eleven ISSR were produced 127 bands of which 100 were polymorphic. Percentage of polymorphism found was 78.74%. The sizes of the amplified products were ranged from 100 to 1,700 bp. Number of scorable bands produced per primers were ranging from 4 to 8. Primer A5 was showing good polymorphic fingerprinting and good resolution of bands among *Garcinia* species. It amplified in all species, *G. gummi-gutta*, *G. indica* and *G. xanthochymus* producing maximum number of bands.

For determining PIC value of each primer, the mean of PIC values were calculated for all loci (Table 2). Highest and lowest PIC values were found in primers A6, A7 and A9. The average PIC calculated for all the ISSR markers was 0.9161. The MI (marker index) for each primer was calculated. The highest MI was found in primer A 809 and marker index (MI) value was 11.68, whereas lowest found in the primer A7 and A9 with 2.22 (Table 2). The obtained average MI found for all primer was 6. 898. The Nei's genetic diversity (h) and Shannon's index (I) among the *Garcinia* species were calculated as 0.2830±0.1358 and 0.4401±0.1815, respectively (Table 4). Observed number of alleles was 1.984±0.016, the value of effective number of alleles was 1.655±0.022, expected heterozygosity (H_E) and unbiased expected heterozygosity (uH_E) were found 0.380±0.009 and 0.402±0.010, respectively. The percentage of polymorphic loci was 91.54% for ISSR primers.

UPGMA dendrogram was constructed from the data obtained from the ISSR primers (Fig. 2). *Garcinia* species were grouped in single cluster with bifurcating nodes. *G. celebica, G. livingstonei, G. elliptica* and *G. talbotii* were present at root level while the remaining five species are from bifurcating nodes.

RAPD polymorphism: Ten RAPD primers were screened for genetic diversity in *Garcinia*. Total of 125 bands were produced. The size of the amplified products ranged from 200 to 1,800 bp. The total number of polymorphic bands was 98 and percentage of polymorphism was 78.4%. RP 2 primer showed well resolved polymorphic and scorable bands in *Garcinia* species. 500 bp size of band was found in *G. morella*, *G. indica* and *G. gummi-gutta* on other hand *G. xanthochymus*, *G. celebica* and *G. talbotii* were sharing around 600 bp of band.

To determine PIC value of each primer, the mean of PIC values was calculated for all loci. High PIC value was found in primer RP 6 and low PIC was found in RP 3 (Table 2). The highest MI was observed with primer RP 6 and the lowest was found in primer RP 1 (Table 2). The average MI of all RAPD primers was 11.140. The Nei's genetic diversity (h) and Shannon's index (I) among all the populations were calculated as 0.2846±0.1237 and 0.4453±0.1638, respectively (Table 4). Observed number of alleles was 2.00±0.00, the value of effective number of alleles was 1.656±0.021, expected heterozygosity (H_E) and unbiased expected heterozygosity (uH_E) were found 0.384±0.088 and 0.406±0.009, respectively. The percentage of polymorphic loci was 93.70% for RAPD primers.

All nine *Garcinia* species were discriminated successfully by RAPD markers. The *Garcinia* were classified into two major clusters (Fig. 3). The

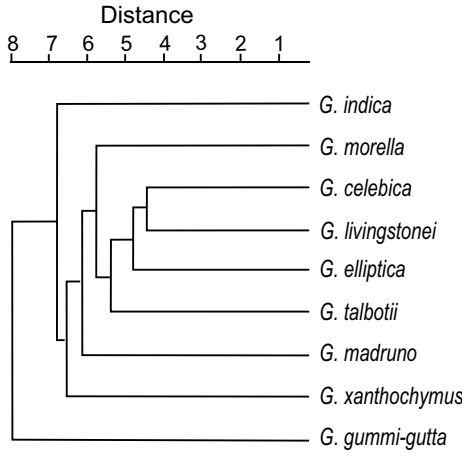

Fig. 2. Dendrogram of *Garcinia* species based on genetic distance obtained from ISSR markers using the UPGMA method

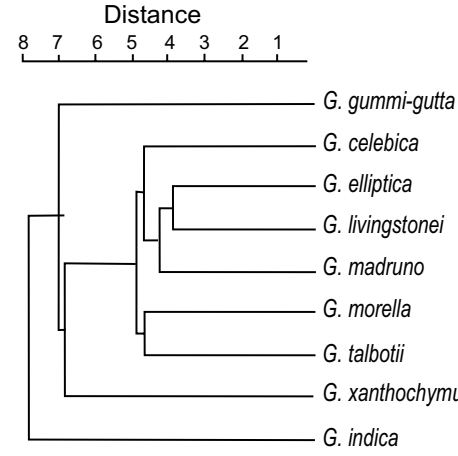

Fig. 3. UPGMA cluster analysis with Jaccard's similarity coefficient of RAPD based markers

first group gathered four species, *G. celebica, G. elliptica, G. livingstonei* and *G. madruno.* Group second involves *G. morella* and *G. talbotii*, while the rest of species bifurcating as basal taxa.

EST-SSR polymorphism: Total ten EST-SSR primers were screened down on *Garcinia* species. Total number of amplified bands was 83 out of which 75 were polymorphic. The percentage of polymorphism was 90.36%. The size of the amplified products ranged from 40 to 1,500 bp. EST-SSR 5 and 7 found good resolution and polymorphic bands in all *Garcinia* species.

EST-SSR primer 5 generated maximum number of bands in *G. madruno*, *G. gummi-gutta*, *G. xanthochymus*, *G. celebica* and *G. elliptica*. *G. madruno* and *G. xanthochymus* were sharing common band of 115 bp. On the other hand *G. xanthochymus* and *G. celebica* found common band of 65 bp. EST-SSR primer 7 revealed clear picture of polymorphism among *Garcinia* species. It generated unique finger printing pattern in each species. *G. morella* found six bands ranging from 150–600 bp, *G. madruno* produced four bands ranging from 150–550 bp. *G. celebica* and *G. talbotii* produced three and one band, respectively. Band sizes were ranging from 140 to 280 bp. *G. elliptica* and *G. livingstonei* produced six and three bands, respectively. The observed bands were ranging from 130 to 350 bp. Economically important species *G. gummi-gutta*, *G. indica* and *G. xanthochymus* produced eight, three and two bands sequentially. Band sizes were ranging from 140 to 350 bp in *G. indica* and *G. xanthochymus,* whereas bands produced by *G. gummi-gutta* were ranging from 230 to 1,400 bp.

Some common bands were found in *Garcinia* species. Band of 140 bp was found common in *G. xanthochymus*, *G. talbotii* and *G. celebica*, whereas band of 150 bp was observed in *G. madruno*, *G. gummi-gutta*, *G. indica* and *G. livingstonei*. Band of 310 bp was shared by *G. morella*, *G. madruno*, *G. indica* and *G. livingstonei*.

The mean PIC values for each primer were counted for all loci. High PIC value 0.9879, calculated in two primers EST-SSR 1 and EST-SSR 2 (Table 2). On other hand low PIC 0.6378 was found in primer EST-SSR 6. The highest MI was found in primer EST-SSR 7 and lowest in the primer 2.5516. The values of highest and lowest MI were 16.6 and 2.5516, respectively (Table 2). The average MI of all EST-SSR primers was obtained. The value of mean MI was 6.228. The Nei's genetic diversity (h) and Shannon's index (I) among the species were calculated as 0.2707±0.1383 and 0.4241±0.1858, respectively (Table 4).

Observed number of alleles was 2.00±0.00, the value of effective number of alleles was 1.670±0.025, expected heterozygosity (H_E) and unbiased expected heterozygosity (uH_E) were found 0.389±0.010 and 0.412±0.011, respectively. The percentage of polymorphic loci was 90.36% for EST-SSR primers. Basing on EST-SSR markers and UPGMA analysis was performed, *G. xanthochymus*, *G. talbotii*, *G. celebica*, *G. livingstonei*, *G. madruno* were included in core group. The remaining four species were distantly separate from core group (Fig. 4).

Table 4
Summary of various genetic diversity indices analysed in *Garcinia* species

Sr. no.	Marker system	PIC	MI	Na	Ne	% of polymorphic loci	Nei	*I* (SD)	H_E (SE)	uHe (SE)
1	ISSR markers	0.9161	6.898	1.984 (0.016)	1.655 (0.022)	91.54	0.2830 (±0.1358)	0.4401 (±0.1815)	0.380 (0.009)	0.402 (0.010)
2	RAPD markers	0.9440	11.140	2.00 (0.00)	1.656 (0.021)	93.70	0.2846 (±0.1237)	0.4453 (±0.1638)	0.384 (0.088)	0.406 (0.009)
3	SSR markers	0.8903	6.228	2.00 (0.00)	1.670 (0.025)	90.36	0.2707 (±0.1383)	0.4241 (±0.1858)	0.389 (0.010)	0.412 (0.011)
4	All markers	0.9168	8.050	1.979 (0.011)	1.663 (0.013)	92.06	0.2806 (±0.1318)	0.4381(±0.1759)	0.385 (0.000)	0.408 (0.006)

PIC = polymorphic information content; MI = marker index; Na = observed number of alleles; Ne = number of effective alleles; Nei = Nei's gene diversity indices; I = Shannon's information index of genetic diversity; H_E = expected heterozygosity; uH_E = unbiased expected heterozygosity

Combined data analysis for all the markers: Cumulative data of eleven ISSR, ten RAPD and EST-SSR were taken into consideration for genetic diversity analysis. Total of 335 bands were considered of all the markers. The overall percentage of polymorphism was 81.49%. The mean of all PIC values calculated from all primers and observed PIC was 0.9168 (Table 4). The average MI of all primers was 8.050. The Nei's genetic diversity (h) and Shannon's index (I) among all the populations were calculated as 0.2806±0.1318 and 0.4381±0.1759, respectively (Table 4).

Cluster analysis was carried out using cumulative data from all the markers. *Garcinia* species were grouped in two clusters. *G. celebica*, and *G. talbotii,* forming one cluster while *G. elliptica* and *G. livingstonei* forming another cluster. Remaining species were diverged from these four species (Fig. 5). A similarity matrix was obtained among all the nine species of the genus *Garcinia* based on Jaccard's coefficient (Supplement Table 1). According to the similarity matrix, the genetic similarity ranged from 0.102 to 0.234. The highest similarity (0.234) was found between *G. gummi-gutta* and *G. indica*. On the other hand, the lowest similarity (0.102) was found between *G. gummi-gutta* and *G. livingstonei*. Genetic diversity indices generated from binary

data of all markers were mentioned in Table 4. Observed number of alleles was 1.979±0.011, the value of effective number of alleles was 1.663±0.013, expected heterozygosity (H_E) and unbiased expected heterozygosity (uH_E) were found 0.385±0.000) and 0.408±0.006, respectively. The percentage of polymorphic loci was 92.06% for all primers.

The Mantel method used to compare the similarity matrices produced correlation coefficients statistically significant for each of the three marker systems. The correlation coefficient between genetic distance matrix of ISSR and RAPD was 0.2709 and between ISSR and EST-SSR was 0.1997. The observed value for correlation coefficient between genetic distance matrix of RAPD and EST-SSR was 0.213. Mantel test revealed significant correlation between molecular markers (r = 0.2709, 0.1997 and 0.213; $p < 0.05$).

Hydroxycitric acid determination

Development of HILIC for HCA: For achieving optimal determination of HCA in short analysis time, HILIC conditions such as the column, mobile phase, flow rate, injection volume and column temperature were optimised. Optimisation results found that mobile phase system consisted of 0.1% (v/v) orthophosphoric acid aqueous solution and acetonitrile afforded optimum efficiency and better peak of HCA. HCA was characterised based on the chromatographic retention time and UV visible wavelength data by comparing the previously identified HCA peaks. The said method can be effectively applied

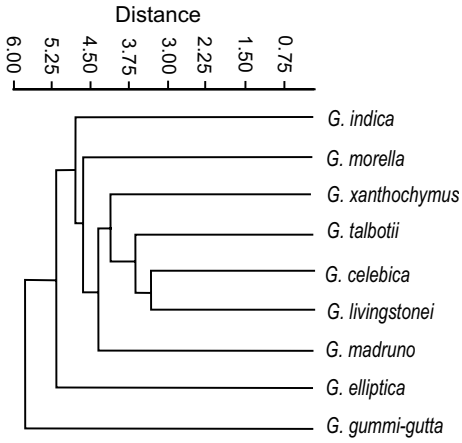

Fig. 4. UPGMA dendrogram of the *Garcinia* species based on the Jaccard's coefficient as calculated from the EST-SSR marker data

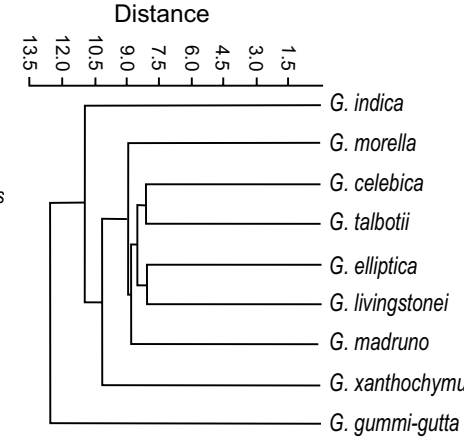

Fig. 5. UPGMA dendrogram based on the genetic distance obtained from ISSR, RAPD and EST-SSR data showing the relationship between nine species of *Garcinia* species

Table 5
Percentage of HCA determined in *Garcinia* species along with commercial product using HILIC-HPLC system

Sr. no.	Species	Location/origin	% of HCA in fruit rinds
1	*G. gummi-gutta*	Kadakeri Karnataka	12.44±1.04
2	*G. xanthochymus*	Dapoli, Maharashtra	6.3±0.286
3	*G. indica*	Dive-Agar, Maharashtra	7.92±0.83
4	*G. morella*	Menasi, Karnataka	0.023±0.012
5	*G. talbotii*	Yana, Karnataka	0.083±0.034
6	*G. celebica*	NBPGR, Kerala	0.34±0.013
7	Commercial product	–	3.99±0.173

for enhanced separation and retention of HCA. This method explains the feasibility of HILIC-HPLC. The retention time (Rt) obtained using HILIC column was 8.88 minutes for standard HCA. Better performance of HCA on HILIC system makes interpretation of analytical data more reliable.

Quantitative analysis of hydroxycitric acid from individual Garcinia species by HILIC: Current method determined HCA from six species of *Garcinia* along with commercial market sample. Chromatograms of HCA from Figures 6 to 10 demonstrated determination of HCA in six *Garcinia* species. These figures showed the obtained chromatograms with very intense well resolved peak at 210 nm, due to HCA. This result confirms our supposition, that in the sample HCA is present and area under curve (AUC) of which was taken for quantitative analysis of HCA content in six species of *Garcinia.* This method proves distinct differences in HCA content of *Garcinia* species (Table 5). The high content of HCA was found in *G. gummi-gutta*, approximately 12.44±1.04% of acid in dried fruit powder. *G. indica* contains 7.92±0.83% of HCA, whereas *G. xanthochymus* contains 6.3±0.286% of HCA. Percentage of HCA was negligible in *G. morella*, *G. talbotii* and *G. celebica*; it was 0.023±0.012%, 0.083±0.034% and 0.34±0.013%, respectively. Presence of HCA in the market sample was found to be 3.99±0.173% of HCA (Fig. 11).

Determination of antioxidant activity, total phenolics and total flavonoids

Antioxidant activity of *Garcinia* fruit powder was analysed using various assays, including DPPH, FRAP in addition to total phenolic and total flavonoid. Percent of free radical scavenging activity using DPPH assay varied from 40.84±0.620 to 95.40±0.720% (Table 6). *G. talbotii* found high percent of free radical scavenging activity, whereas *G. morella* was showing the lowest

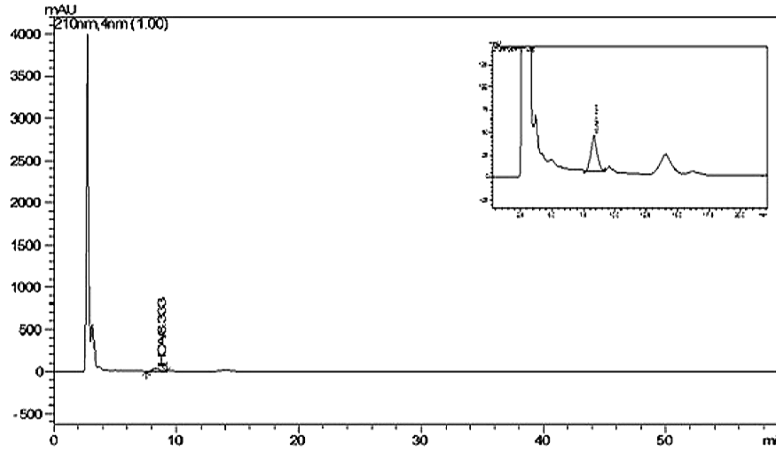

Fig. 6. Chromatogram of *G. gummi-gutta*

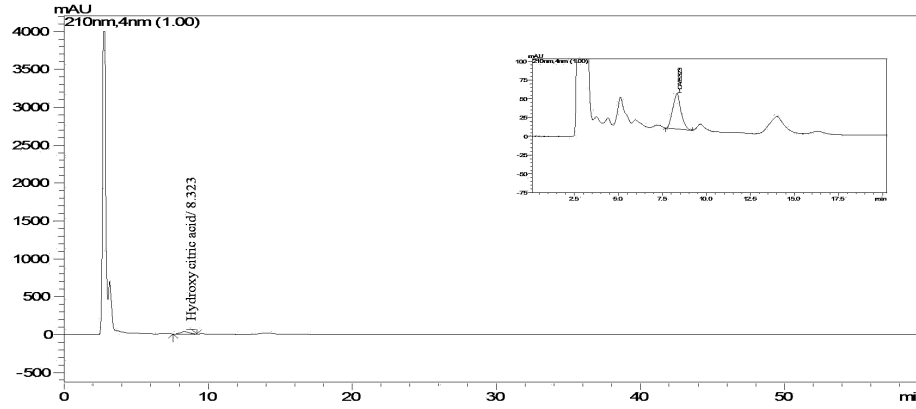

Fig. 7. Chromatogram of *G. xanthochymus*

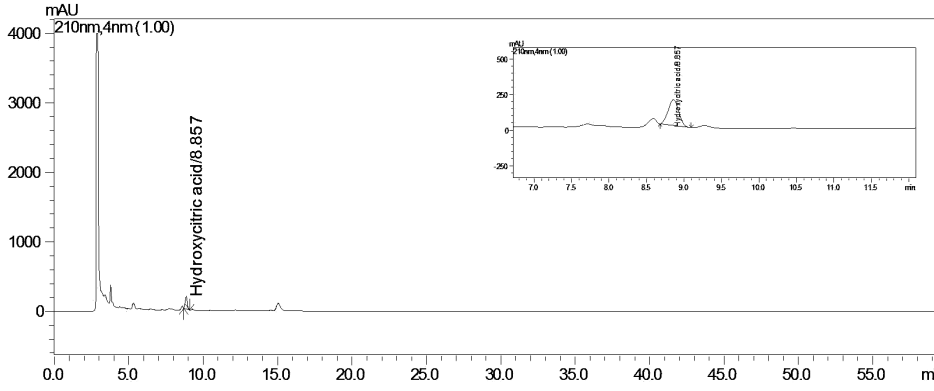

Fig. 8. Chromatogram of *G. indica*

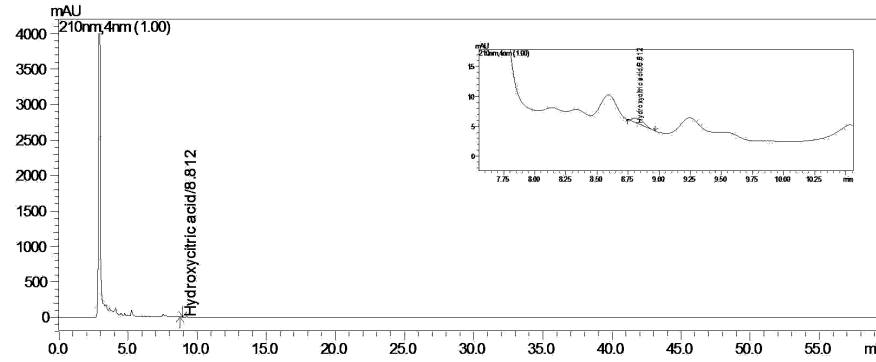

Fig. 9. Chromatogram of *G. morella*

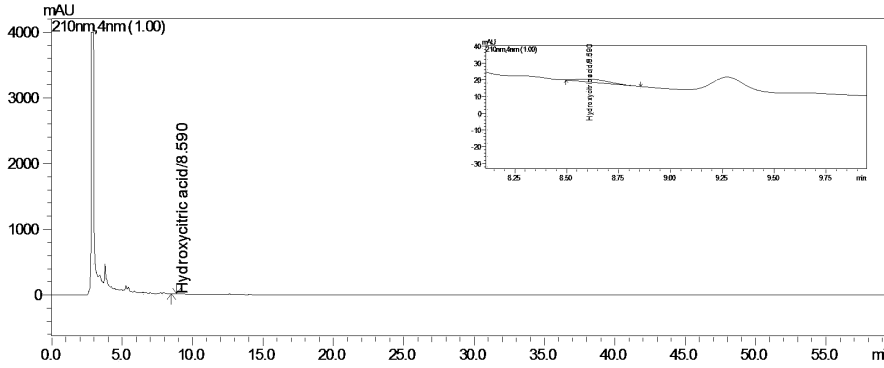

Fig. 10. Chromatogram of *G. talbotii*

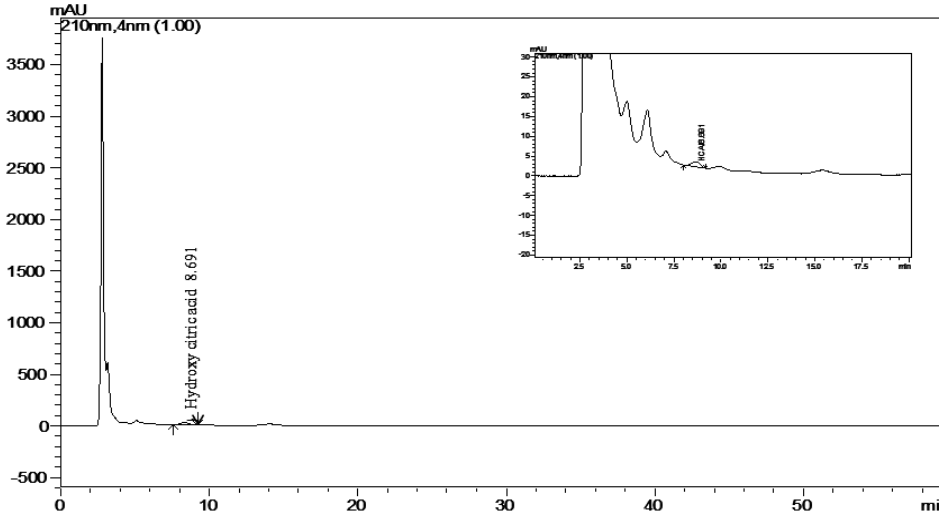

Fig. 11. Chromatogram of *Garcinia celebica*

Table 6
Percentage and content of antioxidant activity, total phenolics and total flavonoids

Sr. no.	Species	Antioxidant activity		Total flavonoids (μg/ml)	Total phenolics (μg/ml)
		% of DPPH activity	FRAP content (μg/ml)		
1	*G. gummi-gutta*	82.54±0.941	3.77±0.185	8.59±0.147	1.22±0.010
2	*G. xanthochymus*	91.37±0.854	6.59±0.157	12.35±0.219	1.07±0.008
3	*G. indica*	90.73±0.976	8.45±0.117	11.17±0.075	1.23±0.015
4	*G. morella*	40.84±0.620	1.76±0.045	3.12±0.067	0.77±0.020
5	*G. talbotii*	95.40±0.720	8.48±0.085	15.14±0.055	1.35±0.027
6	*G. celebica*	60.63±0.562	0.66±0.040	2.84±0.056	0.81±0.006

percent (Fig. 12). In FRAP assay *G. talbotii* reported high content of antioxidant activity (8.48±0.085 μg/ml) slightly more than *G. indica* which was recorded 8.45±0.117 μg/ml. The lowest activity was found in *G. celebica* (0.66±0.040 μg/ml). Concentration of total phenolic and flavonoids was also higher in the *G. talbotii*; it was 1.35±0.027 μg/ml and 15.14±0.055 μg/ml, respectively. Second important species for phenolic and flavonoids contents were *G. xanthochymus* and *G. indica*. *G. xanthochymus* found 12.35±0.219 μg/ml of total flavonoids and *G. indica* found 1.23±0.015 μg/ml of total phenolics.

The percent of free radical scavenging activity and amount of total phenolics and flavonoids distributed in the species were graphically presented in Figure 13. FRAP analysis of antioxidant, total flavonoids and total phenolics were approximately the same in *G. talbotii*, *G. indica* and *G. xanthochymus.* UPGMA

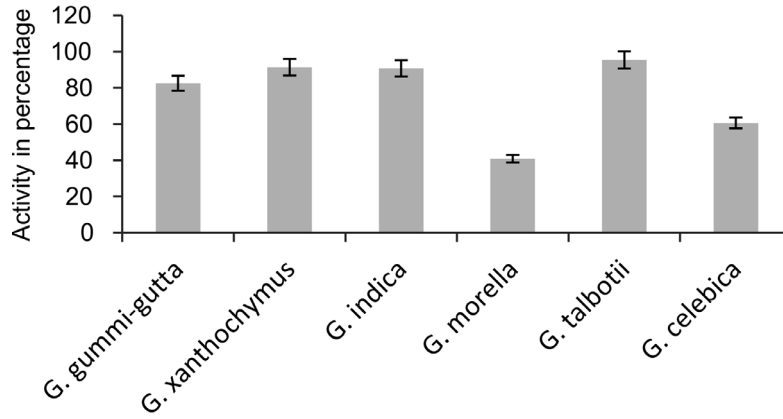

Fig. 12. Comparative graphical analysis of antioxidant activity, DPPH in percentage in six *Garcinia* species

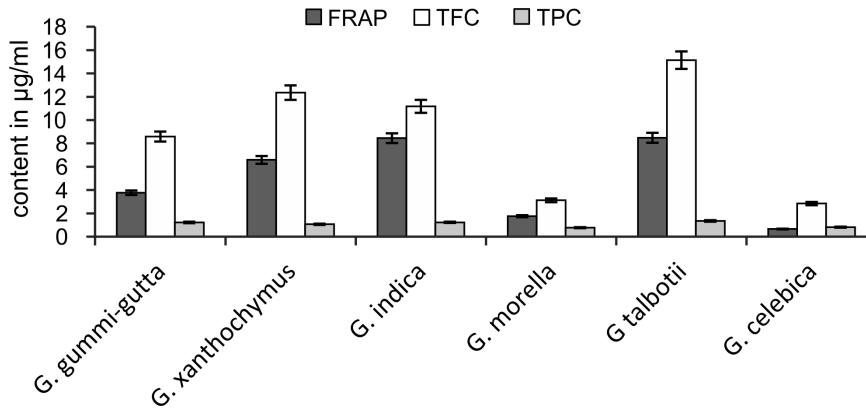

Fig. 13. Comparative graphical analysis of FRAP, TFC and TPC in six *Garcinia* species

dendrogram was developed using percentage of acid content and antioxidant activity of six *Garcinia* species. It has shown that three species having less percentage of HCA, *G. morella* and *G. celebica* were clustered in one group. *G. talbotii* having less HCA content, but showing antioxidant activity was placed in another cluster with *G. xanthochymus*, *G. indica* and *G. gummi-gutta*, which is a rich source of HCA, diverged from these groups (Fig. 14). The principal component analysis (PCA), using molecular and phytochemical data, reflects the diverse distribution of the *Garcinia* species on the scatter plot (Fig. 15). The first two principal components contribute 67.34% and 18.90%, respectively, making a cumulative contribution of 86.24%. The distribution of species was similar to those of obtained in the UPGMA dendrogram.

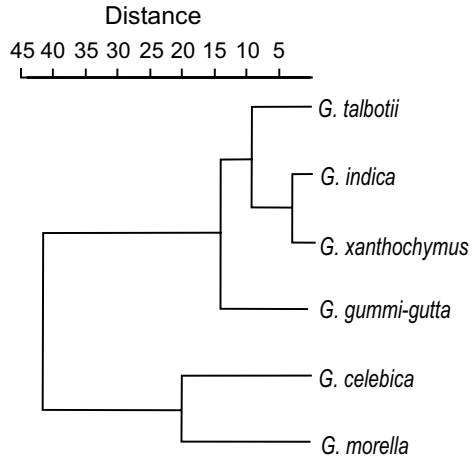

Fig. 14. UPGMA dendrogram of six *Garcinia* species constructed independently based on antioxidant activity and hydroxycitric acid content variation obtained by HILIC-high-performance liquid chromatography

DISCUSSION

Population genetician seeks to understand how variations found in survivorship, fertility, and gene flow contribute to changes in allele frequencies within and among populations. Researchers developed many diverse arrays of chemical and molecular tools and made accessible for high resolution of diversity studies among plant species.

Genetic diversity in Garcinia using molecular markers

The results of the present study, ISSR and RAPD along with EST-SSR found to be efficient for genotypes characterisation. During the characterisation of *Garcinia* species mixture of marker system including dominant, co-dominant and randomly amplifying were screened down. Until now, the characterisation of most important species *G. gummi-gutta*, *G. indica* and *G. mangostana* has been relied on RAPD, AFLP and ISSR markers (Mansyah *et al.* 2010, Qosim *et al.* 2011, Tharachand *et al.* 2015, Thatte *et al.* 2012). High percent of polymorphism have been reported at inter and intra species level using dominant markers. RAPD and ISSR markers are randomly present in the whole genome and there are no evidences that these markers were located in specific regions (Ferreira and Grattapaglia 1998). It is possible to find them in coding or non-coding region where as EST-SSR markers containing both, coding and non-coding region. This variation could be due to the differences of targeted alleles per locus and their distribution in each species. This is in agreement with the previous polymorphism studies in *Garcinia* where it found 52 to 96% (Mansyah *et al.* 2010, Parthasarathy *et al.* 2013, Priyadevi *et al.* 2009).

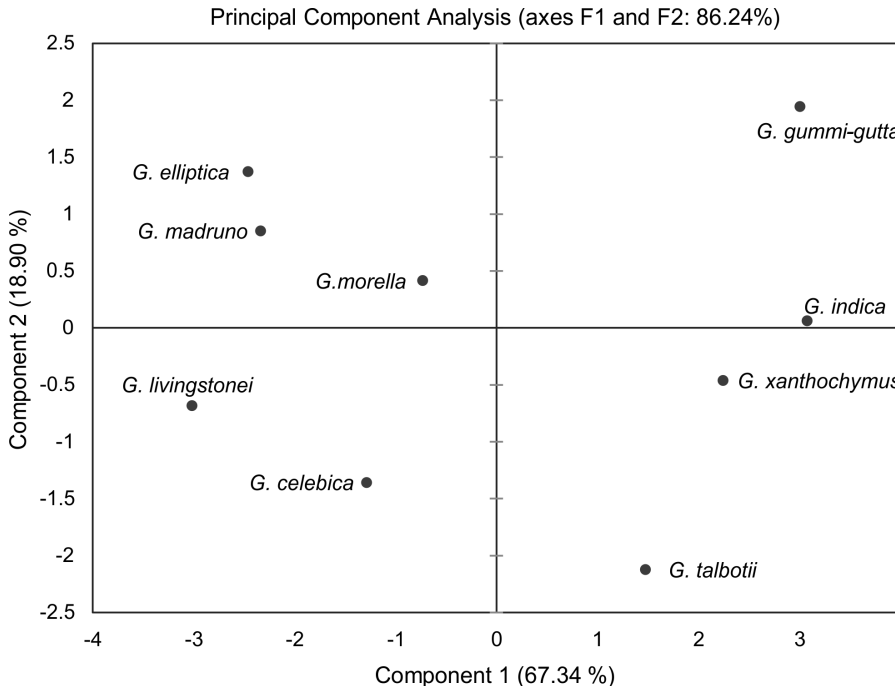

Fig. 15. Two dimensional scatter plot of nine *Garcinia* species by principal component analysis using molecular and chemical assays

Overviewed from all clustering pattern of UPGMA dendrogram, intra-species relationship among *Garcinia* species was clearly revealed. *G. livingstonei*, *G. celebica*, *G. talbotii* and *G. madruno* were closely related while *G. xanthochymus*, *G. indica* and *G. gummi-gutta* were diversified from these species. *G. livingstonei, G. celebica, G. talbotii* and *G. madruno* were sharing some common characteristics. Flowers arise in axillary and terminal position. Leaves are usually egg- or lance-shaped, simple and oppositely arranged, veins are prominent with coriaceous leaf texture, whereas *G. indica* and *G. gummi-gutta* have smooth leaf surface. *G. celebica, G. madruno* and *G. talbotii* have weakly lobes fruit stigma, while stigma lobes are completely divided into rays in *G. gummi-gutta* and *G. indica*. Ovary in *G. gummi-gutta* and *G. indica* is more than five locules. *G. indica* and *G. gummi-gutta* are indigenous species and distributed strictly to the Western Ghats. From the current study supported by the morphological characters and origin and distribution of species, it was confirmed that cluster dispersion of *G. indica* and *G. gummi-gutta* from other species and they were placed in Section *Brindonia* (Jones 1980, Parthasarathy *et al.* 2013, Shameer *et al.* 2019, Singh 2009). Sobir *et al.* (2011) found the same results of clustering in *Garcinia* species from Indonesia. Two species namely *G. celebica* and *G. xanthochymus* utilised in present study were placed in different groups, Section *Garcinia* and Section *Xanthochymus*, respectively (Saleh 2006).

Similarity matrix using all the marker data found, it was ranging from 0.097 to 0.261. *G. indica* and *G. gummi-gutta* were least similar with *G. livingstonei*; it was 0.097 and 0.102, respectively. *G. talbotii* and *G. celebica* were highly similar. *G. indica* and *G. gummi-gutta* were genetically far removed from all accessions studied and that agrees with their unique morphological features that distinguish them from the other species evaluated.

Genus *Garcinia* is characterised by the dioecism of its species. The plants are polygamodioecious and seeds are apomictically developed, therefore plants are homogenous. However, the high genetic diversity observed may have resulted from several events. Richards (1997) well explained the theory and reasons behind polymorphism in obligate apomixis. He discussed the mechanism of variation found in apomictic species. One of the reasons is high ploidy levels that can also trigger the occurrence of mutations causes variability. Several previous researchers reported high number of chromosomes in genus *Garcinia* (Ha 1978, Krishnawary and Raman 1949, Richards 1990, Robson and Adams 1968, Tixier 1960).

PIC values and markers index were determined to compare the ability of various markers to discriminate species and correct genetic diversity. Markers with higher PIC values have a greater ability to discriminate the species. From above values of PIC it has been clear that all the markers are highly informative ($0.5 < PIC$) and can be employed in genetic diversity of plant species. The marker index (MI) represents the efficiency of that marker. The high

MI is nothing but the efficiency of marker to simultaneously analyse a large number of bands, rather than level of polymorphism (Powell *et al.* 1996). MI in EST-SSR was higher as compared to ISSR and RAPD. Possible reason of that is the source ESTs of the EST-SSR loci are homologous to genes with known roles in resistance, secondary/hormone metabolism and flower development, or translation initiation factor. Some of them, or their related genes, are potential candidate genes for adaptation to local climate (Lalagüe *et al.* 2014, Yoder *et al.* 2014). Candidate genes for adaptation to climate gradients have higher genetic diversity than non-candidate genes by a number of loci-climate association studies (Pierro *et al.* 2016, Pluess *et al.* 2016, Sork *et al.* 2016). Current study made it clear that genetic diversity is very high and the values of Nei were slightly higher than for dicotyledons (0.165), long-lived perennial plants (0.180), widespread (0.183) (Hamrick and Godt 1996). It also revealed a high level of genetic variation at the species level. For all the cumulative marker data, Percent of polymorphic loci was 92.06%, which denotes the efficiency of this combined approach of molecular markers. Datta *et al.* (2016) revealed efficiency of combined marker system (ISSR, RAPD and SSR) in chick pea and pigeon pea, and reported high percentage of polymorphism in all the markers.

Principal Component analysis is one of the most practically statistical tools for screening multivariate data with significantly high correlation. The PCA was taken into consideration to determine the consistency of the differentiation among the species defined by the cluster analysis. The analysis found that, the first two principal components accounted for major role in separating species. The PCA analysis of nine *Garcinia* species supported the results of clustering. It showed multiple dimensions of the distribution of the genotypes in a scatter plot. Separation of the species to their respective clusters as well as PCA was observed for all the three markers with the exception of few rearrangements. The Mantel test found a positive correlation between genetic distances with statistically significant for each of the three marker systems used independently.

Chemical diversity

Determination of HCA: According to the functional groups in the HCA, it is found in an alpha, beta di-hydroxy tri-carboxylic acid and therefore it easily lactonises to the corresponding lactones (Lewis and Neelakantan 1965). In earlier methods, determination of HCA using HPLC and LC-MS, the acid content in *G. gummi-gutta* was ranging from 16 to 55% (Jayaprakasha and Sakariah 1998, Upadhyay *et al.* 2013), where in another study it was reported only 5.98 from the fruit rinds (Ashish *et al.* 2008). In the studies on *G. indica* it was ranging from 5.05 to 19% (Ashish *et al.* 2008, Jayaprakasha and Sakariah 2002, Kumar *et al.* 2013). North-east species, *G. oblongifolia* contains 10.13 to

18.59% of HCA, where as in *G. xanthochymus* reported acid content was only 0.07% (Asish *et al.* 2008, Dang *et al.* 2011). To verify this method, we compared the HCA content obtained by current method with the reported HCA content. Though the percentage of HCA determined in *G. gummi-gutta* and *G. indica* is less as compared with some reported methods but resolution of picks is very good and retention time is longer than the previous methods. This is the first time we are reporting the high HCA content in *G. xanthochymus*, which can be an alternate standard protocol for all types of *Garcinia* species.

Here, to study the interspecies variation of HCA, the attempt was made on three endemic species of *Garcinia*, *G. talbotii*, *G. morella* and *G. celebica* whose HCA determination had not been previously reported as well as the processed market sample for the comparison of acid content. From all the studies, it may be confirmed that fruit rinds are the major source of HCA. This method proved to be highly reliable, rapid and sensitive for organic acids estimation. The HCA profile in genus *Garcinia* is vital as a chemical marker for the genus and the content of it specifically demonstrated significant polymorphism at species level. Also, the complex taxonomic issues can be resolved in genus *Garcinia* with HCA profiling. Use of HCA profile would have to be universal and convenient to determine. The compilation of HCA from the six different species along with one market product analysed here provides an example of multidisciplinary approach.

Determination of antioxidant activity: The antioxidant activity found in *Garcinia* species has quite a similar tendency to the results of genetic analysis and hydroxycitric acid content. We found the antioxidant activity of *G. talbotii* is much more than the *G. indica*, *G. gummi-gutta* and *G. xanthochymus*. High content of phenolics and flavonoids was also found in *G. talbotii*. *G. gummi-gutta* and *G. indica* have been considered as the good source of the antioxidant as their fruits are in general use, but the *G. talbotii* contains high free radical scavenging activity. In earlier reports maximum scavenging activity of *G. gummi-gutta* found 49% where in another water extract it was reported 36.20% (Ranjani *et al.* 2014; Subhashini *et al.* 2011). Total phenolics and flavonoids in *G. gummi-gutta* were quite higher than the current findings (Subhashini *et al.* 2011). *G. celebica* showed less antioxidant activity and content of total phenolics and flavonoids as compared to other species. Elya *et al.* (2012) reported methanolic extract of *G. celebica* leaves was showing high antioxidant activity. The results of antioxidant activity and content of total phenols and flavonoids showed significant ($p < 0.05$) variation among the *Garcinia* species. To the best of our knowledge there are no reports evaluating such variability at the species level.

Correlation between genetic diversity and chemical diversity: In current study it has been cleared that genetic diversity among Indian *Garcinia* species determined the phytochemical profile. We found considerable variation exists in the HCA composition and antioxidant activity, which is consistent with the genet-

ic clustering of the species. HCA and free radical scavenging activity showed positive correlation with molecular data (r = 0.112 and 0.59, and 0.938) ($P < 0.01$). Clustering results of all the markers with cumulative data corroborate the cluster analysis performed on HCA content and antioxidant activity assay. Even after clustering of the six species based on HCA and antioxidant activity, *G. gummi-gutta*, *G. indica* and *G. xanthochymus* were being isolated. Interestingly the results of cluster analysis using cumulative data of all the markers were found similar to clustering of using content of HCA and antioxidant. Chemical variation in this group of compounds has been used to define intra- and interspecific variability in a variety of plant species and grouping of plants. These results confirm that the genetically directed production of HCA, phenols and flavonoids were correlated in the *Garcinia* species and could be possible alternative for grouping the species taxonomically. Molecular markers have been successfully employed for the identification of herbal drugs (Ganie *et al.* 2012, Heubl 2010, Irshad *et al.* 2009, Rivera-Arce *et al.* 2007, Rout 2006). Though the combined study on molecular and chemical characterisation is rare in case of medicinal plants, there are some reports made available where both the techniques have been taken into consideration for chemical and molecular profiling (Böszörményi *et al.* 2009, Han *et al.* 2008). It suggests that species in the same grouping may have similar routes of biosynthesising secondary compounds, which results in activation of similar genes.

Currently the results are allowing us to establish a reliable relationship between molecular markers and certain phytochemical traits, such approach of molecular biology is needed to be used to evidence specific markers. This work will support future studies on the genetic and chemical evaluation of *Garcinia* species belonging to the family Clusiaceae, which should include a large number of species so that a more complete study of the chemical, genetic and taxonomic diversity can be performed. Thus the molecular markers are essential in the characterisation of the genetic diversity of *Garcinia* species despite the agronomical and chemical characteristics that distinguish and group genotypes. Moreover, the combined information will reflect applicability of molecular markers in assessing diversity at chemical level. The molecular data obtained in this study is very important, as they identify the diversity present in the *Garcinia* species in addition to separating the species into two genetically and chemically distinct groups, which can be used in the further selection of breeding programs of this genus. The results of polymorphism could provide information regarding genetic structure and distribution of the *Garcinia* species and further help to make an effective conservation strategy for the breeding purposes. Regarding the method development of HCA, application demonstrates that extremely polar compounds can be retained using HILIC columns. In addition, it also suitable for compounds that require detection at a low wavelength.

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Authors' contributions: JA, ND, GS, KM designed the study; JA, VJ, VP and CS conducted lab experiments; JA and GS performed the HPLC method development. JA, ND and KM analysed the data; JA and ND wrote the manuscript, and JA as Principle Investigator, supervised the research project.

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