Chapter 7

Morphological, chemical and molecular taxonomy of a new *Garcinia* species- *Garcinia pushpangadaniana* Sabu *et al.*

P.S. Shameer[1], K. B. Rameshkumar[2], A. R. Sivu[3], T. Sabu[1], N. S. Pradeep[4] and N. Mohanan[1*]

[1]*Garden Management, Education, Information and Training Division*
[2]*Phytochemistry and Phytopharmacology Division*
[4]*Microbiology Division*
Jawaharlal Nehru Tropical Botanic Garden and Research Institute
Palode, Thiruvananthapuram-695562, Kerala, India
[3]*Department of Botany, NSS College Nilamel, Kollam- 691535, Kerala, India*
[*] *Corresponding author*

Abstract

The genus *Garcinia* is an important component of the forest flora of the Western Ghats, and the region hosts a wide diversity with several taxa, including ones yet to be described. The genus is considered as a taxonomically difficult one due to the complexity and diversity in floral characteristics. The present chapter describes the biosystematics of a new *Garcinia* species, *G. pushpangadaniana*, described from the Western Ghats, using chemosystematics and molecular systematics. The HPTLC profile and volatile chemical profiles of the leaves supported the species status and allied nature to *G. xanthochymus* and *G. talbotii*. Molecular taxonomy using the chloroplast coding region *mat*K could demarcate the new taxon as a distinct species, closely allied to the species *G. xanthochymus* and *G. talbotii.*

Keywords: *Garcinia pushpangadaniana*, *Garcinia xanthochymus, Garcinia talbotii,* Chemotaxonomy, Molecular taxonomy

Introduction

The forests of the Western Ghats, with nearly 7500 flowering plants, is a rich repository of plant wealth with several new species having been discovered from the region (Nayar *et al*., 2014). The region hosts wild relatives of many important spice crops and food crops and also is the centre of origin and diversity of several such plant groups. The genus *Garcinia* is an economically important group of plants distributed in the tropical regions of the world. The Western Ghats is a centre of diversity of *Garcinia* species in India. Out of the 37 *Garcinia* species distributed in India, 7 are endemic to the Western Ghats.

The genus *Garcinia* is considered as a taxonomically difficult one due to the complexity and diversity in floral characteristics with many unresolved phylogenic issues surrounding the genus. Characteristic differences in the floral architecture were observed even among closely related taxa of *Garcinia* (Gustafsson *et al*., 2002, Sweeney, 2008). Morphological characters are known to be affected by developmental and environmental factors and in the case of *Garcinia* species, an unusual evolutionary plasticity has been generally observed and the classification of *Garcinia* species and its phylogeny solely depending on morphological characters proved to be more uncertain. The incorporation of biosystematics in such taxonomically difficult groups will allow classifications using new descriptors and methods that yield more robust inter relations.

Biosystematics based on secondary metabolite profile has proven as an efficient supportive tool for plant systematics. The genus *Garcinia* is characterized by the presence of a large number of secondary metabolites such as xanthones, benzophenones and biflavonoids, in addition to volatile secondary metabolites (Hemsekhar *et al*., 2011). Several attempts have been made to evaluate the phylogeny among Clusiaceae members through secondary metabolite profiling (Waterman and Hussain, 1983). Among the secondary metabolites, volatile chemicals can efficiently be utilized for chemotaxonomic purposes (Labra *et al*., 2004). Though the volatile chemical profile reflects the evolutionary history, it is more indicative of the ecological conditions (Nogueira *et al*., 2001).

In the last decade, new valuable tools based on DNA analysis were made available for taxonomic studies (Winfield, 2003; Labra *et al*., 2004). The use of DNA genotyping has been instrumental in solving controversial taxon attributions by comparing genotypes independently from phenotypes. DNA genotyping offers the unique capacity to classify accessions regardless of environmental condition and plant growth stage.

A new taxon of the genus *Garcinia* has been collected from the forests of the Western Ghats. In the present chapter, the efficiency of chemotaxonomy and molecular taxonomy to support the species status of the new *Garcinia* taxon has been evaluated.

1. Morphological studies

The new taxon *Garcinia pushpangadaniana* T. Sabu, N. Mohanan, Krishnaraj, & Shareef (Holotype *TBGT 72601*) was collected from Kadalar forests, Idukki district, Kerala (**Figure 1**). Detailed evaluation of the vegetative and reproductive morphological features revealed the new taxon has distant relation to *G. xanthochymus* and *G. talbotii* with pentamerous flowers and the absence of rudimentary pistils in male flowers (**Table 1**). *G. xanthochymus* Hook. f. ex T. Anderson is an indigenous tree in Indo-Malay region, and its distribution in India is extended to the evergreen to semi-evergreen forests (100-1000m) of North East India and Andaman Nicobar Islands. *G. talbotii* Raizada ex. Santapau is an endemic species to the evergreen to semi-evergreen forests (100-350 m) of the Western Ghats. However, the prominent morphological differences in shape of leaf, pedicel length of male and female flowers, nature of staminodes, number of stigma, ovary and seeds, features in fruits and seeds qualify the new taxa to be a distinct species. The demarcating feature of the new taxon is the large fruits that weigh upto 750 g, with irregular ridges on the fruit surface.

Table 1. Characteristic morphological features of the new taxon in comparison with *G. talbotii* and *G. xanthochymus*

Plant part	*G. talbotii*	*G. xanthochymus*	*G. pushpangadaniana*
Leaf	Ovate, elliptic-oblong or lanceolate. Emarginate or acute at apex 9-22 x 4-8 cm.	Linear- oblong or oblong-lanceolate. Acute or acuminate at apex 12-35 x 4-10 cm.	Elliptic- oblong. Acute or obtuse at apex 14-20 x 6-8 cm.
Flower	Fascicled or pseudo spikes Stamens 8-10 in each of 5 long clawed, spathulate bundles. Stigma 3-4 lobed, peltate Ovary 3-4 locular.	Fascicled Stamens 15-20 in 5 phalanges bundles of 3-5 each. Stigma 5 lobed, oblong Ovary 5 locular	Fascicled Stamens 12-15 in phalanges Stigma 6-8 lobed, oblong Ovary 6-8 locular.
Fruit	Broadly oblong, smooth Up to 4 cm diam. Weight: Upto 45g	Subglobose, smooth. *ca*. 6.5 cm diam Weight: Upto 55 g	Irregular ridges on the surface, *ca*. 12 x 11 cm diam. Weight: Upto 750g
Seeds	Oblong 1-3, up to 2.5 cm	Oblong 1-4, up to 3.5 x 1.8 cm	Plano convex 2-6, ca. 2 x 1 cm
Latex	White or yellowish white	Milky white or pale green turning yellow	Milky white

Figure 1. *G. pushpangadaniana* **A.** Habit, **B**. Stem bark, **C**. Leaf, **D**. Male flower, **E**. Female flower, **F**. Seed and **G**. Fruit

Dichotomous key prepared for *G. pushpangadaniana* and related species

Stamens in 5 phalanges; sepals and petals 5 rarely 4
Leaves more than thrice as long as abroad, over 30 cm long; berry with distinct mamilla or beak…….....*G. xanthochymus*
Leaves less than twice as long as broad, less than 20 cm long; berry without distinct mamilla or beak
Fruit large, without any pulp, irregularly ridged on the surface, seeds planoconvex…….....*G. pushpangadaniana*
Male flowers fascicled or pseudo spikes stigmatic lobes 3- 4…… *G. talbotii*

2. Chemotaxonomy of the new species

The use of distribution patterns of secondary metabolites is well established as a major tool for characterize, classify and describe taxa. The vast information of secondary metabolites can also be utilized for investigating population structures, species and phyletic relationships and evolutionary status. The genus *Garcinia* is characterized by the presence of a large number of secondary metabolites with diverse structural features such as xanthones,

benzophenones, flavonoids, biflavonoids and terpenoids and the vast data on secondary metabolites has been utilised successfully to demarcate species (Waterman and Hussain, 1983).

2.1. Chemotaxonomy based on HPTLC profiles

The versatile and cost effective analytical tool HPTLC allows us to analyze up to 20 plants in a single analytical run and the phytochemical profile can yield valuable information on plant identity. The HPTLC profile can be utilized as very detailed differentiating fingerprints of different species, often closely related species that would otherwise be impossible to distinguish from each other physically (Reich and Schibli, 2007).

In the present study, the leaf methanol extracts were analysed using Camag HPTLC system, using silica gel HPTLC plates (Kieselgel 60 F 254, 20 cm × 20 cm, 0.2 mm thickness, Merck, Germany). The extracts were spotted by means of Camag Linomat V fitted with a Hamilton microlitre syringe. The plates were developed using chloroform: methanol (17:3) in the CAMAG twin-trough glass chamber, previously saturated with the solvent for 30 minutes. The mobile phase compositions were chosen after testing different solvent systems of varying polarity. The flavonoid profile was obtained on exposure of the plate to NH_3 vapour.

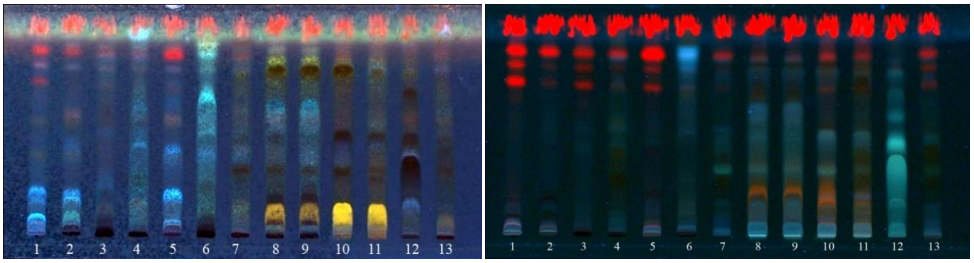

Figure 2. HPTLC profile of the leaf methanol extract along with 11 other *Garcinia* species **A**. 366 nm after exposure to NH_3. **B**. 366 nm after derivatisation (1. *G. gummi-gutta;* 2. *G. cowa,* 3. *G. rubro-echinata,* 4. *G. imberti,* 5. *G. indica,* 6. *G. mangostana,* 7. *G. morella,* **8. *G. pushpangadaniana* (Ist acc.), 9. *G. pushpangadaniana* (IInd acc.),** 10. *G. talbotii,* 11. *G. xanthochymus,* 12. *G. travancrica,* 13. *G. wightii*)

Biflavonoids, xanthones and benzophenones are the major phenolic compounds present in *Garcinia* species and the HPTLC of the methanol extracts represents the phenolic profile, especially the biflavonoids that shows intense fluorescence under exposure to NH_3 vapour. The secondary metabolite profile revealed that *G. xanthochymus, G. talbotii* and the new taxon comes under the same group and the presence of characteristic spots to the new taxon supports its species status (**Figure 2**).

2.2. Chemotaxonomy based on leaf volatile chemical profiles

Standardized descriptors based on volatile oil constituents have been proposed as an efficient tool for differentiation of plants. However, the use of volatile oil constituents for species differentiation is limited by the fact that several environmental factors may influence the plant chemical composition (Labra *et al*., 2004 and Grayer *et al*., 1996).

Volatile chemical profiles of the leaves were studied using GC-MS analysis of the essential oils. The essential oils were isolated from fresh leaves by hydrodistillation for 3h using Clevenger type apparatus. The oils were analyzed by gas chromatography methods. The GC-FID analysis was carried out on a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID) and a CP Sil 8CB fused silica capillary column (30m × 0.32mm, film thickness- 0.25µm). The GC/MS analysis was done on a Hewlett Packard 6890 gas chromatograph fitted with a cross-linked 5% phenyl methyl siloxane HP-5 MS capillary column (30m × 0.32mm, film thickness- 0.25µm) coupled with a 5973 series selective mass detector. The constituents were identified by retention indices calculated using homologues of n-alkanes (C_8-C_{22}) (Dool and Kratz 1963), comparing mass spectra with published data (Adams, 2007) and by mass spectra library search (Wiley 275 and NIST).

Gas chromatography- mass spectrometry (GC-MS) studies of the leaf essential oils resulted in the identification of 58 volatile compounds in all the three species (**Table 2**). The major volatile constituents of all the three species, the sesquiterpenoids, were derived from trans, trans farnesyl pyrophosphate (FPP), through mevalonic acid pathway, pointing to the allied nature of the species. However, in the new taxon compared to other species, monoterpenoids (2.8%) biosynthesized through trans geranyl pyrophosphate (GPP) were also present, while in *G. xanthochymus,* diterpenoids (4.4%) biosynthesized through trans geranyl geranyl pyrophosphate (GGPP) were exclusively present. The presence of monoterpenoids formed from a distinct biosynthetic pathway support the species status for the new taxon, as elucidated through morphological studies. The presence of more complicated diterpenoids ($C_{20}H_{32}$) in *G. xanthochymus* compared to the simple monoterpenoids ($C_{10}H_{16}$) and sesquiterpenoids ($C_{15}H_{24}$) suggests that *G. xanthochymus* is more evolved in the group.

Table 2. Essential oil composition of the leaves of *Garcinia pushpangadaniana, Garcinia xanthochymus* and *Garcinia talbotii*

Compound	RRI	*G. xan* (%)	*G. pus* (%)	*G. tal* (%)
Z-β-Ocimene	1032	--	0.2	--
Linalool	1095	--	1.8	--
Terpineol	1186	--	0.4	--
Geraniol	1249	--	0.4	--
δ-Elemene	1338	0.3	0.3	--
α-Cubebene	1348	0.9	0.7	0.7
Cyclosativene	1371	0.4	--	--
α-Ylangene	1373	--	0.8	--
α-Copaene	1376	13.0	3.1	27.0
β-Bourbonene	1387	3.2	6.8	0.1
β-Cubebene	1387	--	0.4	--
β-Elemene	1390	4.6	--	--
β-Caryophyllene	1419	17.0	11.4	30.4
β-Copaene	1430	1.6	--	0.1
β-Gurjunene	1433	--	--	2.2
γ-Elemene	1434	0.1	0.4	--
Aromadendrene	1439	0.3	1.1	1.6
α-Humulene	1452	6.6	3.2	10.7
cis-Cadina-1(6)-4-diene	1461	--	1.4	0.1
α-Acoradiene	1464	--	--	0.1
γ-Gurjunene	1475	1.2	--	3.1
γ-Muurolene	1478	12.5	11.7	3.8

Amorpha-4,7 (11)-diene	1479	0.1	--	--
α-Amorphene	1483	--	--	1.3
β-Selinene	1489	0.1	0.6	--
δ-Selinene	1492	3.2	0.9	--
trans-Muurola-4(14)-5-diene	1493	9.0	--	--
γ-Amorphene	1495	--	2.6	--
α-Muurolene	1500	1.2	3.7	--
δ-Amorphene	1511	--	1.2	--
γ-Cadinene	1513	2.7	12.4	--
δ-Cadinene	1522	4.6	13.1	--
trans Cadina 1,4-diene	1533	0.1	1.0	0.1
Cadina-1(2),4-diene	1535	--	--	0.9
α-Cadinene	1537	0.4	1.4	0.1
Cadala-1(10),3,8-triene	1540	--	--	0.3
α-Calacorene	1544	0.3	1.2	--
Germacrene B	1559	0.5	0.4	--
Nerolidol	1561	--	0.4	--
Epiglobulol	1576	--	--	0.2
Spathulenol	1577	0.1	--	--
Caryophyllene oxide	1582	2.3	0.8	2.6
Globulol	1590	--	--	0.1
Cubeban-11-ol	1595	--	--	0.1
Humulene epoxide II	1608	0.4	--	0.5
1,10-di epi Cubenol	1618	--	--	1.2
α-Corocalane	1622	--	0.2	--
1-epi-Cubenol	1627	0.1	1.5	0.1
cis-Cadina-4-en-7-ol	1635		0.9	--
allo Aromadendrene epoxide	1639	0.4	--	--
Caryophylla-4(12),8(13)-diene	1639	--	--	0.1
α-Muurolol	1644	--	0.5	0.2
Cubenol	1645	0.1	--	0.8
α-Cadinol	1652	0.5	0.9	0.1
Cis-calamenen-10-ol	1660	0.1	--	--
14-Hydroxy 9-epi-Z-caryophyllene	1666	--	--	0.5
14-Hydroxy 9-epi-E-caryophyllene	1668	--	--	0.1
3E-Cembrene A	1947	4.4	--	--
Total (%)		92.3	87.8	89.2
Monoterpenoids		Nil	2.8	Nil
Sesquiterpene- Hydrocarbons		83.9	79.8	82.6
Sesquiterpene-Oxygenated		4	5.2	6.6
Diterpenoids		4.4	Nil	Nil

RRI: Relative retention index calculated on HP-5 column

Similarity and cladistic analyses performed statistically based on the distribution of 58 volatile chemicals using SPSS software (ver.16.0) showed *G. pushpangadaniana* distinct from other two species (**Figure 3, Table 3**). The species is more related to *G. xanthochymus* 62%), compared to *G. talbotii* (39%).

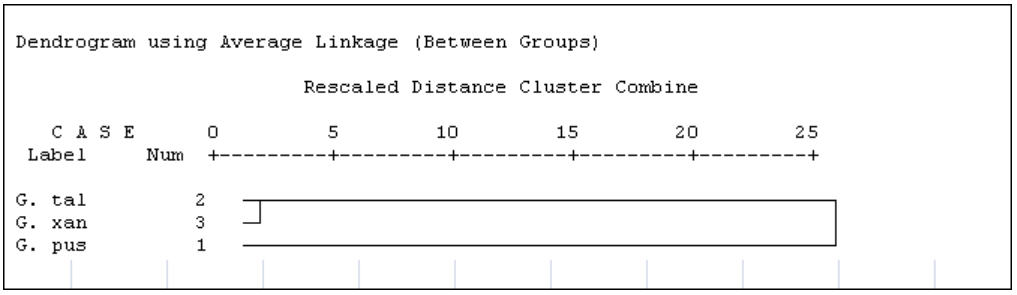

Figure 3. Dendrogram based on essential oil constituents of the leaves of *Garcinia pushpangadaniana, Garcinia xanthochyma* and *Garcinia talbotii.*

Table 3. Similarity matrix between three *Garcinia* species of the Western Ghats based on volatile chemical profile.

Case	Correlation between vectors of values		
	1:1	2:2	3:3
1:1	1.000	.391	.622
2:2	.391	1.000	.794
3:3	.622	.794	1.000

3. Molecular taxonomy

Molecular taxonomic approaches may be defined as DNA based methods that permit an exact and rapid method of distinguishing specimens based on their variation in genetic composition. Molecular markers are a direct assay of hereditary material and unlike morphological markers, molecular markers are not prone to environmental influences and can complement data from descriptors such as morphological characters (Mba and Tohme, 2005). Molecular systematics has become a major tool used in conservation biology for describing biodiversity, discriminating among taxa and establishing likely paths of evolution through phylogenetic analysis (Avise, 1989; Soltis *et al*., 1999).

In the present study, Genomic DNA was isolated from young leaves using DNeasy plant DNA isolation kit (Qiagen). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v 5.6. The phylogenetic analyses of 28 accessions of 10 *Garcinia* species were done using *matK* with *Clusia criuva* of Clusiaceae family as the out group member (ncbi-TNS:SK08071206). The analysis involved 28 nucleotide sequences. In the present study, *G. pushpangadaniana, G. talbotii* and *G. xanthochymus* comes under separate clad, in congruence with the morphological and chemical classifications. The dendrogram clearly delimits the species status of *G. pushpangadaniana* and is more allied to *G. talbotii* (**Figure 3**).

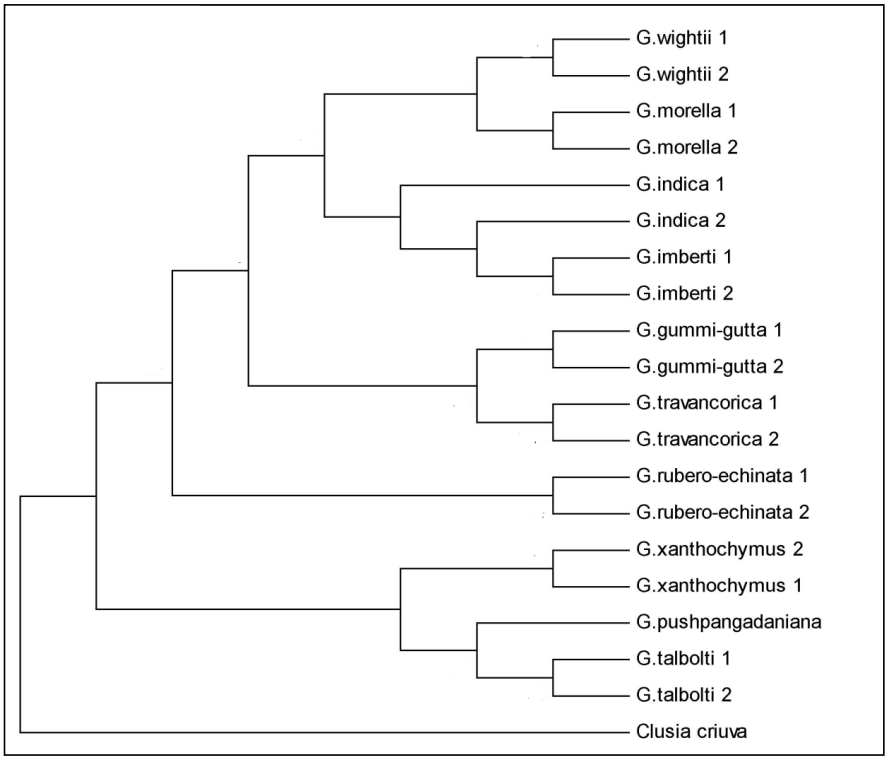

Figure 4. Phylogram based on *matK* loci of 28 accessions of 10 *Garcinia* species and the out group *Clusia criuva*

Conclusions

The HPTLC profile as well as the biosynthetic evaluation of the volatile terpenoids supported the species status for the new taxon. The molecular phylogeny also points to its proximity to *G. talbotii* and *G. xanthochymus* as elucidated through morphological evaluation. The present study highlights the importance of combined analysis of morphological traits, chemical profiles and genetic diversity that represents the optimal approach to assign species status to a new taxon.

References

1. Adams RP. **2007**. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Fourth edition. Allured Pub. Co., Carol Stream, IL.
2. Avise JC. **1989**. A role for molecular genetics in the recognition and conservation of endangered species. *Trends Ecol. Evol.* 4, 279-281.
3. Dool VH and Kratz PD. **1963**. A generalization of the retention index system including linear temperature programmed gas liquid partition chromatography. *J. Chromatogr.*, 11, 463-471.
4. Grayer RJ, Kite GC, Goldstone FJ, Bryan SE, Paton A and Putievsky E. **1996**. Infraspecific taxonomy and essential oil chemotype in sweet basil, *Ocimum basilicum*. *Phytochemistry*, 43, 1033-1039.

5. Gustafsson MHG, Bittrich V and Stevens PF. **2002**. Phylogeny of Clusiaceae based on rbcL sequences. *Internat. J. Plant Sci*. 163, 1045- 1054.
6. Hemshekhar M, Sunitha K, Santhosh MS, Devaraja S, Kemparaju K, Vishwanath BS and Girish KS. **2011**. An overview on genus *Garcinia:* phytochemical and therapeutical aspects. *Phytochem. Rev.*, 10(3), 325-351.
7. Labra M, Miele M, Ledda B, Grassi F and Mazzei M. **2004**. Morphological characterization, essential oil composition and DNA genotyping of Ocimum basilicum L. cultivars. *Plant Sci*., 167, 725-731.
8. Mba C and Tohme J. **2005**. Use of AFLP markers in surveys of plant diversity. *Meth. Enzymol.,* 395, 177-201.
9. Nayar TS, Beegam AR and Sibi M. **2014**. Flowering plants of the Western Ghats, India. JNTBGRI, Thiruvananthapuram.
10. Nogueira PC, Bittrich V, Shepherd GJ, Lopes AV and Marsaioli AJ. **2001**. The ecological and taxonomic importance of flower volatiles of *Clusia* species (Guttiferae). *Phytochemistry*, 56(5), 443-452.
11. Reich E and Schibli A. **2007**. High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants. Thieme, New York.
12. Soltis PS, Soltis DE and Chase MW. **1999**. Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature,* 402, 402-404.
13. Sweeney PW. **2008**. Phylogeny and floral diversity in the genus *Garcinia* (Clusiaceae) and relatives. *Int. J. Plant Sci*., 169(9), 1288-1303.
14. Waterman PG and Hussain RA. **1983**. Systematic significance of xanthones, benzophenones and biflavonoids in *Garcinia*. *Biochem. Syst. Ecol.,* 11(1), 21-28.
15. Winfield MO, Wilson PJ, Labra M and Parker JS. **2003**. A molecular analysis of *Gentianella* ssp. in Britain. *Plant Syst. Evol*., 267, 137-151.