Volatile and Non-Volatile Chemical Profiling of Eleven Hitherto Uninvestigated *Thottea* Species (Aristolochiaceae) of the Western Ghats

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***Abstract*—The genus *Thottea* Rottb. (Aristolochiaceae) is represented in the Western Ghats by eleven species, most of which have so far remained uninvestigated for their chemical constituents. In the present study the leaf essential oils of all eleven species were obtained by hydrodistillation and analysed by GC–MS. Oil yields ranged from 0.05% to 0.7% v/w and a total of 108 compounds, accounting for 90.4% to 98.5% of the oils, were identified. Sesquiterpenoids predominated over monoterpenoids in every species and phenylpropanoids were almost entirely absent. Bicyclogermacrene and spathulenol were the compounds most widely shared across the genus, followed by β-elemene, E-caryophyllene and globulol, while individual species were characterised by distinctive major constituents such as cubenol in *T. abrahamii* (28.8%) and *T. dinghoui* (41.1%), γ-cadinene in *T. dalzelli* (57.2%), γ-muurolene in *T. barberi* (30.7%), germacrene B in *T. adichilthottiana* (25.2%) and α-costol in *T. ponmudiana* (30.9%). A new chemotype of *T. siliquosa*, dominated by E-β-ocimene (31.7%) and bicyclogermacrene (15.1%) and completely lacking spathulenol, is reported. Cluster analysis of the volatile data (SPSS 16.0) generated a dendrogram that sub-grouped the species and supported their delimitation on chemical grounds. From the leaf methanol extract of *T. siliquosa*, pentadecanal was isolated and characterised by ^{1}H and ^{13}C NMR; this is the first report of the compound from the species.**

***Index Terms*—Aristolochiaceae, bicyclogermacrene, chemotaxonomy, chemotype, essential oil, GC–MS, pentadecanal, spathulenol, Thottea, Western Ghats.**

I. INTRODUCTION

Thottea Rottb. is a genus of perennial herbs and small undershrubs belonging to the birthwort family Aristolochiaceae. The members of the genus occur in the shaded understorey of wet evergreen forests and are distributed across South and South-East Asia, with the Western Ghats of peninsular India forming an important centre of diversity for the genus. Eleven species have so far been described from this region, several of them only in recent years, and a number of them are narrowly endemic.

Species delimitation in *Thottea* rests almost exclusively on floral morphology. Because flowering in several taxa is intermittent and seasonal, and because vegetative characters vary widely and inconsistently within populations, morphologically similar species are extremely difficult to separate outside the reproductive phase. Recently described taxa such as *T. abrahamii* and *T. idukkiana* are practically indistinguishable from their closest relatives on vegetative characters alone. In such situations, secondary metabolite profiles, and volatile terpenoid profiles in particular, provide an additional and largely independent set of characters that can be used to corroborate morphological delimitation.

Despite this potential, the genus has attracted very little chemical attention. Apart from the leaf oil of *T. ponmudiana*, in which pregeijerene B and 8-α-acetoxyelemol were reported as the major constituents together with terpinolene, elemol and bulnesol, and which was also shown to be active against Gram-positive bacteria [1], the essential oils of the Western Ghats species remain unexplored. The present study was therefore undertaken to (i) characterise the leaf essential oils of all eleven *Thottea* species occurring in the Western Ghats by GC–MS, (ii) examine the chemotaxonomic value of the volatile profiles through similarity and cladistic analysis, and (iii) isolate and characterise a major non-volatile constituent from the leaves of *T. siliquosa*.

II. MATERIALS AND METHODS

A. Plant Material
Fresh mature leaves of eleven *Thottea* species were collected from natural populations in the southern Western Ghats . The identity of each collection was confirmed using standard floras and voucher specimens were deposited in the herbarium

B. Isolation of the Essential Oil
Fresh leaves (500g) of each species were chopped and subjected to hydrodistillation for 3h in a Clevenger-type apparatus. The oil obtained was separated, dried over anhydrous sodium sulphate and stored in sealed amber vials at 4 °C until analysis. Oil yield was calculated on a fresh weight basis and expressed as % v/w.

C. GC–MS Analysis
Identification of the constituents was based on the comparison of their relative retention indices (RRI), computed against a homologous series of n-alkanes injected under identical conditions, with literature values, and on the comparison of their mass spectra with those held in the NIST and Wiley libraries and with published spectra [2]. Relative percentage amounts were calculated from the total ion chromatogram peak areas without the use of correction factors.

D. Statistical Analysis
Similarity and cladistic analyses were performed on the basis of the distribution of volatile constituents among the eleven species using SPSS software, version 16.0. A proximity (dissimilarity) matrix was generated and hierarchical cluster analysis was used to construct the dendrogram.

E. Extraction and Isolation of the Non-Volatile Constituent
Shade-dried leaves of *T. siliquosa* were extracted with methanol in a Soxhlet apparatus. On removal of the solvent the extract yielded a white precipitate which gave a single strong spot on TLC (solvent system: 100% acetone). The precipitate (250 mg) was washed with hexane, dried and subjected to column chromatography over silica gel (100–200 mesh), eluting with hexane:chloroform (50:50) and gradually increasing the polarity up to 100% chloroform. Fractions of 10 mL were collected and pooled on the basis of their TLC profiles. Fractions F34 to F58, which showed a single strong spot, were combined and, on evaporation of the solvent, furnished a white amorphous solid in pure form. The compound was characterised by ^{1}H and ^{13}C NMR spectroscopy (Bruker Avance III, 400 MHz).

III. RESULTS AND DISCUSSION

A. Essential Oil Yield and General Composition
The essential oil yield of the eleven *Thottea* species varied from 0.05% to 0.7% v/w. A total of 108 compounds were identified from the eleven oils (Table I, Appendix), the total identified content ranging between 90.4% and 98.5%. Among the volatile constituents, monoterpenoids were present only in small amounts and phenylpropanoids were almost completely absent from the oils of the species studied. Bicyclogermacrene and spathulenol were the compounds common to most of the species, followed by β-elemene, E-caryophyllene and globulol. The distribution of the major constituents across the eleven species is summarised in Fig. 1.

B. Species-wise Distribution of Volatile Constituents
Spathulenol was the major compound in *T. siliquosa* (22.1%) and *T. sivarajanii* (27.9%). E-caryophyllene (12.0%), α-pinene (6.5%) and 7,14-anhydro-amorpha-4,9-diene (5.3%) were the characteristic compounds of *T. siliquosa*, whereas globulol (6.2%) and 2-α-acetoxy-11-methoxy-amorpha-4,7-diene (8.6%) were the secondary compounds of *T. sivarajanii*. Cubenol was identified as the major compound in *T. abrahamii* (28.8%) and *T. dinghoui* (41.1%); in addition to cubenol, bicyclogermacrene and spathulenol were also among the main compounds in *T. abrahamii* (17.8% and 7.2%) and *T. dinghoui* (12.6% and 6.0%), respectively. Germacrene B was the major volatile compound in the oil of *T. adichilthottiana* (25.2%), followed by α-pinene (17.2%) and bicyclogermacrene (12.7%).

Volatile chemical analysis of *T. barberi* revealed γ-muurolene (30.7%) as the major compound, followed by bicyclogermacrene (10.4%), β-elemene (8.4%) and E-caryophyllene (7.7%). In contrast with the other species, the oil of *T. dalzelli* was dominated by γ-cadinene (57.2%) together with bicyclogermacrene (9.4%). Germacrone was the main volatile component of *T. duchartrei* (21.6%), while it occurred as a secondary compound in *T. idukkiana* (11.2%). Conversely, bicyclogermacrene was the major compound in *T. idukkiana* (21.8%) and accounted for 14.2% of the oil of *T. duchartrei*. Cyclocolorenone (13.1%) and Z- and E-β-ocimene (7.0% and 8.2%, respectively) were also present in appreciable amounts in *T. duchartrei*, whereas higher contents of sabinene (7.0%) and E-caryophyllene (6.6%) were the highlights of *T. idukkiana*.

The major volatile chemicals in the leaves of *T. ponmudiana* were α-costol (30.9%), selina-3,11-dien-6-α-ol (13.6%) and selina-3,7(11)-diene (12.0%). Similarly, α-cadinol (14.5%), δ-cadinene (13.8%), bicyclogermacrene (10.7%) and germacrene-D-4-ol (8.5%) were the characteristic compounds of *T. sasidharaniana*.

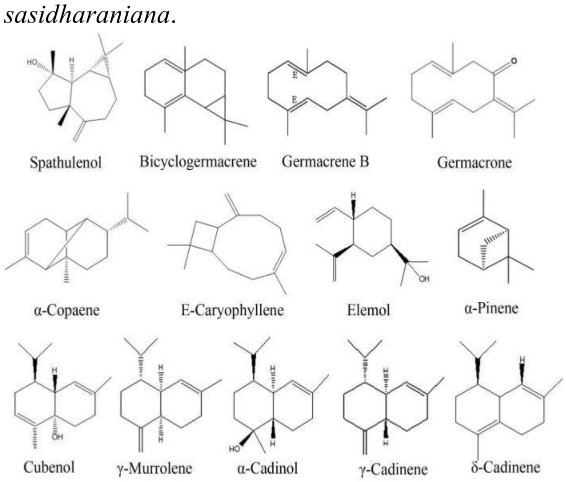

Fig. 1. Major volatile chemical constituents in *Thottea* species.

C. A New Chemotype of T. siliquosa from the Western Ghats

GC–MS analysis of the essential oil of a *T. siliquosa* population, followed by database matching and interpretation of the data, resulted in the identification of 11 compounds comprising 95.9% of the total oil composition. Monoterpenoids, amounting to 63.1%, were the major class of compounds in this oil, followed by sesquiterpenoids, which comprised 32.8% of the oil. The major compounds were identified on the basis of their mass spectra as E-β-ocimene (31.7%) and bicyclogermacrene (15.1%) (Table II, Fig. 2). Previous studies reported that the leaf essential oil of *T. ponmudiana* contains 8-α-acetoxyelemol and pregeijerene B as the major compounds along with terpinolene, elemol and bulnesol, and that these oils exhibited significant antimicrobial activity against Gram-positive bacteria [1]. In the present study, E-caryophyllene (12.7%) and terpinolene (8.9%) were the other characteristic components of the oil. More importantly, spathulenol, the characteristic component of all the other *Thottea* species examined, was completely absent, while the high content of bicyclogermacrene corroborates the earlier reports. The oil composition therefore represents a distinct chemotype of *T. siliquosa*. Mass spectra and structures of the major compounds of this oil are shown in Fig. 3 and Fig. 4.

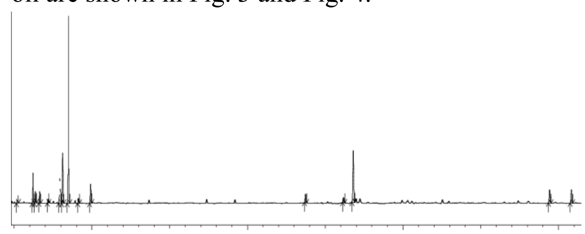

Fig. 2. Gas chromatogram of the leaf essential oil of the *Thottea siliquosa* chemotype.

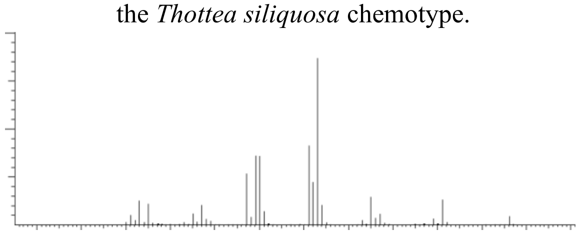

Fig. 3. Mass spectrum and structure of E-β-ocimene.

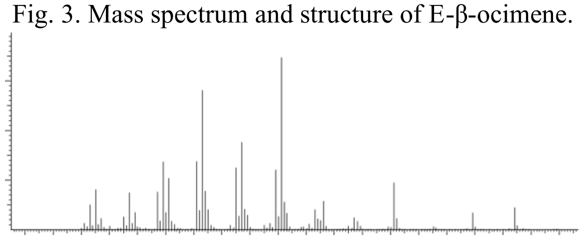

Fig. 4. Mass spectrum and structure of bicyclogermacrene.

TABLE II ESSENTIAL OIL COMPOSITION OF *THOTTEA SILIQUOSA* LEAVES

Sl. No.	RT (min)	RRI lit.	RRI cal.	Compound	%
1	5.207	932	932	α-Pinene	5.1
2	6.207	969	970	Sabinene	2.3

Sl. No.	RT (min)	RRI lit.	RRI cal.	Compound	%
3	6.637	988	987	Myrcene	3.4
4	7.183	1002	1006	α-Phellandrene	1.5
5	7.923	1024	1027	Limonene	4.6
6	8.113	1032	1032	(Z)-β-ocimene	5.6
7	8.483	1044	1043	(E)-β-ocimene	31.7
8	9.917	1086	1083	Terpinolene	8.9
9	23.733	1417	1415	(E)-Caryophyllene	12.7
10	26.800	1500	1489	Bicyclogermacrene	15.1
11	39.427	1823	1828	Khusinol acetate	5.0
				Total identified	95.9
				Monoterpene hydrocarbons	63.1
				Oxygenated monoterpenes	0
				Sesquiterpene hydrocarbons	27.8
				Oxygenated sesquiterpenes	5.0

RT: retention time; RRI: relative retention index.

D. Chemotaxonomic Classification of Thottea Species

The taxonomic identification of *Thottea* species generally relies on floral morphology, and morphologically similar species are difficult to distinguish during the non-reproductive stages. Flowering in some *Thottea* species is intermittently seasonal. Furthermore, *Thottea* species of the Western Ghats exhibit a wide range of morphological diversity, and the important diagnostic features vary inconsistently among the species. Newly reported species such as *T. abrahamii* and *T. idukkiana* are found to be indistinguishable from their closest relatives. In a recent study, *Thottea* species from the Western Ghats were grouped into different classes on the basis of floral morphological characters: on the nature of the sepals, *T. duchartrei* and *T. idukkiana* are allied, while on the number and arrangement of the stamens, *T. abrahamii* and *T. dinghoui* group together. *Thottea barberi* was found to be unique with respect to its floral characteristics. Although *T. ponmudiana* and *T. sivarajanii* share some floral characters, they fall under different clades.

The inter-specific relationships of the eleven species can be elaborated equally well on the basis of their volatile chemical composition. Bicyclogermacrene and spathulenol serve as common marker compounds for the genus *Thottea* in the southern Western Ghats, as is also evident from earlier reports. The major compound of *T. ponmudiana* is α-costol (30.9%), with elemol (12.0%) as another important component, whereas spathulenol (22.1%) and E-caryophyllene (12.0%) were the major compounds of *T. siliquosa*. The volatile profiles of *T. ponmudiana* and *T. siliquosa* thus reveal that the two taxa are chemically distinct, and chemotaxonomy supports distinct species status for both. The dominance of the more complex sesquiterpenoids in *T. ponmudiana* further suggests the primitive nature of this species relative to *T. siliquosa*. The present study also shows that sesquiterpenoids predominate over monoterpenoids throughout the genus.

Cluster analysis based on the distribution of the volatile constituents was carried out to sub-group the species. Similarity and cladistic analyses performed with SPSS 16.0 yielded the dendrogram presented in Fig. 5 and the proximity (dissimilarity) matrix given in Table III. The results demonstrate that the eleven species can be differentiated on the basis of their volatile chemical profiles, and highlight the value of combining morphological traits with chemical profiles for a better understanding of species status and phylogeny in the genus.

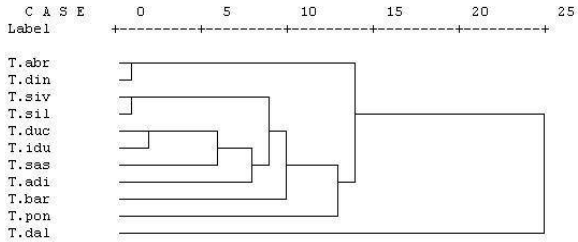

Fig. 5. Dendrogram depicting the sub-grouping of *Thottea* species based on volatile chemical profiles.

TABLE III PROXIMITY MATRIX (DISSIMILARITY MATRIX) OF *THOTTEA* SPECIES BASED ON VOLATILE CHEMICAL PROFILES

	Squared Euclidean Distance										
Case	1:1	2:10	3:11	4:2	5:3	6:4	7:5	8:6	9:7	10:8	11:9
1:1	.000	1.817E3	2.058E3	2.199E3	2.223E3	4.545E3	379.170	1.951E3	1.486E3	2.730E3	1.972E3
2:10	1.817E3	.000	1.693E3	1.628E3	1.645E3	3.623E3	2.394E3	1.486E3	1.046E3	2.056E3	1.524E3
3:11	2.058E3	1.693E3	.000	1.964E3	2.062E3	3.918E3	2.589E3	1.844E3	1.560E3	2.206E3	453.120
4:2	2.199E3	1.628E3	1.964E3	.000	2.103E3	4.294E3	2.786E3	1.712E3	1.203E3	2.345E3	1.621E3
5:3	2.223E3	1.645E3	2.062E3	2.103E3	.000	3.954E3	2.854E3	1.921E3	1.335E3	2.576E3	1.856E3
6:4	4.545E3	3.623E3	3.918E3	4.294E3	3.954E3	.000	5.118E3	3.851E3	3.313E3	4.579E3	4.097E3
7:5	379.170	2.394E3	2.589E3	2.786E3	2.854E3	5.118E3	.000	2.482E3	2.161E3	3.199E3	2.485E3
8:6	1.951E3	1.486E3	1.844E3	1.712E3	1.921E3	3.851E3	2.482E3	.000	538.460	2.317E3	1.616E3
9:7	1.486E3	1.046E3	1.560E3	1.203E3	1.335E3	3.313E3	2.161E3	538.460	.000	2.011E3	1.237E3
10:8	2.730E3	2.056E3	2.206E3	2.345E3	2.576E3	4.579E3	3.199E3	2.317E3	2.011E3	.000	1.983E3
11:9	1.972E3	1.524E3	453.120	1.621E3	1.856E3	4.097E3	2.485E3	1.616E3	1.237E3	1.983E3	.000

E. Non-Volatile Chemical Analysis of Thottea siliquosa

T. siliquosa is widely distributed throughout the Western Ghats but has been little investigated for its chemical potential. In addition to the volatile analysis, the leaf methanol extract prepared by Soxhlet extraction yielded, on removal of the solvent, a white precipitate that gave a strong single spot on TLC. Purification of this material by silica gel column chromatography as described above afforded a white amorphous solid. On the basis of its ^{1}H and ^{13}C NMR spectra (Fig. 6 and Fig. 7), the compound was characterised as pentadecanal, $CH_3(CH_2)_{13}CHO$. This is the first report of pentadecanal from the leaves of *T. siliquosa*.

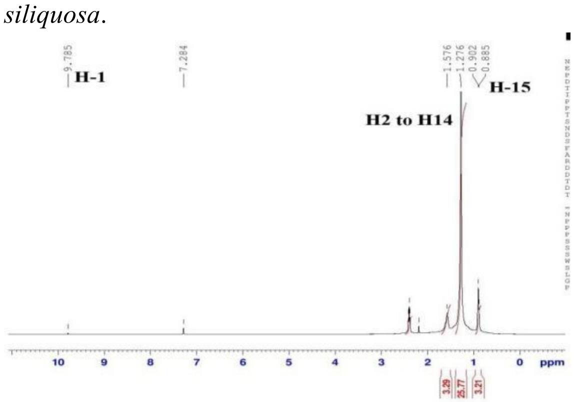

Fig. 6. ^{1}H NMR spectrum of pentadecanal.

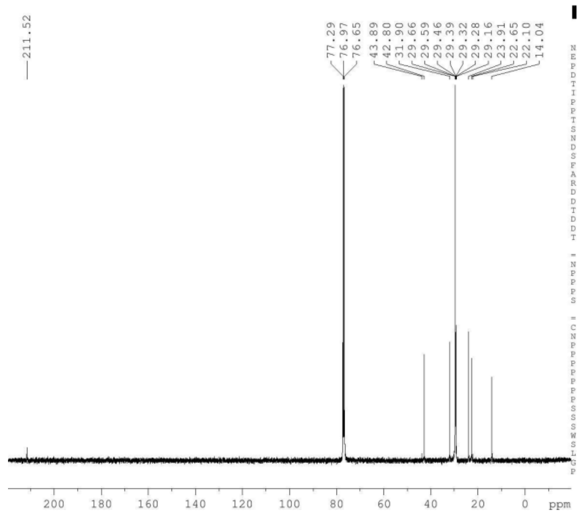

Fig. 7. ^{13}C NMR spectrum of pentadecanal.

IV. CONCLUSION

The leaf essential oils of all eleven *Thottea* species of the Western Ghats were characterised for the first time in a single comparative study. The oils are sesquiterpenoid-rich and virtually devoid of phenylpropanoids, with bicyclogermacrene and spathulenol acting as genus-level markers and with individual species carrying distinctive major constituents that permit their chemical differentiation. A new chemotype of *T. siliquosa*, characterised by a high content of E-β-ocimene and the complete absence of spathulenol, is reported. Cluster analysis of the volatile data grouped the species in a manner that can be read alongside the floral morphological classification, showing that volatile profiling is a useful supplementary tool for the delimitation of morphologically difficult taxa in the genus. The isolation of pentadecanal from the leaves of *T. siliquosa* is reported here for the first time and adds to the limited knowledge of the non-volatile chemistry of the genus. Further work on the biological activity of these oils and of the isolated constituent is in progress.

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Authors: please insert the remaining citations used in the Introduction and Discussion — in particular the floral-morphology classification of the Western Ghats Thottea species and the earlier reports on bicyclogermacrene and spathulenol as genus markers — following the same IEEE-style numbering.]