The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
20(3): S.I(3). 2158-2162, 2025 www.thebioscan.com

CHEMICAL PROFILING AND DENDROGRAPHIC REPRESENTATION OF 16 MEMBERS OF ORDER MYRTALES COLLECTED FROM DIFFERENT REGIONS OF MAHARASHTRA, INDIA

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DOI: 10.63001/tbs.2025.v20.i03.S.I(3).pp2158-2162

KEYWORDS
Chemotaxonomy; Myrtales; High-performance thin-layer chromatography (HPTLC); Secondary metabolites; Pairing affinity; Phytochemical profiling,

Received on:
15-10-2025

Accepted on:
12-11-2025

Published on:
22-12-2025

ABSTRACT

Chemical profiling was employed to delineate phylogenetic relationships among 16 taxa of the angiosperm order Myrtales collected from distinct biogeographic regions of Maharashtra, India. Leaf methanolic extracts were analyzed via High Performance Thin-Layer Chromatography (HPTLC) to generate comprehensive phytochemical fingerprints. Binary presence–absence matrices derived from distinct chromatographic bands were used to compute Pairing Affinity Indices, quantifying interspecific chemical similarity and divergence. Distance matrices informed construction of dendrograms to visualize chemoprofile-based phylogenetic patterns across seasonal cohorts. The highest affinity values were observed between Eucalyptus globulus and Terminalia mantaly (52%) in Lonavala (summer) and between Combretum indicum and Gnetonia floribunda (50%) in Mumbai (winter), indicating pronounced chemotaxonomic divergence. Lower affinity values (25–26%) were recorded among other species pairs, reflecting varied degrees of secondary metabolite overlap. Cladistic interpretation of chemoprofiles revealed discrete clusters consistent with family-level differentiation within Myrtaceae, Lythraceae, Lecythidaceae, Combretaceae and Melastomataceae. These findings demonstrate that HPTLC-derived secondary metabolite distribution provides informative chemotaxonomic markers that can complement morphological and molecular data in resolving evolutionary relationships. The results support the integration of chemical data into systematic frameworks and highlight the potential of chemoprofiling for enhancing the resolution of phylogenetic inference in Myrtales.

INTRODUCTION

The most deep-rooted insights in scientific history, was cognizance all organisms on earth being interconnected or interdependent to some or the other common decent (Hinchliff et al. 2015). The advancement of phylogenetic biology has reformed the study of molecular and developmental evolution. (Donoghue et al. 2008). Earlier, phylogenetic analysis was based on morphological comparison among the fossils, but the information from fossils was limited. Molecular phylogenetic analysis has now become popular using molecular data such as DNA, proteins, Mitochondrial DNA, RNA etc. (Horiike 2016). In general, molecular methods are considered far superior since the actions of evolution ultimately reflected in genetic sequence. With evolution of science in intellectual and computational advancements the integration of phylogenetics, ecology, body character evolution biogeography and enabling evolutionary biologists etc, has been at its ease. (Crisp et al. 2009; Rabosky et al. 2013; Givnish et al. 2014; Linder et al. 2014; Rose et al. 2016). The analysis of DNA and protein sequences also provides unrivalled opportunities to gene phylogenies, which may not always be represented in a phylogeny form, and in many cases may not correspond to the organism. (Ziemert et al. 2012).

The concept of Phylogenetic analysis is not new however, in the last decade the concept has been considered robustly, especially in animal sciences. But unfortunately, this approach is becoming derelict (Johnson and Briggs et al. 1984). With the development of plant and natural products chemistry, potential value of plant secondary metabolites to taxonomy has been recognized (Fairbrothers et al. 1975; Conti et al. 1997). The very first study on chemical taxonomy carried by Mc Nair (1935) explained the distribution of volatile oil, fixed oils, and alkaloids in the angiosperm plant consecutively, the first comparative study, mainly on volatile oil of Myrtaceae. These studies thus confirmed the individuality of bioactive of different taxa, which may further contribute to the identification of possible variation in intraspecies. The prime method for separation of these compounds were majorly established using chromatography i.e., paper chromatography. (Wink et al. 2010). With the further advancement

in different field of science, botanists and chemists together proposed the possibility of enlisting the chemical constituents to characterize, classify, and describe in the various taxa. A good knowledge of chemical composition leads to better understanding of plants for its medicinal value (Hussein et al. 2019). The relative complex mixture of major secondary metabolites are the representatives of different class of organic compounds which are the characteristics of many taxa. (Stojanović et al. 2015). In the earlier work quantification of Ursolic acid, a secondary metabolite from different geographical locations from order Myrtales (Nidhi Pathak and Anil Avhad 2023, Nidhi Pathak et. al 2022 and Nidhi Pathak et. al 2023) In this current study, different taxa of order Myrtales for its chemical phylogeny evaluation have been considered.

Members of Myrtales are a group of dicotyledonous plants, found across the tropical and temperate regions of the world, reveals the character of woody, herbaceous, terrestrial ranging from huge trees of small annual herbs (Bell et al. 2010; Dahlgren et al. 1984) inferior ovary that shows transition from perigyny to epigyny, retained the large number of stamens and five carpels (Daniel, 2009). In Bentham and Hooker's classification of order Myrtales shows its position in Polypetalae, subclass calyciflorae. As per of Bentham and hooker's classification order Myrtales is embraced six families, viz., Myrtaceae, Lythraceae, Rhizophoraceae, Combretaceae, Melastomataceae, and Onagraceae (Bentham et al. 1862). With further studies and advancement, further 3 more families Punicaceae, Lecythidaceae and Alangiaceae were added in order Myrtales by Engler and Prantle in the year 1887 (Morley 1984). However, in the year 1973, a well-known taxonomist Carl Linnaeus, regarded 10 families in order Myrtales, further to these three families were added of which Barringtoniaceae, Asteranthaceae group separated from Lecythidaceae and Sonneratiaceae of family Lythraceae later Alangiaceae and Onagraceae were removed by from order Myrtales by Hutchinson (Hutchinson,1973). Using "Relaxed clock methodology" a recent study on the lineage for order Myrtales was identified between 89 to 99 million years ago (Bell et al. 2010). Consecutively in the year 2000, the order Myrtales were divided into three suborders Melastomatineae, Myrtineae and Lythrineae. Of these suborders, Melastomatineae, seven different families were included namely Penaeaceae, Oliniaceae, Rhynchocalycaceae, Alzateaceae, Crypteroniaceae, Melastomataceae and Memecylaceae. Whereas suborder Myrtainae includes three families namely Myrtaceae, Onagraceae and Vochysiaceae and suborder Lythrineae includes three families namely Lythraceae, Onagraceae and Combretaceae (Thorne, 2000). Thus, many studies on identifying the deep lineage divergence in the order Myrtales were carried, although the members of this order vary in different classifications with many different opinions (Sytsma et al. 2004).

One such study on phylogenetic analysis of about 19 taxa using 77 binary code of order Myrtales was reported by the author Johnson and Briggs (1984), using CLAX method, a new numerical technique, by which an attempt to understand an evolutionary shift was focused. The conclusion from the study reveals that family Myrtaceae form a coherent and holophyletic group, and that of family Heterophyxidaceae and Psiloxylaceae are now diverged from the lineage of Myrtaceae. The other set of study on phylogenetic analysis was carried out by the author Maurin in the year 2021, concluded the relationship in Myrtales at various taxonomic levels. The study of total 9 families and approximately 400 genera almost 14,000 species of different continents expect Antarctica, were carried out using probe kit for the following family namely Alzateaceae, Combretaceae, Crypteroniaceae, Lythraceae, Melastomaceae, Myrtaceae, Onagraceaae, Penaeaceae, and Vochysiaceae. The result of study come out to be a comprehensive phylogenetic hypothesis, using High – throughout sequencing and Angiospermae 353 probe kit one of the powerful tools for phylogenomic analysis. For better understanding of these trees a proper genetic data is required that probably link the incomplete lineage or hybridization event (Maurin et al. 2021).

Moving forward with the other such studies documented, basis the rbcL sequences of 50 taxa of 39 members of Myrtales and 11 rosid groups were analysed using parsimony and likelihood to provide a phylogenetic hypothesis of interfamily relationship in Myrtales. With an existing data on congruence between the family from an earlier study and the rbcL topology was considered to identify the probable synapomorphies that would validate the clades supported by the molecular tree. The rbcL harmonize two major clades of the first clade covers Myrtaceae lineage to a Melastomataceae whereas, the second clade covers family Onagraceae, with Lythraceae and Combretaceae lineage. Phenotypic characters concluded with the suggestion on the ancestor of first clade involves flowers with stamens which is directly inserted on the edge of hypanthium. Although, the support for the basal split of Myrtales is weak, possibly because of rapid early radiation in the order. Well according to author, these findings provide new framework for interpretation of morphological characters. (Conti et al. 1997).

So far studied relationship of order Myrtales were notified using numerous phenotypic studies namely Morphological, palynological, cytological and anatomical. The current study aims to identify the relationship between different members basis the chemical phylogeny using pairing affinity formula.

MATERIAL AND METHODS

Collection of Plant Material

The study was conducted by collecting the leaves from Mumbai and Lonavala, Maharashtra, India pertaining to three different seasons *Viz.*, Winter, Monsoon and Summer. The collected leaves were thoroughly washed and dried at room temperature for a day, further dried using a hot air oven at 55 ºC for 48 hours. The dried plant material was pulverized to a coarse powder using a grinder, sieved using a 180-micron mesh; it was collected and stored in air-tight containers for the further analysis.

Preparation of Methanolic extracts

Methanolic solutions were prepared by Weighing about 0.1g of dried leaves powder in 10 ml of methanol, refluxed on boiling water bath at 600c for 1 hour. The extract was filtered using Whatman filter paper 41. The fresh filtrate was used for further analysis.

Chromatogram development using HPTLC

The chromatograms were developed using HPTLC, the samples were spotted with a Camag microliter syringe (100µl) on a pre-coated silica gel aluminium plates 60F -254(20 cm X 10 cm) with 250µm thickness, (E.Merck, Darmstadt, Germany) using a Camag linomat IV (Camag, Switzerland) applicator. Linear ascending development was carried out in 20 cm X 10 cm twin turf glass chamber (Camag, Switzerland) using mobile phase, Toluene: Ethyl acetate: Glacial acetic acid (11:05:0.5). The length of solvent run was 8 mm the plates were air dried and were derivatized using 10% ethanolic sulphuric acid and developed in a hot air oven at 1100C for 5 minutes. The TLC plate were observed under visible range, short and long UV wavelength, photographed using U.V. Cabinet.

Paring Affinity

Comparative study on the presence of chemical compounds in different members of same family were considered (Vajha et al. 2011). The complementary chromatogram with different Rf values, keeping triterpenes as a reference compound were further used for calculating the affinities between the species.

The method adopted by (Ellison et al. 1962) was followed, to study the comparisons in the form of qualitative relationships by HPTLC method. Hence, by determining the total number of compounds

present in a particular species, and the number of compound common in both species. Which was expressed as percentage by determining the Pairing affinity index.

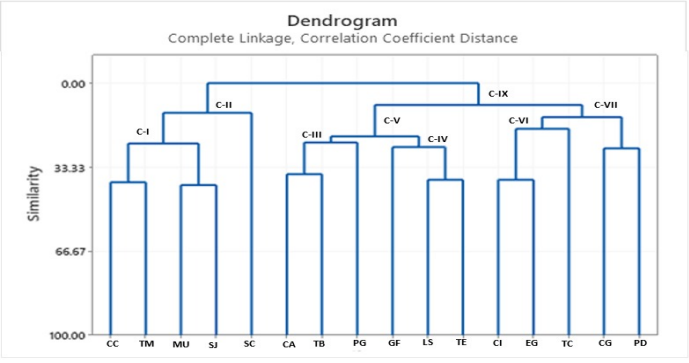
$$Pairing\ Affinity = (\frac{Spot\ common\ in\ Species\ A\ and\ B}{Total\ spot\ in\ Species\ A+B})X\ 100$$

MINI TAB®

MiniTab® statistical software 21.3.1.0 is data analysis software tool for statistical analysis used by different organizations worldwide. It is well known tool used by Six Sigma practitioners, statisticians, and quality engineers to analyze the data which help to solve real-world problems. The tool is widely used in a variety of industries, education, manufacturing, and healthcare. It provides users with tools to perform statistical analysis, regression analysis, hypothesis testing, and ANOVA.

RESULTS

The idea of representing the plants species under a particular family is ideally based on its phylogeny, morphology, taxonomy, Molecular and genetic identity. Besides these parameters presence of chemicals in the plant plays significant role. Hence, looking forward to the scope of chemical taxonomy in various pharmaceutical industries this study was conducted. On the basis of obtained chemical fingerprint obtained, phylogenetic tree was constructed. The very first step of the study was to identify the presence of number of distinct phytochemical bands in all species. The pioneer part of the study involves the identification of identical phytochemical band within different plant species of same order. The results thus obtained were further used to calculate the Paring affinity between the species using the formula, to develop a phylogenetic tree. Our final super matrix has a taxon coverage of 16 species of order Myrtales. Belonging to 5 different families Myrtaceae, Lytheraceae, Lecythideaceae, Combretaceae and Melastomaceae.. For all the season studied above, the chemical profile from the Lonavala region during summer and Mumbai region during winter were found to be effectively distributed chromatogram for phylogenetic tree preparation.



Higher the pairing affinity value, more the distance between the species. The highest affinity value obtained from the data suggests the distance matrix between the species belonging to the same order. Based on the calculative value, the highest affinity value encountered from the data is 52% between the species Eucalyptus globulus and Terminalia mantaly from the region of Lonavala whereas, in Mumbai the highest value encountered between Combretum indicum and Gentonia floribunda with 50% affinity. And lowest with 25% between Callistemon citrinus and Terminalia mantaly, Terminalia bellirica and Careya arborea, Eucalyptus globulus and Combretum indicum in Lonavala. From the region of Mumbai, the lowest affinity documented were between Lagerstroemia speciosa and Combretum indicum, Memecylon umbellatum and Eucalyptus globulus, Pimenta dioica and Eucalyptus globulus, Lagerstroemia speciosa and Terminalia mantaly with 26% affinity.

FIGURE 1: Dendrogram representation from the region of Lonavala during summer

The Above dendrogram represents the paring affinity between the species, considering there chemical profiling. To understand the above dendrogram the data is represented in different clades. The above dendrogram represents chemical profiling in Lonavala region during summer, nine clades were identified to understand the affinity between species of Myrtales. Four species were identified as the first branching taxa, clade (C-I) *Callistemon citrinus*, *Terminalia mantaly*, *Memecylon umbellatum* and *Syzygium jambos*. Clade II along with Clade I with *Syzygium cuminii*. Clade five however represents two Clades III and IV. Where, Clade III constitute three species *Careya arborea* showing close affinity with *Terminalia bellirica* and *Psidium guajava* Clade IV representing close relationship between *Lagerstroemia speciosa* and *Terminalia crenulata* along with *Getonia floribunda*. Moreover, Clade VI represents *Combretum indicum*, *Eucalyptus globulus* and *Terminalia catappa* based on the calculated affinity. Clade VIII between *Couroupita guanensis* and *Pimenta dioica*. Clade IX here represents the group of two different clade constituting 11 species, having close affinity with each other.

The below dendrogram represents the paring affinity between the species, considering there chemical profiling. To understand the above dendrogram the data is represented in different clades. Of the above dendrogram from Mumbai region during winter, ten clads were identified to understand the affinity between species of Myrtales. Clade I represent the affinity of three different species namely *Callistemon citrinus*, *Pimenta dioica* and *Terminalia crenulata* whereas, Clade II represents *Couroupita guanensis* from the allied family, *Eucalyptus globulus* and *Getonia floribunda* species. Under the Clade III, six different species with its closest affinity are placed. Likewise, Clade VI, with Clade IV and V represents the affinity of four species namely *Careya arborea*, *Terminalia bellirica*, *Syzygium jambos* and *Terminalia catappa*. The shortest clade, Clade VIII represents two species *Combretum indicum* and *Memecylon umbellatum*. Whereas Clade XI represents two different clad IX and X with four different species namely *Lagerstroemia speciosa*, *Terminalia mantaly*, *Psidium guajava* and *Syzygium cuminii*. Clade VII represents the highest clad with 10 species with its closest species affinity, the other six species with less similarity observed under different clad, Clade-XII.

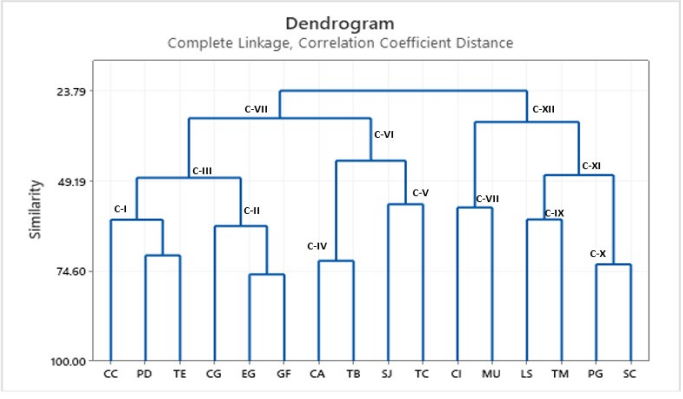


FIGURE 2: Dendrogram representation from the region of Mumbai during Winter

DISCUSSION & CONCLUSION

The blend of chemical profiling for targeted plant species in taxonomy leads to edge cutting tool for evolutionary study. The field of chemotaxonomy evolved through the merge of chemo + taxonomy, which explains "the classification of plant species on the basis of their chemical constituents" (Misra et al. 2016). Most chemical studies primarily were designated to the systemic issues in plants named as secondary metabolites, consisting of alkaloids, flavonoids, phenols, steroids, glycosides, terpenes, and various pigments. Certain groups of secondary metabolites were delimited to specific families e.g. The isothiocyanates an alkaloid form Senecio, hydroxycinnamic acid from Cruciferae member. Although thousands of secondary metabolites are produced by plants, very few are known to be significant for its role (Alston et al. 1963). Knowledge about chemistry of plants greatly increased during the nineteenth century, the great interest in the chemotaxonomic research has been developed in almost all areas/disciplines of science (Gurucharan Singh, 2004). Other significance of chemotaxonomy is in providing a rationale to a study for identifying and quantifying the specific class of natural compounds. The long existing concept of chemotaxonomy from past centuries were certainly approached to understand the variations in metabolic profile of plant species (Misra et al. 2016). Though the plants are categorized in different families, the chemical profile reunited with few similarities. Our phylogeny provides a significant step towards chemotaxonomy from order Myrtales considering Bentham and hookers classification system. The present analysis and proposals will not be the last word, but the data generated will be useful for its significant contribution in the growing field of chemotaxonomy.

Acknowledgement

The author wishes to thank Dr. (Mrs.) Usha Mukundan Director of RJ college and Dr. Himanshu Dawda Principal of RJ college for their guidance throughout the work.

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