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Nidhi Pathak
Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai, Maharashtra, India

Anil Avhad
Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai, Maharashtra, India

Rajkumar Diwakar
Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai, Maharashtra, India

Himanshu Dawda
Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai, Maharashtra, India

Usha Mukundan
Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai, Maharashtra, India

Corresponding Author:
Nidhi Pathak
Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai, Maharashtra, India

Phylogenetic screening of secondary metabolites and identification of biomarkers in selected members of family myrtaceae

Nidhi Pathak, Anil Avhad, Rajkumar Diwakar, Himanshu Dawda and Usha Mukundan

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Abstract
Application of medicinal plants for the benefit of humans is perpetual and is positively increasing every day. Hence, for a better understanding of plant community, origin, and their association with each other their taxonomic categorization is essential. It is fascinating to observe the increasing endearment of the researcher's towards chemotaxonomy owing to the nature and the structure of the chemical moiety of secondary metabolite. Morphological parameters and the wield of biochemical compounds in resolving the taxonomic disputes are gaining importance with the help of these bioactive/ biochemical compounds. A remarkable contribution of chemotaxonomy is providing a philosophical study for identification and quantification of a specific class of natural compound/chemical affiliation in a particular taxon and hence became advantageous in further study of unknown species lying within the same genera/taxon. The present study reveals the affinity between the species of the same family by concluding phytochemical screening and HPTLC analysis to establish the relative relationship between active compounds.

Keywords: Affinity, biochemical marker, chemotaxonomy, dendrogram, Myrtaceae, ursolic acid

Introduction
Myrtaceae (Myrtle, Eucalypts, clove, or *guava* family) is a large family of dicotyledonous woody plants laid in the order Myrtales comprising of 5,650 species organized in approximate 130 to 150 genera (Govaerts *et al.,* (2008) [13]. It has been recognized as the eighth largest family, with excellent ecological and economic importance worldwide (Grattapaglia *et al.,* (2012) [14]. The members of this family are rarely herbs, mostly shrubs or trees, some being the tallest trees, distributed in forests or woodlands. The fundamental features among myrtaceae member is the presence of oil glands in their leaves and are chemically dominated by Terpenes. Economically, Myrtaceae is a very important family, where some plant species are cultivated, for instance *Eucalyptus* spp., for its wood, used to produce paper pulp, lamppost and charcoal; many species are used for aesthetic purpose and some are used as spices, such as *Syzygium aromaticum* (L.) Merr. & L.M. Perry, known as "clove". Several species produce edible fruits that are utilized for making juice, jelly, and sweets, such as *Psidium guajava* L. ("guava"), *Eugenia uniflora* L. ("Barbados cherry"), *Syzygium* spp. ("jambo"). Myrtaceae members are also used in folk medicine to treat several diseases like anti-inflammatory, anticancer, antifungal etc (Porter *et al.,* (2000) [22]; Cruz *et al.,* [8] (2012); Cascaes *et al.,* (2015) [6]. The most cited traditional use from myrtaceae family is *Syzygium aromaticum* (L.) Merr. & L.M. Perry & *Syzygium cumini* (L.) Skeels, ("*Jamun*") widely used for its antifungal, anti-protozoal, antiviral, antidiarrheal, antidiabetic, analgesic, antimalarial, antioxidant etc properties (Cock *et al.,* (2018) [7]. *Myrtus communis* L ("Myrtle") for the treatment of gastric ulcer, diarrhea, dysentery, cancer, rheumatism, hemorrhage etc. (Sisay *et al.,* (2017) [24] *Pimenta dioica* (L.) Merr. ("All spice") used in menstrual cramps, upset stomach (Zhang *et al.,* (2012) [32] *Lagerstroemia speciosa* (L.) Pers ("*Jarul*" or "*Taman*") well known for antidiabetic activity (Rajkumar *et al.,* (2016) [23].
The total estimated species in the family ranges from 3,600 (Gadek *et al.,* (1996) [12], 4000 (Berry *et al.,* (2004) [1] to 5,500 (Govaerts *et al.,* (2008) [13]; Ed Biffin *et al.,* (2010) [10]. The deviation in the number of species is mainly attributed to the complex taxonomical features of the family and new systematic investigations performed worldwide (Lucas *et al.,* (2005) [19]. Family Myrtaceae is the eighth largest family of angiosperms in terms of species number (Snow *et al.,* (2012) [26]. Phylogenetic analysis of unlike species of plants have been critically important for the current systematic studies (Soltis *et al.,* (2005) [28]; Judd *et al.,* (1999) [17];

Doyle *et al.,* (2013) [9] and they have largely contributed to recent taxonomic changes in Myrtaceae, at both family and genus level (Wilson *et al.,* (2005) [31]; Craven *et al.,* (2010) [3]; Snow *et al.,* (2011) [25]; Murillo-A J *et al.,* (2012) [21]; Mazine Ff *et al.,* (2014) [20]. The earlier classification of Myrtaceae using morphological phylogenetics derive in an alliances-sub-alliances system, regarded as relatively equivalent to a formal tribes-subtribes system (Briggs *et al.,* (1979) [4]; Johnson *et al.,* (1984) [16]. Many phylogenetic analyses (Johnson *et al.,* (1984) [16]; Gadek *et al.,* (1996) [12]; Wilson *et al.,* (2005) [31] have indicated that fruit consistency is not a reliable character to delimitate tribes or subfamilies in Myrtaceae. Most of the fleshy-fruited species from Australasia (mainly the genus Syzygium) have been confirmed as a lineage independent to fleshy-fruited species from Central America, South America and eastern Australia (Briggs *et al.,* (1979) [4]; Gadek *et al.,* (1996) [12]. Myrtaceae, has been divided into three widely recognised subtribes based on embryo morphology; Eugeniinae and Mrytinae which occur in both the Old and New World tropics and the Myrciinae which are confined to the New World (Landrum *et al.,* (1981) [18].

Chemotaxonomy involves the expanding field of study concerning to the chemical variation in divergent types of plants. Chemotaxonomy investigates the distribution of chemical compounds or groups of biosynthetically related compounds in series of related or supposedly related plants (Bhargava *et al.,* (2013) [2]. Knowledge about chemistry of plants greatly increased during the eighteenth and nineteenth centuries the great interest in chemotaxonomic research has been developed in almost all areas/disciplines of science now a days. In neoteric years, interest towards study of Biochemicals and realization of an idea that, the animal kingdom and the plant kingdom have experienced a chemical co evolution (Gurucharan, (2004) [15] Since former, the essential oil and terpenoids of many aromatic plants turned out to be used as bioactive ingredients in drug, food, perfumery and cosmetic formulations throughout the world and hence it is worthy to study their role in chemotaxonomy. Families like Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Umbelliferae, Verbenaceae and Piperaceae are commonly known for its volatile oil and oil glands also presence of biologically active compounds. (Bhargava *et al.,* (2013) [2].

The current research aims to assess the correlation of secondary metabolites composition and recognition of biomarker present in selected member of family myrtaceae using HPTLC analysis. Following plant member *viz, Pimenta dioica, Syzygium lanceolatum, Syzygium jambos, Eucalyptus globulus, Melaluca leucodendron, Callistemon citrinus, and Psidium guajava* were screened. The study shall make exiguous contribution in chemotaxonomy.

Materials And Methods Collection of plant material

The healthy plant was collected from Mumbai, Byculla from Rani Baugh. The plant material was air dried in the shade and by using hot air oven at 600c for 4 hours. Later, pulverized to a coarse powder by using grinder and sieved and was stored in airtight container until further use.

Sample Preparation of Methanolic and Aqueous extracts

The solution were prepared by weighing about 1.0g of dried leaves powder in 10ml of alcohol and water and refluxed on boiling water bath at 600c for 1 hour. The extract was filtered using filterol paper. The filtrate was evaporated to dryness. The residue obtained after evaporation was weighed and reconstituted with the extraction solvent. The diluted extract was further used for the preliminary analysis of phyto-constituents and quantification of Ursolic acid using HPTLC method.

Preliminary phytochemical screening: Collection of plant material

The plant was collected from a healthy plant in Mumbai, Byculla from Rani Baugh. The plant material was air dried in the shade also by using hot air oven at 600c for 4 hours and pulverized to a coarse powder by using grinder and sieved and was stored in airtight container until further use.

Preparation of Methanolic and aqueous extracts

Three grams of each plant were refluxed using Soxhlet apparatus twice with 200ml of Alcohol and water for 8 hours. All extracts were collected, and vacuum dried under reduced pressure. The dried extracts were weighed in glass bottle; the extract was diluted ten times. The diluted extract was used for preliminary analysis of phyto-constituents and quantification of ursolic acid using HPTLC method.

Preliminary phytochemical screening

Phytochemical screening of the plants was carried out using (Sofowora *et al.,*(1993) [27]; Trease *et al.,* (1989) [29]; Carson *et al.,* (1995) [5] method. Where, screening of different secondary metabolites composition was performed. Test for Alkaloid was screened using Mayer's Reagent followed by the test for Flavonoids using NaOH solution, Phenol by Folin's reagent, Terpenoid using Anisaldehyde reagent, Tannins using FeCl3 solution, Steroids by Salkowski's test and Glycoside by Killer Killani's method.

HPTLC Specifications

The samples were spotted with a Camag microliter syringe on a 100µl 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 through glass chamber (Camag, Switzerland) using mobile phase consisting of Toluene: Ethyl acetate: Glacial acetic acid (11:05:0.5). The length of the chromatogram run was 8mm. the plate was air dried and sprayed with 10% ethanolic sulphuric acid and was developed in a hot air oven at 1100C for 5 minutes. The TLC plates was scanned using a Camag TLC scanner at 366nm, controlled by winCATS software version 4.03. Evaluation was performed using peak areas with linear regression.

Calibration curve of standard Ursolic acid

Standard solution of Ursolic acid (1mg/mL each) prepared in a methanol (stock solution). Standard working solutions were prepared by diluting standard stock solution with methanol in the concentration range 50–200 µg/mL and 100– 1000 µg/mL. Each working standard solution was spotted on the TLC plate to obtain final concentration range 2–10µg spot (Ursolic acid). Calibration curves were generated by linear regression based on the peak areas.

Estimation of Ursolic acid

To estimate the amount of Ursolic acid present in different members of family Myrtaceae, 4 µl of Leaf extract and standard Ursolic acid were spotted on the TLC plates (band length 8mm). Post development solvent system, air dried and was subjected to derivatization followed by development of chromatogram. The plate was scanned at 540 nm wavelength

of light using CAMAG TLC scanner. The Ursolic acid was calculated with respect to the calibration curve.

Analysis of chemical phylogeny by HPTLC fingerprints

The method adopted by (Ellison *et al.*, (1962) [11] was followed to study the comparisons in the form of qualitative relationships by HPTLC. Species were compared based on their biochemical affinities. (Vajha *et al.*, (2011) [30]. A comparison of spots observed in ultraviolet light with those appearing after use of the triterpene as reference compound showed chromatogram with different Rf values were generally complementary. The comprehensive data from the chromatograms was calculated for each sample. These data provided at once an opportunity to compare pairs of species in terms of chromatographic affinity (or presumptive biochemical affinity). Hence, by determining the total number of compound present in a particular species, then the number of compound common in both species. Which was expressed as percentage by determining the Pairing affinity index (PA): paired affinity index = spots in common for species A and B X 100 (Ellison *et al.*, (1962) [11] Using the PA indices accordingly, the calculation was executed for each species among 7 species considered in the current studies.

UPGMA

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) is an online method of tree construction. Purpose of the set-up is to construct taxonomic phenograms, which reflects the similarities between the operational taxonomical units (OUT's). The method uses a sequential clustering algorithm, where all sequences are differentiated through pairwise alignment to evaluate the distance matrix. The two sequences with minimum distance are identified and clustered as a single pair. However, the distance between this pair and all other sequences are recalculated to form a new matrix. Using this new matrix, the sequence that is closest to the first pair is identified and clustered. This process is thus repeated until all sequences have been incorporated in the cluster. Slightly different clustering may also be seen when the data is presented to the algorithm in a different order. The WPGMA (Weighted Pair Group Method with Arithmetic Mean) algorithm is similar to its unweighted variant, method using the distance between clusters, calculated as a simple average.
The UPGMA calculate the matrix by referring different statistical methods such as Pearson coefficient, Jaccard similarity coefficient, Dice coefficient, Euclidean distance, Manhattan metric etc.

Results

Phytochemical screening: Preliminary phytochemical screening of resulted into presence of Terpenoids and phenol in the analyzed plant group.

Table 1: Phytochemical screening

Plant	Aqueous extract								Methanolic extract							
	A	F	P	Ter	T	S	Sap	Gly	A	F	P	Ter	T	S	Sap	Gly
Pimenta dioica	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-
Melaluca lucodendron	-	+	+	+	-	-	+	+	-	-	+	+	-	-	+	+
Syzygium lanceolatum	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
Psidium guajava	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	+
Syzygium jambos	-	+	+	+	-	+	+	-	-	+	+	+	-	+	+	-
Eucalyptus globulus	-	+	+	+	+	-	+	-	+	-	+	+	+	-	+	-
Callistemon citrinus	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-
Pimenta dioica	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-

Where, A: Alkaloids, F: Flavonoids, P: Polyphenol, Ter: Terpenoids, S: Steroids, Sap: Saponin, Gly: Glycosides

Quantitative Estimation of Ursolic Acid: The total amounts of UA were calculated using their respective calibration curves. The aggregate quantity of UA found in *Eucalyptus globulus* was relative more.

Table 2: Quantification of ursolic acid

Plant Sample	mg of UA/gm
Callistemon citrinus	1.26
Syzygium jambos	0.11
Syzygium lanceolatum	0.51
Eucalyptus globulus	1.32
Pimenta dioica	0.13
Melaluca leucodendron	0.95
Psidium guajava	0.38

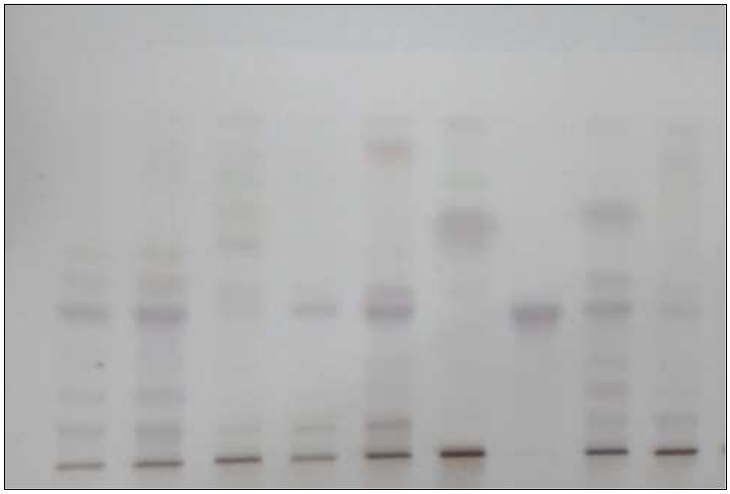

Fig 1.1: HPTLC Fingerprint at 540 nm, Track I and II: *Callistemon citrinus*, Track III: *Syzygium jambos*, Track IV: *Syzygium lanceolatum*, Track V: Eucalyptus *globulus*, Track VI: *Pimenta dioica*, Track VII: Ursolic Acid, VIII: *Melaluca leucodendron*, *IX: Psidium guajava*

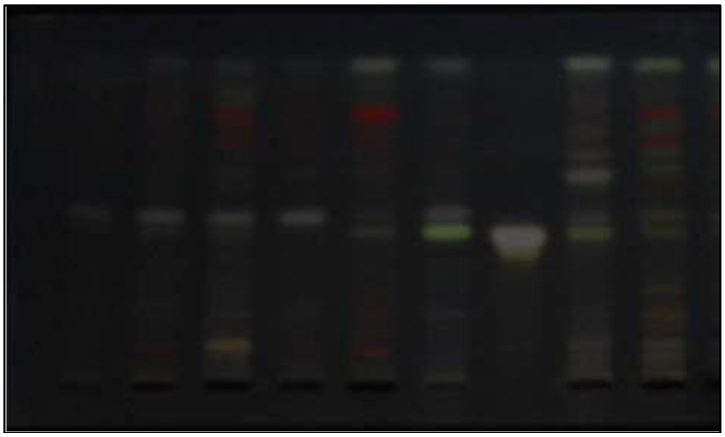

Fig 1.2: HPTLC Fingerprint at 366 nm, Track I and II: *Callistemon citrinus*, Track III: *Syzygium jambos*, Track IV: *Syzygium lanceolatum*, Track V: Eucalyptus *globulus*, Track VI: *Pimenta dioica*, Track VII: Ursolic Acid, VIII: *Melaluca leucodendron*, *IX :Psidium guajava*.

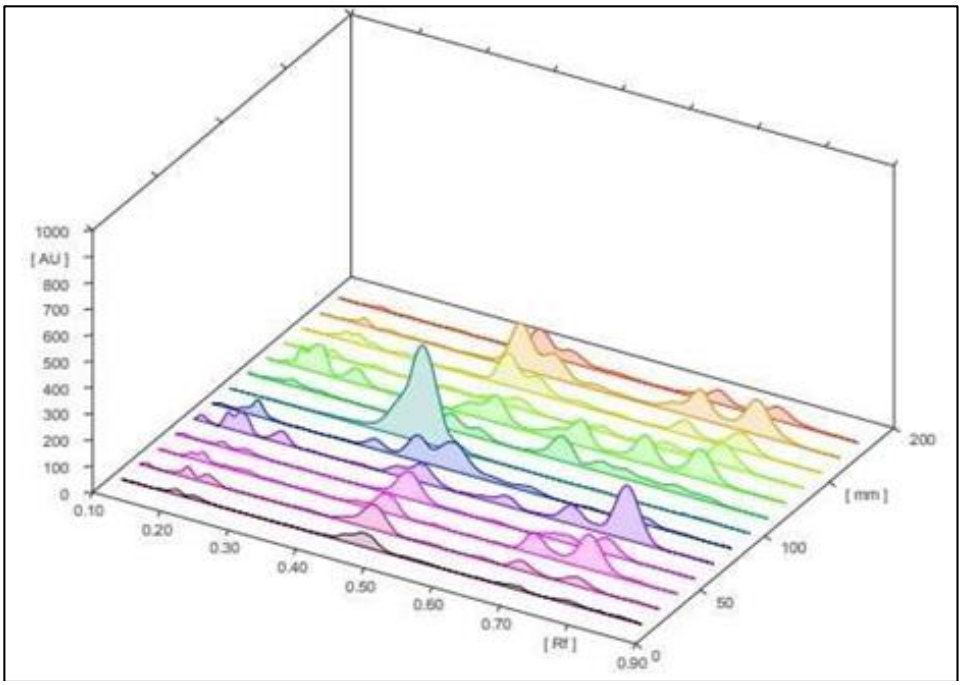

Fig 1.3: Typical chromatogram, Track I and II: *Callistemon citrinus*, Track III: *Syzygium jambos*, Track IV: *Syzygium lanceolatum*, Track V: Eucalyptus *globulus*, Track VI: *Pimenta dioica*, Track VII: Ursolic Acid, VIII: *Melaluca leucodendron*, *IX: Psidium guajava*

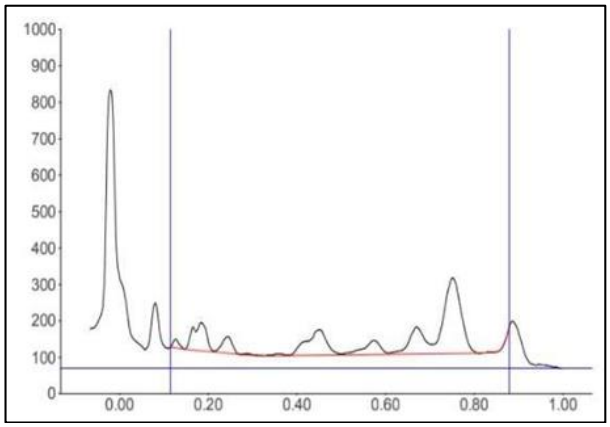

Fig 1.4: Typical chromatogram of *Pimenta dioica*

Table 3: Biochemical Affinity between the species of family Myrtaceae

Plants	*Callistemon citrinus*	*Syzygium jambos*	*Syzygium lanceolatum*	*Eucalyptus globulus*	*Pimenta dioica*	*Melaluca leucadendron*	*Psidium guajava*
Callistemon citrinus	-	23.07%	25%	30.76%	30.76%	23.07%	28.57%
Syzygium jambos	23.07%	-	23.07%	28.57%	35.71%	42.85%	33.33%
Syzygium lanceolatum	25%	23.07%	-	23.07%	30.76%	46.15%	28.57%
Eucalyptus globulus	30.76%	28.57%	23.07%	-	35.71%	35.71%	33.33%
Pimenta dioica	30.76%	35.71%	38.46%	35.71%	-	42.85%	33.33%
Melaluca leucadendron	23.07%	42.85%	30.76%	35.71%	42.85%	-	40%
Psidium guajava	28.57%	33.33%	28.57%	33.33%	33.33%	40%	-

Higher PA value was believed as an indication of close affinity between different species. PA value of 46 % was considered as marker of close relationship. The PA value revels the relationship of the species from same family. Highest PA value 46.15% was found between *Syzygium jambos* and *Melaluca leucadendron* and lowest value 23.07% was found between *Callistemon citrinus* and *Melaluca lucodendron* followed by *Syzygium jambos* and *Callistemon citrinus* etc (The detail values of pairing affinity is shown in table 4). These values predict the close relationship of different species of same family. The relationship was study by preparing the dendrogram using the values of paring affinity. The dendrogram was prepared using UPGMA Software.

With reference to the result obtained by calculating the affinities among the members of same family the dendrogram here represents the close relationship of both *Syzygium* species followed by *Callistemon citrinus* and *Melaleuca leucadendron* and the other species. Scope for the extended studies on dendrogram can be attained to study the relation between same family with different chemical composition.

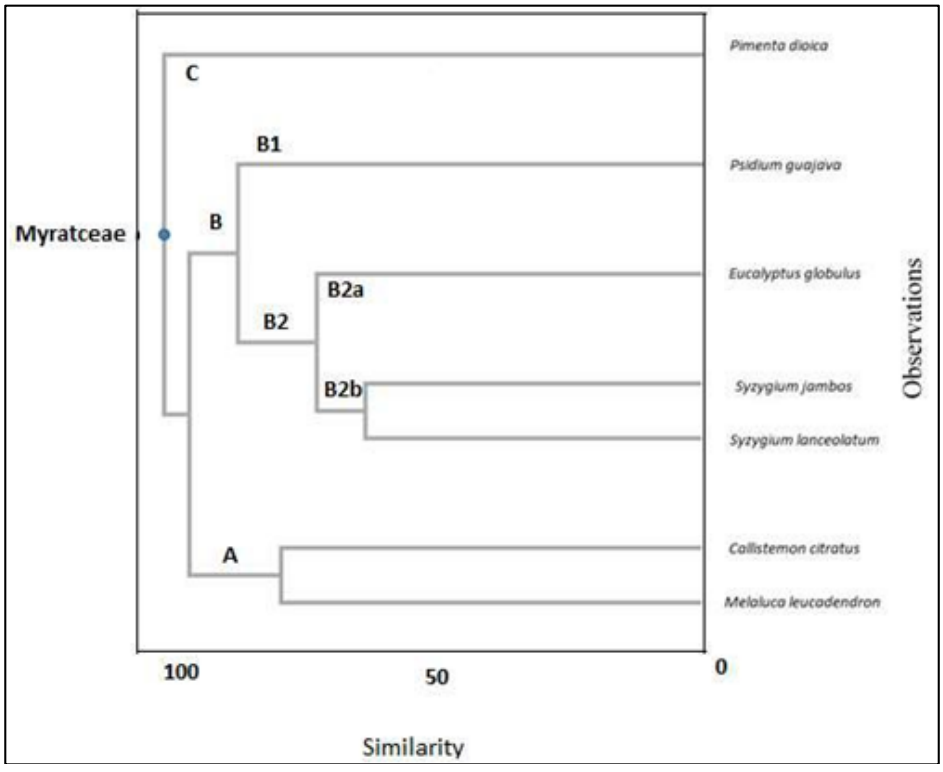

Fig 1.5: Affinity relationships amongst different members of Myrtaceae

The Dendrogram analysis of 7 plants considering their biochemical affinities, represents three major clusters (A, B and C), *Pimenta dioica* being a separate cluster included C, all other species are collected in cluster A and B, cluster A represents closely linked *Callistemon citrinus* and *Melaluca lecodendron*, while the cluster B is again branched into B1 and B2, branch B1 represents *Psidium guajava*. Branch B2 is again differentiated into cluster B2a representing *Eucalyptus globulus*, while B2b shows a close connection between *Syzygium jambos* and *Syzygium lanceolatum.*

An endeavor to construct a dendrogram, based on pairing affinity between the species of same family was executed using UPGMA online software. This software allows to analyze similarity matrix by using 3 different statistical method. That is Pearson's coefficient correlations, Jaccarad similarity coefficient and Dice coefficient. The Pearson's coefficient measures the linear correlation between two sets of variables and gives value between +1 and -1 inclusive, where 1 corresponds to an ideal positive correlation. Whereas, Jaccarad similarity coefficient also noted as Tanimoto coefficient, that measures the similarity between two sets of binary data. It is defined as the size of the intersection divided by the size of the union of the sample sets gives a value between 0 and 1. Lastly, Dice coefficient which measures the similarity between two sets of binary data and it also ranges from 0 to 1, moreover like Jaccard coefficient but gives twice the weight to agreements. For current study, Pearson's coefficient statistical method was used to find the similarities between the species, where coefficient of correlation is the degree of which two variables are inter- related.

Discussion & Conclusion

Based on above studies, it can be culminated that chemotaxonomy plays a vital role in relating the plant with each other though they belong to same family and same order the chemical composition of the plants is different from each other. The well-known effective constituent in family Myrtaceae is eugenol mentioned in other literature but from the above studies it is infer that a terpenoidal compound ursolic acid possibly be a potential biochemical marker for phylogenetic studies in family Myrtaceae. the dendrogram was prepared from the biochemical affinities introduced in the table (3) of seven Myrtaceae members the result divulge many species linked together, The Dendrogram analysis of 7 plants considering their biochemical affinities, represents three major clusters (A, B and C), *Pimenta dioica* being a separate cluster included C, other species are collected in cluster A & B, cluster A represents closely linked *Callistemon citrinus* and *Melaluca lecodendron*, while the cluster B is again branched into B1 and B2, branch B1 represents Psidium guajava. Branch B2 is again differentiated into cluster B2a representing *Eucalyptus globulus*, while B2b shows a close connection between *Syzygium jambos* and *Syzygium lanceolatum.*

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