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Metabolic profiling and discrimination of *Cymbopogon* species using direct analysis real time mass spectrometry and principal component analysis

Pratibha Singh, Vikas Bajpai, Sunil Kumar, Mukesh Srivastava and Brijesh Kumar

Abstract
Several *Cymbopogon* species such as *C. citratus, C. flexuosus, C. nardus* and *C. khasianus*×*C. pendulus* are extensively used for flavors, fragrance and as folk medicines worldwide due to their volatile essential oils (VEOs). Direct analysis in real time mass spectrometry (DART-MS) method was developed for identification of VEOs from the intact plant parts of ten *Cymbopogon* species. Total sixteen compounds including VEOs, phenolics and flavonoids were tentatively identified on the basis of their exact mass measurement and molecular formula using DART-MS chemical fingerprint of *C. citratus, C. citronella, C. flexuosus, C. pendulus, C. commutatus, Cymbopogon. jwarancusa, C. nardus, C. khasianus, C. jwarancusa*× *C. nardus* and *C. khasianus*× *C. pendulus*. DART-MS data was analyzed by principal component analysis and 19 marker peaks were identified which can discriminate among these selected *Cymbopogon* species. *C. citratus, C. jwarancusa, C. commutatus, C. khasianus* and *C. jwarancusa*×*C. nardus* were approximately overlapping each other while *C. flexuosus, C. nardus* and *C. khasianus*×*C. pendulus* were closer to each other whereas *C. pendulus* and *C. citronella* were much apart from all the species. The identified marker peaks could be used for authentication, discrimination and quality control of *Cymbopogon* species.

Keywords: DART-MS, Volatile essential oils, *Cymbopogon* species, PCA

1. Introduction
Genus *Cymbopogon* (Family; Poaceae) is well known tropical perennial shrub [1-2]. The plants of this family are distributed worldwide [1-2]. *Cymbopogon* species has been reported to possess various pharmacological activities such as analgesic [2], antibacterial [3], anticarcinogenic [3], cardioprotective [3], antifungal [3], anti-inflammatory [4], antileishmanial [5], antioxidant [6], antiprotozoal [7], antipyretic [8], antirheumatic [9], antitrypanosomal [10], antiseptic [11], antispasmodic [12], antitussive [12] and antiviral [13] activities. They also possess some pharmacological properties such as diuretic and sedative [14]. *C. citratus* belonging to the genus *Cymbopogon* has traditionally been used for the treatment of anxiety, diabetes, dyslipidemia, fever, flu, gastrointestinal disturbances, malaria, and pneumonia [15]. It has also been used to inhibit platelet aggregation [16]. Phytochemicals such as volatile essential oils (VEOs), tannins, saponins, flavonoids, alkaloid and terpenoids are reported in *Cymbopogon* species [17]. The essential oil, Cryptomeridiol shows antispasmodic activity [18]. Myrcene, citral, cis-linalool oxide and cryptomeridiol are the major commercially available component of VEOs from *Cymbopogon* species (commonly from *Cymbopogon citratus, Cymbopogon flexuosus, Cymbopogon pendulus, Cymbopogon commutatus, Cymbopogon jwarancusa, Cymbopogon nardus, Cymbopogon khasianus, Cymbopogon jwarancusa*× *Cymbopogon nardus* and *Cymbopogon khasianus*× *Cymbopogon pendulus*) and are used as fragrance and flavour in pharmaceutical industries [19], aromatherapy [20] and chemotherapy [21].
Various analytical methods such as electrospray ionization mass spectrometry (ESI-MS) [22], fourier transform infra-red spectroscopy (FTIR) [23], nuclear magnetic resonance (NMR) [24] were used to analyze the cryptomeridiol a major component of VEOs of *Cymbopogon* species. The other essential oils component such as citronellol and citral were also studied by high performance thin layer chromatography (HPTLC) [25] and quantified by using a high performance liquid chromatography (HPLC) method [26] in *Cymbopogon citratus.*

Due to the volatile nature, the analysis of essential oils from *Cymbopogon* species is mainly reported by gas chromatography-mass spectrometry (GC-MS).[27] However, the HPLC ESI-MS based identification and characterization of flavonoids in *C. citratus* leaves has been also reported.[22,28] These methods are tedious and need time-consuming sample preparation and chromatographic separation steps.

Direct analysis in real time (DART) mass spectrometry is an ionization technique which ionizes samples under ambient conditions [29]. It can ionize solid, liquid and gas samples directly without any samples preparation [30]. This technique has been successfully used for identification of pesticides, explosives on solid surfaces and in liquids, chemical warfare agents in solvents, food packaging additives, flavored contents of food, contaminant in soil, cocaine in urine with vast range of applications in forensics [31, 32]. Recently DART-MS followed by multivariate analysis were also used for discrimination of plant species [33-37] and cultivars [38], authentication of animal fats [39] detection of adulteration and geographical variation [39-40].

This manuscript aimed to develop an efficient DART MS method for identification of VEOs from *C. citratus, C. citronella, C. flexuosus, C. pendulus, C. commutatus, C. jwarancusa, C. nardus, C. khasianus, C. jwarancusa*×*C. nardus* and *C. khasianus*×*C. pendulus*. The PCA was used to discriminate the selected *Cymbopogon* species and identification of marker peaks which can be used for authentication and quality control of this plant.

2. Materials and Methods

2.1 Materials

The leaves of *C. citratus, C. citronella, C. flexuosus, C. pendulus, C. commutatus, C. jwarancusa, C. nardus, C. khasianus, C. jwarancusa*×*C. nardus* and *C. khasianus*×*C. pendulus* were collected in June 2015 from CSIR-India Institute of Integrated Medicine (CSIR-IIIM), Jammu. Voucher specimens of *C. citratus*-RRL-52923, *C. citronella*-RRL-52926, *C. flexuosus*-RRL-52925, *C. pendulus*- RRL-52932, *C. commutatus*- RRL-52919, *C. jwarancusa*-RRL-52927, *C. nardus*-RRL-52924, *C. khasianus*-RRL-52930, *C. jwarancusa*×*C. nardus*-RRL-52931 and *C. khasianus*×*C. pendulus*-RRL-52928 were deposited in medicinal plant herbarium of CSIR-IIIM, Jammu. Standard samples of *p*-coumaric acid, Kaempferol, Cholorogenic acid and Orientin were purchased from Sigma Aldrich. The leaf samples were thoroughly washed with tap water followed by distilled water to remove foreign particles from its surface and dried at room temperature (approximately 26-28°C).

2.2 DART MS operating parameters

The mass spectrometer used was a JMS-T100LC, Accu TOF atmospheric pressure ionization time-of-flight mass spectrometer (Jeol, Tokyo, Japan) fitted with a DART ion source. The mass spectrometer was operated in positive-ion mode with a resolving power of 6000 (full-width at half-maxima). The orifice 1 potential was set to 28 V, ring lens and orifice 2 potentials were set to 13 and 5 V, respectively. Orifice 1 was set at 100°C and RF ion guide potential at 300 V. The DART ion source was operated with helium gas flowing at approximately 4.0 L/min and gas heater was set at 300°C. The potential on the discharge needle electrode of the DART source was set to 3000 V, electrode 1 at 100 V and the grid at 250 V. Data acquisition was from *m/z* 50 to 1050. All the leaf samples were analyzed in 15 repeats to check the reproducibility of spectra. Mass calibration was accomplished by including a mass spectrum of neat polyethylene glycol (PEG) (mixture of PEG 200 and PEG 400) in the data file. The mass calibration was accurate to within ±0.002 u. Using the Mass Centre software, the elemental composition were determined on selected peaks.

2.3 Statistical analysis

Principal component analysis (PCA) was performed with the STATISTICA software, windows version 7.0 (Stat Soft, Inc., USA). Data for PCA analysis was extracted from DART-MS spectra of fifteen repeats of each sample. All ions having ≥5% peak intensity were selected for statistical analysis.

3. Results and Discussion

3.1 Screening of phytochemicals in *Cymbopogon* species

Comparative DART-MS fingerprint spectra of the *Cymbopogon* species are shown in Fig. 1. All spectra showed common peaks (*m/z*) with different relative abundance. Sixteen phytochemicals were tentatively identified based on their exact mass, molecular formula and literature reports [27, 41-42] as shown in (Table 1) and structure of all the identified phytochemicals are given in Fig 2. The peaks at *m/z* 137.1337 ($C_{10}H_{16}$), 153.1263 ($C_{10}H_{16}O$), 157.1596 ($C_{10}H_{20}O$), 170.1374 ($C_{10}H_{18}O_2$) and 205.1957 ($C_{15}H_{24}$) were identified as myrcene (2), citral (3), citronellol (5), cis-linalool oxide (7) and γ-1-cadinene (9) respectively. All the identified compounds were again confirmed by their exact mass and fragmentation patterns using HPLC-ESI-QTOF-MS/MS study. Fragmentation patterns of compounds *p*-coumaric acid (6), Cis-Linalool oxide (7), Caffeic acid (8), Caryophyllene oxide (10), Cryptomeridiol (12), Kaempferol (13), Cymbodiacetal (14) Cholorogenic acid (15) and Orientin (16) are also compared from literature and/or standard compounds for authentication Fig. S1 A and B, Figure. S2 and Table S1 and S2. Details of experimental method and figures are provided in supplementary.

3.2 Comparison of DART-MS fingerprint of *Cymbopogon* species

All these VEOs were detected in relatively high abundance in the leaves of *Cymbopogon* species. Myrcene (2) *m/z* 137.1337 ($C_{10}H_{16}$) was detected in high abundance in *C. citratus, C. citronella, C. commutatus, C. jwarancusa, C. nardus, C. khasianus* and *C. jwarancusa*×*C. nardus* while citral (3) *m/z* 153.1263 ($C_{10}H_{16}O$) was found abundant in all the species of *Cymbopogon* except *C. citronella*. Similarly, citronellol (5) *m/z* 157.1596 ($C_{10}H_{20}O$) was identified in high abundance in the leaves of *C. citratus, C. citronella* and *C. flexuosus* while γ-1-cadinene (9) *m/z* 205.1957 ($C_{15}H_{24}$) was detected high abundance in the leaves of *C. citratus, C. citronella* and *C. jwarancusa*×*C. nardus*. The peak at *m/z* 223.2068 ($C_{15}H_{26}O$) identified as β-eudesmol (11) was found relatively high in *C. citratus* followed by *C. commutatus* and *C. khasianus*×*C. pendulus*. The peak at *m/z* 241.2172 ($C_{15}H_{28}O_2$), identified as cryptomeridiol (12), was found relatively in low abundance in *C. citratus* followed by *C. commutatus* and *C. khasianus*×*C. pendulus*. The peak at *m/z* 449.1088 ($C_{21}H_{20}O_{11}$) was identified as Orientin (16) with high intensity in *C. citratus, C. citronella, C. pendulus* and *C. commutatus*. All the identified phytochemicals were present in *C. citratus* except *p*-coumaric acid (6) at *m/z* 165.0561 ($C_9H_8O_3$) and citral (3) at *m/z* 153.1263 ($C_{10}H_{16}O$) was detected in all the *Cymbopogon* species except *C. citronella* Table 1.

The DART MS spectra revealed the variation in the relative intensities of some of the most common essential oils in the

leaves of studied species Fig. 3. It was obtained as the ratio of the expression of the peak to the sum of all the expressions within the spectra ranging from *m/z* 95-550 as shown in Fig. 1. All the ions with a relative intensity above 5% were taken and compared on the basis of these relative intensities. Fifteen repeats were carried out for each sample and the averaged result was utilized for analysis. The results indicated significant variations of bioactive compounds among the leaves of all the ten *Cymbopogon* species Fig. 3. Approximately similar relative content of myrcene (2) at *m/z* 137.1337 ($C_{10}H_{16}$) was detected in *C. khasianus* and *C. jwarancusa*×*C. nardus* followed by *C. commutatus* and *C. citronella* while it was found in relatively low abundance in *C. jwarancusa* and *C. nardus.* Similarly, citral (3) at *m/z* 153.1263 ($C_{10}H_{16}O$) was detected approximately in same abundance in *C. khasianus*×*C. pendulus*, *C. citratus, C. flexuosus, C. pendulus, C. jwarancusa* and *C. nardus* while it was detected relatively in low abundance in *C. khasianus* and *C. jwarancusa*×*C. nardus*. Whereas cis-linalool oxide (7) at *m/z* 170.1374 ($C_{10}H_{18}O_2$) was abundant in *C. citronella, C. pendulus, C. jwarancusa* and *C. citratus.* γ-1-cadinene (9) at *m/z* 205.1957 ($C_{15}H_{24}$) and orientin (16) at *m/z* 449.1088 ($C_{21}H_{20}O_{11}$) were abundant in *C. citronella* and *C. commutatus* respectively.

3.3 Discrimination of *Cymbopogon* species using principle component analysis

PCA is an unsupervised procedure that determines the directions of the largest variations in the data set and the data are generally presented as a two dimensional plot (score plot) where the coordinate axis represents the directions of the two largest variations [33-34]. DART-MS data combined with principal component analysis (PCA) served as an efficient and powerful tool to identify the chemical markers and to discriminate among *Cymbopogon* species [33-34]. The DART-MS data from fifteen repeats of each species (*C. citratus, C. commutatus, C. flexceosus, C. pendulus, C. jwarancusa, C. citronella, C. nardus, C. khasianus, C. jwarancusa*×*C. nardus* and *C. khasianus*×*C. pendulus*) were subjected to PCA.

The first two principal components PC1 and PC2 hold 31.76% and 30.12% respectively of the total variability. Thus, the PCs were able to explain 61.88% of the total variability. To obtain the best expression some peaks having low scores were dropped to get the best possible results. Finally, the first two principal components PC1 and PC2 hold 32.48% and 31.65% respectively of the total variability on the basis of 19 peaks at *m/z* 95.1022, 123.1415, 135.1439, 151.1754, 153.1263 (Citral), 155.1407 (citronellal), 157.1596 (citronellol), 169.1555, 170.1374 (cis-Linalool oxide), 183.1255, 200.1608, 205.1957 (γ-1-Cadinene), 221.1965 (Caryophyllene oxide), 228.2416, 237.2230, 305.3056, 312.3203, 409.4548 and 449.1088 (Orientin) Fig. 4A. Out of 36 peaks only 19 peaks showed 64.13% total variance. Peak at *m/z* 95.1022 (32.48%) gave a higher contribution for discrimination followed by peak at *m/z* 123.1415 (31.65%).

The PCA discriminated all the ten *Cymbopogon* species in to four categories. *C. citronella* and *C. pendulus* were standing isolated in the bi-plot which indicated these two species have entirely different pattern than the rest species studied. The remaining eight species were further divided in two sub groups. In the first sub group the species were *C. nardus, C. flexuosus* and *C. khasianus*×*C. pendulus,* this sub groups has peaks at *m/z* 95.1022, 135.1439, 153.0757 and 305.3056 which were detected in all the three species of the group.

In the second sub group there were five species namely *C. khasianus, C. jwarancusa*×*C. nardus, C. citratus, C. jwarancusa* and *C. commutatus* which have close similarities. This group was dominated by peaks at *m/z* 183.1255 and 200.1608 which was detected in all the five species except *C. jwarancusa*×*C. nardus* in which peak at *m/z* 200.1608 was not detected.

The major difference in these two sub groups was presence and absence of peaks at *m/z* 95.1022, 153.1263 and 305.3056. The species *C. citronella* was dominated by the high intensity of peak at *m/z* 205.1957 which was not detected in *C. pendulus.* The abundance of myrcene (*m/z* 137.1337) and citral (*m/z* 153.1263) were detected approximately five and two fold higher in *C. jwarancusa*×*C. nardus* and *C. khasianas*×*C. pendulus* compared to their parents, *C. jwarancusa, C. nardus* and *C. khasianus, C. pendulus* respectively. Hence these hybrid species may be selected for the isolation of myrecene (2) and citral (3) respectively. It is evident from this study that PCA effectively served the desired purpose.

3.4 Comparative chemical fingerprint of *C. jwarancusa, C. nardus* and *C. jwarancusa* × *C. nardus*

Out of 36 studied peaks, 22 peaks were present in either *C. jwarancusa* and *C. nardus* or its hybrid (*C. jwarancusa*×*C. nardus*). The distributions of these peaks are shown by the help of Venn diagram in Fig. 5A. Seven peaks were detected (at *m/z* 95.1022, 135.1439, 167.1392, 169.1555, 284.2937, 287.2944 and 305.3056) in *C. nardus* whereas two peaks (at *m/z* 110.0992 and 200.1608) were detected only in *C. jwarancusa* while only one peak at *m/z* 170.1374 was detected in both the species but absent in *C. jwarancusa*×*C. nardus*. Total of twelve abundant peaks (at *m/z* 127.1131, 137.1337, 153.1263, 154.1919, 183.1172, 205.1957, 273.3163, 279.2169, 393.5278, 409.4548, 459.5511 and 503.1269) were detected in hybrid. Out of twelve peaks only 6 peaks (at *m/z* 183.1172, 279.2169, 393.5278, 409.4548, 459.5511 and 503.1269) were common with *C. jwarancusa* and *C. jwarancusa*×*C. nardus*. Only two peaks (at *m/z* 137.1337 and 153.1263) were detected in all the three species. The remaining four peaks (at *m/z* 127.1131, 154.1919, 205.1957 and 273.3163) were detected only in *C. jwarancusa*×*C. nardus* which were completely absent in *C. jwarancusa* and *C. nardus.*

3.5 Comparative Chemical fingerprint of *C. khasianus, C. pendulus* and *C. khasianus* × *C. pendulus*

The DART-MS analysis of *C. khasianus, C. pendulus* and its hybrid (*C. khasianus*×*C. pendulus*) produced total 22 peaks. The distributions of these peaks are shown by the help of Venn diagram in Fig. 5 B. Four peaks (at *m/z* 137.1337, 273.363, 296.1506 and 445.3061) were detected only in *C. khasianus*. Six peaks (at *m/z* 123.1415, 151.1754, 228.2416, 312.3203, 409.4548 and 449.1088) were detected only in *C. pendulus* while only two peaks (at *m/z* 183.1172 and 200.1608) were detected in both the species but absent in *C. khasianus*×*C. pendulus.* Total ten abundant peaks (at *m/z* 95.1022, 104.1123, 110.0992, 135.1439, 153.1263, 170.1374, 279.2169, 305.3056, 393.5278 and 503.1269) were detected in *C. khasianus*×*C. pendulus.* Out of these ten peaks only two peaks (at *m/z* 135.1439 and 170.1374) were common with *C. pendulus* and three peaks (at *m/z* 110.0992, 153.1263 and 279.2169) were detected in all the three species. The remaining five peaks (at *m/z* 95.1022, 104.1123, 305.3056, 393.393.5278 and 503.1269) were detected in the *C. khasianus*×*C. pendulus* but completely absent in *C. khasianus*

and *C. pendulus*. There was no common peak in *C. khasianus* and *C. khasianus*×*C. pendulus* individually.

Only two common peaks (at *m/z* 137.1337 and 153.1263) were detected in *C. jwarancusa*, *C. nardus* and *C. jwarancusa*×*C. nardus.* Similarly three common peaks (at *m/z* 110.0992, 153.0757 and 279.2169) were identified in *C. khasianus*, *C. pendulus* and *C. khasianus*×*C. pendulus*.

4. Conclusions

The volatile essential oils were successfully analyzed and identified in ten *Cymbopogon* species by DART-MS analysis. The 16 bioactive compounds including eleven VEOs components such as 6-Methylhept-5-en-2-one (1), myrcene (2), citral (3), citronellal (4), citronellol (5), cis-Linalool oxide (7), γ-1-Cadinene (9), caryophyllene oxide (10), *β*-Eudesmol (11), cryptomeridiol (12) and cymbodiacetal (14), three phenolics such as *p*-coumaric acid (6), caffeic acid (8) and chlorogenic acid (15) and two flavonoids, kaempferol (13) and orientin (16) were successfully identified in the *Cymbopogon* species. Present study provided information which may help in selection of *Cymbopogon* species on the basis of their relative abundance of bioactive or commercially useful VEOs components such as myrcene (2), citral (3), citronellal (4), citronellol (5). DART-MS followed by PCA showed the similarity and dissimilarity among the *Cymbopogon* species (*C. citratus, C. citronella, C. flexuosus, C. pendulus C. commutatus, C. nardus, C. jwarancusa, C. khasianus, C. jwarancusa*×*C. nardus* and *C. khasianus*×*C. pendulus)*. This is first study by DART-MS for identification of volatile essential oils and discrimination of *Cymbopogon* species by PCA.

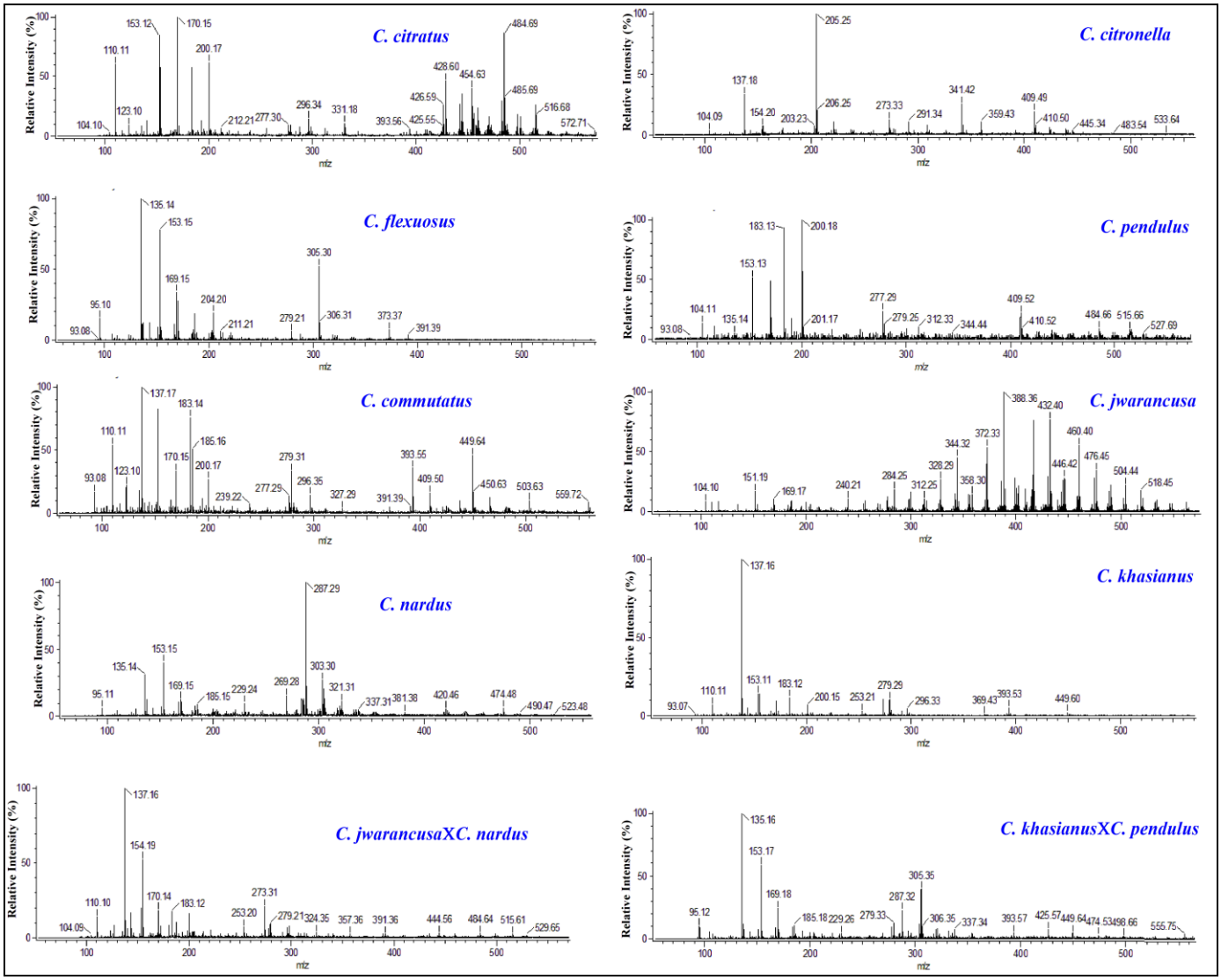

Fig 1: DART-MS fingerprint of ten *Cymbopogon* species (*C. citratus, C. citronella, C. flexuosus, C. pendulus, C. commutatus, C. jwarancusa, C. nardus, C. khasianus, C. jwarancusa*×*C. nardus* and *C. khasianus*×*C. pendulus).*

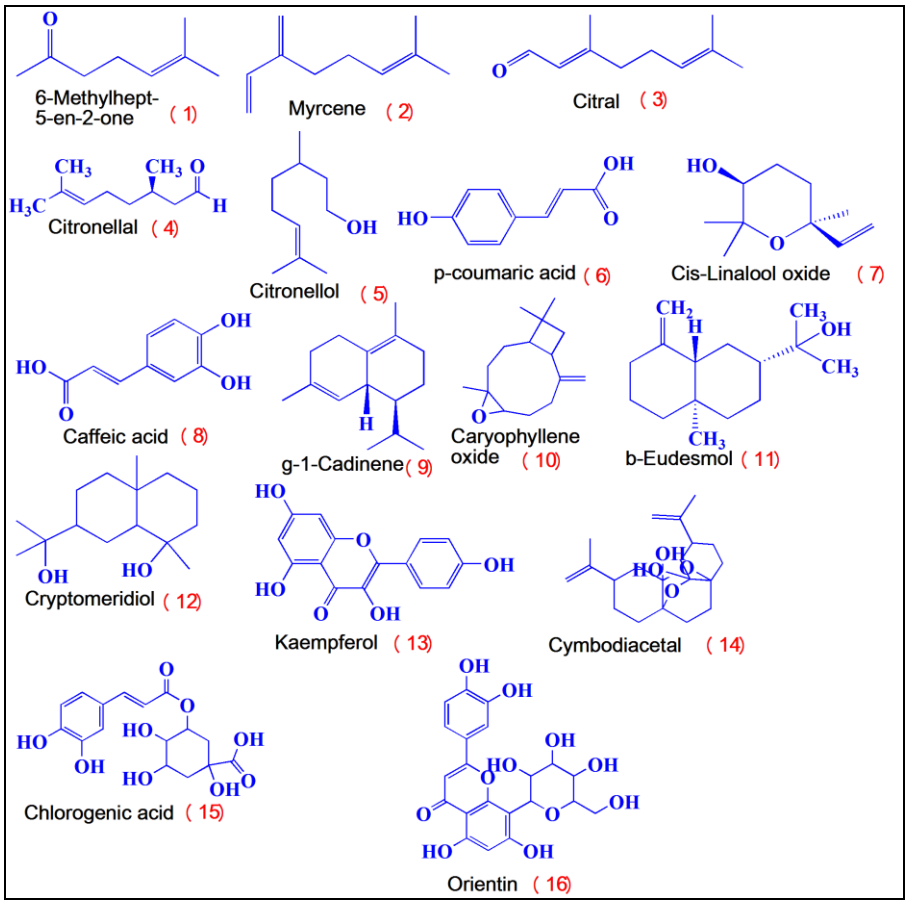

Fig 2: Chemical structures of identified compounds (1-16) in *Cymbopogon* species

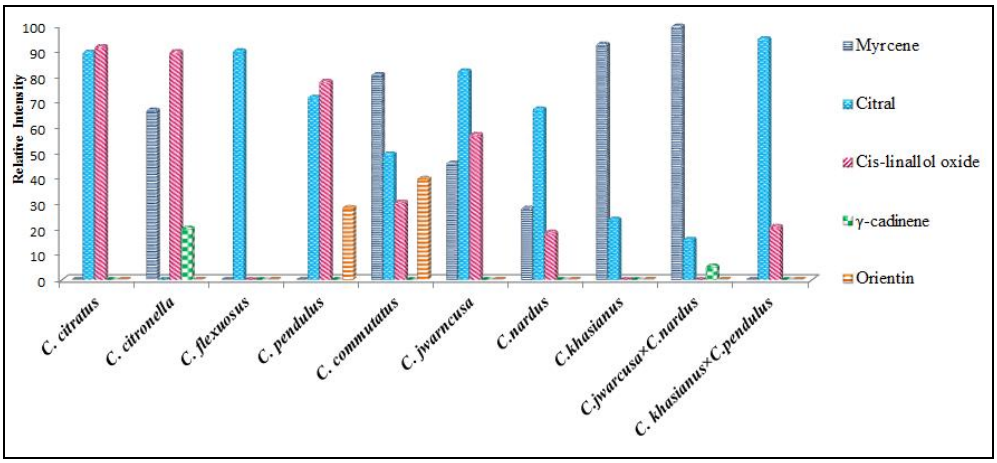

Fig 3: Relative intensity of bioactive compounds in *Cymbopogon* species.

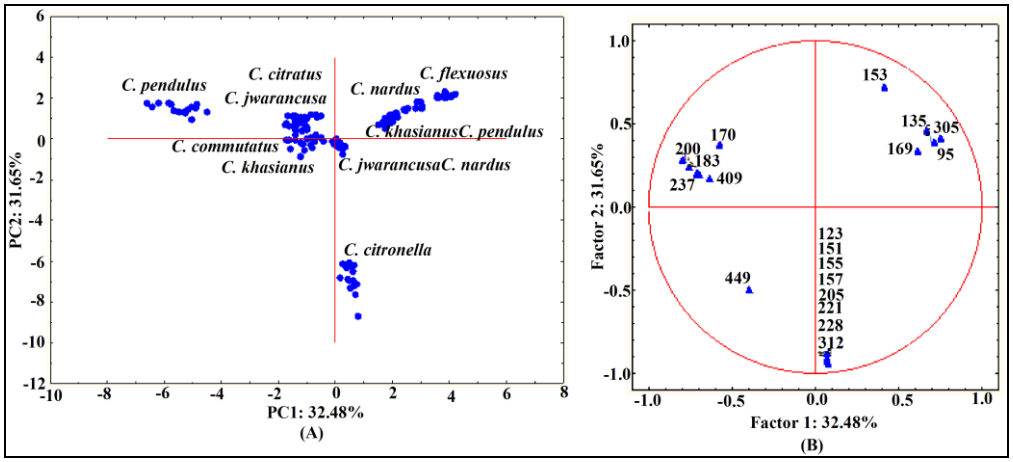

Fig. 4: (A) PC1 *vs* PC2 plot showing discrimination among the leaves of *Cymbopogon* species. (B) PC1 *vs* PC2 score plot showing loading of variables.

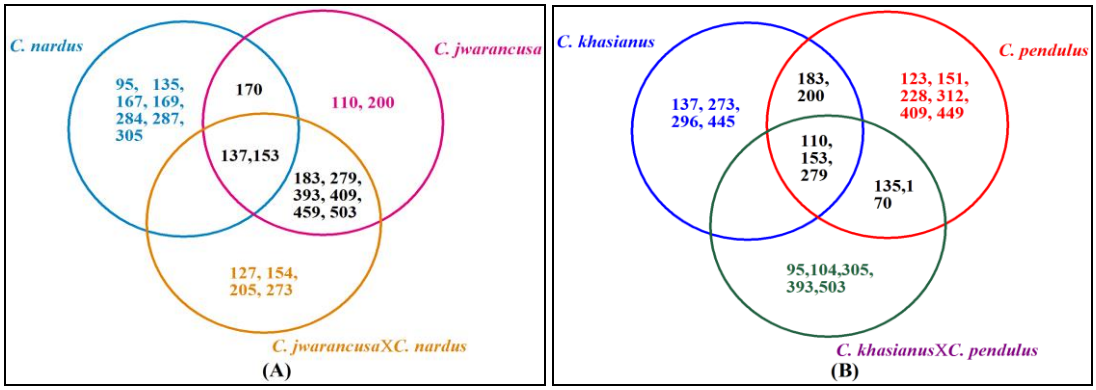

Fig. 5: (A) Distribution of peaks (*m/z*) among the *C. jwarancusa*, *C. nardus and C. jwarancusa*×*C. nardus* (A) and *C. khasianus*, *C. pendulus and C. khasianus*×*C. pendulus* (B).

Table 1: DART-MS mass measurements of phytochemicals in leaves of *Cymbopogon* species

S. No	Compounds name	Calculated mass (*m/z*)	Measured Mass (*m/z*)	Molecular formula	Error (Δmmu)	Distribution									
						Cc	Cct	Cf	Cp	Ccm	Cj	Cn	Ck	Cj×n	Ck×p
	6-Methylhept-5-en-2-one	127.1123	127.1131	$C_8H_{14}O$	0.81	+	-	-	-	-	-	-	-	+	-
	Myrcene	137.1330	137.1337	$C_{10}H_{16}$	-0.71	+	+	-	-	+	+	+	+	+	-
	Citral	153.1279	153.1263	$C_{10}H_{16}O$	0.61	+	-	+	+	+	+	+	+	+	+
	Citronellal	155.1436	155.1407	$C_{10}H_{18}O$	-2.90	+	+	-	-	-	-	-	-	-	-
	Citronellol	157.1592	157.1596	$C_{10}H_{20}O$	-0.40	+	+	+	-	-	-	-	-	-	-
	p-Coumaric acid	165.0552	165.0561	$C_9H_8O_3$	0.91	-	-	+	-	-	-	+	-	-	-
	cis-Linalool oxide	170.1385	170.1374	$C_{10}H_{18}O_2$	1.08	+	-	-	+	+	+	+	-	-	+
	Caffeic acid	181.0501	181.0509	$C_9H_8O_4$	-0.61	+	-	-	-	-	-	-	-	-	-
	γ-1-Cadinene	205.1956	205.1957	$C_{15}H_{24}$	1.37	+	+	-	-	-	-	-	-	+	-
	Caryophyllene oxide	221.1905	221.1965	$C_{15}H_{24}O$	3.20	+	+	-	-	-	-	-	-	-	-
	β-Eudesmol	223.2062	223.2068	$C_{15}H_{26}O$	-1.81	+	-	-	-	+	-	-	-	-	+
	Cryptomeridiol	241.2168	241.2172	$C_{15}H_{28}O_2$	3.69	+	-	-	-	+	-	-	-	-	+
	Kaempferol	287.0556	287.0575	$C_{15}H_{10}O_6$	-1.27	+	-	-	-	-	-	+	-	-	-
	Cymbodiacetal	335.2222	335.2226	$C_{20}H_{30}O_4$	-0.40	+	-	-	-	+	-	-	-	-	-
	Chlorogenic acid	355.1029	355.1028	$C_{16}H_{18}O_9$	0.25	+	-	-	-	+	+	-	-	-	+
	Orientin	449.1084	449.1088	$C_{21}H_{20}O_{11}$	1.10	+	+	-	+	+	+	-	-	-	-

[a](+): detected, (-): not detected, Cc: *C. citratus*, Ct: *C. citronella*, Cf: *C. flexuosus*, Cp: *C. pendulus*, Ccm: *C. commutatus*, Cj: *C. jwarancusa*, Cn: *C. nardus*, Ck: *C. khasianus*, Cj×n: *C. jwarancusa*×*C. nardus* and Ck×p: *C. khasianus*×*C. pendulus*

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