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Biochemical profiling of aroma and flavonoid compounds in certain genotypes of pummelo

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ABSTRACT

Pummelo fruit volatile compounds are mainly comprised of esters, alcohols, aldehydes, ketones, lactones, hydrocarbons, terpenoids and acids. A total of 50 volatile aroma compounds were identified in pummelo juice which includes 9 hydrocarbons, 10 monoterpenoids, 20 sesquiterpenoids, 9 alcohols, 2 aldehydes and ketones. Limonene was the most abundant compound, representing 0.750 to 49.331 per cent of the total aroma volatiles identified in the juice of pummelo. Among flavonoids 12 compounds identified, Naringenin was found to be high followed by catechin and other compounds. Naringenin levels play an important role in determining the bitterness of pummelo and limonene contributes for the pleasant orange flavour of the juice. Among all the accessions observed, the pink pulp accession 22(4) had high amount of naringenin (601.66 ng/ml) and the low level (100.02ng/ml) was recorded in white pulp accession 20(1) which may be useful as a parent for development of less bitter pummelo.

Key words*: Citrus maxima,* volatile compounds, flavonoids, GC-MS, LC-MS.

INTRODUCTION

Citrus maxima Merr. belonging to the *Rutaceae* family, commonly known as 'Pummelo'. It was reported to grow wild on river banks of Fiji and other islands. It was introduced to China around 100 B.C and widely cultivated in southern China, Japan, southern Thailand, Taiwan, India, Indonesia and Malaysia. *C. maxima* fruit has faint aroma, sour and sweet flavour, high nutritional and medicinal value. The fruit pulp is used as an appetizer and also responsible for antitoxic, cardiac stimulant, stomach tonic properties; which have been reported in ancient and medieval literature (Arias and Ramon, 2). Citrus fruits and their products provide a number of nutrients such as ascorbic acid, citric acid, folic acid, vitamin B_6 and flavonoids **(**Arayjo, 1).

Flavonoids are found throughout all fruits and best known flavonoidss are quercetin and kaempferol. If flavonoids occur in high concentrations or form molecules with metal ions, they can contribute to the plant tissue colour. Flavones and flavonols have also been shown to be antioxidants and free radical scavengers. It has been proposed that the expressions of genes and activities of enzymes related to flavonoid biosynthesis and metabolism can be regulated and altered in different cultivation regions under different temperatures, precipitations, and sunshine exposures, which eventually impact the accumulation of the flavonoids in fruits (Majo *et al*., 10). Flavonoids are the major group of phenolic compounds found in citrus fruits. Flavonoids can be classified as flavanones, flavones and flavonols that occur either in the free form and or as glycosides in various parts of citrus fruits. Most of these compounds cannot be found commercially and their synthesis is very costly due to complex structural characteristics **(**Ooghe and Detavernier, 15).

Moreover, the natural variability in phenolic composition of citrus fruits and products not only can be ascribed to genetic characteristics, but also to factors such as stages of maturity, climatic conditions or post-harvest processing **(**Vanamala *et al.,* 18, Galaverna and Dall, 6). The genetic characteristics of citrus varieties, thus mainly influence the micro constituents and consequently the phenolic and flavonoid content and composition **(**Mouly *et al.,* 12, Marini and Balestrieri, 11, Ooghe and Detavernier, 15). The objectives of the present research were to determine various aroma volatile compounds, and flavonoids from eleven pummelo accessions.

MATERIALS AND METHODS

Eleven accessions of pummelo (Accession -8 (4), Accession - 19(1), Accession -10(5), Accession - 19(4), Accession - 18(1), Accession - 20(1), Accession - 18(3), Accession-22(4), Accession-18(5), Accession -24(1) and Accession - 25(5)) were collected from the field gene bank maintained at ICAR-IIHR, Bangalore. The present investigation includes biochemical analysis of pummelo having the characteristic features such as flavonoids and strong aroma. 50 gm of sample was homogenized and kept

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the sample for 3 min in room temperature, after that 0.5 g of solid NaCl was added to inhibit further enzyme activity and to increase the releasing rate of the analyzes in to the head space. Then the sample was kept for 20 minutes with stirring continuously to increased the transferred analytes and then allowed to equilibrated. After equilibrium sampling was done by inserting the preconditioned SPME fiber into the headspace of the vial for 2 hrs with continuous stirring at 25 ± 1°C (Shivashankara et al, 18). Gas chromatography/ Mass spectrometry (GC/MS) analysis was carried out using a Varian-3800 gas chromatograph coupled with Varian-4000 MS system on a DB-5 column (30 m × 0.25 mm) i.e., 0.25 µm film thickness. The carrier gas was helium with a flow rate of 1 ml min^{-1}; injector and detector temperatures were 250 and 270°C, respectively. For the qualitative identification of volatile substances and comparative variation of retention time and index, the following standard (ethyl acetate, propanol, hexanol, 1-octene-3-ol) were co-chromatographed. Volatile compounds were identified by comparing the retention index, which was determined by using the homologous series of n-alkanes (C5 to C32) (Jennings and Shibamoto, 8, Kovats, 9) and by comparing the mass spectra data with the NIST and Wiley libraries. 25 ml of sample was homogenised with ethyl acetate and evaporated. Then added 4ml NaOH (2N) kept it overnight. 68Then Adjusted pH to 2 with 2N HCl and extracted with 40 ml of ethyl acetate (4 times × 10 ml). Collected the ethyl acetate layer washed with distilled water till the pH becomes 6.5 to 7 and dried with Na_2SO_4 and evaporated to dryness. Dissolved this residue in 1 ml of HPLC grade methanol / 2ml of HPLC mobile phase. Filtered through membrane and injected to LCMS. The mobile phase for flavonoids analysis consists of an aqueous phase of 0.1 per cent formic acid in water (A) and organic phase of 0.2 per cent formic acid in methanol (B). The initial gradient was composed of 90 per cent aqueous phase and 10 per cent organic phase, held for 1.0 min. At 3.0 min, the gradient was changed to 60 per cent aqueous phase and 40 per cent organic phase, held for 5.5 min. At 8.5 min, linear gradient was followed arriving at 80 per cent aqueous phase and 20 per cent organic phase, held for 5.0 min. At 10.0 min the gradient was changed to 10 per cent aqueous phase and 90 per cent organic phase, held for 0.5 min and final step with 70 per cent aqueous phase and 30 per cent organic phase for 2.0 min.

The system was then returned to the initial conditions at 12 min and this condition was maintained for 1 min for equilibrating before the next injection. The flow rate was 0.4ml/min. the analytical column 2.1 × 50 mm UPLC BEH-C18 column (Waters) with 1.7um particles, protected by a Vanguard BEH C-18 column (Waters) with 1.7µm guard column (Waters) and the column temperature was maintained at 25^0 C. The sample injection volume was 2 µl for flavonoids. The metabolites eluted were monitored using the UPLC column effluent was pumped directly without any split into the TQD-MS/MS (Waters, USA) system, optimized for the flavanoid analysis. The evaluation parameters used to validate the methodology for the determination of all the flavonoids were linearity, the detection and quantification limits and repeatability. Linearity was determined by constructing calibration curves with standard solutions of the individual flavonoids. The multiple reaction monitoring (MRM), the most sensitive detection mode was employed for the analysis. The mass spectra obtained using negative ionization mode (ESI^-) full scan showed the most abundant forms of de-protonated M-H molecules of flavonoid standards namely catechin (m/z= 289.0), hesperetin (m/z= 300.96), apigenin (m/z= 268.96), naringenin (m/z= 271.03), myrcetin (m/z= 371.03), rutin (m/z= 609.16), luteoline (m/z= 284.9), quercetin (m/z= 301.032), umbelliferone (m/z= 166.968). Metabolites were identified by running the known standards and comparing the retention time and the fragmentation ions. LC-MS is fast developing into versatile method for the analysis of metabolites in biological matrices. The advantage lies in its capacity to analyse non-volatile compounds without any derivatization.

The Principal Components Analysis was then conducted on the correlation matrix (SAS, 2012). The first two significant principal components were plotted on a bi-plot graph.

RESULTS AND DISCUSSION

In the present study, a total of ten monoterpenoids were found in pummelo juice. Among them, limonene was maximum which ranged from 0.75 – 49.331%. High concentration of monoterpenoids was found in the accession 20 (1), whereas, the low concentration was in the accession 18. The concentration of monoterpenoids and other groups in different accessions (Table 1). Among the identified 20 sesquiterpenoids, *trans*-caryophyllene (3.45 – 44.65 %) was present in relatively high amount. Maximum amount of sesquiterpenoids were present in accession-25(5) (82.680%) whereas, minimum amount was present in accession-20(1) (35.177%) (Fig. 1 & 2 and Table 2). Similar results were obtained by (Cheong *et al.,* 4) reported that 60 volatile compound were identified in the *Citrus microcarpa* juices. Monoterpenoids, PC 1 and PC 2 which accounted for 99.9% and 0.12% of the variance, respectively. Illustrates the PCA biplots of pink and white pummelo juices of all the accessions (Fig. 3).

Table 1. Monoterpenoids and its quantity (%) in juice of selected pummelo accessions.

RT	CAS no	Compound	K.I	18(1)	18(3)	18(5)	19(4)	20(1)	19(1)	22(4)	8(4)	24(1)	25(5)	10(5)
				(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
8.134	80568	α-Pinene	932	N.D	0.011	N.D	0.147	0.038	0.002	0.006	0.085	0.001	0.000	0.001
10.053	3387415	(+)-Sabinene	976	N.D	0.061	N.D	0.357	N.D	0.002	0.009	0.003	N.D	N.D	0.001
10.738	127913	β-Pinene	978	N.D	0.124	N.D	0.259	0.027	N.D	0.014	0.719	0.001	N.D	0.005
14.958	499978	1(7), 8-p-Menthadiene	1003	N.D	0.016	0.000	N.D	N.D	0.001	0.021	0.000	0.002	0.001	0.160
11.396	99832	α-Phellandrene	1005	N.D	0.030	0.011	0.024	0.156	0.012	0.002	0.070	0.024	0.036	0.007
22.997	13466789	δ-3-Carene	1010	0.004	0.006	0.004	0.008	N.D	0.003	0.003	0.001	0.002	0.001	0.002
14.783	99865	α-Terpinene	1019	N.D	0.004	N.D	0.060	0.090	0.002	0.002	0.101	0.005	0.001	N.D
12.378	138863	Limonene	1031	0.750	7.451	0.759	27.110	49.331	4.319	6.567	39.296	5.169	1.165	1.363
23.183	99854	γ- terpinene	1062	0.004	0.007	0.005	0.003	0.001	0.002	0.000	0.001	0.002	0.001	0.001
11.868	586629	α- Terpinolen	1089	N.D	0.015	N.D	0.058	0.049	0.014	0.003	0.034	0.003	0.010	0.000

Table 2. Sesquiterpenoids and its quantity (%) in juice of selected pummelo accessions.

RT	CAS no	Compound	K.I	18(1)	18(3)	18(5)	19(4)	20(1)	19(1)	22(4)	8(4)	24(1)	25(5)	10(5)
				(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
24.186	17699148	(-) - α - Cubebene	1345	1.543	1.294	0.985	0.897	0.653	1.689	0.512	0.082	0.123	0.587	1.535
24.919	14912448	(+) - Ylangene	1370	5.906	5.515	3.951	3.542	2.179	5.928	1.430	0.125	4.832	1.728	2.974
26.05	95910364	Isoledene	1393	0.034	0.041	0.029	0.050	0.011	0.020	0.810	0.672	0.008	0.613	0.464
25.248	27862073	(+) - 9 - Aristolene	1424	0.115	0.107	0.085	N.D	0.015	0.017	0.012	0.002	N.D	0.010	0.016
24.421	3691121	α - Guaiene	1437	0.161	0.323	0.131	0.252	0.042	0.191	1.968	0.086	0.023	0.624	0.425
26.553	87445	*Trans* - Caryophyllene	1440	5.599	3.501	3.925	11.91	3.784	9.353	38.995	44.658	3.458	26.737	34.980
23.684	30824670	γ - Elemene	1445	0.066	0.124	0.068	0.070	N.D	0.012	0.159	0.000	N.D	0.008	0.021
27.156	489394	(+) - Aromadendrene	1447	1.157	0.968	0.558	0.673	0.290	0.717	N.D	0.202	0.969	0.059	0.446
26.897	23986745	Germacrene D	1464	1.485	1.907	1.352	1.303	3.327	1.733	1.341	0.001	4.874	30.630	0.873
27.403	54324037	(+) - Epi bicyclosesqui phellandrene	1471	2.098	1.385	1.043	0.816	0.335	1.773	0.425	0.008	1.005	0.654	0.869
28.977	30021740	ĩ - Muurolene	1486	3.672	6.126	3.694	3.664	1.074	2.414	1.651	0.335	1.704	1.576	1.453
27.033	31983229	α - Muurolene	1501	0.493	0.517	0.319	0.318	0.062	0.230	0.186	0.026	0.054	0.098	0.446
27.498	39029419	γ-Cadinene	1510	13.593	6.169	36.003	3.617	6.973	15.694	10.199	0.120	26.772	1.985	13.548
29.65	483761	δ-Cadinene	1524	13.474	14.981	10.838	11.68	7.744	14.105	4.983	1.337	16.989	6.187	9.682
29.514	39029419	α-Cadinene	1542	6.943	8.059	5.978	5.694	3.726	7.320	2.356	0.024	6.771	3.608	5.551
25.626	15423571	Germacrene B	1582	0.465	0.733	0.345	0.145	0.086	0.185	1.943	0.010	0.039	1.093	0.233

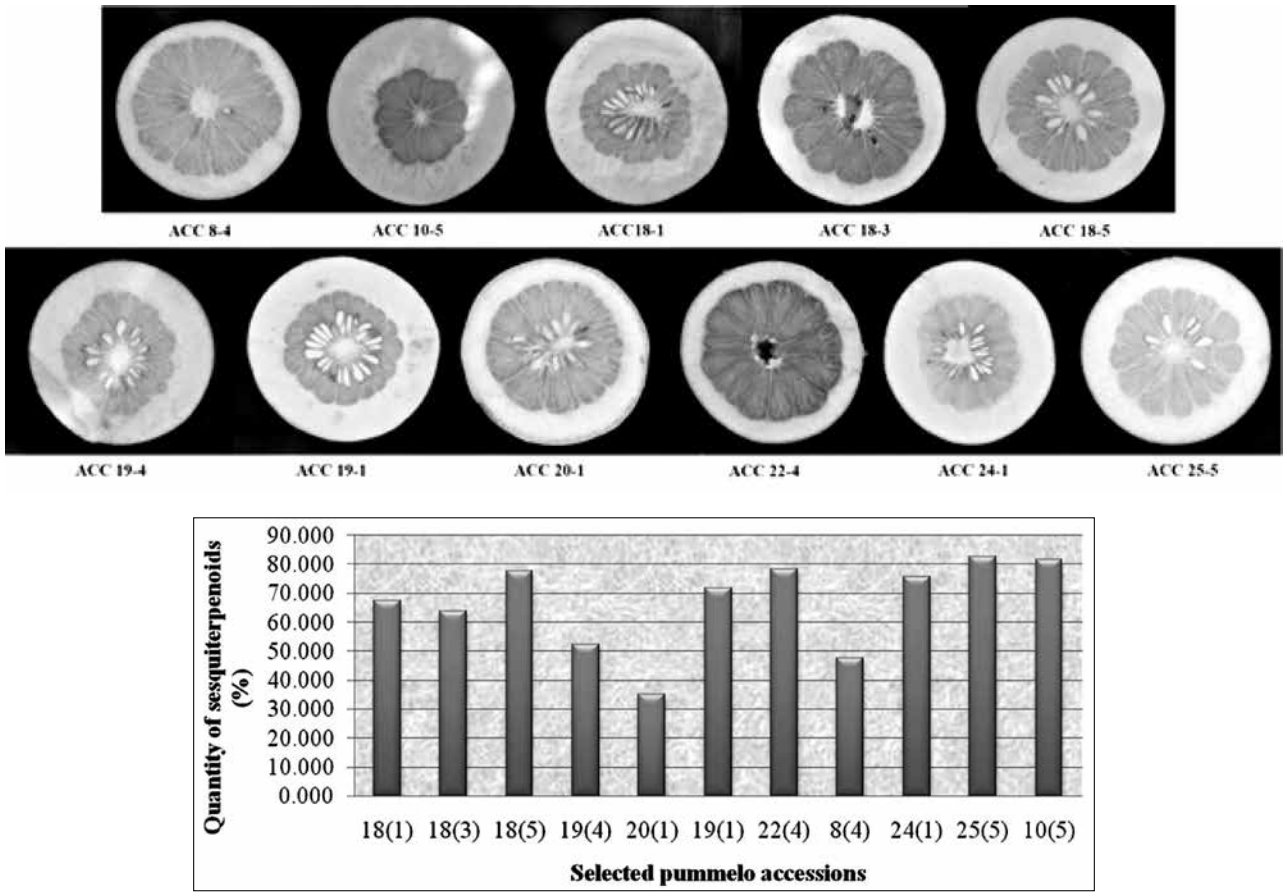

Fig. 1. Distribution of volatile compounds in selected pummelo accessions.

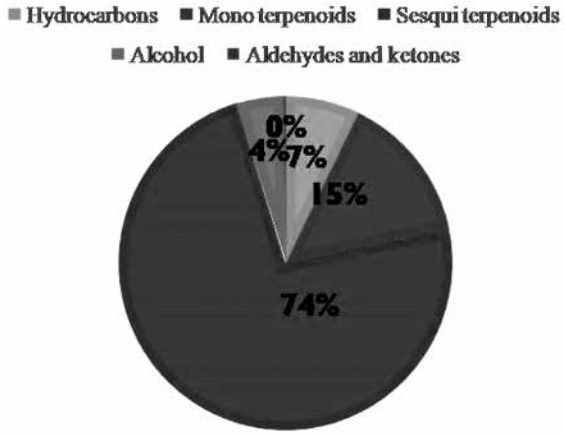

Fig. 2. Amount of sequiterepenoids present in juice of selected pummelo accessions.

As the chemical profiles of all the accessions of white & pink pummelo juices were not significantly different so all the score points were relatively nearer.

Sesquiterpenoids, PC 1 and PC 2 which accounted for 61.5% and 25.8% of the variance, respectively. illustrates the PCA biplots of pink and white pummelo juices of all the accessions As the chemical profiles of all the accessions pummelo juices were significantly different so all the score points were relatively distant.

Under PCA analysis among all the volatile groups, monoterpenoids were having high variable component in which the principle volatile was limonene and it was highest in accession 8(5) followed by accession 20(1) which may be due to genetic makeup of accessions. The volatile compounds were found to be relatively higher in white coloured juice accessions. PCA also showed that accession 8(4) (Khanapura local) is distinct from the others especially with respect to some of alcohols, hydrocarbons and sesquiterpenes.

A total of nine hydrocarbon compounds were identified in pummelo in which five compounds were found to be high 0.143- 2.25 % in pummelo. Maximum amount of hydrocarbons were present in accession-18 (1) with 11.981 % whereas minimum was in accession- 8(4) with 0.673 %. The concentration of hydrocarbons in different accession of pummelo

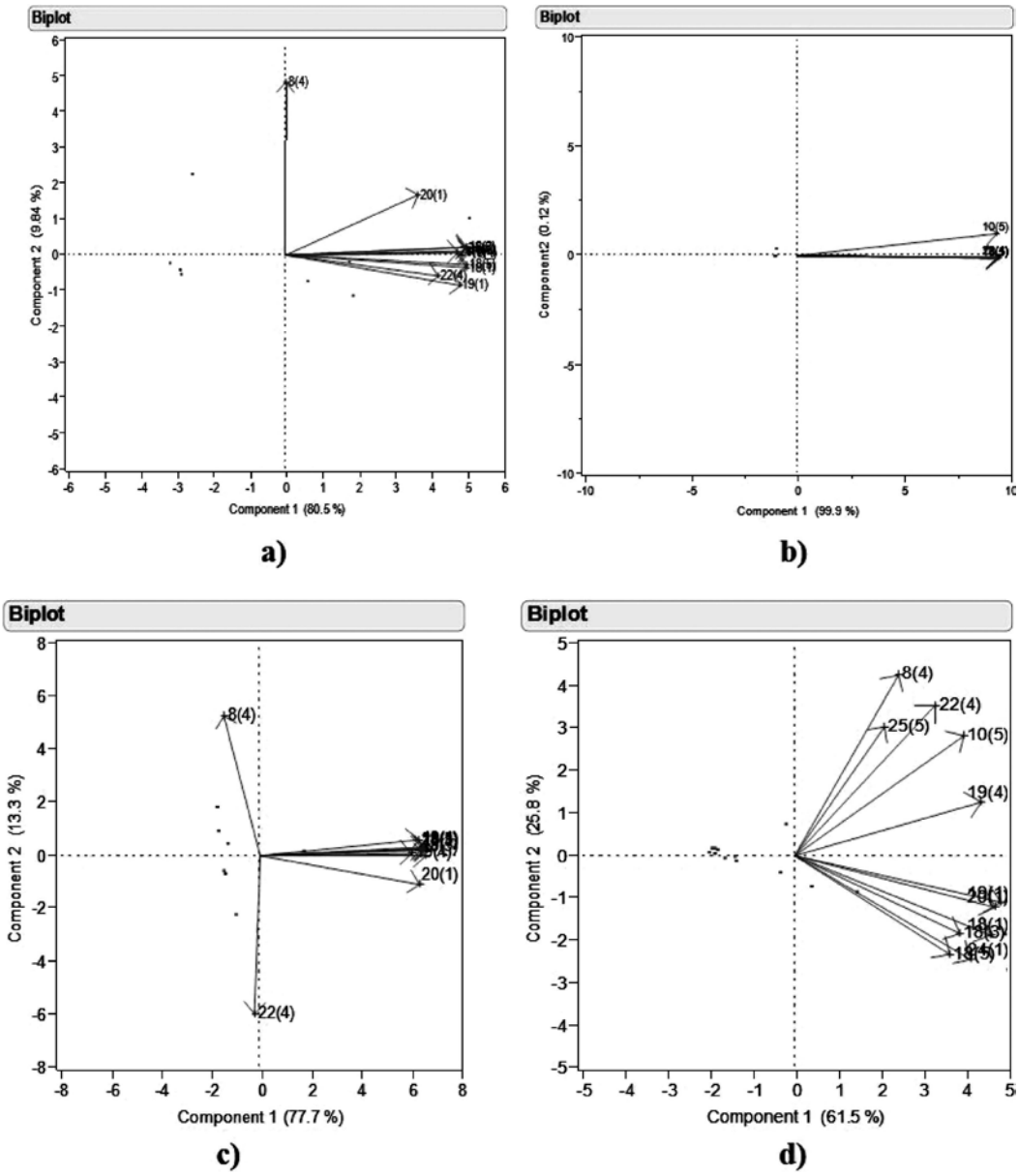

Fig. 3. PCA Biplot of volatile representing a) Hydrocarbons, b) Monoterpenoids, c) Alcohols and d) Sesquiterpenoids in eleven pummelo accessions.

(Table 3). The maximum amount of alcohols was present in accession 18(1) (6.73%). Among the nine alcohols identified, cis-α-copaene-8-ol (0.097- 3.791 %) and khusinol (0.006-0.484 %) were present in relative high amount (Table 4). Among the aldehydes and ketones identified, nootakatone (0.002-2.357 per cent) was present in relatively high amount. Maximum amount of aldehydes and ketones were present in accession 18(1) (2.96 %) whereas minimum amount was present in accession- 20(1) (0.003 %). The accessions 18(5), 19(4), 8(4), 24(1) aldehydes and ketones were not detected (Table 5).

Pummelo juice consists of nine alcohol compounds among them cis-α-copaene-8-ol and khusinol were present in high concentration. Around 6 alcohols were reported in organge juices including the ones reported in this study (Qiao *et al.,* 17).

Among aldehydes and ketones, nootakatome was recorded in the pumello juice of accessions. Aldehydes were reported in a relatively higher concentration white and pink pummelos (Perez-Cacho and Rouseff, 16). The amount of aldehydes detected in the pink pummelo juice (c.a. 800 ppm) was 2-fold higher than that of the white pummelo

Table 3. Hydrocarbons and its quantity (%) in juice of selected pummelo accessions.

RT	CAS no	Compound	K.I	18(1)	18(3)	18(5)	19(4)	20(1)	19(1)	22(4)	8(4)	24(1)	25(5)	10(5)
				(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
25.839	28375862	3-Methyldiadamantane	1238	0.278	0.325	0.157	0.192	0.135	0.101	0.124	0.303	0.039	0.199	0.106
30.081	16728997	Naphthalene,1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	1528	2.345	1.675	1.592	1.434	0.678	1.612	0.561	0.099	1.352	0.741	0.866
27.813	24406051	1-Isopropyl-4,7-dimethyl-1,2,4a,5,6,8a-hexahydronaphthalene	1537	1.850	1.630	1.169	1.154	0.557	1.117	0.191	0.000	1.145	0.399	0.508
29.772	79718835	Cadina-1(10),4-diene	1538	2.910	3.381	1.954	2.413	3.505	1.836	0.701	0.120	1.995	1.066	1.134
29.168	112362740	1(10),4-aromedenedradiene	1560	1.994	1.750	1.257	1.407	0.234	1.691	0.956	N.D	0.615	0.828	0.672

Table 4. Alcohols and its quantity (%) in juice of selected pummelo accessions.

RT	CAS no	Compound	K.I	18(1)	18(3)	18(5)	19(4)	20(1)	19(1)	22(4)	8(4)	24(1)	25(5)	10(5)
				(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
38.152	55784708	4-Bromo-1,7,7-trimethylbicyclo [2.2.1]heptan-2-one	1391	0.016	0.245	0.127	0.094	0.054	0.035	0.024	0.294	0.031	0.024	0.012
32.764	142507	(+)-Nerolidol	1537	0.002	0.168	0.157	0.147	0.014	0.069	0.186	0.002	0.089	0.115	0.064
31.627	77171552	(-)-Spathulenol	1576	0.097	0.016	0.097	0.053	0.094	0.062	0.191	0.295	0.127	0.049	0.095
34.383	19912620	tau.-Muurolol	1623	2.159	1.670	1.100	0.000	0.635	1.960	0.079	N.D	1.382	0.640	0.610
26.281	58569258	cis-α-Copaene-8-ol	1659	3.791	3.454	2.778	2.862	2.043	3.225	0.204	0.097	2.080	1.685	2.052
32.1	11031451	Santalol	1668	0.112	0.123	0.040	0.147	0.001	0.094	0.116	0.111	0.042	0.555	0.064
33.206	18319352	Cedr-8-en-13-ol	1668	0.229	0.178	0.145	0.141	0.027	0.106	0.257	0.045	0.054	0.154	0.020
32.988	24268346	Khusinol	1678	0.196	0.329	0.181	0.176	0.680	0.150	0.484	0.006	0.165	0.032	0.213

Table 5. Aldehydes and ketones and its quantity in juice of selected pummelo accessions.

RT	CAS no	Compound	K.I	18(1)	18(3)	18(5)	19(4)	20(1)	19(1)	22(4)	8(4)	24(1)	25(5)	10(5)
				(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
38.689	6902916	Germacrone	1694	0.608	N.D	N.D	N.D	0.001	0.173	0.000	0.000	N.D	0.000	0.000
42.08	4674504	Nootkatone	1847	2.357	0.029	N.D	N.D	N.D	0.131	0.003	N.D	N.D	0.003	0.002

juice (c.a. 409 ppm). Monoterpenic and unsaturated aliphatic aldehydes were identified in the peels of white and pink color pummelo **(**Cheong, 3). Nootkatone are expensive aromatic of grapefruit and is in high demand for its flavour and fragrance. **(**Furusawa *et al.,* 5).

Two principal components (PCs) were obtained from the extracted pummelo juice data set of Hydrocarbons, PC 1 and PC 2 which accounted for 80.5% and 9.84% of the variance, respectively. Fig. 3 (a) illustrates the PCA biplots of pink and white pomelo juices of all the accessions. As the chemical profiles of white pummelo juices (8(4) & 20(1)) were significantly different and the score points were distant from pink pummelo juices which were not significantly different so all the score points were relatively nearer.

Alcohols, PC 1 and PC 2 which accounted for 77.7% and 13.3% of the variance, respectively. Fig. 3 (c) illustrates the PCA biplots of pink and white pummelo juices of all the accessions. As the chemical profiles of white (8(4) & pink 22(4)) pummelo juices were significantly different and the score points were distant from other accessions which were having score points relatively nearer.

Several flavonoid compounds were identified *i.e.,* Rutin, Hesperetin, Myrcetin, Quercetin, Apigenin, Naringenin, Kaempferol and Epigallocatechin. Naringenin is the flavanone or aglycone responsible for bitterness in pummelo and it was higher in accession 22(4) (Table 6).

The Citrus fruits like orange and sweet orange are known for highest amount flavanoids. A total of 12 flavanoids were identified in pummel juice. Among the 12 identified flavanoid components, naringenin constitutes 63% followed by hesperitin (10%) and umbelliferone (5%). Similar, results were reported (Nogata *et al.,* 14) in *C. clementina* juice, (Gattuso *et al.,* 7) in *C. paradise.* Among all the accessions observed, accession 22(4) consists of higher naringenin in which the pulp was pink in colour and lowest was present in accession 20(1) which has the white pulp.

Pummelo has been considered as an important fruit which has got lot of medicinal properties. The fruit volatile compounds are mainly comprised of esters, alcohols, aldehydes, ketones, lactones, hydrocarbons, terpenoids and acids. A total of 50 volatile aroma compounds were identified in pummelo juice which includes 9 hydrocarbons, 10 monoterpenoids, 20 sesquiterpenoids, 9 alcohols, 2 aldehydes and ketones. Limonene was found to be 0.750 to 49.331 per cent of the total aroma volatiles identified in the juice of pummelo. Among flavonoids of which the Naringenin was highest followed by catechin. Naringenin content plays an important role in bitterness of pummelo juice and limonene contributes for the pleasant orange flavour of the juice. Among all the accessions observed, accession 22(4) consists of higher naringenin in which the pulp was pink in colour and lowest was present in accession 20(1) which has the white pulp. The accession 20(1) could be used in the breeding programme to develop less bitter/ sweet type of pummelo which has got good market potential.

Table 6. Flavonoids profiling and its quantity (ng /ml) among the 11 pummelo accessions.

Flavanoid components	8(4) (ng/ml)	10(5) (ng/ml)	18(1) (ng/ml)	18(3) (ng/ml)	18(5) (ng/ml)	19(1) (ng/ml)	19(4) (ng/ml)	20(1) (ng/ml)	22(4) (ng/ml)	24(1) (ng/ml)
Catechin	92.45	47.94	13.70	11.98	3.42	50.08	132.7	70.19	17.98	12.84
Umbellifero	3.47	73.54	63.49	0.56	3.00	48.11	25.50	12.84	10.66	7.40
Luteoline	17.64	4.41	5.88	5.88	17.64	8.82	20.6	13.23	10.29	5.47
Rutin	78.17	20.85	15.16	5.69	5.69	9.95	13.3	7.58	21.79	17.06
Hesperitin	2.46	2.26	1.44	3.49	1.64	2.15	2.9	0.82	2.87	1.23
Myrcetin	27.68	30.75	20.50	18.45	8.20	52.28	18.5	14.35	12.30	19.48
Quercetin	12.32	16.43	11.73	17.60	18.77	3.52	8.2	7.04	14.08	10.56
Apigenin	0.90	6.69	27.34	0.63	1.20	1.65	1.4	0.90	1.70	1.80
Naringenin	367.09	341.20	218.83	212.23	344.75	168.31	370.1	100.02	601.66	182.02
Kaempferol	0.19	0.19	0.16	0.08	0.21	0.17	0.1	0.29	0.16	0.17
Epicatechin	9.92	8.82	15.43	17.63	19.84	13.22	26.4	15.43	19.84	16.53
Epigallocatechin	30.15	15.87	7.40	4.23	6.35	11.11	5.3	11.64	4.23	7.93

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