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IDENTIFICATION OF VARIETIES AND BIOMARKERS ANALYSES ON ESSENTIAL OILS FROM PEELS OF *CITRUS* L. COLLECTED IN PAKISTAN

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Abstract

Five kinds of *Citrus* L. were collected from Sheringal, Dir Upper, Khyber Pakhtunkhwa (KPK), Pakistan, and their fruits, branches, and leaves were used to identify their correct nomenclature. In addition, the dried peels were gotten from fruits of *C. reticulata* var. *Kinnow* purchased from the bazaar of Sheringal. The essential oils (EOs) were extracted from their peels by hydrodistillation or cold-pressing, and then were detected by GC-MS, respectively. As a result, 2 kinds were both identified as *C. karna* Raf. (Khatta), 1 kind was deduced as *C. jambhiri* Lush. (Rough lemon), the other 2 kinds should belong to the varieties of *C. medica* (citron), *C. limon* (lemon), or *C. aurantifolia* (lime), but still needs to be confirmed further. In total, 36 major components were qualified and quantified in 8 prepared samples, accounting from 59.9-99.5%. Thirteen compounds such as limonene (22.8-97.6%), *β*-pinene (0-2.5%), *β*-myrcene (0-1.2%), terpinen-4-ol (0-2.1%), *α*-bergamotene (0-1.4%), *β*-bisabolene (0-1.6%), E-nerolidol (0-5.3%), *γ*-eudesmol (0-0.6%), *α*-cadinol (0-1.3%), nootkatone (0-14.1%), n-hexadecanoic acid (0-8.5%), tricosane (0-0.8%), and squalene (0-0.6%) are prominent. The other important biomarkers in peels EOs of *Citrus* such as *γ*-terpinene (0-0.2%) and linalool (0-0.5%) were relatively less. *α*-bergamotene and *β*-bisabolene are two important biomarkers for discriminating citron, lemon, and lime from the other species of *Citrus*. To the best of authors' knowledge, there is no report on the detection of nine chemicals including *β*-pinene, terpinen-4-ol, *α*-bergamotene, *δ*-elemene, E-nerolidol, *γ*-eudesmol, *α*-cadinol, tricosane, and squalene from peels EOs of *Citrus* produced in Pakistan previously.

Key words: *Citrus* L., Peels, Essential oils, Biomarkers, *α*-Bergamotene, *β*-Bisabolene.

Introduction

Genus *Citrus* L. including about 20 species (Huang, 1997), is the most important genus of Rutaceae (Kamal *et al*., 2013). Eight species including mandarin (*C. reticulata*), sweet orange (*C. sinensis*), lime (*C. aurantifolia*), lemon (*C. limon*), grapefruit (*C. paradisi*), pummelo or shaddock (*C. grandis* or *C. maxima*), sour orange (*C. aurantium*) and citron (*C. medica*) are important (Huang, 1997; González-Mas *et al*., 2019). They are produced in many countries with tropical or subtropical climate such as Brazil, USA, China, India, Japan, Mexico, Pakistan, *etc*. (Anwar *et al*., 2008; Singh, 1981). *Citrus* is mainly originated from south-east and south Asia, mainly from China and India (Huang, 1997; Singh, 1981). There are a number of lemon like fruits found in India but the detailed study of these is still awaited (Singh, 1981). Some varieties such as *C. jambhiri* Lush. (Rough lemon) (Khan and Shaukat, 2006; Singh, 1981) and *C. karna* Raf. (Khatta) (Khan and Shaukat, 2006) are just indigenous in India and Pakistan.

Citrus has important medicinal usage. In China, the peels collected in different growth stages from some varieties of *C. reticulata* can be used as two kinds of Chinese materia medica (CMM) such as Citri Pericarpium Reticulate (CPR) and Citri Pericarpium Reticulate Viride (CPRV). CPR and CPRV are usually used as the Qi-regulated CMM due to their special pharmacological activities and low-cost (Pharmacopoeia committee of the People's Republic of China, 2020). *Citrus* peels have many important and valuable medicinal ingredients such as essential oils (EOs), pectins, flavonoids, limonoids, alkaloids, fatty acids, coumarins, *etc*. (Rafiq *et al*., 2018). The peels EOs extracted from *Citrus* have a wide range of potential activities in food, perfumery, sanitary, cosmetics and pharmaceutics (Rehman *et al*., 2008; Kamal *et al*., 2013; González-Mas *et al*., 2019). However, in Pakistan, the utilization of *Citrus* peels is relatively less (Yousafzai *et al*., 2021).

Citrus peels are rich in EOs. Forty-nine chemicals common in these 8 species, nearly 90% belonging to terpenoids, can be thought as the biomarkers of these EOs in some extent. The representative ones include limonene, *γ*-terpinene, linalool, terpinen-4-ol, *α*-terpineol, (E, E)-*α*-farnesene, (E)-nerolidol (González-Mas *et al*., 2019). Besides these, thymol (Duan *et al*., 2016; González-Mas *et al*., 2019), methyl methylanthranilate (González-Mas *et al*., 2019), n-hexadecanoic acid can also be thought as the biomarkers of peels EOs from *Citrus*. The EOs from peels of different *Citrus* varieties have different chemotype due to the different content of these biomarkers (Lota *et al*., 2000; Fanciullino *et al*., 2006; Wang, 2014).

There are many varieties of the above 8 species, sometimes even the planters cannot discern them clearly. In consequence, the correct identification of these varieties from the fruits, branches and leaves is the first issue, which can guarantee the following research.

In this study, five different kinds of *Citrus* plants were collected in Upper Dir, Khyber Pakhtunkhwa (KPK), Pakistan. First, the identification were conducted by their fruits, branches, and leaves. Then the EOs were extracted from the peels by hydro-distillation or cold-pressing, then analyzed by GC-MS (Gas chromatography-Mass spectrometer), and then the biomarkers in these EOs were ascertained. These biomarkers will further make sure the identification. In addition, the EO from peels of Kinnow was also studied.

Materials and Methods

Plant materials: Five kinds of *Citrus* fruits with No. as 1, 2, 3, 5, and 6 were collected in May 23, 2021. Among them, 2 and 3 were collected from Usheri (35°07'26.4"N 71°57'39.6"E), 1, 5, and 6 from Gandigar (35°06'46.8"N 71°59'38.4"E), both area are in Upper Dir, KPK, Pakistan. The branches and leaves corresponding to these fruits were provided by Naseeb Zada in June 14, 2021. The dried peels of fruits of *C. reticulata* var. *Kinnow* purchased from the bazaar in Sheringal, Upper Dir, KPK, Pakistan, in April of 2021 was provided by Anwar Ul Haq in June 2, 2021.

Chemicals: Distillated water freshly prepared in the Pharmacognosy Lab of Department of pharmacy, Shaheed Benazir Bhutto University (Sheringal), n-hexane extra pure (Sigma-Aldrich), n-hexane HPLC grade (Merck), chloroform HPLC grade (Merck).

The varieties identification: The varieties identification of 5 kinds samples were based on the analyses of their fruits, branches, and leaves from their characters of appearance, respectively.

Extraction of the EOs: The peels were dried at ambient open air with temperature from 20°C to 45°C prior to extraction. The dried *Citrus* peels from 1, 2, 3, 5, 6, and Kinnow, and the dried peels after cold-pressing of 3 were subjected to hydro-distillation for 1-7 h, twice, using a Clevenger apparatus, respectively. The fresh peel of 3 was extracted by cold-pressing. The remained less water in these EOs was removed by anhydrous sodium sulfate, then the EOs were stored at -4°C until analyzed. In the end, 8 kinds of EOs were gotten corresponding to 1, 2, 3, 3 cold-pressing, 3 after cold-pressing, 5, 6, and Kinnow, respectively.

The analyses of EOs

The physical analyses: The extracted rate and density of EOs were determined.

The samples preparation: The sample of EO extracted from 5 was diluted as V_{EO}: $V_{chloroform\ (HPLC)}$ 1: 25 (3.85%, v/v). The sample of EO extracted from 6 corresponding to about 10 μL was diluted by 100 μL n-hexane (HPLC) directly. The samples of EOs extracted from 1, 2, 3, and Kinnow were diluted as V_{EO}: $V_{n\text{-}hexane}$ (HPLC) 1: 10 (9.09%, v/v), respectively. The EO mass 0.014 g extracted from 3 by cold-pressing was added 100 μL n-hexane (HPLC) with the concentration as 140 mg/mL. The EO mass 0.03 g extracted from the dried peels after cold-pressing of 3 was added 500 μL chloroform (HPLC) with the concentration as 60 mg/mL.

GC-MS detection: A Thermo Scientific (DSQⅡ) GC-MS (USA) with instrument software version: 2.0.71, matched with a NIST (National Institute of Standards and Technology) 2.3 MS database, equipped with a column TR-5MS (30 m × 0.25 mm i.d., 0.25 μm film thickness), was used for GC-MS detection. The oven temperature was programmed from 50°C held for 2 min to 150°C at 8°C min^{-1}, and further increased to 300°C at 15°C min^{-1}, and then held for 5 min. The solvent delay was 3 min. The injector was operated in split ratio 20: 1 with temperature 250°C. Electron impact mass spectra, scan from m/z 50-650 amu. The injection volume was 1 μL. The carrier gas was Helium with flow rate 1 mL/min.

The identification and quantitation of compounds: The peaks in total ion chromatograms (TICs) obtained by GC-MS were identified by probability based matching (PBM) first. The linear retention indices (LRIs) of identified compounds from semi-apolar column were gotten from NIST 17 database. The peak area normalization was used to calculate the relative area percentage of each compound.

PCA (principal components analyses) and CA (clustering analyses): The chemicals detected in EOs were to be done PCA by SPSS-26 (Statistical Product and Service Solutions). The 8 samples were to be done CA by SPSS-26.

Results and Discussion

Varieties identification: The detailed analyses information of five kinds of *Citrus* with No. 1, 2, 3, 5, and 6 by their fruits, branches and leaves was listed in (Tables 1 and 2). The detailed information of deduced varieties from literatures and websites was presented in (Table 3).

The 5 was deduced as *C. jambhiri* Lush. (Rough lemon) since its appearance characterization is very similar with that of rough lemon. The appearance characterization of 2 is close to that of 5, but the rind surface is smooth, and the leaves are some larger, the flesh not so acid, the serrate is larger. It should be a kind of citron. The 1 and 3 presented some characters related to *C. karna* Raf. (Khatta), and they were deduced as *C. karna* Raf. The 6 should belong to lime or lemon. The 2 and 6 belonging to which varieties still need to be confirmed further.

Yield, density of EOs: For 1, 2, 3, 5, 6, and Kinnow, the extraction rate (Volume of EO mL/mass of dried peel g) by hydro-distillation is 3.06%, 1.97%, 0.94%, 0.86%, 1.20%, 1.76%, respectively, the corresponding density (ρ) is 0.81, 0.83, 0.86, 0.92, 0.7, and 0.79, respectively. The extracted rate (mass of EO g/mass of fresh peel g) of 3 by cold-pressing is 0.12%. The extraction rate (mass of EO g/dried peel g, %) of 3 after cold-pressing by hydro-distillation is 0.09%. Previously, the hydro-distilled EOs content (mass of EO g/mass of fresh peels g) of *C. reticulata* var. *Kinnow*, *C. sinensis* var. *Mosambi* and *C. paradisii* were 0.30%, 0.24%, and 0.20%, respectively (Kamal *et al*., 2013).

Table 1. Analyses of fruits.

Five kinds of *Citrus*	No.				
	1	**2**	**3**	**5**	**6**
Number of fruits	**28**	**6**	**10**	**2**	**1**
Pictures					
Description	Fruit is of medium size, globose, with a finely pebbled rind texture. External colour is predominantly orange but occasionally has the slightest of blushes. The rind often feels soft, is of medium thickness and is easily peeled. Basal end commonly collared and with radial furrows.	Fruit large in size, globose; basal with radial furrows; with broad apical nipple surrounded by a deep irregular areolar furrow. Rind medium-thick; surface smooth; easily separable; color lemon-yellow.	Fruit is of medium size, globose, with a finely pebbled rind texture. External colour is predominantly orange but occasionally has the slightest of blushes. The rind often feels soft, is of medium thickness and is easily peeled. Basal end commonly collared and with radial furrows.	Round, with a rough rind, and a broad nipple surrounded by a **irregular areolar furrow**. Thick rind. Fruit medium in size. Rind surface typically deeply pitted, and rough or bumpy, ribbed; easily separable; color lemon-yellow.	Fruit is oblate in shape. The colour is pale yellow. The rind is medium, tough and the surface is smooth and even. The pulp sweet and moderate acid. Albedo not pigmented.
The average mass of fresh fruits (g)	136.54	192.57	119.37	181.88	122.91
The average mass of fresh peels (g)	59.59	112.99	61.58	107.32	65.20
The average mass of dried peels (g)	> 11.86 (about 15.80)	17.88	15.79	16.45	12.46
The ratio of dried peels compared with fresh (%)	> 19.90 (about 26.51)	15.82	25.64	15.33	19.11
The ratio of fresh peels compared with fresh fruits (%)	43.65	55.18	51.59	59.00	53.05
The ratio of dried peels compared with fresh fruits (%)	> 8.68 (about 11.57)	8.96	13.23	9.04	10.14
The taste of pulps	Slight sweet, strong acid	Sweet and moderate acid	Sweet and moderate acid	Sweet and **strong** acid	Sweet and moderate acid
The width of dried flavedo (mm)	1	1	1	1	1
The width of dried mesocarp (mm)	2-4	1-4	6	1-4	1-5

Note: "-" means no related information.

Table 2. Analyses of branches and leaves.

Five kinds *Citrus*		No.				
		1	**2**	**3**	**5**	**6**
Pictures						
Leaves	Mean Length (cm)	8.6	11.2	11.2	7.6	8.8
	Mean Width (cm)	3.1	5.3	6.0	2.9	3.7
	Mean length/Mean width	2.8	2.1	1.9	2.6	2.4
	Number of Leaves	5	7	7	7	15
	Color of leaves	Darker green	Darker green	Darker green	Light green	Darker green
	Leaflets (Yes/No)	Yes	No	Yes	No	Yes
	Sawteeth (Yes/No)	No	Yes	No	Yes (small)	Yes (very small)
	Leaf apex	Blunt	Blunt	Slightly blunt	Blunt	Slightly blunt
Branches	Thorns (Yes/No)	Yes	-	-	Yes (long)	Yes (very short)
	Young/old (Shape of stem)	Flat/round	-	-	Three prism curve/round	Flat/round

Table 3. The information of deduced *Citrus* cultivars planted in Pakistan.

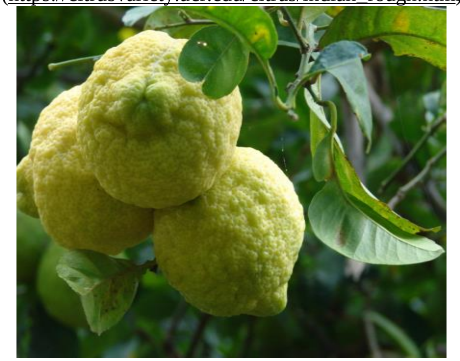
No.	Common name	Species	Varieties	Production area	Characterization	Pictures
1	Rough lemon (Huang, 1997) Jatti Khatti, Jhambheri (Khan & Shaukat, 2006; https://citrusvariety.ucr.edu/citrus/indian_rough.html; Hajra, Nair & Daniel. 1997)	*C. limon*	*C. jambhiri* Lush. (Huang, 1997; Khan & Shaukat, 2006; Hajra, Nair & Daniel. 1997)	The plains of Sindh, Balochistan and the Punjab (Khan & Shaukat, 2006).	Fruit medium in size, of highly variable form but usually oblate to elliptic-oblong; commonly with irregularly furrowed or lobed basal collar or neck; usually with broad apical nipple surrounded by a **deep irregular areolar furrow**. Rind medium-thick; **surface typically deeply pitted**, and rough or bumpy, sometimes ribbed; easily separable; color lemon-yellow to brownish-orange. Tree **with numerous small thorns**; leaves medium-small, **blunt-pointed**, and light green (https://citrusvariety.ucr.edu/citrus/indian_rough.html; Hajra, Nair & Daniel. 1997). They have very less amount of juice and pulp. (https://indiagardening.com/growing-fruits/lemon-varieties-in-india-types-of-lemons/) Leaves are **small**, round **faintly serrated**; fruits **deep yellow, is sharply acidic.** (http://www.fruitipedia.com/2018/11/rough-lemon_citrus-jambhiri/) **Relatively less spiny**. (https://www.flowersofindia.net/catalog/slides/Indian%20Rough%20Lemon.html)	 (https://citrusvariety.ucr.edu/citrus/indian_rough.html)  (https://upload.wikimedia.org/wikipedia/commons/8/85/Starr_061105-1366_Citrus_limon.jpg)  **B. 3 : Citrus jambhiri** (Singh, 1981)

Table 3. (Cont'd.)..

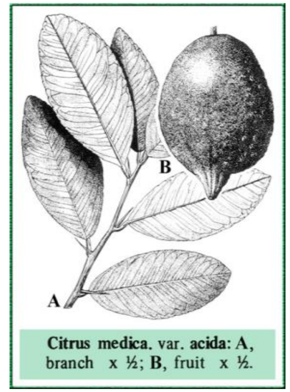
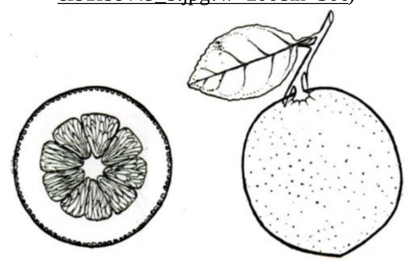
No.	Common name	Species	Varieties	Production area	Characterization	Pictures
2	**Khatta** (Marwat *et al.*, 2013; http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=250071509; Singh, 1981) Indian lemon (https://www.plantnames.unimelb.edu.au/Sorting/Citrus_1.html)	*C. medica* (Khan & Shaukat, 2006; http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=250071509) *C. aurantium* (https://citrusvariety.ucr.edu/citrus/kharna.html) *C. karna* Raf. (Singh, 1981)	*C. medica* var. *acida* Brandis or var. *tarung* (Khan & Shaukat, 2006; http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=250071509) *C. aurantium* var. *Khatta* (https://citrusvariety.ucr.edu/citrus/kharna.html) *C. karna* Raf. (Singh, 1981)	Balochistan and plains. (Khan & Shaukat, 2006) Northern Punjab and the foothills (http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=250071509).	Suspected of being a hybrid of sour orange and lemon. The fruits are typical sour oranges. Round to oblong, looks like a sour orange with a **rough rind**, and **a nipple**. Fruits: large 6-10 cm in diameter, great variation in shape and surface. Rind moderately thick, firm; surface smooth, warty or ribbed; tightly adherent; color golden yellow to deep orange. Rind cadmium-yellow when ripe. Relatively tender flesh, **pulp very acid**. Low seed count, seeds numerous, somewhat slimy, and moderately polyembryonic; orange flesh, coarse-textured, only moderately juicy. Tree vigorous, medium to large in size, 15-20 feet, upright-spreading, spiny; foliage lemon-like but darker green. New growth purple-tinted. The leaves and flowers are similar to those of rough lemon, but larger in size. Leaves medium sized 55-110 x 30-75 mm, narrowly petiole winged, articulated margin, serrate, apex obtuse. (https://citrusvariety.ucr.edu/citrus/kharna.html; http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=250071509; Singh, 1981)	 (http://www.efloras.org/object_page.aspx?object_id=60153&flora_id=5)  (https://hemlocklily.files.wordpress.com/2014/05/4699301850_cfb1fbe449_b.jpg?w=200&h=300)  FIG. 5 : *Citrus karna* (Singh, 1981)

Table 4. The identification and quantitation of EOs isolated from *Citrus* peels collected in Sheringal, Upper Dir, KPK, Pakistan.

No.	Chemicals	CAS	LRIs	Samples							
				1	2	3	3 cold-pressing	3 after cold-pressing	5	6	Kinnow
				%							
1.	α-Pinene	80-56-8	937	0.1	0.1	0.1	tr	tr	0.2	-	0.2
2.	Benzene, 1-ethyl-4-methyl-	622-96-8	954	-	-	-	-	-	-	0.9	-
3.	β-Pinene	127-91-3	979	0.6	0.6	1.4	0.5	0.7	2.5	-	-
4.	Decane	124-18-5	1000	-	-	-	-	-	-	0.7	-
5.	β-Myrcene	123-35-3	991	1.1	1.0	1.0	0.6	tr	0.2	-	1.2
6.	Limonene	138-86-3	1030	96.3	94.6	94.8	60.5	31.0	78.2	22.8	97.6
7.	γ-Terpinene	99-85-4	1060	-	-	-	-	-	-	0.2	-
8.	*cis*-Linalool oxide	5989-33-3	1074	0.1	-	0.1	-	-	-	-	-
9.	Undecane	1120-21-4	1100	-	-	-	-	-	-	0.3	-
10.	Linalool	78-70-6	1099	0.4	-	0.5	0.1	-	-	-	tr
11.	Terpinen-4-ol	562-74-3	1177	-	0.8	-	-	tr	2.1	-	0.1
12.	α-Terpineol	98-55-5	1189	0.3	-	0.3	0.3	0.6	0.2	0.3	-
13.	Decanal	112-31-2	1206	-	-	0.4	-	-	-	-	-
14.	*cis*-Carveol	1197-06-4	1229	-	0.1	-	-	-	-	-	0.1
15.	Linalyl acetate	115-95-7	1257	0.2	-	0.2	-	-	-	-	-
16.	Citral	5392-40-5	1276	tr	-	0.1	-	-	-	-	-
17.	δ-EIemene	20307-84-0	1338	-	tr	tr	0.1	tr	0.3	-	-
18.	Nerol acetate	141-12-8	1364	0.1	-	-	-	-	-	-	-
19.	Geranyl acetate	105-87-3	1382	0.1	tr	0.2	-	-	-	-	-
20.	(E)-β-caryophyllene	87-44-5	1419	0.1	-	0.1	0.1	0.2	tr	0.2	-
21.	α-Bergamotene	17699-05-7	1435	-	0.2	-	-	tr	1.4	-	-
22.	Germacrene D	23986-74-5	1481	tr	tr	0.1	0.2	0.1	0.2	-	-
23.	Eremophilene	10219-75-7	1499	-	-	-	-	-	-	-	0.1
24.	β-Bisabolene	495-61-4	1509	-	0.2	-	-	tr	1.6	-	-
25.	2,4-Di-tert-butylphenol	96-76-4	1519	-	-	-	-	-	-	0.6	-
26.	E-Nerolidol	40716-66-3	1564	tr	-	0.1	0.1	5.3	tr	0.7	-
27.	γ-Eudesmol	1209-71-8	1631	-	tr	-	-	-	-	0.6	tr
28.	α-Cadinol	481-34-5	1653	-	tr	-	-	0.7	tr	1.3	tr

Table 4. (Cont'd.).

No.	Chemicals	CAS	LRIs	Samples							
				1	2	3	3 cold-pressing	3 after cold-pressing	5	6	Kinnow
				%							
29.	Nootkatone	4674-50-4	1808	tr	-	0.2	0.3	10.6	tr	14.1	tr
30.	n-Hexadecanoic acid	57-10-3	1968	-	-	-	-	8.5	tr	-	-
31.	Phytol	150-86-7	2114	-	tr	-	0.1	-	-	-	tr
32.	Osthole	484-12-8	2144	-	-	tr	0.3	-	-	-	-
33.	Tricosane	638-67-5	2300	-	tr	tr	-	tr	0.1	0.8	-
34.	Diisooctyl phthalate	131-20-4	2543	tr	0.2	tr	32.4	2.2	0.1	26.3	tr
35.	Bis(2-ethylhexyl) isophthalate	137-89-3	2707	tr	0.2	-	0.1	-	-	-	tr
36.	Squalene	111-02-4	2832	-	-	-	0.2	-	-	0.6	tr
	MHs (5)			98.1	96.3	97.2	61.5	31.7	81.0	23.0	99.0
	MAlcs (5)			0.8	0.9	0.9	0.3	0.6	2.3	0.3	0.2
	MAlds (1)			tr	-	0.1	-	-	-	-	-
	MEsters (3)			0.3	tr	0.4	-	-	-	-	-
	SHs (6)			0.1	0.4	0.2	0.3	0.4	3.6	0.2	0.1
	SAlcs (3)			tr	tr	0.1	0.1	6.0	tr	2.7	tr
	SKets (1)			tr	-	0.2	0.3	10.6	-	14.1	tr
	DAlcs (1)			-	-	-	0.1	-	-	-	-
	THs (1)			-	-	-	0.2	-	-	0.6	-
	Fatty acids (1)			-	-	-	-	8.5	-	-	-
	Phenols (1)			-	-	-	-	-	-	0.6	-
	Phenol ethers (1)			-	-	tr	0.3	-	-	-	-
	Aromatic esters (2)			0.1	0.4	tr	32.5	2.2	0.1	26.3	0.1
	Alkanes (3)			-	-	tr	-	-	0.1	1.9	-
	Benzenes (1)			-	-	-	-	-	-	0.9	-
	Aliphatic Alds (1)			-	-	0.4	-	-	-	-	-
	Coumarins (1)			-	-	tr	0.3	-	-	-	-
	In total (36)			99.5	98.2	99.4	95.6	59.9	87.1	70.4	99.4

Note: The "-" denotes there is no related data. "tr", trace, means the content is less than 0.05%. In the abbreviations such as "MHs, MAlcs, MAlds, MEsters, SHs, SAlcs, SKets, DAlcs, THs, and Aliphatic Alds", the "M, H, Alc, Ald, S, Ket, D, and T" refer to "Monoterpene, Hydrocarbon, Alcohol, Aldehyde, Sesquiterpene, Ketone, Diterpene, and Triterpene", respectively

Chemical analyses of the EOs: The detailed information of qualified and quantified results can be seen in (Table 4). The total quantitation of 36 compounds ranged from 59.9-99.5%, in which, the 3 after cold-pressing (59.9%) and 6 (70.4%) are relatively less.

Limonene (22.8-97.6%) is relatively less in 3 after cold-pressing (31.0%) and 6 (22.8%), which is the most abundance compound with content 35.0-95.0% in peels EOs from *Citrus* (González-Mas *et al*., 2019). It can down to 34.8% in peels EO from lime (Ndiaye *et al*., 2017), down to 38.1% in peels EO from *C. limon* (González-Mas *et al*., 2019; Lota *et al*., 2002), which means 6 should be a kind of lime or lemon. Limonene is 1 (96.3%) and 3 (94.8%), which was detected in peels EO (56.3%) of *C. karna* (Lota *et al*., 2002). In this point, it is a contradiction the 1 and 3 were induced as *C. karna*. *β*-pinene (0-2.5%) was undetected in the peels EOs from *Citrus* planted in Pakistan previously, to the best of authors' knowledge. *β*-Myrcene (0-1.2%) was relatively high in 1 (1.1%), 2 (1.0%), 3 (1.0%), and Kinnow (1.2%). *γ*-Terpinene (0-0.2%) was only detected in 6 (0.2%), which was undetected in peels EOs from *Citrus* in Pakistan to the best of our knowledge.

Terpinen-4-ol (0-2.1%) was relatively high in 2 (0.8%) and 5 (2.1%), which was undetected in peels EOs from *Citrus* planted in Pakistan to the best of our knowledge. It is up to 1.4% in peels EOs from lime (González-Mas *et al*., 2019; Lota *et al*., 2002). *α*-terpineol (0-0.6%), which was detected in peels EOs from Kinnow, *C. sinensis* var. Mosambi, *C. sinensis* (Malta), and grapefruit, with the content >1%, 12.2%, 8.5%, and 10.2%, respectively (Kamal *et al*., 2013; Javed *et al*., 2014), was undetected in Kinnow in this study. Linalool (0-0.5%) was relatively high in 1 (0.4%) and 3 (0.5%).

Two important SHs including *α*-bergamotene (0-1.4%) and *β*-bisabolene (0-1.6%) (structure displayed in Fig. 1), were relatively high in 5 (1.4% and 1.6%, respectively), were also detected in 2 (0.2% and 0.2%, respectively), and in 3 after cold-pressing (tr and tr, respectively). *α*-bergamotene was undetected in the peels EOs from pummelo, was usually trace in peels EOs from mandarin, grapefruit, sweet orange, and bitter orange, was a characteristic component in peels EOs from lime (up to 1.0%), citron (up to 0.5%) and lemon (up to 1.7%). *β*-bisabolene was usually trace in the peels EOs from pummelo, sweet orange, bitter orange, mandarin, and grapefruit, was a characteristic component in peels EOs of lime (up to 2.1%), citron (up to 0.7%), and lemon (up to 2.8%) (González-Mas *et al*., 2019). Previously, *α*-bergamotene and *β*-bisabolene were detected in rough lemon (0.3% and 0.5%) and *C. karna* Raf. (Khatta) (1.0% and 1.6%), respectively (Lota *et al*., 2002). In this point, it's also a contradiction that the 1 and 3 were deduced as *C. karna* Raf.. Bisabolene was also detected in peels EOs extracted by cold-pressing from Eureka lemon produced in Pakistan (Rehman *et al*., 2008). To the best of authors' knowledge, there was no report on the detection of *α*-bergamotene from peels EOs of *Citrus* produced in Pakistan.

δ-eIemene (0-0.3%) was relatively high in 5 (0.3%), which to the best of authors' knowledge, was undetected in peels EOs from *Citrus* produced in Pakistan, undetected in rough lemon and *C. karna* Raf. (Lota *et al*., 2002). *δ*-eIemene, is up to 0.4% in peels EOs of mandarin (Fanciullino *et al*., 2006), and *C. medica* (González-Mas *et al*., 2019; Aliberti *et al*., 2016), respectively. (E)-*β*-caryophyllene (0-0.2%) was relatively high in 3 after cold-pressing (0.2%) and 6 (0.2%), which was detected in peels EOs of *C. karna* Raf. (0.6%) and rough lemon (0.2%) (Lota *et al*., 2002). (E)-*β*-Caryophyllene in peels EOs of lime is up to 0.6% (Lota *et al*., 2002), of *C. aurantium* is up to 0.9% (Lota *et al*., 2001), of lemon is up to 0.7% (González-Mas *et al*., 2019; Lota *et al*., 2002). Germacrene D (0-0.2%) was relatively high in 3 after cold-pressing (0.2%) and 5 (0.2%), which was detected in rough lemon (0.1%) and *C. karna* Raf. (0.9%) (Lota *et al*., 2002). Germacrene D in peels EOs of lime is up to 1.0% (Lota *et al*., 2002), of *C. aurantium* up to 2.1% (Lota *et al*., 2001), of *C. medica* up to 0.5% (González-Mas *et al*., 2019; Petretto *et al*., 2016).

In this study, three SAlcs including E-nerolidol (0-5.3%), *γ*-eudesmol (0-0.6%), and *α*-cadinol (0-1.3%) were detected. E-nerolidol was relatively high in 3 after cold-pressing (5.3%) and 6 (0.7%), *γ*-eudesmol was relatively high in 6 (0.6%), *α*-cadinol was relatively high in 6 (1.3%) and 3 after cold-pressing (0.7%). To the best of authors' knowledge, these three chemicals were undetected in peels EOs from *Citrus* produced in Pakistan previously, were undetected in rough lemon and *C. karna* Raf. (Lota *et al*., 2002). E-nerolidol in peels EOs of *C. aurantium* is up to 3.2% (González-Mas *et al*., 2019; Lota *et al*., 2001).

Nootkatone (0-14.1%) was relatively high in 3 after cold-pressing (10.6%) and 6 (14.1%), which was undetected in rough lemon and *C. karna* Raf. (Lota *et al*., 2002).

n-hexadecanoic acid (0-8.5%) was relatively high in 3 after cold-pressing (8.5%), which was only detected in peels EOs of *C. sinensis* (Malta) (0.9%) from *Citrus* planted in Pakistan to the best of authors' knowledge (Javed *et al*., 2014), was undetected in rough lemon and *C. karna* Raf. (Lota *et al*., 2002). Tricosane (0-0.8%) was relatively high in 6 (0.8%), which was undetected in peels EOs of *Citrus* planted in Pakistan to the best of authors' knowledge. Squalene (0-0.6%) was relatively high in 3 cold-pressing (0.2%) and 6 (0.6%), which was undetected in peels EOs of *Citrus* planted in Pakistan to the best of authors' knowledge, was undetected in rough lemon and *C. karna* Raf., either (Lota *et al*., 2002).

Diisooctyl phthalate (tr-32.4%) was detected in each sample, and was relatively high in 3 cold-pressing (32.4%) and 6 (26.3%), which was unreported in the peels EOs from *Citrus* produced in Pakistan to the best of authors' knowledge. Phthalates, especially bis(2-ethylhexyl) phthalate, are commonly used plasticizers in synthetic polymers. Unfortunately, they are extracted from the polymers upon exposure to solvents such as dichloromethane, chloroform, or toluene, e.g., from syringes, tubing, vials *etc*. Therefore, they are often detected as impurities (Gross, 2015; Wang, 2018).

The 36 chemicals were classified into 7 components through PCA (Fig. 2). Eight samples can be divided into 2 clusters (Fig. 3). In one cluster, the sample 5 is somewhat different with the other four samples; In another cluster, the sample 3 cold-pressing is somewhat different with the other two samples.

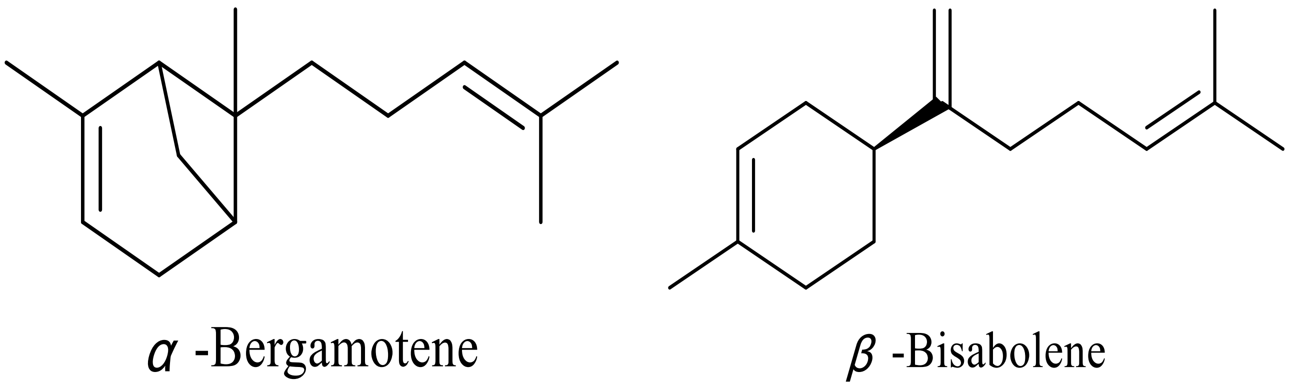


Fig. 1. The structure of α-bergamotene and β-bisabolene.

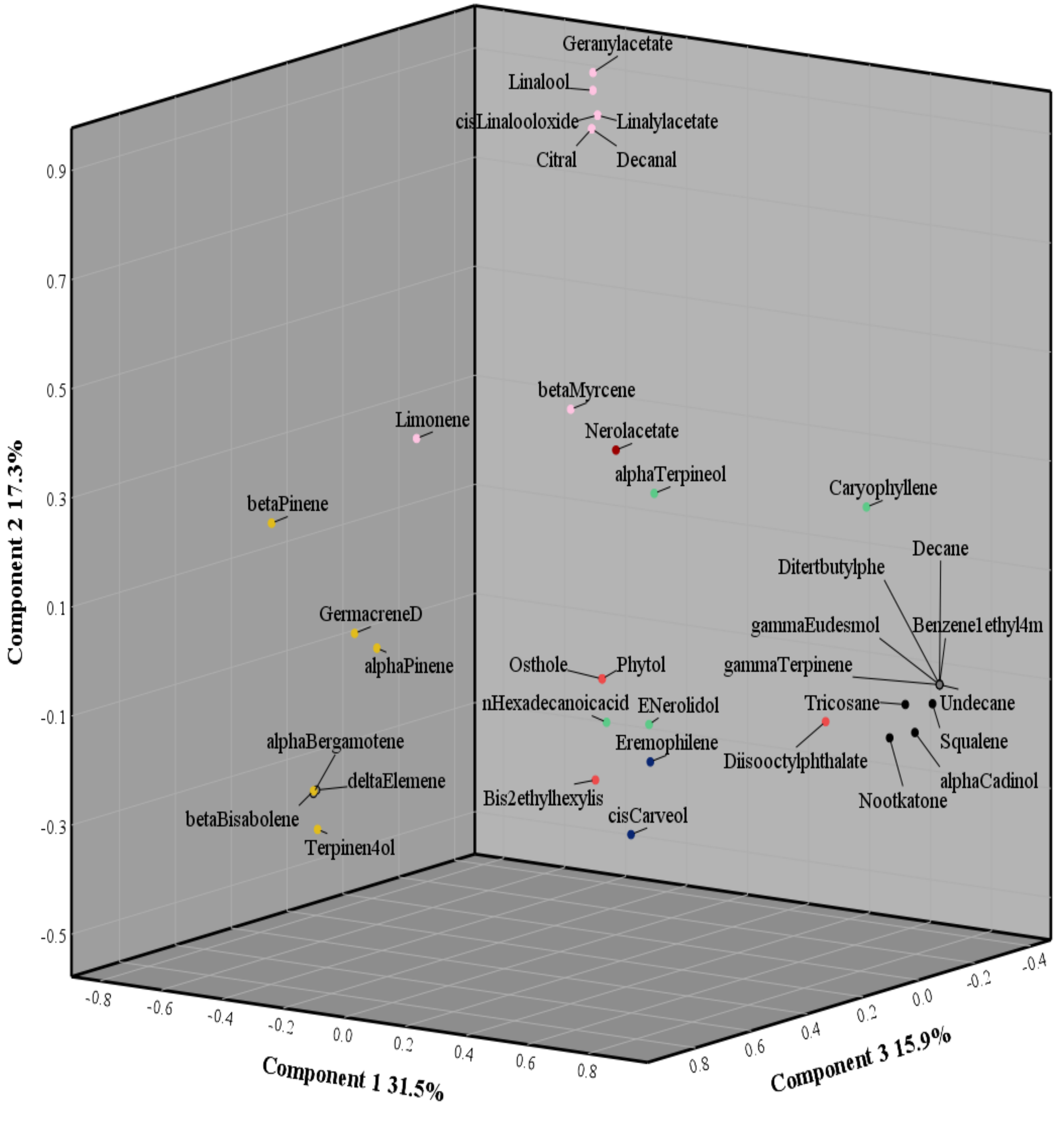


Fig. 2. The PCA result of 36 chemicals. The different color corresponding to different component.

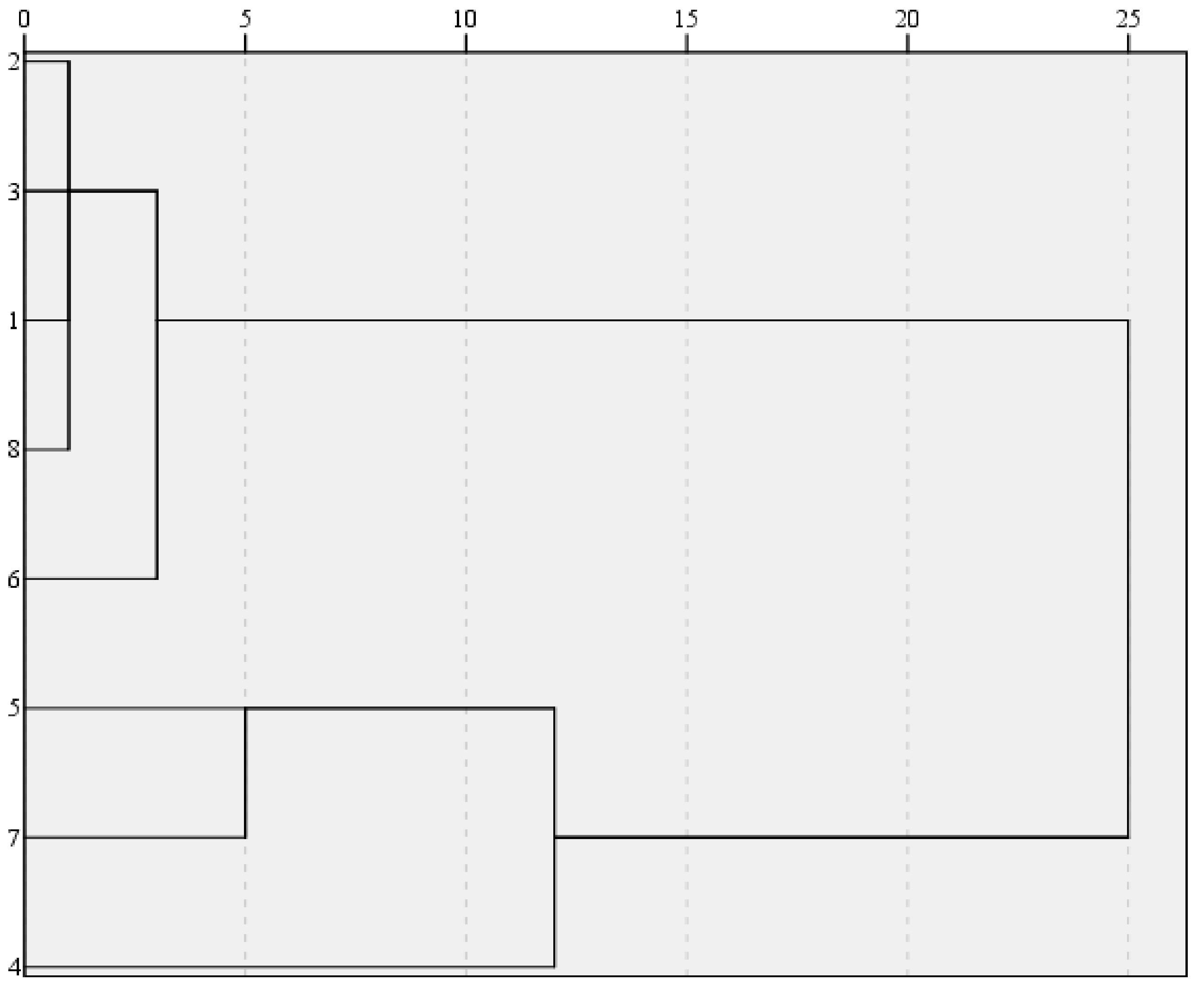


Fig. 3. The CA result of 8 samples. The numbers "1, 2, 3, 4, 5, 6, 7, 8" in the left side correspond to samples "1, 2, 3, 3 cold-pressing, 3 after cold-pressing, 5, 6, Kinnow", respectively.

Conclusion

No. 1 and 3 were both identified as *C. karna* Raf. (Khatta), 5 was identified as rough lemon. The 2 should be a kind of citron, 6 should be a kind of lime or lemon, as to which varieties there still needs to be confirmed further. In total, 36 components were qualified and quantified in 8 samples. Thirteen compounds including limonene, *β*-pinene, *β*-myrcene, terpinen-4-ol, *α*-bergamotene, *β*-bisabolene, E-nerolidol, *γ*-eudesmol, *α*-cadinol, nootkatone, n-hexadecanoic acid, tricosane, and squalene are prominent. To the best of authors' knowledge, there is no report on the detection of 9 chemicals inclduing *β*-pinene, terpinen-4-ol, *α*-bergamotene, *δ*-elemene, E-nerolidol, *γ*-eudesmol, *α*-cadinol, tricosane, and squalene from peels EOs of *Citrus* produced in Pakistan. *α*-Bergamotene and *β*-bisabolene are two important biomarkers for discriminating citron, lemon, and lime from the other species of *Citrus*.

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