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Chemical composition and biological activities of peels and leaves essential oils of four cultivars of *Citrus deliciosa* var. *tangarina*

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ABSTRACT

The hydrodistilled essential oils of peels and leaves of four cultivars of *Citrus deliciosa* var. *tangarina* (Fina Clementine, Nour Clementine, Spinosa Clementine and Thornless Clementine) belonging to family Rutaceae were analyzed by GC/MS. Limonene was the major constituent of all the oil samples under investigation except in case of Nour Clementine leaves, where sabinene was the major component. Generally, total hydrocarbons were of higher percentage than total oxygenated compounds, also total monoterpenes showed higher percentage than total sesquiterpenes. The studied essential oils showed a dose-dependent antioxidant activity. Thornless leaves oil showed the highest antioxidant potential, it recorded the same EC_{50} as ascorbic acid. The antimicrobial activity of *Citrus* peels and leaves oils was screened against Gram-positive and Gram-negative bacteria, yeast and fungi. Leaves oils showed higher antimicrobial activity than peels oils against all the tested micro-organisms among them Thorny Clementine leaves oil was the most potent. Peels oils were tested for cytotoxic activity against liver, cervix, breast carcinoma and normal baby hamster kidney fibroblast cell lines. Peels oils showed potent cytotoxic activity against the tested carcinoma cell lines comparing to doxorubicin, while they exhibited no effect on normal baby hamster kidney fibroblast cell line which indicated their safety. The antiviral activity of the studied essential oils was tested by hemagglutination test of washed chicken erythrocytes. The allantoic amniotic fluid of embryonatic eggs inoculated with different *Citrus* essential oils under investigation, suspension of H5N1 virus and antibiotic gave a positive test, this indicates inactivated H5N1 virus.

Keywords: *Citrus deliciosa* var. *tangarina*, Fina Clementine, Nour Clementine, Spinosa Clementine, Thornless Clementine, antioxidant, antimicrobial, cytotoxic, antiviral.

1. Introduction

Citrus is an ancient crop, with records of human cultivation extending back to 2100 BC[1]. Early studies of *Citrus* have a great mission, as they often revealed a rich harvest of biologically active principles including essential oil, flavonoids[2-4] coumarins[5-8] and limonoids[9, 10].

Due to the great nutraceutical and economic importance of *Citrus* essential oils, numerous investigations have been performed aimed at identifying their chemical composition. Limonene was the prominent component in most of the analyzed peels and leaves essential oil samples of different C*itrus* species[11-16]. *Citrus* essential oils showed a broad spectrum of biological activities mainly antimicrobial[17-19], antioxidant[20, 21] and cytotoxic[22, 23] activities. This study was initiated on *Citrus deliciosa* var. *tangarina* cultivars: Fina Clementine, Nour Clementine, Spinosa Clementine and Thornless Clementine belonging to Family Rutaceae in order to through light on these species. The study includes qualitative and quantitative estimation of hydrodistilled peels and leaves essential oils components by GC/MS and investigation of certain biological activities of the oils including antioxidant, antimicrobial, cytotoxic and antiviral activities to evaluate their medicinal value.

2. Materials and methods

2.1. Plant material

Samples of *Citrus deliciosa* var. *tangarina* cultivars: Fina Clementine, Nour Clementine, Spinosa Clementine and Thornless Clementine breeded and evaluated in Horticulture Research Institute (Cairo - Alexandria road, 107 km, near to Ali Mubarak village, Egypt) were collected during November on the years 2008 and 2009 and were kindly identified by Prof. Dr. Mohamed Yehia Hegab and Prof. Dr. Ramadan Abo Serie, Department of *Citrus* researches, Horticulture Research Institute.

2.2. Material for antioxidant study

DPPH (1,1-diphenyl-2-picrylhydrazyl radical): Sigma Chemical Company, St. Louris, Mo, USA.
Ascorbic acid: (Sigma Aldrich)

2.3. Material for microbiological study

Micro-organisms: Gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus luteus*), Gram-negative bacteria (*Escherichia coli and Proteus vulgaris*), yeast (*Saccharomyces cerevisiae*) and fungi (*Aspergillus flavus*) available in stock culture of Microbiology Department, National Organization of Drug Control and Research (NODCAR).
Discs of augmentin, erythromycin, gentamicin and ketoconazole: 5 µg/disc, Oxoid Chemical Co., UK.

2.4. Material for cytotoxicity study

Liver carcinoma (HEPG2), cervix carcinoma (HELA), breast carcinoma (MCF7) and normal baby hamster kidney fibroblast cell lines (BHK) were obtained from National Cancer Institute, Kasr El Ainy, Cairo, Egypt.

2.5. Material for antiviral study

Embryonating eggs: 9-11 day old
Candle device for eggs examination.
H5N1 virus in Tris-buffered tryptose broth (1.21 g Tris per liter of tryptose broth).
Antibiotic: pencillin, (10,000 IU/ml).
Disinfectant: 70% ethyl alcohol containing 3.5% iodine and 1.5% sodium iodide.
Syringe fitted with a 25-gauge 5/8-inch (16-mm) needle.
Washed chicken erythrocytes suspension (5%).
Egg incubator: Temperature 38-39 °C and relative humidity 60-70%.
Biological safety cabinet.

2.6. Preparation of the essential oils

Fresh leaves and fruits of the four cultivars under investigation were collected, fruits were peeled and both leaves and peels were stored in deep freezer till subjected to hydrodistillation. About 2 kg of leaves and peels of the each cultivar under investigation were subjected to hydrodistillation[24]. The essential oils obtained were separately dried over anhydrous sodium sulfate and kept in a refrigerator for analysis. The percentage of the oils was calculated on fresh weight bases and physical characters including, color, odor, refractive index and specific gravity were determined and recorded in table 1.

Table 1: Physical characters of Clementine peels and leaves essential oils

Citrus species	Organ	Physical characters				
		percentage yield (%w/v)	Color	Odor	R.I.	Sp. gr.
Fina Clementine	Peels	0.68	Yellow	Mandarin like odor	1.4807	0.96
Spinosa Clementine		0.96	Yellow		1.4343	0.68
Nour Clementine		1.4	Yellow		1.4244	0.98
Thornless Clementine		0.8	Yellow		1.463	0.68
Fina Clementine	Leaves	0.65	Pale yellow	Characteristic, strongly aromatic	1.4661	0.80
Spinosa Clementine		0.5	Pale yellow		1.4256	0.74
Nour Clementine		0.5	Pale yellow		1.4256	0.90
Thornless Clementine		0.43	Pale yellow		1.4760	0.95

2.7. Analysis of the essential oils

GC/MS analysis of the essential oils was carried out on a Hewlett-Packard gas chromatograph Model 5890, coupled to a Hewlett-Packard MS model 5989B, equipped with an HP1 MS column (12 m × 0.2 mm, 0.33 µm); programming from 60 (5 min) to 300 ˚C at 5 ˚C/min, 5 min hold; the carrier gas was helium at 1.0 ml/min flow rate; injection in split mode was (60:1); injector and detector temperatures were 225 and 300 ˚C, respectively. The EIMS mode was at 70 eV; electron multiplier was 2500 V; ion source temperature was 180 ˚C; mass spectral data were acquired in the scan mode in the *m/z* range 33 to 450. The essential oils components were identified by comparing their mass fragmentation patterns with those of the available reference[25]. Identification was also confirmed by electronic Wiley and NIST mass spectral data base. The retention indices (RI) of the volatile oils components were determined relative to the retention times of series of hydrocarbons. Results are given in tables (2) and (3). The percentages of different classes of chemical constituents was calculated and compiled in tables (4) and (5).

2.8. Antioxidant activity

Different dilutions of the oil samples were prepared in methanol. Ascorbic acid, 2.5, 10, 12.5, 25 and 50 µl/ ml in methanol were used as standard antioxidant. Antioxidant activity was determined by measuring the decrease in absorbance of DPPH solution (0.0001M) at 517 nm upon the addition of different dilutions of the oil samples[26]. EC_{50} (the concentration of the solution required to give a 50% decrease in the absorbance of the test solution) were determined. The results obtained are compiled in table (6).

2.9. Antimicrobial Screening

The antimicrobial activity of *Citrus* essential oils was screened using agar diffusion technique[27]. Filter paper discs (Whatman No. 1, 5 mm diameter) containing 15 µl of each essential oil were applied to the surface of trypticase soy agar (Difco) plates inoculated with the bacterial or fungal strain of the test organisms. Discs of augmentin, erythromycin and gentamicin were used as positive control for antibacterial activity while ketoconazole discs were used as positive control for antifungal activity. The plates were incubated at 37 °C for 24-48 hours in case of bacteria and yeast and at 25 °C for 48 hours in case of fungi. After incubation, the diameters of inhibition zones were recorded in mm. The results are recorded in tables (7 and 8).

2.10. Cytotoxic activity

Cytotoxicity determinations were carried out in National cancer institute, Cairo University, Cairo, Egypt. The studied peels essential oils were dissolved in DMSO in ratio of (1:1). Cytotoxic activity was tested using Sulforhodamine-B assay according to the method of Skehan, 1990[28]. IC_{50}

was determined and compared with that of doxorubicin as reference standard. Results are recorded in table 9.

Table 2: Results of GC/MS analysis of Clementine peels essential oils

Peak No.	Compound	Kovats index	Area %			
			Fina	Spinosa	Nour	Thorn-less
1	*n*-Hexane	600	0.74	--	0.85	5.32
2	Toluene	760	0.07	--	--	--
3	*α*-pinene	939	0.89	0.88	0.92	0.67
4	Sabinene	975	0.88	1.44	0.80	0.56
5	Myrcene	991	2.97	2.82	3.53	2.4
6	*δ*-3-Carene	1010	0.42	0.54	--	0.1
7	Limonene	1029	88.73	77.55	89.95	87.53
8	*Trans-β*-ocimene	1050	--	--	0.57	--
9	*γ*-Terpinene	1060	0.51	0.78	0.11	0.29
10	Terpinolene	1089	0.15	0.26	1.58	0.09
11	Not identified	1090	2.37	--	--	--
12	1,3,8-*p*-menthatriene	1110	0.07	--	--	--
13	Not identified	1113	0.06	1.72	0.11	0.8
14	Not identified	1131	--	1.44	0.27	--
15	Citronellal	1153	0.18	--	--	--
16	Terpinen-4-ol	1177	--	2.43	--	0.41
17	Not identified	1188	0.59	--	0.50	--
18	*α*-Terpineol	1189	--	2.18	--	--
19	Decanal	1202	0.56	--	0.45	--
20	Not identified	1241	--	--	--	0.19
21	Carvone	1243	0.22	0.26	--	0.12
22	Perillaldehyde	1272	0.32	0.1	0.11	--
23	*α*-Copaene	1377	0.05	--	0.05	--
24	*n*-Dodecanal	1409	--	3.03	--	--
25	Not identified	1461	--	0.89	--	--
26	Not identified	1500	--	1.32	--	--
27	*δ*-Cadinene	1523	0.08	0.04	0.07	--
28	Not identified	1705	--	--	--	1.29
29	Sinensal	1757	0.15	0.14	0.11	0.08
30	Not identified	1788	--	0.96	--	--
31	Not identified	1801	--	1.21	--	--

Table 3: Results of GC/ MS analysis of Clementine leaves essential oils

Peak No.	Compound	Kovats index	Area %			
			Fina	Spinosa	Nour	Thorn-less
1	*n*-Hexane	600	0.61	1.84	0.88	0.6
2	Toluene	760	--	--	0.31	--
3	Xylene	867	0.22	--	0.4	--
4	Styrene	897	--	--	0.33	--
5	*α*-Thujene	930	0.46	--	0.7	--
6	*α*-Pinene	939	1.49	1.63	1.92	2.55
7	Sabinene	975	16.93	12.61	19.76	9.94
8	Myrcene	991	2.98	2.64	3.72	3.21
9	*α*-Phellandrene	1003	0.61	--	0.66	0.74
10	*δ*-3-Carene	1010	8.57	7.75	8.04	12.52
11	Limonene	1029	21.13	20.02	11.54	11.86
12	*Trans-β*-ocimene	1050	5.84	2.69	5.01	10.22
13	*γ*-Terpinene	1060	1.93	1.98	3.14	0.34
14	Not identified	1071	0.71	1.11	0.95	--
15	Terpinolene	1089	1.89	1.35	2.21	2.67
16	Linalool	1097	12.58	14.04	18.78	10.23
17	Not identified	1128	0.28	--	0.48	0.33
18	Citronellal	1153	5.39	6.81	3.5	1.72
19	Terpinen-4-ol	1177	4.33	2.85	5.36	4.87
20	*α*-Terpineol	1189	1.03	1.54	1.27	0.76
21	Citronellol	1226	1.57	2.47	0.7	0.75
22	Geraniol	1253	2.03	5.14	0.52	0.85
23	Methyl geranate	1325	0.24	0.4	--	--
24	Geranyl acetate	1381	--	--	--	0.79
25	Not identified	1394	0.27	--	--	--
26	*Trans-β*-Caryophyllene	1419	1.58	2.55	1.13	3.83
27	*α*-Humulene	1455	0.22	--	0.15	0.57
28	*Trans-β*-farnesene	1457	0.27	--	0.28	0.7
29	Bicyclogermacrene	1500	0.28	0.66	0.28	1
30	*E,E- α-* Farnesene	1506	--	--	--	0.41
31	*δ*-Cadinene	1523	0.21	--	--	0.47
32	Nerolidol	1533	0.14	--	--	0.25
33	Not identified	1536	--	--	--	0.34

34	Spathulenol	1578	--	0.71	--	--
35	Caryophyllene oxide	1583	--	0.67	--	--
36	Not identified	1592	--	2.25	--	--
37	β-Sinensal	1700	3.44	4.65	4.46	8.21
38	α-Sinensal	1757	2.77	1.64	2.37	9.23

Table 4: The calculated percentage of different classes of components of Clementine peels essential oils

Oil constituents	***Clementine***			
	Fina	**Spinosa**	**Nour**	**Thorn-less**
Identified components	97.04	92.46	99.12	97.72
Unidentified components	2.96	7.54	0.88	2.28
Monoterpene hydrocarbons	94.62	84.27	97.46	91.64
Sesquiterpene hydrocarbons	0.13	0.04	0.12	0
Other hydrocarbons	0.81	0	0.85	5.32
Total hydrocarbons	95.56	84.31	98.43	96.96
Oxygenated monoterpenes	0.4	4.87	0	0.53
Oxygenated sesquiterpenes	0.15	0.14	0.11	0.08
Other oxygenated constituents	0.88	3.13	0.56	0
Total oxygenated compounds	1.43	8.14	0.67	0.61
Total monoterpenes	95.02	89.14	97.46	92.17
Total sesquiterpenes	0.28	0.18	0.23	0.08

Table 5: The calculated percent of different classes of components of Clementine leaves essential oils

Oil constituents	***Clementine***			
	Fina	**Spinosa**	**Nour**	**Thorny**
Identified components	98.74	96.94	98.57	99.11
Unidentified components	1.26	3.36	1.43	0.89
Monoterpene hydrocarbons	61.83	50.67	57.87	59.27
Sesquiterpene hydrocarbons	2.56	3.21	1.84	2.99
Other hydrocarbons	0.83	1.84	1.92	0.37
Total hydrocarbons	65.22	55.72	61.63	62.63
Oxygenated monoterpenes	27.17	33.25	30.13	28.92
Oxygenated sesquiterpenes	6.35	7.67	6.83	7.53
Other oxygenated constituents	0	0	0	0
Total oxygenated compounds	33.52	40.92	36.96	36.45
Total monoterpenes	89	83.92	88	88.19
Total sesquiterpenes	8.91	10.88	8.67	10.52

Table 6: EC_{50} of Clementine peels and leaves essential oils

	Cultivar	**EC_{50} µl/ml**
Clementine Peels oils	Fina	18
	Spinosa	28
	Nour	68
	Thornless	118
Clementine leaves oil	Fina	16
	Spinosa	12
	Nour	20
	Thornless	8
Ascorbic acid		8

Table 7: Results of antimicrobial activity of Clementine peels and leaves essential

	Citrus species and positive control	Staphylo-coccusaureus	Micro-coccusluteus	Escherichia coli	Proteus vulgaris	Saccharomyces Cervaceae
Clementine peels oils	Fina	++	+	--	+	++
	Spinosa	+	+	+++	+	++
	Nour	--	+	–	--	--
	Thornless	+	++	++	++	++
Clementine leave oils	Fina	++	+	++++	+	+++
	Spinosa	+++	+++	+++	++	+++
	Nour	++	++	+++	+++	+++
	Thornless	+++	++	+++	++	++
Augmentin (positive control)		+++	+++	+++	++++	++++
Erythromycin (positive control)		++++	+++	+++	+++	+++
Gentamicin (positive control)		++	+++	+++	+++	++++

oils (--) no zone of inhibition
\+ diameter of inhibition zone = 8 mm
++ diameter of inhibition zone = 9-16 mm
+++ diameter of inhibition zone = 16-20 mm
++++ diameter of inhibition zone = 21-30 mm

Table 8: Results of antifungal activity of Clementine leaves essential oils

Sample	*Aspergillusflavus*
Fina Clementine	--
Spinosa Clementine	++
Nour Clementine	--
Thornless Clementine	++
Ketoconazole (positive control)	+++

(--) no zone of inhibition
+ diameter of inhibition zone = 2-8 mm
++ diameter of inhibition zone = 12-6
+++ diameter of inhibition zone = 16–20 mm
++++ diameter of inhibition zone = 21-24 mm

Table 9: Results of cytotoxic activity of Clementine peels essential oils

Citrus peel oils and drug	IC_{50}µl/ml			
	HEPG2	HELA	MCF7	BHK
Fina Clementine	0.656	4.64	0.852	No effect
Spinosa Clementine	0. 965	0.856	0.828	No effect
Nour Clementine	0.581	0.856	0.732	No effect
Thornless Clementine	0.544	2.17	0.694	No effect
Doxorubicin	0.6	0.85	0.7	0.4

2.11. Antiviral activity

Was carried out at Central Laboratory for Evaluation of Veterinary Biologics, Section: Inactivated Viral Poultry Vaccine and Lab animal, Farm Department, Viruses laboratory at Al- Abbasya, following the method of Swayne, 1998[29]. The method based on the ability of allantoic amniotic fluid (AAF) to cause hemagglutination (HA) of washed chicken erythrocytes. Embryonatic eggs were inoculated with *Citrus* essential oils under investigation, suspension of H5N1 virus and antibiotic. Then, hemagglutination test was carried out on the allantoic amniotic fluid to detect live (negative HA test) and inactivated (positive HA test) viruses [30].

3. Results and discussion

3.1. Physical characters of Clementine peels and leaves essential oils

The percentage yield of the essential oils calculated on fresh weight bases, table (1), showed certain variation, the maximum yield of peels oils (1.4%) was obtained from Nour Clementine, while the maximum yield (0.65%) of leaves oils was obtained from Fina Clementine. No significant difference on the refractive index and specific gravity of the different oils was found.

3.2. GC/MS analysis of Clementine peels and leaves essential oils

From tables (2-5), it was found that limonene is the major constituent of all peels and leaves oils under investigation except in case of Nour Clementine leaves, where sabinene was the major component (19.76%). In general the percent of hydrocarbons is higher than the percent of oxygenated compounds in all the oil samples under investigation, also the percent of monoterpenoid compounds is higher than that of sesquiterpenes. Clementine peels oils have 8 common components including: *α*-pinene, sabinene, myrcene, limonene, *γ*-terpinene, terpinolene, *α*-sinensal and unknown compound.

Nour Clementine peels oil has the highest percent of total hydrocarbons (98.43%) which constituted mainly of monoterpenoid compounds (97.46%). Clementine leaves oils showed 19 common compounds including: *n*-hexane, *α*-pinene, sabinene, myrcene, *δ*-3-carene, limonene, *trans*-*β*-ocimene, *γ*-terpinene, terpinolene, linalool, citronellal, terpinen-4-ol, *α*-terpineol, citronellol, geraniol, *trans*-*β*-caryophyllene, bicyclogermacrene, *β*-sinensal, and *α*-sinensal.

Leaves oils have higher percent of oxygenated compounds than peels oils but hydrocarbons still represent the major component. Spinosa Clementine contains the highest percent of oxygenated compounds (40.92%).

The total sesquiterpenes content is higher in the leaves oils than the peels oils, it reaches up to 24.67% in Thornless Clementine leaves oil while the highest percent in peels oils is 0.28% in Fina Clementine.

3.3. Antioxidant activity of Clementine peels and leaves essential oils

All samples showed a dose-dependent manner in scavenging DPPH radical. In general leaves oils showed higher antioxidant activity than peels oils. Thornless leaves oil showed the highest antioxidant potential, it recorded the same value of EC_{50} as ascorbic acid (8 µl/ml). These results indicated that these essential oils may contain constituents with strong proton-donating abilities[16].

3.4. Antimicrobial screening of Clementine peels and leaves essential oils

Citrus peels and leaves oils exerted variable effects against the tested Gram-positive bacteria, Gram-negative bacteria and yeast. Generally, leaves oils showed higher antimicrobial activity than peels oils against all the tested micro-organisms.

3.5. Antifungal activity of Clementine peels and leaves essential oils

Citrus peels oils had no zone of inhibition against *Aspergillus flavus*, while Spinosa Clementine and Thornless Clementine leaves oils had moderate antifungal activity when compared to ketoconazole as standard.

The observed antimicrobial activity was probably due to combination of more than one constituent. The various components may act synergistically[31].

3.6. Cytotoxic activity of Clementine peels essential oils

The four tested peels oils showed potent cytotoxic activity against liver, cervix and breast carcinoma cell lines comparing to doxorubicin. Thornless Clementine peels oil showed the most potent effect on liver and breast carcinoma cell lines, while Spinosa Clementine and Nour Clementine peels oils showed the highest cytotoxic effect on breast carcinoma cell line. The peels oils under investigation have no effect on normal baby hamster kidney fibroblast cell line which indicating its safety.

3.7. Antiviral Activity of Clementine peels and leaves essential oils

All peels and leaves essential oils showed a positive hemagglutination test in comparison with positive control, this indicates inactivated H5N1 virus, but death of the embryos was observed during three days after inoculation. Further research is needed, however, to confirm these possibilities and to study the toxicological effect of these oils.

4. Conclusion

A real comparative study of the composition of peels and leaves oils of different *Citrus* species could be carried out since all trees are grown in the same pedoclimatic and cultural conditions. Similarly, extraction conditions were identical for all samples. So, the influence of environmental and technical parameters on the chemical composition of the essential oils was considered negligible and qualitative and quantitative differences between studied cultivars were closely related to genetic background.

Leaves oils of all cultivars under investigation exhibited more potent antioxidant, and antimicrobial activities. This could be attributed to their higher percent of oxygenated compounds and sesquiterpenes as the results of GC/MS revealed. The observed cytotoxic activity of peels essential oils may be attributed to the presence of limonene, *α*-pinene, *β*-myrecene and caryophyllene which had been previously proved to be the cytotoxic components in other essential oils[32]. Other constituent viz. *β*-pinene, *α*-terpineol, *γ*-terpinene and *transα*-bergamotene may have synergistic effects with limonene[33].

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