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Research Article

Volatile Constituents, Antimicrobial and Cytotoxic Activities of *Citrus reticulata* Blanco Cultivar Murcott

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ABSTRACT

Hydrodistilled essential oils isolated from the leaf, ripe and unripe rinds as well as flower hexane extract of Murcott mandarin were analysed by GLC-MS to identify their constituents. The identified compounds were 48, 41, 40 and 46 from the mentioned organs, respectively. Monoterpenes represented the highest percentage for the identified components of ripe rind (94.76%), unripe rind (97.05%) and flower hexane fraction (50.97%) while oxygenated monoterpenes (45.94%) were the highest for leaf oil. Limonene was the major components in all samples followed by terpinene-4-ol and linalool in leaf oil, geranial, γ-terpinen and neral in flower hexane extract. Myrcene represented 2.43% and 2.69% for the ripe and unripe rind, respectively. Moreover, the major constituents were quantified by GLC-FID using a calibration curve of limonene. All tested samples showed high concentration of limonene which reached its highest concentration in flower hexane fraction (527.54 µg/ml). The tested samples were evaluated for their antimicrobial activities by using agar well diffusion assay and determination of minimum inhibitory concentration (MIC) using gentamicin, ampicillin and amphotricin B as positive controls. Flower hexane extract showed the best activity against *Enterococcus faecalis* while leaf oil exhibited its highest activity against *Enterobacter cloacae* and *Bacillus subtilis*. Additionally, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Aspragillus fumigatus* were the most sensitive to ripe rind oil while *Saccharomyces cerevisiae* was greatly inhibited by unripe rind (MIC = 1.95 µg /ml for all). *Escherichia coli* showed equal responses towards ripe and unripe rind oils (MIC=3.9 µg/ml). Also, ripe rind and leaf oils exhibited equal inhibitory effect against *B. subtilis*. MTT assay was used to evaluate cytotoxic activity compared to doxorubicin. Leaf oil showed the most potent effect on human lung carcinoma (A-549) cell line with IC_{50} = 2.5 µg, while unripe rind oil exhibited the highest activity on human colon carcinoma (HCT-116) and hepatocellular carcinoma (Hep-G2) cell lines with IC_{50} = 2.98 and 3.62 µg, respectively. The results recommend the use of Murcott mandarin oils as food preservatives and need further studies for the possible use as anticancer agents.

Keywords: Murcott mandarin, Rutaceae, volatile oils, antimicrobial, cytotoxicity.

INTRODUCTION

Citrus peels and leaves are considered as industrial and agricultural waste. These wastes are potential source for secondary metabolites in particular essential oils (EO) and flavonoids. Citrus EO are characterized by the presence of monoterpenes (as limonene, cymene), their oxygenated derivatives including aldehydes (citral), ketones, acids, alcohols (linalool), esters and sesquiterpenes[1]. They are considered one of the potential sources for the screening of anticancer, antimicrobial, antioxidant, and free radicals scavenging agents. They can be used as analgesic, sedative, anti-inflammatory and spasmolytic remedies in addition to their antiparastic and insecticidal properties[1,2]. They are also used in pharmaceuticals, perfumery and cosmetics in addition to its use in food industries as preservatives and in confectionary, cookies and desserts, and drinks[2-4].

Limonene (the major component of citrus oils) is known for its medicinal and pharmacological actions such as antitumor, anti-inflammatory, digestive and larvicidal activities. It eases constipation, relives water retention, promotes circulation and increases absorption of vitamin C to fight cold and flu. It also strengthens skin and it has many industrial uses in cosmetic products as a fragrant in perfume industry, in food manufacturing as flavoring agent and it is added to cleaning products to give orange- or lemon-like fragrance[5,6].

Murcott mandarins (Rutaceae) is most likely a tangor which is a hybrid between *Citrus reticulata* and *Citrus sinensis*. The fruit is marketed under the name Honey Tangerine; however, its official name is Murcott. The fruit is large, bright orange, very juicy and having many seeds with thin adhered peel. It is of high commercial value on the international fruit market because of its size, sweet taste, little acidic flavor, and attractive internal and external color. The fruit matures in January-March making it the latest maturing mandarin type fruit[7-10].

Concerning the current available literature, commercially available Brazilian Murcott rind oil was investigated by GLC-MS analysis[11] while more recent report studied

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oleoresin of Taiwan Murcott rind by supercritical liquid extraction[12]. Additionally, proteins, volatiles, sugars, organic acids, carotenoids were identified and gene expression levels were measured in Floridan Murcott fruits[13]. Nothing was reported about the chemical constituents and biological activities of Murcott mandarins cultivated in Egypt. The aim of our study is to identify the volatile constituents of leaf, flower, ripe and unripe rinds of Murcott mandarin cultivated in Egypt as well as its antimicrobial and cytotoxic activities.

MATERIALS AND METHODS

Plant materials

Citrus reticulata Blanco Cultivar Murcott fresh leaves and ripe fruits were collected from private Citrus garden in El Nagah village, Kom Hamada, El Baheira Governorate, Egypt in February 2014, while the flowers and unripe fruits were collected in April and December 2015, respectively. The plant was identified by Dr. B. Holyel, Prof. of Pomology, Faculty of Agriculture, Benha University. Voucher specimens were deposited (accession no. CR-134) in Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

Essential oils extraction

Fresh leaves, ripe as well as unripe rinds (100 g each) were hydro-distilled for 6 h in Clevenger's apparatus. The obtained oils were collected and dried over anhydrous sodium sulphate. Fresh flowers (100 g) were extracted with *n*-hexane (200 ml x 3), filtered and concentrated under stream of nitrogen. All samples were kept in refrigerator at 4º C till use.

Gas liquid chromatography-mass spectrometry (GLC-MS)

1µL of each tested sample (100 µl/ml hexane) was injected into an Agilent 6890 gas chromatography (USA) equipped with PAS-5MS capillary column (30 m x 0.32 mm; 0.25 µm film thickness), splitless injector attached to an Agilent 5973 quadrupole mass spectrometer. The injector temperature was 250°C and the temperature program started at 45°C isothermal for 3 min and raised to 280°C at 8°C/min, 10 min isothermal. Helium was the carrier gas (1 ml/min). The mass spectrophotometry detector was operated in electron impact ionization mode and ionizing energy of 70 eV scanning from m/z 40 to 500. The temperature of ion source was 230°C. For flower extract, 1 mg was dissolved in 0.5 ml hexane. Kovats indices (RI) were calculated with respect to a set of co-injected standard hydrocarbons (C_8-C_{24})

Gas liquid chromatography flame ionization detector analysis (GLC-FID)

Quantification of major components of investigated samples was carried out by injection of 1 µl of each sample into Trace GC Ultra (Italy) equipped with TR-WAXMS column (30 m x 0.25 mm; 0.25 µm film thickness) and splitless injector. The temperature program started at 50°C isothermal for 2 min and raised to 260°C at 8°C/min, 5 min isothermal. The used carrier gas was helium (1.5 ml/min). The injector temperature was 250°C while detector temperature was 280°C. The integration was carried out using Chrom-Card software. The identification was based upon comparison of retention time of the samples peaks and available authentics of *α*-pinene, myrcene, *α*-phellandrene, *p*-cymene and limonene. For quantification of major components, calibration curve was carried out using serial dilution of limonene (0.0007- 0.016 µg/µl).

Antimicrobial activity

EO of leaf, ripe rind, unripe rind and flower hexane extract were evaluated for their antibacterial activities against *Staphylococcus aureus* RCMB 010027, *Enterococcus faecalis* RCMB 010063 and *Bacillus subtilis* RCMB 010067 as Gram-positive bacteria and *Enterobacter cloacae* RCMB 010072, *Klebsiella pneumoniae* RCMB 010093 and *Escherichia coli* RCMB 010052 as Gram-negative bacteria. The antifungal activity was evaluated using *Aspragillus fumigatus* RCMB 02568, *Candida albicans* RCMB 05036 and *Saccharomyces cerevisiae* RCMB 05177. All microorganisms were obtained from the Regional Center for Mycology and Biotechnology, Egypt. Well diffusion method was performed on nutrient agar medium for bacterial strains and Saboroud dextrose agar for fungi[14]. The samples were dissolved in dimethyl sulfoxide (DMSO) at concentration of 500 µg/ml. Ampicillin, gentamicin and amphotricin B (100 µg/ml water) were used as positive control for Gram positive bacteria, Gram negative bacteria and fungi, respectively. The wells were filled with 100 µl from stock solution of each sample, the standards and DMSO as a negative control. The cultures were incubated at 37ºC for 24 hours for bacteria and for 2-7 days for fungi. All the assays were done in triplicate and results were expressed as mean zone of inhibition diameter in mm ± standard deviation (SD)

Agar plate dilution method was used to determine the minimum inhibitory concentration (MIC) of each sample (5 to 250 µg/ml)[15,16]. Inocula were obtained from a suspension containing approximately $1\text{-}2\times10^8$ colony-forming unit (cfu/ml). The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity comparable to that of the 0.5 McFarland standards.

Cytotoxic activity

EO of leaf, ripe rind, unripe rind and flower hexane extract were tested for their cytotoxic activity against human lung carcinoma (A-549), human colon carcinoma (HCT-116), and human hepatocellular carcinoma (Hep-G2) cell lines. These mammalian cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cytotoxicity was evaluated using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide which is known as MTT assay against DMSO and doxorubicin as negative and positive controls, respectively[17,18]. The optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to estimate the number of viable cells.

Cell viability % = [1-(ODt/ODc)] x100% where,

ODt is the mean optical density of wells treated with the tested sample; ODc is the mean optical density of untreated cells.

The relation between surviving cells and each sample concentration (0.39-50 µg/ml) is plotted to get the survival curve of each tumor cell line after treatment with the tested sample. The 50% inhibitory concentration (IC_{50}) was

calculated from graphic plots of the dose response curve for each applied concentration.

Statistical analysis

All experiments were repeated at least three times. Results are reported as means ±SD.

RESULTS AND DISCUSSION

Identification of volatile constituents by GLC-MS analysis

The flower hexane extract and the hydrodistilled oils of leaf, ripe rind and unripe rind yielded 0.8, 1.3, 1.8 and 0.8% v/w, respectively. The highest oil content was obtained from the ripe rind. The identified components and their relative percentage are given in Table 1 according to the order of their elution. The compounds were identified by comparison of their Kovats retention indices and mass spectra of each component with those of reported data[19-22]. Identification was also confirmed by electronic NIST mass spectral data base. Most of the non-identified components are present as traces with relative percentage less than 0.01.

It is clear that monoterpene hydrocarbons represented the major percentage in flower hexane extract and hydrodistilled EO of ripe and unripe rind accounting 50.97, 94.76 and 97.05%, respectively while oxygenated monoterpenes were the major for leaf EO (45.94%).

Altogether 110 components were identified, representing 86.02, 79.29, 99.25 and 99.94% in flower hexane extract, leaf, ripe and unripe rinds, respectively. In flower hexane extract, 46 compounds were identified comprising 73.14% of monoterpenes and 3.52% of sesquiterpenes. The flower hexane extract is characterized mainly by the presence of limonene (44.09%), geranial (5.82%) and *γ*-terpinene (4.87%) as major constituents. Additionally, 48 components were identified in the leaf oil, representing 61.26% of monoterpenes and 13.38% of sesquiterpenes with limonene (13.9%), terpinen-4-ol (13.78%), linalool (11.98%), caryophyllene oxide (7.47%), *α*-terpineol (3.36%) and neryl acetate (2.70%) as the major components. The ripe rind oil contained high percentage of monoterpene hydrocarbons (94.76%) where limonene (92.03%) is the most abundant components and 1.4% of sesquiterpenes. For the unripe rind oil, 40 components were identified with limonene (93.71%) and myrcene (2.69%) as the major monoterpene hydrocarbons and only 0.39% of sesquiterpenes.

In all samples the main compound was limonene (13.9-93.71%) (Table 1). Sabinene was absent in the flower hexane extract and was present in small percentages in all other oils. *β*-pinene was present only in the flower hexane fraction. The oxygenated monoterpenes ranged from 1.93 to 45.94% and the main polar compounds were terpinene-4-ol, linalool and *α*-terpineol (13.78, 11.98 and 3.36%, respectively) in leaf oil. Geranial (5.82%) was the major in flower hexane extract while, *α*-terpineol (1.17%) in ripe rind and linalool (0.94%) in oil of unripe rind. Linalool and terpinene-4-ol, were identified in all the analyzed oils but their highest quantity were signaled in leaf oil. The *α*-terpineol displayed its highest concentration in leaf oil and flower hexane extract (3.36 and 2.17 %, respectively). Neral and geranial display its highest rate in flower hexane extract (3.84 and 5.82 %, respectively), however both of them are missing in other oils. The sesquiterpene hydrocarbons fraction represented about 0.17, 0.13 to 0.33 % of the oils of the unripe, ripe rind and leaf, respectively while it is higher in the hexane extract of the flower (0.83%). Among ssesquiterpene hydrocarbons, *E*-caryophyllene was the major component followed by *α-trans* bergamotene in flower hexane fraction, and they were absent in all other oils, while *α-cis* bergamotene was found only in the leaf essential oil (0.33%).

During ripening, catabolic reactions predominate and the production of volatiles occurs during a short period and is influenced by internal and external factors[23]. So, the detailed composition may differ according to maturity and growing conditions as shown in variation of oil composition in ripe and unripe rinds.

α-Phellandrene, *α*-terpinene, *γ*-terpinene, linalool oxide (*cis* and *trans* forms), *p* -menth-2-en-1-ol (*cis* and *trans* forms), *trans*-piperitol, *2β*-hydroxy-1,4-cineole, piperitone, *trans*-ascaridol glycol, *neo*-3-thujanol acetate, *p*-cymene-7-ol, limonene-1,2-diol, *α- cis* bergamotene, *E*-nerolidol, caryophyllene oxide, humulene epoxide II, caryophylla-4 (12), 8 (13)-diene-5-*α*-ol, *α*-cadinol, *Z*-14-hydroxycaryophyllene, *E*- sesquilavandyl acetate, sabina ketone, decyl acetate, 3*Z*-hexenyl benzoate, and heptacosane were only detected in leaf oil. Such components as *β*-pinene, terpinolene, *cis*-chrysanthenol, neral, geranial, *E*-caryophyllene, *α-trans*bergamotene, *n*-undecane, *n*-tridecane, *n*-tetradecane, (1-butylheptyl) benzene, (1-pentylheptyl) benzene, undecylbenzene, dodecylbenzene, tangritin, co-elution of eugenol acetate with 1-phenyl heptane-3-one and methyl linoleate with *n*-heneicosane were only detected in flower hexane extract. Although these components were not major in other oils, these results suggest difference in the volatile profiles between flower extract fraction and leaf oil.

GLC-MS analysis of commercially available rind oil of Brazilian Murcott showed the identification of 88 components where limonene was represented by 94.6% of oil constituents and 31 compounds were detected as traces by using three different conditions of analysis[11]. Additionally, analysis of oleoresin extracted by supercritical fluid extraction of Murcott rind cultivated in Taiwan, revealed the presence of 33 volatile compounds where limonene represented only 76.34%[12]. Upon comparison of our results with these two reports, qualitative and quantitative differences were detected for the identified compounds, which may be attributed to the ecological variations and difference in methods of extraction and analysis.

Quantitative determination of major oil components by GLC-FID

The concentration of major components for the tested samples were determined through GLC-FID analysis by using calibration curve of limonene, which exhibited high linearity where $y = 6E^{+09}x + 2E^{+07}$with coefficient of determination (R^2) of 0.9877 at the used concentrations. Data shown in Table 2 were expressed as µg/ml oil or extract. Limonene was chosen as external standard because of its high availability in addition to the presence of

monoterpenes as the major identified components in all tested samples. Concentrations of *α*-pinene, *β*-pinene, myrcene, *α*-phellandrene, limonene, *γ*-terpinene, linalool, terpinen-4-ol and butylated hydroxyl toluene were determined in all tested samples.

Limonene represented the highest concentration in leaf, ripe rind and unripe rind oils and hexane fraction of flower (4.13, 435.09, 226.77 and 527.54 µg/ml, respectively). Limonene was followed by butylated hydroxytoluene (15.98 µg/ml) in ripe rind oil, myrcene (4.98 µg/ml) in unripe rind oil, *α*-pinene (3.88 µg/ml) in flower hexane extract and terpinen-4-ol (3.74 µg/ml) in leaf oil.

To conclude, the characteristic volatile profile of rind oils (either ripe or unripe) seem to have been associated with a much higher proportion of monoterpene hydrocarbons including limonene, various oxygenated monoterpenes and the relatively higher level of linalool and terpinene-4-ol are factors characterizing the volatile composition of leaf essential oil. As has been reported, linalool, *α*-terpineol and terpinen-4-ol were very important to the flavor of *citrus* oils[24]. The current study showed that the levels of linalool, *α*-terpineol and terpinen-4-ol being higher in leaf oil and flower hexane fraction than rind oils (either ripe or unripe).

Obviously, we conclude that the chemical composition of isolated essential oils from different plant parts of Murcott mandarin cultivated in Egypt showed quantitative and qualitative differences in the main components.

Antimicrobial activities

Results of antibacterial and antifungal activities of leaf, ripe and unripe rinds EO and flower hexane extract against different microorganisms by well diffusion technique, and MIC values indicated that all tested samples showed potential activity against the tested strains except *C. albicans* which showed resistance against flower hexane extract.

The inhibition zone diameter ranged from 17.8 ± 0.63 to 23.5 ± 0.48 mm for Gram negative bacteria and from 19.4 ± 1.2 to 23.9 ± 1.5 mm for Gram positive bacteria. The measured inhibition zone diameter for fungi ranged from 17.6 ± 1.2 to 22.6 ± 1.2 mm. On the other hand, inhibition zone diameters for standards were 20.2 ± 0.12 - 27.3 ± 0.44 mm for gentamycin, 25.3 ± 0.58 - 28.9 ± 0.14 mm for ampicillin and 21.9 ± 0.12-27.8 ± 0.58 mm for amphotericin B.

MIC values for tested oils and flower hexane extract (Table 3) ranged from 1.95 to 125 µg/ml. This study revealed that rind oil showed maximum activity with MIC values ranging from 1.95 to 7.81 µg/ ml against all the tested strains.

The results showed that the tested samples exhibited relatively strong antibacterial activities specially; on *K. pneumonia* where ripe rind oil exceeded the activity of gentamicin as an antimicrobial standard as illustrated by MIC values (1.95 and 3.9 µg for ripe rind oil and gentamycin, respectively). Hexane extract of the flower was superior to inhibit the growth of *E. facecalis* as indicated by its MIC value (1.95 µg). Oil of unripe rind was found to be more effective as antifungal than that of ripe rind against *S. cerevisiae*, while they nearly have the same antimicrobial activities against *E. coli* (MIC=3.9 µg). Weak antifungal activity against *C. albicans* in comparison with amphotricin B was only shown by oil of ripe rind (MIC=7.81 µg). Resistance of *C. albicans* was reported before against *C. limon* oil[25].

The MIC assay is generally more accurate than agar well diffusion assay for EO. The limitation of the oils' activity can be explained by the low water solubility of the oil and its components, which limits their diffusion through the agar medium. Only the more water-soluble components diffuse into the agar.

The hydrocarbon components either remain on the surface of the medium or evaporate[26].

When comparing data obtained in different studies, most publications provided generalization about whether or not a plant oil or extract possesses activity against Gram-positive and Gram-negative bacteria and fungi. Some publication also show the relative activity of plant oils and extracts by comparing results from different oils tested against the same organisms[27].

Comparison of the data is problematic. First, the composition of plant oil and extracts vary according to local climatic and environmental conditions. Furthermore, some oils with the same common name may be derived from different plant species. Secondly, the method used to assess antimicrobial activity, and the choice of test organisms, varies between publications[27]. It was reported that the major components of essential oils with antibacterial properties are geranyl acetate, carvacrol, geraniol, *p*-cymene, limonene, *γ*-terpinene, carvone, citral, citronellal, *α*-terpineol, terpinene-4-ol and perillaldehyde[1,28].

Limonene, *α*- pinene and linalool exhibited strong antibacterial activity[2]**.** Moreover, oxidized *d*-limonene is more active than the freshly distilled product[29]. There is a positive correlation between monoterpenes, limonene and sesquiterpenes content of the oils and the pathogen fungi inhibition[1].

Broad fungitoxic effect against *A. fumigatus* is due to presence of *dl*-limonene[30] while antifungal activity against *C. albicans* could be related to the synergistic action of the oxidized essential oil components, formed mainly of *α*-terpineol, terpinene-4-ol and linalol[31]. Yeast and fungi are markedly inhibited by oils rich in aldehydes, and alcohols[32]**.**

Low molecular weight compounds of EO as monoterpenes allow them to easily penetrate through cell walls and affect various biochemical processes[1,2]. EO sensitize the cell membrane, causing an increase in permeability and leakage of vital intracellular constituents, the impairment of bacterial enzyme system and cell respiration as well as coagulation of cell contents[32].

Gram-positive bacteria were, in general, more sensitive to essential oils than gram-negative bacteria[1,29,33], but orange and lemon oils were found to be equally effective against both Gram-positive and Gram-negative organisms[34]. Terpineol and other terpeneless fraction of *citrus* oils appeared to have greater inhibitory effect on food-borne bacteria than the other citrus oils[29].

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Table 1: Chemical composition of flower hexane extract and essential oils of leaf, ripe and unripe rinds of Murcott mandarin.

No.	Name	Reported RI	Calculated RI	M^+ (m/z)	Base peak (m/z)	Relative %			
						F	L	R	UR
1	*α*- Pinene	939	939	136	93	0.26	0.02	0.25	0.58
2	Sabinene	975	975	136	93	--	0.12	0.05	0.07
3	*β*- Pinene	979	979	136	93	1.57	--	--	--
4	Myrcene	990	990	136	93	0.09	0.32	2.43	2.69
5	*α*-Phellandrene	1002	1004	136	93	--	0.19	--	--
6	*α*-Terpinene	1017	1014	136	121	--	0.12	--	--
7	Limonene	1029	1027	136	68	44.09	13.9	92.03	93.71
8	*γ*-Terpinene	1059	1058	136	93	4.87	0.65	--	--
9	*n*- Octanol	1068	1066	130	41	--	--	0.09	0.35
10	*cis*- Linalool oxide	1072	1075	170	59	--	1.3	--	--
11	*trans*- Linalool oxide	1086	1083	170	59	--	0.71	--	--
12	Terpinolene	1088	1086	136	93	0.09	--	--	--
13	Linalool	1096	1095	154	71	2.39	11.98	0.17	0.94
14	*n*-Undecane	1100	1106	156	43	0.1	--	--	--
15	*cis*-*p*-Menth-2-en-1-ol	1121	1116	154	43	--	0.6	--	--
16	*trans*-*p*-Mentha-2,8-dien-1-ol	1122	1119	152	43	--	--	0.06	0.01
17	*cis*- *p*-Mentha-2,8-diene-1-ol	1137	1133	152	43	--	--	--	0.01
18	*trans*-*p*-Menth-2-en- 1-ol	1140	1136	154	43	--	1.72	--	--
19	*cis*-Verbenol	1141	1139	152	41	--	--	0.07	--
20	Citronellal	1153	1154	154	41	0.55	--	0.10	0.05
21	Sabina ketone	1159	1158	138	81	--	0.89	--	--
22	*cis*-Chrysanthenol	1164	1165	152	41	0.16	--	--	--
23	Terpinen-4-ol	1177	1177	154	71	1.19	13.78	0.07	0.11
24	*α*-Terpineol	1188	1188	154	59	2.17	3.36	1.17	0.38
25	*n*-Decanal	1201	1197	156	41	--	--	0.30	--
26	*trans*- Piperitol	1208	1204	154	84	--	Tr.	--	--
27	*trans* – Carveol	1216	1216	152	109	--	--	0.15	--
28	Citronellol	1225	1221	156	41	--	--	0.11	--
29	*2β*- Hydroxy- 1,4-cineole	**	1222	170	43	--	1.53	--	--
30	Nerol	1229	1228	154	41	1.00	--	--	Tr.
31	Neral	1238	1237	152	41	3.84	--	--	--
32	Carvone	1243	1242	150	82	--	1.09	0.05	--
33	Geraniol	1252	1249	154	69	1.01	--	--	Tr.
34	Piperitone	1252	1253	152	82	--	0.46	--	--
35	*2E*- Decanal	1263	1255	154	41	--	--	0.04	--
36	Geranial	1267	1265	152	41	5.82	--	--	--
37	*trans*- Ascaridol glycol	1269	1271	170	109	--	1.67	--	--
38	*n*- Decanol	1269	1272	158	41	--	--	0.03	--
39	Perilla aldehyde	1271	1274	150	67	--	--	0.09	0.25
40	*neo*-3-Thujanol acetate	1276	1278	196	43	--	0.55	--	--
41	Limonen- 10-ol	1289	1284	152	67	--	--	0.05	0.07
42	*p*-Cymen-7-ol	1290	1286	150	135	--	1.66	--	--
43	*2E, 4Z*- Decadienal	1293	1303	152	81	--	--	0.04	--
44	*n*-Tridecane	1300	1303	184	57	0.1	--	--	--
45	*p*- Vinyl guaiacol	1309	1309	150	135	--	--	--	Tr
46	Limonene-1, 2- diol	**	1350	170	43	--	0.91	--	--
47	Citronellyl acetate	1352	1352	198	43	0.06	--	0.07	0.02
48	Neryl acetate	1361	1357	196	69	2.53	2.7	0.05	--
49	*cis*-Carvyl acetate	1367	1361	194	43	--	--	0.02	--
50	Geranyl acetate	1381	1378	196	69	1.31	1.92	0.07	0.09

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51	*β*- Cubebene	1388	1388	204	161	--	--	0.04	0.04
52	*n*-Tetradecane	1400	1391	198	57	0.53	--	--	--
53	Decyl acetate	1408	1398	200	43	--	0.28		--
54	Dodecanal	1408	1417	184	57	--	--	0.05	--
55	*α- cis* Bergamotene	1412	1418	204	93	--	0.33	--	--
56	*E*- Caryophyllene	1419	1424	204	41	0.49	--	--	--
57	*p*- Menth-1-en-9-ol acetate	1423	1428	196	94	--	--	0.03	--
58	*α-trans* Bergamotene	1434	1429	204	93	0.34	--	--	--
59	Spirolepechinene	1451	1450	204	91	--	--	--	0.01
60	Sesquisabinene	1459	1458	204	41	--	--	--	0.03
61	Germacrene *D*	1485	1476	204	161	--	--	0.01	--
62	*E-β*- Ionone	1488	1480	192	177	--	--	0.11	--
63	Valencene	1496	1496	204	161	--	--	0.01	--
64	*n*- pentadecane	1500	1500	212	57	0.75	--	0.04	0.04
65	Bicyclogermacrene	1500	1504*	204	121	--	--	0.04	--
66	Epizonarene	1501		204	161				
67	*E, E- α*- Farnesene	1505	1508	204	41	--	--	--	0.02
68	Butylated hydroxytoluene	1515	1517	220	205	1.38	1.07	1.22	--
69	*δ*- Cadinene	1523	1524	204	161	--	--	0.03	0.07
70	Eugenol Acetate	1522	1530*	206	164	0.05	--	--	--
71	1- Phenyl heptane-3-one	1526		190	91				
72	Elemol	1549	1541	222	59	--	--	--	0.03
73	*E*-Nerolidol	1563	1563	222	69	--	1.12	--	--
74	Bornyl angelate	1565	1566*	236	83	0.14	--	--	--
75	8- Acetoxy-Carvotanacetone	1565		210	43				
76	*3Z*-Hexenyl benzoate	1566	1569	204	105	--	0.33	--	--
77	Dendrolasin	1572	1572	218	69	--	--	--	Tr.
78	Caryophyllene oxide	1583	1583	220	41	--	7.47	--	--
79	*n*- Hexadecane	1600	1586	226	57	0.64	--	0.02	--
80	Humulene epoxide II	1608	1602	220	43	--	0.78	--	--
81	(1-butylheptyl) benzene	**	1629	232	91	0.09	--	--	--
82	*epi-α*- Cadinol	1640	1642*	222	161	--	0.85	--	--
83	Caryophylla-4(12), 8(13)-diene-5-*α*-ol	1640		220	41				
84	*α*-Cadinol	1654	1654	222	43	--	0.83	--	--
85	*Z*-14-Hydroxy caryophyllene	1667	1667	220	41	--	0.46	--	--
86	*β*- Sinensal	1699	1699	218	93	0.5	--	0.02	0.12
87	*n*-Heptadecane	1700	1700	240	57	0.76	0.79	--	--
88	*2E, 6Z*- Farnesal	1713	1699*	220	43	--	0.21	--	--
89	Cedroxyde	1713							
90	*E*- Nerolidyl acetate	1717	1723	204	41	--	--	--	0.01
91	(1-pentyl heptyl) benzene	**	1716	246	91	0.28	--	--	--
92	*E*- Sesquilavandyl acetate	1740	1724	264	43	--	0.26	--	--
93	*α*- Sinensal	1756	1734	218	93	0.81	--	0.03	0.06
94	Undecylbenzene	**	1778	232	92	0.06	--	--	--
95	*n*- Octadecane	1800	1791	254	57	0.76	0.47	--	0.01
96	Dodecylbenzene	**	1859	246	92	0.07	--	--	--
97	*n*- Nonadecane	1900	1900	268	57	0.51	--	--	0.01
98	Methyl hexadecanoate	1921	1919	270	74	0.5	0.13	--	0.02
99	Hexadecanoic acid (palmitic acid)	1960	1960	256	41	0.04	1.09	--	0.05
100	*E,Z*- Geranyl linalool	1987	1988	290	69	--	--	--	Tr.
101	Eicosane	2000	1964	282	57	0.66	--	0.01	--
102	Methyl linoleate	2095	2085	294	67	1.26	--	--	0.05

103	*n*-Heneicosane	2100	2100	296	57	--	0.11	0.01	--
104	*n*- Docosane	2200	2130	310	57	0.37	--	--	0.01
105	*n*- Tricosane	2300	2318	324	57	--	0.12	0.02	0.01
106	*n*- Tetracosane	2400	2401	338	57	0.34	0.13	--	0.01
107	*n*- Pentacosane	2500	2500	352	57	1.42	0.25	--	0.01
108	Hexacosane	2600	2539	366	57	--	--	--	Tr.
109	Heptacosane	2700	2694	380	57	--	0.06	--	--
110	Tangeritin	**	2846	372	357	0.07	--	--	--
% of	Monoterpene hydrocarbons					50.97	15.32	94.76	97.05
	Oxygen containing monoterpenes					22.17	45.94	2.33	1.93
	Sesquiterpene hydrocarbons					0.83	0.33	0.13	0.17
	Oxygen containing sesquiterpenes					2.69	13.05	1.27	0.22
	Others					9.36	4.65	0.76	0.57
	Total identified compounds					79.29	79.29	99.25	99.94
Number of identified compounds						46	48	41	40

*: Co-eluted; **: Identified by mass fragmentation; Tr. ≤0.01; F: flower; L: leaf; R: Ripe rind; UR: Unripe rind.

Table 2: Quantification of major components of flower hexane extract and essential oils isolated from leaf, ripe rind and unripe rind of Murcott mandarin.

Compounds	Conc. (µg/ml)			
	F	L	R	UR
α-Pinene	3.88	-	3.65	0.97
β-Pinene	0.03	-	-	-
Myrcene	1.04	0.13	8.12	4.98
α-Phellandrene	-	0.05	-	-
Limonene	527.54	4.13	435.09	226.77
γ-Terpinene	0.02	0.16	-	-
Linalool	0.24	3.04	1.01	1.49
Terpinene -4- ol	-	3.74	-	0.13
Butylatedhydroxy toluene	-	0.50	15.98	-

F: Flower hexane extract; L: Leaf oil; R: Ripe rind oil; UR: Unripe rind oil

It was reported that mandarin EO showed a wide spectrum of antimicrobial activity, being strongly active against *E. coli*. Mandarin activity may be attributed to the presence of oxygenated monoterpenes (as carvone and limonene oxide) (13.6% in mandarin essential oils) or due to the synergistic interaction of other constituents present in smaller amounts[35].

Soković et al.[36] stated that there was no significant correlation between the antibacterial activity and the relative percentage of the major constituents. This finding suggested major components are not necessarily responsible for the total activity. The different antibacterial activity of the oils, compared with those of their major components, can be explained by either the synergistic effect of the different components in the oil and/or by the presence of other active constituents in small concentrations. This activity of leaf EO can be attributed not only to limonene but also to other components as caryophyllene oxide, which exhibited significant antimicrobial activity against S. *aureus*, *E. cloacae*, *K. pneumonia* and *E. coli* in a previous study[37] in addition to the synergistic effect of other components as linalool,

Table 4: IC_{50} of Murcott mandarin EO and flower hexane extract against A-549, HCT-116 and Hep-G2 cell lines.

Tested sample	IC_{50} (µg/ml)		
	A-549 cell line	HCT-116 cell line	Hep-G2 cell line
Rind oil	4.57	5.07	9.37
Leaf oil	2.5	3.02	4.54
Flower hexane extract	22.6	19.2	20.3
Unripe rind oil	3	2.98	3.62
Doxorubicin	0.85	0.46	0.47

Table 3: MIC values (µg/ml) leaf, ripe and unripe rinds EO and flower hexane extract of Murcott mandarin

Tested material	MIC (µg/ml)								
	Gram -ve			Gram +ve			Fungi		
	E. cloacae	*K. pneumoniae*	*E. coli*	*S. aureus*	*E.faecalis*	*B. subtilis*	*A. fumigatus*	*C. albicans*	*S. cerevisiae*
Ampicillin	-	-	-	0.24	0.49	0.49	-	-	-
Gentamicin	0.49	3.9	0.24	-	-	-	-	-	-
Amphotricin B	-	-	-	-	-	-	0.49	0.98	0.24
Ripe rind oil	3.9	1.95	3.9	1.95	3.9	1.95	1.95	7.81	3.9
Leaf oil	1.95	3.9	15.63	3.9	3.9	1.95	62.5	125	62.5
Flower hexane extract	31.25	15.63	15.63	3.9	1.95	15.63	62.5	NA	125
Unripe rind oil	15.63	7.81	3.9	15.63	15.63	31.25	3.9	15.63	1.95

NA= No Activity.

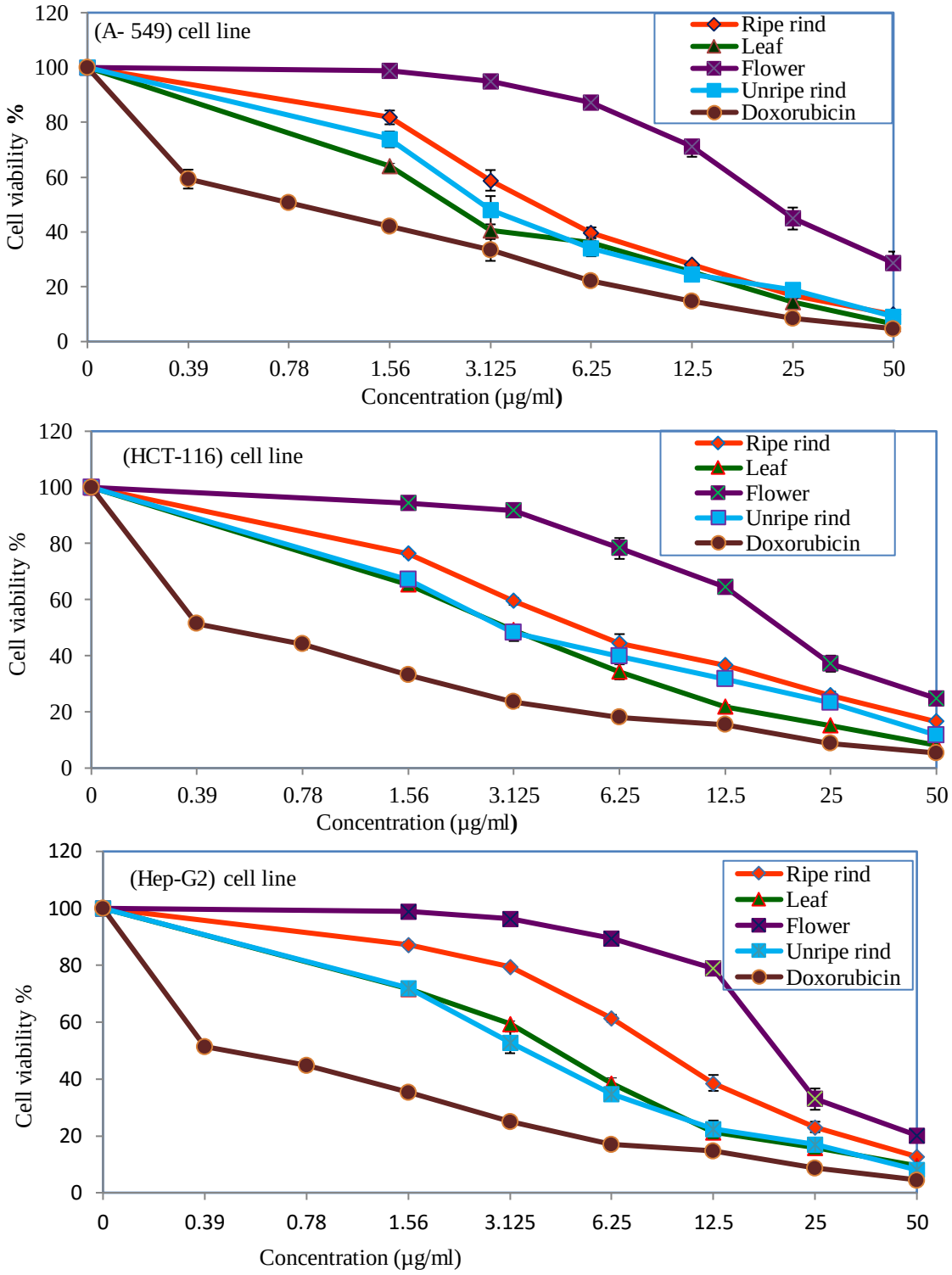

Figure 1: *In vitro* cytotoxic activity of Murcott mandarin oils of different organs and flower hexane extract against A-549, HCT-116 and Hep-G2 cell lines.

terpinene-4-ol, γ-terpinene, α-terpineol, geranyl acetate, carvone and other minor constituents.

Cytotoxic activity

In vitro cytotoxic activity of the applied samples against tested cell lines showed decrease in cell viability in dose-dependent manner as illustrated in Figure 1. Evaluation was based on IC_{50} values as follows: $IC_{50} \leq 20$ µg/ml = highly active, IC_{50} 21-200 µg/ml = moderately active, IC_{50} 201-500 µg/ml = weakly active and IC_{50}> 501 µg/ml = inactive which is in a good accordance with the protocol of the American National Cancer Institute[38].

It is clear that the flower hexane extract was less potent than the hydrodistilled oils isolated from different organs as indicated by the values of IC_{50}. The cytotoxicity of the applied samples against A-549 cell line was arranged as follow: Leaf EO > unripe rind EO > ripe rind EO > flower

hexane extract. The higher activity of leaf oil (IC_{50}= 2.5 µg) may be due to the presence of limonene, terpinen-4-ol and linalool as major compounds (13.9, 13.78 and 11.98%, respectively). The activity could be also attributed to some specific components found in the oil as caryophyllene oxide which was reported to have a potent cytotoxic activity against a wide range of cell lines[39]. EO of unripe rind is more cytotoxic to A- 549 cells (IC_{50} = 3 µg) than that of ripe rind IC_{50} = 4.57) although both of them have nearly similar concentrations of the major compound limonene.

As indicated by IC_{50} values, the cytotoxicity of tested samples against HCT-116 cell are arranged as follow:

Unripe rind oil > leaf oil > ripe rind oil > flower hexane extract. EO of unripe rind and leaf showed close cytotoxic effect on HCT-116 cell (IC_{50} =2.98 and 3.02 µg, respectively)

As shown in Table 4 oil of unripe rind showed the most cytotoxic effect against Hep-G2 cell with IC_{50} = 3.62 µg which may be correlated to the presence of limonene and myrcene. All of the tested oils showed high cytotoxic activity against all tested cell lines while flower hexane extract was less active against A-549 and Hep-G2 cell lines as indicated by its IC_{50} values (22.6 and 20.3 µg, respectively).

Our results are in agreement with that reported for the cytotoxicity of monoterpens and correlated with the effect of limonene, myrcene, linalool and terpinene-4-ol [40,41] which are major constituents identified in Murrcot mandarin in this study.

Peel oils of four cultivars of *Citrus deliciosa* var. *tangarina* showed potent cytotoxic activity against liver carcinoma (Hep-G2) which was attributed to the presence of limonene, *α*-pinene, *β*-myrecene and caryophyllene[21]. Other constituents as *β*-pinene, *α*-terpineol, *γ*-terpinene and trans-*α*-bergamotene may be responsible for the cytotoxic activity due to synergistic effects with limonene[42].

Our previous study reported that limonene and myrcene exhibited strong cytotoxic activities against HCT-116 (IC_{50}=2.97 and 1.27 µg, respectively) and Hep-G_2 (IC_{50}=2.95 and 0.93 µg, respectively) in dose dependent matter when evaluated by MTT assay[43]. Many monoterpenes (as limonene, myrcene, linalool, terpinene-4-ol, citronellal, perillyl alcohol, carveol, carvone, geraniol, *α*- terpinolene, , ….etc) have been proposed to exert potent cytotxic activity[44-46] which explain the cytotoxic effect of EO of Murcott leaf as it contains significant quantity of terpinene-4-ol (13.78%) in addition to high contents of limonene and linalool (13.9 and 11.98 %, respectively). D-Limonene is metabolized into perillic acid, dihydroperillic acid and limonene1, 2-diol which have a higher bioavailability which explain its possible mechanism as antiproliferative effect[44]. Cytotoxic effect of tested samples could be due to presence of limonene as major compound. EO with higher percentage of limonene, showed greater cytotoxicity due to the induction of carcinogen metabolizing enzymes, growth factor receptor expression, and inhibition of 3-hydroxyl-3- methyl glutraryl CoA reductase.

Additionally, D-limonene oxygenated derivatives, e.g. perillyl alcohol, carveol, carvone, geraniol and menthol, exhibited biological activity *in vivo* against certain types of malignant tumors. Perillyl alcohol, a hydroxylated limonene analog, exhibits chemopreventive activity against liver, colon, mammary gland and pancreas cancer in rodents. As limonene, linalool, *α*- terpinolene, carvone, citronellal and camphene; exhibited potent antitumor and antioxidant activities, ingestion of these aroma compounds may help to prevent *in vivo* oxidation damage such as lipid peroxidation, which is associated with cancer, premature aging and diabetes[45].

CONCLUSION

A total of 110 volatile constituents were identified from hexane flower extract as well as hydrodistilled EO of leaf, ripe rind and unripe rind of Murcott mandarin where limonene was the major compounds. The tested samples exhibited potential antimicrobial activities against different bacteria and fugi which recommend their use as food preservatives. They also exhibited strong *in vitro* cytotoxic effects against lung, liver and colon carcinoma cell lines in dose-dependent manner.

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