Chemical composition and cytotoxic activity of petitgrain essential oil of *Citrus aurantium* L. "Russian colon"

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Abstract: The essential oil isolated by hydrodistillation from the fresh leaves of the locally cultivated *Citrus aurantium* L. "Russian colon" has been analyzed by GC-MS. Twenty three components accounting to 94.38% of the total detected constituents were identified. The major ones were: linalool (49.90%), linalyl acetate (13.09%), *α*-terpineol (8.81%), geraniol (4.69%) and geranyl acetate (4.49%). Screening of the cytotoxic effect of the oil on two malignant cell lines of hepatic origin (HepG2), and breast tissue origin (MCF-7) was studied. The oil exhibited a moderate activity against HepG-2 cell line only.

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1. Introduction

Genus *Citrus* comprises a great number of species; most of them are evergreen aromatic shrubs to small size trees. They are widely cultivated mainly for their highly appreciated tasty fresh fruits as well as their processed products which have a large market worldwide. Egypt is ranked among the top ten largest citrus producers in the world, with an annual production estimated as 3,461,000 tons (season 2011-2012) representing approximately 3.0% of that of the total world's (FAO, 2012).

Citrus essential oils and their versatile major components are considered safe and thus extensively processed as flavoring agent in nutritive materials like candies, marmalades, sweets, dairy products, beverages……etc. They are much needed in the pharmaceutical industry to mask the unpleasant tastes of drugs *via* their strong flavoring effect. Furthermore and because of their pleasant and characteristic fragrance, they are widely used in the perfumery industry (Rouseffand Perez-Cacho, 2007).

Every year, or more regularly every other year, Citrus trees are subjected to the pruning process to avoid crowding, allow light penetration through the trees, optimize flowering, increase yield, improve fruit size and quality and assist pest disease control (Pittaway, 2002). This process is done from post-harvest to the pre-bloom stage and ends with the removal of massive amounts of the green foliage parts (leaves and branches) among other wastes.

Therefore, one of the objectives of the present study was to investigate a possible alternative for the disposal of citrus agricultural wastes in order to maximize benefits from them. So it was found of interest to analyze the essential oil of the leaves of bitter orange and further, investigate its cytotoxic effect.

Citrus aurantium L. (syn. bigarade orange, Seville orange, bitter orange or sour orange) is a small tree, about five to six meters tall, with pleasant odor and white flowers. Unlike most other *Citrus* species, its fruit juice is seldom used because of the presence of the bitter tasted flavanone, naringin, in addition to the high levels of citric acid responsible for the strong sour taste. However, bitter orange tree gifted us with three distinct oils, viz. bitter orange peel oil expressed from the oil glands filling the outer flavedo layer of the peel; neroli oil from the freshly picked flowers and petitgrain oil from the fresh leaves and twigs of the tree (Rouseffand Perez-Cacho, 2007).

Globally, bitter orange neroli oil is highly appreciated and is described as one of the top pearls of perfumery (Bonaccorsi *et al*., 2011) beside its use as a flavoring agent in versatile food and beverage products. The production of neroli oil is surprisingly less than the world demand, about 2000 Kg/year at a market price of more than 4,500 USD/Kg (Bonaccorsi, *et al.*, 2011). Consequently and as expected, this pure and high quality, high priced product will be subjected to adulteration with oils obtained from flowers of other *Citrus* species or more commonly, oils from leaves of *C. aurantium* L. In fact, the latter because of its pleasant and attractive fragrance, is extensively used in cosmetics, perfumes and soaps (Lota *et al*., 2001).

Bitter orange leaf oil has been subjected to extensive analytical studies over the past several decades. The published articles have been successively reviewed by Lawrence (1993), Mondello *et al*. (1996), Dugo *et al*. (2002) and Dugo *et al*. (2011). During the last few years several articles have also been published including Azadi *et*

al. (2012), Majnooni *et al*. (2012), Periyanayagam *et al*. (2013), Darjazi (2013), Sarrou *et al*. (2013), Trabelsi *et al*. (2014), Abderrezak *et al*. (2014), Ellouze and Abderrabba (2014) and Ouedrhiri *et al*. (2015).

In general, the chemical composition of the leaf oil revealed distinct variation among the reported articles. Nevertheless, the linalool/linalyl acetate chemotype (12-66% / 13-62%) is the most prominent (Lota *et al*, 2001).

Essential oils extracted from different parts of bitter orange were found therapeutically effective in treatment of various diseases. They aid in digestion, cardiovascular diseases, anti-cancer, treatment of stroke, antianxiety, antiviral and obesity (Suryawanshi, 2011). Majnooni *et al*. (2012) studied the cytotoxic activity of the essential oil of the leaves of *C. aurantium* L. on six tumor cell lines (human promyelocytic leukemia HL60, human chronic myelogenous leukemia K562, Jurkat adult T cell leukemia, prostate adenocarcinoma PC3, human colon adenocarcinoma HT29 and human cervix carcinoma Hela) and on a normal cell line human umbilical vein endothelial cell (HUVEC) using LDH assay. They found that the oil had the highest activity against Jurkat and HL60. Furthermore, lower effects were noticed on PC3 and HUVEC. They attributed the cytotoxic activity of the oil to limonene and linalool.

In Egypt, hepatocellular carcinoma keeps on being one of the most noteworthy reasons for tumor occurrence and mortality in men (Lehman, 2008), while breast cancer disease is the most well-known dangerous tumor among ladies (Zawilla, 2011).So, we decided to carry out a cytotoxicity screening of the oil on two malignancy cell lines, of hepatic origin (HepG2), and breast tissue origin (MCF-7).

2. Experimental

2.1. Plant material

The fresh healthy leaves of *Citrus aurantium* L. cultivar "Russian colon" were collected early in the morning in February 2014 from trees growing in the Fruit Experimental Station, Mansoura University. The plant identity was kindly authenticated by Dr. Mohsen Fahmy Prof. of Pomology, Faculty of Agriculture, Mansoura University, Egypt.

2.2. Isolation of the essential oil

The fresh leaves (700 g) were subjected, immediately after collection, to hydrodistillation for 3 h. using a Clevenger-type apparatus. The oil was dried over anhydrous sodium sulfate then stored at +4 ºC in the dark until tested.

2.3. GC-MS analysis

GC/MS analysis of the essential oil was carried out at the Faculty of Pharmacy, Cairo University. A Shimadzu Model GC-17A gas chromatograph interfaced with a Shimadzu model QP-5000 mass spectrometric detector and a Shimadzu AOC-20i auto-injector module (Japan) was used. The injection volume was 1 µL. The instrument was controlled by the Shimadzu Class-5000 version 2.2 software containing a NIST62 (National Institute of Standards and Technology) MS library. The components were separated on a DB5 column (30 m length, 0.25 mm inner diameter and 0.25 µm film (J&W Scientific, Santa Clara, Calif. U.S.A.). Injections were made in the split mode for 30 s and the gas chromatograph was operated under the following conditions: injector 220ºC, column oven 40ºC for 3 min then programmed at a rate of 12ºC/min to 180ºC, kept at 180ºC for 5 min and finally ramped at a rate of 40 ºC/min to 220ºC and kept for 2 min, He carrier gas at 1 mL/min. The transfer line and ion-source temperatures were adjusted at 230 and 180ºC, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 ev. The scan range was set at 40-500 m/z. Volatile components were identified using the procedure described in Farag & Wessjohann (2012) and peaks were first deconvoluted using AMDIS software (www.amdis.net) and identified by their retention indices (RI) relative to n-alkanes (C8-C20) (Adams, 2007) as well as mass spectrum matching to NIST, WILEY library database.

2.4. Cell lines and cell culture

Two human tumor cell lines namely; hepatocellular carcinoma (*HepG2)* and breast cancer carcinoma (MCF-7) were obtained from ATCC *via* a Holding company for biological products and vaccines (VACSERA, Cairo, Egypt). They were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100 µg/ml streptomycin at 37ºC in a 5% CO_2 incubator.

2.5. Chemicals used in MTT assay

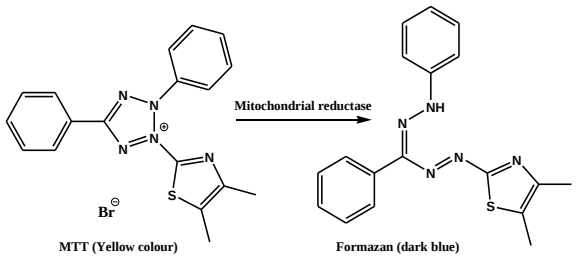
MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide], Doxorubicin, and Dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA).

2.6 Cytotoxic activity by MTT assay:

The assay was carried out according to the methods described earlier by Mosmann, 1983, Mauceri *et al*., 1998 and Lee *et al*., 2008.

2.7. Statistical analysis:

The results are expressed as the mean values ± standard deviation SD from three separate experiments. Significant difference from the control value and each compound was determined by student's t-test. The differences were considered statistically significant from the control at $p < 0.05$.



3. Results and discussion:

3.1. Chemical Composition of the essential oil

Bitter orange leaves yielded 0.71 % v/w of a clear, faint yellow essential oil, lighter than water and having a fresh, pleasant sweet aroma. The GC/MS analysis of this local oil sample is presented in Table 1. Comparison of the data with those reported for oils from several Mediterranean countries; three European, one Asian and two African, in addition to an Iranian one are also listed in the same Table.

Twenty three components, accounting for 94.38 % of the oil were identified. These same components almost represent the top ones as reported in numerous articles regardless of the continent or the country as illustrated in details in Table 1. The oxygenated fraction of this local bitter orange leaf oil sample is 85.26%. Alcohols (65.11%) are the most abundant, followed by esters (19.76%) then aldehydes (0.39%). The monoterpene hydrocarbons contribute about 7.26% and are represented in a descending order by trans-*β*-ocimene (2.18%), myrcene (1.97%), *β*-pinene (0.93%), cis-*β*-ocimene (0.87%), terpinolene (0.49) and limonene (0.47%) in addition to four minors. Linalool is the dominant alcohol component (49.9 %) followed by *α*-terpineol (8.81%), geraniol (4.69%) and nerol (1.69%). Linalyl acetate is the major ester (13.09%) followed by geranyl acetate (4.49%) and neryl acetate (2.18%).

The oil in general, reveals a good homogeneous composition with those reported in Table 1 and several others described in the literature and classified under the linalool/linalyl acetate chemotype. However, it should be noted that the linalool/linalyl acetate ratio in the present study is highly in favor of linalool (49.9/13.09) and very close to that found in the young leaves oil of the Greek sample (58.21/12.42). Furthermore, those oil samples extracted by steam distillation e.g. (Table 1, columns 7-9) revealed much higher ratios in favor of linalyl acetate as compared to those prepared by hydrodistillation (Table 1; columns 1 to 6). The Italian group has also reported the same notice on their investigation of several samples of bitter orange neroli oil (Bonaccorsi *et al*., 2011). Moreover, the same observation can be extended to the ratios of geraniol/geranyl acetate and nerol/neryl acetate. Unfortunately, we are still ignoring a lot of the impact of the variable reactions that possibly taking place along the 3-hrs hydrodistillation of a crude aromatic sample on its produced oil.

The Egyptian bitter orange leaf oil was subjected to previous studies including the one referred to in Table 1, column 8 (Dug*o et al*., 2011). The authors didn't declare any information about the cultivar, place and month of collection. The results were almost identical with those reported by the Italian team for Italian petitgrain oil (Mondello *et al*., 1996). Prager and Miskiewicz (1981) analyzed different commercial samples of petitgrain oils produced in the Mediterranean countries. The results of the Egyptian sample quantified linalool 33.7% and linalyl acetate 38.4% thus exhibiting the same globally dominant chemotype. On the other hand, atypical data for oil samples collected also from local bitter orange leaves were reported: Limonene (26.79%), linalool/linalyl acetate (4.95/3.45) (Karawaya *et al*., 1970); linalool/linalyl acetate (18.37/zero), *α*-terpineol (16.30%), geranyl acetate (12.45%), neryl acetate (12.4%), apiole (4.05%) (Haggag *et al*., 1999); limonene (29.9%), linalool/linalyl acetate (12.3/0.1), geraniol (4.2%) (Hifnawy *et al*., 2004).

It is worth to note that among the recently reported studies on the same subject, some atypical or even abnormal data are evident. The following are only examples: an Iranian sample (Majnooni *et al*., 2012), limonene (57.57%), linalool (8.01%) and absence of any ester; a Tunisian sample (Trabelsi *et al*., 2014) *β*-fenchyl alcohol (8.4%) instead of *α*-terpineol and a Moroccan sample (Quedrhiri *et al*., 2015); linalyl 2-aminobenzoate (41.8%) instead of linalyl acetate.

3.2. Cytotoxicity assay

In the cytotoxicity assay, two cell lines, HepG-2 and MCF-7 were used. The results showed that bitter orange leaf oil had moderate activity against HepG-2 cell line. For the other cell line, MCF-7, the oil did not exhibit a promising activity (Table 2).

Table 1. Comparative chemical composition (% peak area) of the present oil sample (column 1) with those reported in certain selected publications (columns 2-9)*

Component	1 Present sample		2 Tunisian	3 Algerian	4 Greek		5 French***		6 Iranian	7 Italian****		8 Egyptian	9 Turkish
	RI**	%			Young leaf	Old leaf	Min.	Max.		Min.	Max.		
α-Pinene	937	0.07	0.20	0.19	0.19	-	0.10	2.20	0.23	0.03	0.30	0.08	0.10
Sabinene	977	0.12	0.20	1.58	0.37	0.22	0.30	0.80	0.47	0.13	0.23	0.17	0.20
β- Pinene	982	0.93	1.30	2.06	3.85	1.90	2.10	5.90	3.10	0.65	1.15	0.71	0.80
Myrcene	990	1.97	2.30	1.82	1.63	2.73	2.10	2.80	2.29	0.56	1.24	1.23	1.80
Car-3-ene	1013	0.07	-	0.20	-	-	-	-	-	0.21	0.67	0.71	0.30
Limonene	1034	0.47	0.70	1.25	0.53	0.77	0.60	0.90	0.82	0.44	2.17	1,91	2.50
Cis-*β*-ocimene	1037	0.87	0.90	-	0.71	1.22	0.90	1.10	0.99	0.20	0.44	0.42	0.30
Trans-*β*-ocimene	1049	2.18	2.30	1.92	4.08	3.11	2.20	3.40	2.70	0.57	1.76	1.61	1.90
Cis- sabinene hydrate	1063	0.09	-	-	-	-	-	-	0.02		0.01	-	trace
Terpinolene	1091	0.49	0.50	0.30	0.40	0.70	0.50	0.60	0.51	0.08	0.22	0.26	0.20
Linalool	1104	49.90	36.80	36.10	58.21	36.03	28.90	37.70	29.34	21.70	32.55	27.82	24.80
Terpinolene-4-ol	1181	0.02	0.20	0.42	0.17	0.13	0.00	0.02	0.14	0.05	0.08	0.05	0.10
α- Terpineol	1195	8.81	11.70	6.80	7.11	12.89	9.10	11.80	9.55	3.09	5.63	2.97	6.20
Nerol	1230	1.69	2.40	1.51	1.45	2.89	1.80	2.30	1.95	0.75	0.99	0.66	0.60
Neral	1245	0.08	-	0.12	-	-	0.00	0.10	0.04	0.21	0.43	0.19	0.30
Linalyl acetate	1252	13.09	22.10	28.94	12.42	23.00	21.50	31.50	31.24	50.68	62.57	54.64	50.10
Geraniol	1256	4.69	7.10	3.97	-	-	5.20	6.70	5.74	0.71	0.95	-	1.20
Geranial	1274	0.31	-	-	-	-	0.00	0.20	-	0.38	0.64	0.30	0.60
Neryl acetate	1359	2.18	3.20	-	2.18	4.46	2.30	2.90	2.58	1.04	1.73	1.31	1.90
Geranyl acetate	1379	4.49	6.00	3.54	4.49	8.70	4.40	5.50	4.83	1.90	3.16	2.75	3.40
β- Caryophyllene	1430	1.14	0.30	0.20	1.09	0.22	0.10	0.30	0.23	0.48	0.61	0.51	0.70
α- Humulene	1466	0.20	-	-	0.10	trace	Trace	Trace	-	0.04	0.06	0.05	0.10
Bicyclogermacrene	1506	0.52	-	-	0.18	0.20	-	-	-	0.04	0.30	0.16	-

*Data in columns: **2** (Boussaada and Chemli, 2006); **3** (Baalionamer and Meklati, 1986); **4** (Sarrou *et al*., 2013); **5** (Lota *et al*., 2001); **6** (Darjazi, 2013);**7** (Mondello *et al*., 1996); **8** (Dugo *et al*., 2011);**9** (Kirbaslar and Kirbaslar, 2004).
**Retention indices.
***The data listed represent the minimum and maximum figures among 24 different cultivars of sour orange trees grown under the same pedoclimatic and cultural conditions. The extraction and analytical procedure were also identical.
****The data listed represent the minimum and maximum figures among 5 samples.

Table 2. Results of cytotoxicity assay of the tested oil on different cancer cell lines*

	MCF-7	HepG-2
Doxorubicin	74.3±9.63	78.3±4.2
Bitter orange	90.1±11.8	84.11±7.87

* Values are expressed as mean ± SD, n=3.

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