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REGULAR ARTICLE

THE CYTOTOXIC EFFECT OF ESSENTIAL OILS *CITRUS AURANTIUM* PEELS ON HUMAN COLORECTAL CARCINOMA CELL LINE (LIM1863)

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ABSTRACT

Citrus essential oils (EOs) contain different terpens that have been shown to possess antitumor effects. We determined the cytotoxic effect of essential oils of *Citrus aurantium* L. subsp*amara* peels on a colorectal cancer cell line (Lim1863). Three samples were harvested from three locations in Syria. EOs were extracted by hydrodistilation and analyzed by GC-MS. EOs content of Limonene was96-97.7 % while α-pinene and β-myrcenewere0.35-1% and 0.9-1.4% respectively. Various concentrations of EOs (0.25-48 µl/ml) were added to cultured cells and incubated for 72 h. Cell viability was evaluated using the MTT-based cytotoxicity assay. The obtained IC50 value range of *C. aurantium* Eos was 2.18-2.44 µl/ml. In conclusion, *C. aurantium* peels Eos obviously reduced the cell viability and it might have cytotoxic effect against colorectal cancer cell line.

Keywords: *Citrus aurantium,* Peels, Essential oils, cytotoxicity, Lim1863

INTRODUCTION

Colon or colorectal cancer is a cancer that starts in the large intestine (colon) or the rectum (end of the colon). Colon cancer in most cases is adenocarcinoma. According to National Cancer Institute (NCI) and American Cancer of Society, colorectal cancer is the third most common cancer diagnosed in both men and women in the United States. The American Cancer Society's most recent estimates for the number of colorectal cancer cases in the United States for 2012are (103,170) new cases of colon cancer and (40,290) new cases of rectal cancer.NCI also estimated that in 2010 colorectal cancer care costs approximately 14.14 billion US dollar in United States only and this costs also are likely to increase. The aim of scientist is to find non-toxic compound that can eradicate cancer cells without harming normal, healthy cells in the same organ. Natural compounds usually have fewer side effects and cost less.

The importance of medicinal plants as remedies has been discovered as early as 5000 years ago **(Umezu et al, 2002)**. They can be used for maintaining health, enhancing overall immune status, and prevention and treatment of chronic diseases **(Spencer, 1999)**. *Rutaceae* plants were often implicated in medical treatments because of their high content of active ingredients, especially essential oils **(Blumenthal, 2002)**. *Citrus aurantium* is a species of citrus genus which has been cultivated in Syria. Current chemical, medical, and pharmacological literature suggest that substances derived from *C.aurantium* can be used as antispasmodic, sedative, tranquilliser, appetizer, cholagogue, and vascular stimulant **(Arias and Ramón-Laca, 2005)**. Among Bioactive derived from *C.aurantium*, EOs is found to be the most prominent in antitumour efficacy. Several studies indicated that EOs have antitumor effects, monoterpenes have been reported to have a chemopreventive effect against rodent mammary, skin, liver, lung and forestomach cancers (Crowell, 1999). EOs from Citrus plants contain a high percentage of monoterpene hydrocarbons (70-95%) along with smaller amounts of sesquiterpene hydrocarbons, oxygenated derivatives and aromatic hydrocarbons **(Mohamed et al, 2010)** and **(Siddique et al, 2011)**. Citrus oil mainly consists of Limonene, α-pinene, β-pinene, β-myrceneand linalool **(Siddique et al, 2011)** and **(Lota et al, 2001)**. Despite the

many publications studying the compositions of *Citrusaurantium* peels EOs, no report on its cytotoxic effect is yet available. In this study, the composition of *Citrus aurantium*peels EOs, obtained from three different locations in Syria, was determined, and their cytotoxic effect against colorectal cancer cell line (lim1863) was studied.

MATERIAL AND METHODS

Plant materials

Citrus aurantium fruits of adult trees were collected from three different locations in two cities; Sample 1: was collected from Tartus-Safita. Sample 2 was collected from an agricultural region in Damascus-Adawi. Sample 3 was collected from a crowded residential area in Damascus-Tijara. Samples were collected on Jan 2011. The peels were ripped carefully using a sharp knife.

EOs extraction

EOs was extracted from fresh (within one day) peels (300 g) collected by hydrodistillation for 3 h using a Clevenger-type apparatus. The oils were dried over anhydrous sodium sulfate and stored at -20 ^{O}C for subsequent analysis.

EOs analysis

The oils were analyzed by GC/MS using an Agilent® 7890A gas chromatograph coupled with an Agilent MS: 5975 mass detector. GC was equipped with a non-polar capillary column Agilent DB-1 (30 m×0.25 mm, film thickness 0.25 µm). Operating conditions were as follows: carrier gas, helium with a constant pressure of 9.43 psi; column temperature, 60-275^{O}C at a rate of 3^{O}C/min; injector temperature, 280^{O}C; injected volume, 1 µl of the oil; and split ratio, 1:25. The MS operating parameters were as follows: ionization potential, 70 eV; ion source temperature 200^{O}C; and resolution 1000. Identification of components present in the oil was based on computer matching with the Nist 08 library.

Cell line

Human colorectal cancer cell line (Lim1863) was obtained by courtesy of Prof. NizarMhaidat from Jordan University of Science and Technology. They were maintained in D-MEM cell culture medium supplemented with 10% fetal calf serum and penicillin/streptomycin (100 IU/ml and 100 µg/ml). Cells were grown at 37°C in a humidified atmosphere of 5% CO2 using LabTech® CO_2 incubator (LCO-065 AI).
The cell line was maintained and grown in D-MEM to 10 subcultures. Samples of the cell line were cryopreserved in liquid nitrogen.

MTT-based cytotoxicity assay

EOs were diluted using Ethanol to the following concentrations (0.25, 0.5, 1, 2, 4, 8, 16, 24, 32 and 48 µl/ml). Assessment of cell viability was carried out by a modified method of Mosmann **(Mosmann, 1983)** using 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). 200 µl cell suspension (5×104 cell/ml) were seeded in 96 microplates and incubated for 24 h (37°C, 5% CO2, air humidified), then 20 µl of various EOs concentrations was added (one of previous concentrations to one well). Microplates containing cells and EOs were incubated for another 72 h, under the same condition. To indicate the accuracy of the experiment, 20 µg/ml of Doxorubicin (Ebewe®) was used as a positive control. The first row of each microplate was assumed as a negative control (containing neither EOs nor doxorubicin). To evaluate cell survival, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 3 h. 150 µl of a medium containing MTT was replaced by DMSO and pipetted to dissolve any formazon crystals formed. Absorbance value was determined at 540 nm by an Enzyme-Linked Immunosorbent Assay (ELISA) plate reader (SCO diagnostic®).

Each oil concentration was repeated eight times. Standard curve of each sample (absorbance value against cell number) were also plotted. Percentage of cell survival was determined, assuming a 100% survival value for negative control. IC50 was calculated using analysis of regression.

Statistical analysis

Microsoft Office Excel 2007 and SPSS 17.0 were used to perform statistical tests. Standard Deviation (STDEV) and Coefficient of Variation (CV) were calculated using Microsoft Office Excel.Spearman Correlation Coefficients and Tukeytest ware performed using SPSS 17.0 with the level of significance set at $P < 0.01$

RESULTS AND DISCUSSION

Chemical composition of the EOs

The essential oil yield was 2.5% (V/W) in sample 1, while yield was 2.1% (V/W) in samples 2 and 3. GC-MS analysis (Figure 1) resulted in the following components described in table 1. Limonene is the major component and its highest percentage was in sample 3. α-pinene, β-myrcenewere detected in all samples while linalol was not detected in any sample. In sample 3 we notice that β-pinene, Terpinolene, delta-3-carene and Terpinene-4-ol were not detected.

Table 1 EOs composition of Syrian *C.aurantium* peels

Component	RT	Sample 1	Sample 2	Sample 3
α-pinene	**5.252**	**0.83**	**0.66**	**0.67**
β-pinene	**6.461**	**0.10**	**0.16**	
β-myrcene	**7.268**	**1.3**	**1.41**	**1.29**
Terpinolene	**8.111**	**0.10**	**0.07**	
dl-limonene	**8.994**	**96.28**	**96.82**	**97.24**
delta-3-carene	**12.421**	**0.46**	**0.61**	
Terpinene-4-ol	**16.975**	**0.13**	**0.12**	
Bisaboline	**37.649**	**0.79**	**0.27**	**0.25**

RT: Retention time

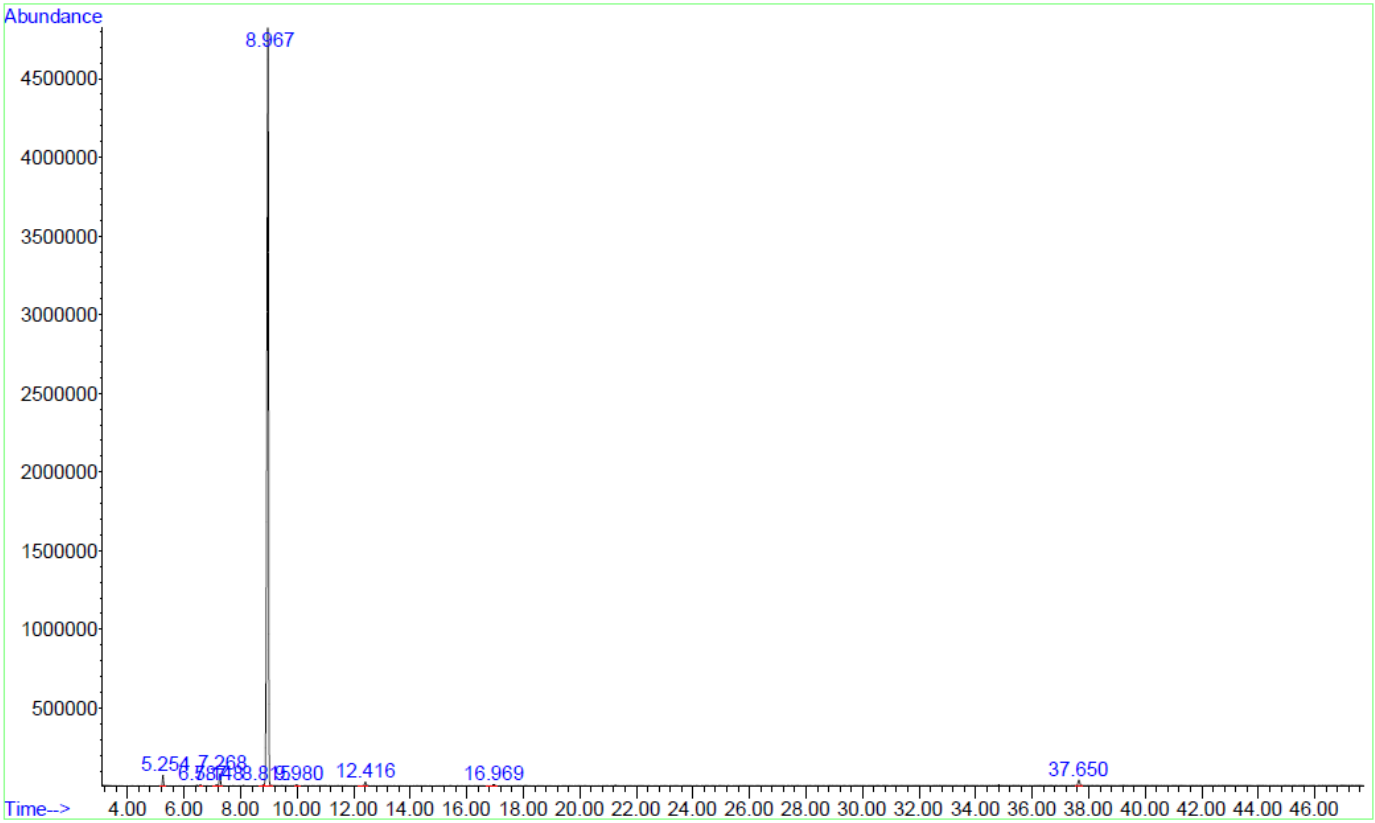

Figure 1 GC-MS chromatogram showing chemical analysis of essential oil from *C.aurantium* peels sample 1

Cytotoxic effect of EOs

A preliminary study showed significant positive correlation between living cell count and absorbance value for MTT using 540nm wavelength for measurement (Figure 2).

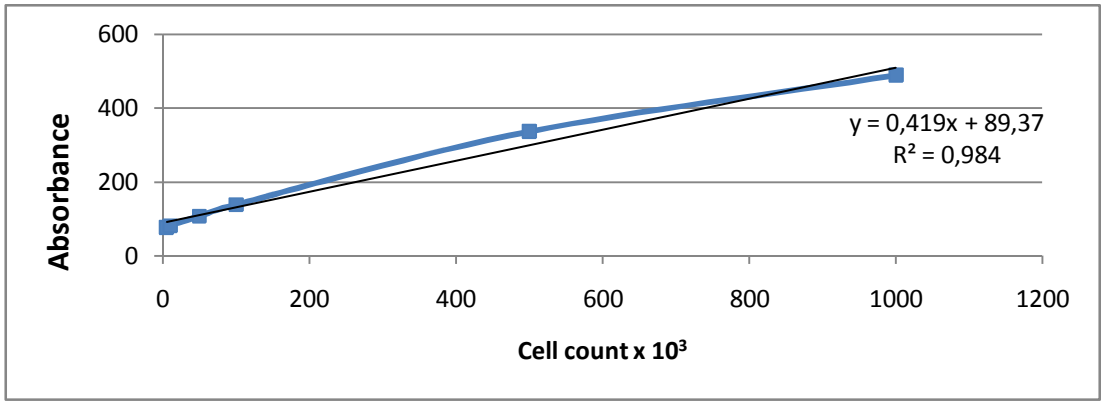

Figure 2 Relationship between living cell count and absorbance value for MTT at wavelength 540nm

Lim cells showed high resistance against Doxorubicin which was used as a positive control (20 µg/ml) and the cell viability was 91.6%. Ethanol used to dilute EOs showed no cytotoxicity. Our study revealed that the inhibitory concentration IC_{50} of sample 1 was 2.18 µl/ml, while IC50 for sample 2 and sample 3 was 2.27 µl/ml and 2.44 µl/ml respectively (Figure 3).

DISCUSSION

EOs composition

Our study revealed a slight difference in the yield of EOs among samples; the highest yield was in sample 1 (2.5%). This value is in conformity with (Arias and Ramón-Laca, 2005) study (2%) and with what was mentioned in **(Jeffrey et al, 2001)** (0.5-2.5), but it differ from that of **(Siddique et al, 2011)** study (0.622%).

In an investigation reported by **(Lota et al, 2001)** on *C.aurantium*, limonene was the most abundant compound of the peels EOs (87.6-95.1%) while other compounds were present at low amounts; α-pinene (0-0.6%), β-pinene (tr-2.4%) and myrcene (1.3-1.8%). In an investigation reported by **(Leite et al, 2008)** on *C. auranitum*, peels EOs contain limonene 96.24%, α-pinene 0.53%, myrcene 2.24%, linalool 0.44%. In this study **(Lota et al, 2001)** we can notice an obviously different in EOs composition even the cultural condition and extraction method were the same.

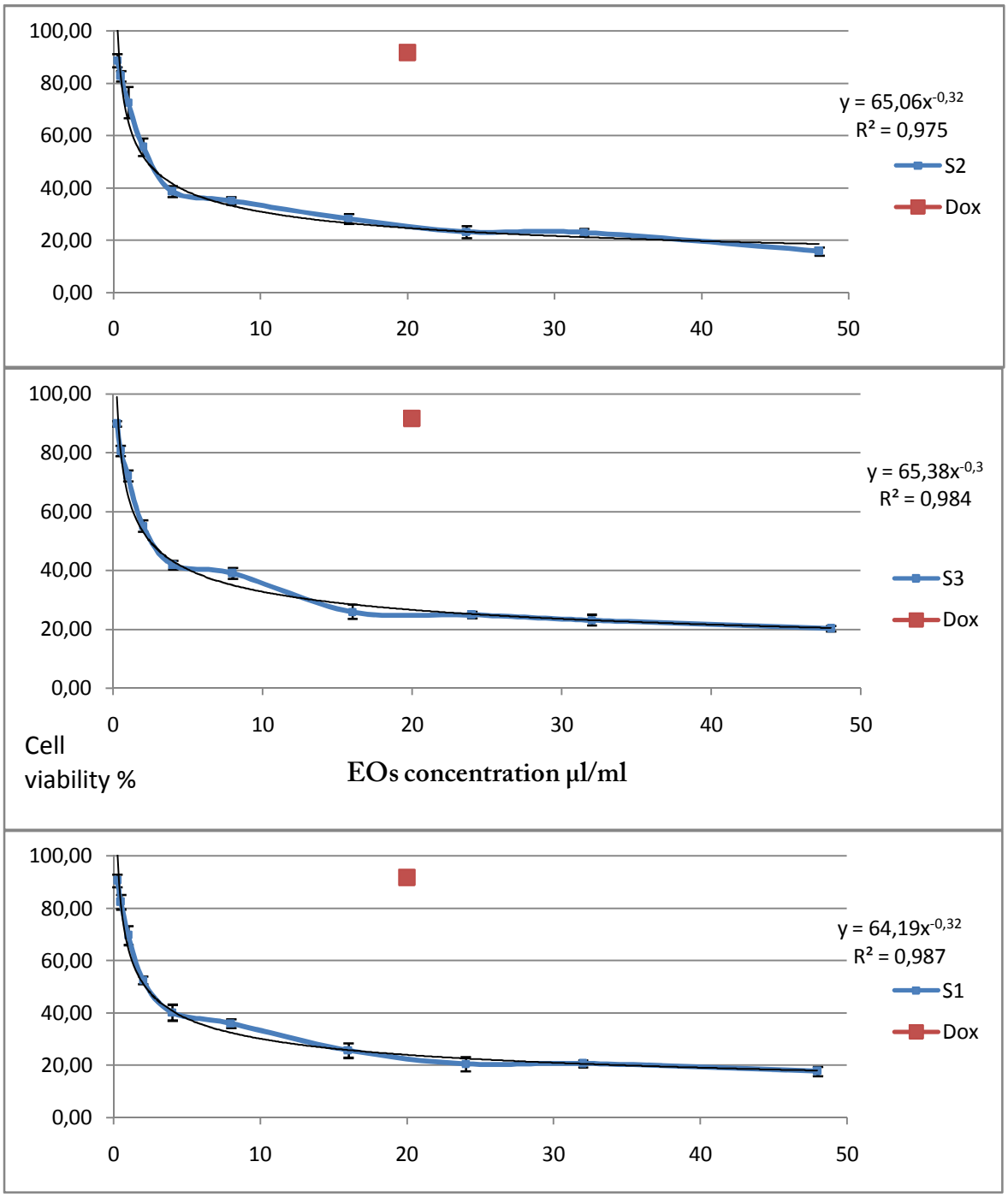

Figure 3 Effects of *C. aurantium* EOs on lim 1863 cell line. S1: sample 1, S2: sample 2, S3: sample 3, Dox: Doxorubicin (20 µg/ml)Y: cell viability, X: EOs concentration

The composition of EOs is slightly difference among our study samples, especially in sample 3. But in general, our investigation is largely in agreement with these previous reports.Actually we cannot know exactly the reason of these slight differences since we know that identical plants might have different EOs composition and those differencesare caused by the effects of light, soil,

temperature, and weather conditions. **Hassiotis et al. (2010)** and **Sangwan et al. (2001)** suggested through there studied that environmental conditions and time of collection can affect the composition and yield of EOs. So, the slight differences in composition among samples might be due to the variation in environmental cultivation conditions, and/or timing of sample collection.

Cytotoxic effect of the EOs

Lim colorectal carcinogenic cells show high resistance against Doxorubicin as demonstrated by a cell viability of 91.6%. This result is in conformity with (Serpe et al, 2004) study and this result reflect the accuracy of the method employed in this experiment. EOs of *C. aurantium* peels decreased viability of the colorectal carcinoma cell line by over 80% at rather low concentration (IC50= 2.18-2.44 µl/ml). Intraday and interday variations for standard curves were in the acceptable range (CV%<20). Spearman Correlation Coefficients reflects significant correlation (0.983) at the 0.01 level between cell viability and EOs concentration. While Tukey test reflects no significant difference among the three samples at the 0.01 level.

Many reports have investigated the cytotoxic effect of different Citrus EOs on human cancer cell lines, but there is no report investigating the cytotoxic effect of *C. aurantium* peels EOs. Moreover, there is no reportstudying the cytotoxic effect of anyof *C. aurantium* EOs against Lim1863 cell line. In an investigation reported by **(Majnnoni et al, 2012)**, *C. aurantium*leaves EOs show cytotoxic effect against six types of cancer cell lines (HL60, K562, Jurkat, PC3, HT29 and HeLa) with IC59 range (17-35 µg/ml). This study **(Majnnoni et al, 2012)**claims that C.aurantium EOs has low toxicity against normal cell lines (HUVEC) and probably against the body. In an investigation reported by (Monajemi et al, 2005), Cytotoxic effect of *C. medica*, *C. limon* and *C. sinensis* peels EOs on Hela and MCF-7 cancer cell line were studied, the variation of IC50 was obvious (0.5-17 µg/ml).

The cytotoxic effect of *C.aurantium* peels EOs may be attributed to a specific and/or synergetic effect of the many components present in the EOs **(Bakkali et al, 2008)**. We know that limonene is one of the most abundant naturally occurring monocyclic monoterpenes found in the oil of Citrus fruit peels and it has a chemoprotective effect against rodent and human tumor **(Crowell, 1999)**. Limonene was found to induce apoptosis, it can also induce phase 1 and phase 2 carcinogen-

metabolizing enzymes (cytochrome p450). These enzymes metabolize carcinogens to less toxic forms and prevent the interaction of chemical carcinogens with DNA **(Sun, 2007)**. Beside limonene, myrcene is also known to possess cytotoxic effect **(Majnooni et al., 2012)**.

CONCLUSION

This study revealed that limonene and myrcene are the main components of local *C. aurantium* peels EOs. Beside, Since *C. aurantium* EOs decreased the viability of Lim cell by over 80%, *C. aurantium* EOs might have a good potential antitumor effect and it might be useful in testing its further potential treatments of colorectal cancer and other cancers. Further study is suggested.

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