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The Cytotoxic Effect of Essential Oil of Syrian Citrus limon Peel on Human Colorectal Carcinoma Cell Line (Lim1863)

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Abstract

Background: Essential oils are the volatile fraction of aromatic and medicinal plants created after extraction by steam or water distillation. Species of the genus *Citrus* (Rutaceae) have been widely used in traditional medicine as volatile oils and are currently the subject of numerous research. *Citrus* essential oil consists of different terpens that have antitumor activities. This study determines the cytotoxic effect of the essential oils of *Citrus limon* L. peels on a colorectal cancer cell line (LIM1863).

Methods: We harvested four samples from four locations in Syria. Essential oils were prepared by hydrodistillation and analyzed by Gas chromatography-mass spectrometry (GC-MS).Various concentrations ofessential oils (0.5-48 µg/ml) were added to cultured cells and incubated for 72 h. Cell viability was evaluated byMTT-based cytotoxicity assay.

Results: We noted 18 components that represented 98.81% of the total oil content. The major components were: *limon*ene (61.8%-73.8%), γ-terpinene (9.4%-10.4%), β-pinene (3.7%-6.9%), O-cymene (1%-2.4%),and citral (0.8%-5.4%).The obtained IC_{50} value range of *Citrus limon* essential oils was 5.75-7.92 µg/ml against LIM1863.

Conclusion: This study revealed that Syrian *Citrus limon* essential oil has a cytotoxic effect on the human colorectalcarcinoma cell line LIM1863 when studied *in vitro*.

Keywords: *Citrus limon*, Essential oils, Cytotoxicity, LIM1863

Introduction

The *Citrus* species (Rutaceae) have been used in Arabic Traditional Medicine assedatives, analgesics, anti-arrhythmics, a stomachic, anti-tachycardic, anti-rheumatic, and for skin care.[1,2] Presently, both the extract and essential oil (EOs)of the *Citrus* genus are widely used in the cosmetic, food and pharmaceutical industries.[3] EOs derived from the *Citrus* species have been reported to have anti-microbial, antioxidant, anti-cancer and anti-fungal activities.[4-7]

Citrus limon EOs contain terpens, aliphatic sesquiterpene, oxygenated

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derivatives and aromatic hydrocarbons. The composition of the terpenic mixture varies according to the species and variations of the examined *Citrus* fruit. This mixture consists of varying proportions of *limon*ene, α–pinene, β-pinene, myrcene, linalol, and terpinen. Monoterpenes are important constituents of Eos from *Citrus* fruits and other plants. A number of these monoterpenes have demonstrated antitumor activities. For example, d-*limon*ene which comprises >90% of orange peel oil has a chemo-preventive activity against rodent mammary, skin, liver, lung and fore stomach carcinomas.[8] It has been reported to induce apoptosis on tumor cells.[9] Perillyl alcohol, a hydroxylated *limon*ene analog, exhibits a chemopreventive activity against liver, mammary gland, pancreas and colon carcinomas in rodents.[10]

Although many studies have shown the cytotoxic effects of *limon*ene,[11] there are few studies on the cytotoxicity of other components of *Citrus* EOs.[12] To the best of our knowledge there has been no prior study on the cytotoxicity of EOs of Syrian *Citrus limon* peels nor any cytotoxic study of *Citrus limon* against the colorectal carcinoma cell line (LIM1863). In this study, the composition of *Citrus limon* peel EOs, obtained from four different locations in Syria was determined. We studied their cytotoxic effects against the colorectal carcinoma cell line, LIM1863.

Materials and Methods

Fresh *Citrus limon* fruits from adult trees were collected from four different locations in Syria: Damascus (sample 1), Tartous (sample 2), Talkalakh (sample 3), and Kirnaz (sample 4) during Winter 2010 and Spring 2011. These fruits were harvested from commercial orchards in Syria. The peels were carefully torn by a sharp knife.

Essential oil extraction

EOs were extracted from fresh (within one day) peels (350 g) and collected by hydrodistillation for 3 h using a Clevenger-type apparatus. The oils were filtered through a 20 µM pore size filter syringe to eliminate microbial contamination. The oils were dried over anhydrous sodium sulfate and stored at -20°C for subsequent analysis.

Essential oil analysis

The oils were analyzed by Gas chromatography-mass spectrometry (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

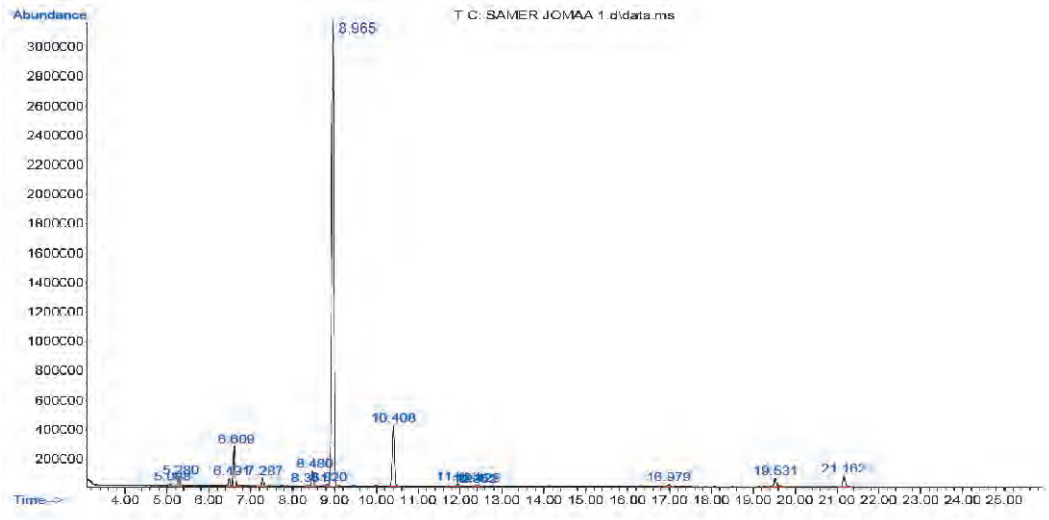

Figure 1. GC-MS chromatogram showing chemical analysis of essential oil (EO) from *Citruslimon* peel sample 1.

Table 1. EOs composition of Syrian *Citruslimon* peels.

Compounds	Retention time	Damascus	Talkalakh	Tartous	Kirnaz
α-phellandrene	5.083	0.261	0.215	0.276	0.349
α-pinene5.278	0.957	0.661	1.124	1.496	
β-phellandrene	6.49	0.987	0.682	1.182	1.173
β-pinene6.607	5.034	3.635	6.768	6.921	
β-myrcene	7.284	1.135	0.747	1.140	1.355
Terpinolene	8.345	0.162			
O-cymene	8.476	2.366	0.996	2.067	1.329
*Limon*ene	8.863	73.843	61.818	66.619	72.415
γ-terpinene	10.404	9.761	9.836	10.382	10.304
Linalol	11.953	0.437	0.524	0.561	0.486
Terpinene-4-ol	16.332	0.520	0.251	0.697	0.264
α-thujone	16.982		0.502		
Citral	19.546	2.240	5.404	2.136	0.748
(±)-lavadulol acetate	21.159		0.598	0.272	0.245
Caryophyllene	26.816		1.418	0.611	0.257
α-bergamotene	27.78		2.840	1.350	0.629
β-bisabolene	9.728		4.901	2.259	0.971

thickness 0.25 µm). Operating conditions were as follows: helium carrier gas at a constant pressure of 9.43 psi; column temperature, 60-275°C at a rate of 3°C/min; injector temperature, 280°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°C; and resolution 1000 Micron. Identification of components present in the oil was based on computer matching with Mass seprctral ibrary [Nist 08 library (Software Version 2.0f)].

Cell lines

Human colorectal carcinoma cell line (LIM1863) was obtained by courtesy of Professor Nizar Mhaidat from Jordan University of Science and Technology. Cells were maintained in D-MEM cell culture medium supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in a LabTech CO_2 incubator (LCO-065 AI).The cell line was maintained and grown in D-MEM to ten subcultures. Samples of the cell line were cryopreserved in liquid nitrogen.

MTT-based cytotoxicity assay

Essential oils were diluted with ethanol to the following concentrations (0.5, 1, 2, 4, 8, 16, 24, 32, and 48 µg/ml). Assessment of cell viability was performed according to a modified method by Mosmann[13] that used 3-(4, 5-dimethylthiazol-2-

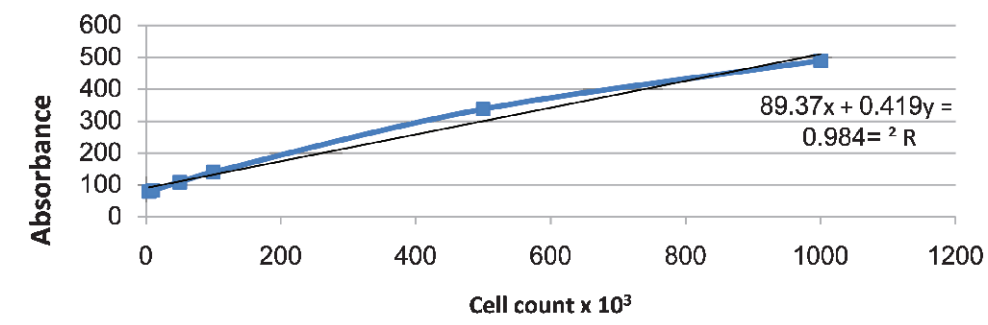

Figure 2. Relationship between living cell count and absorbanceat 540nm.

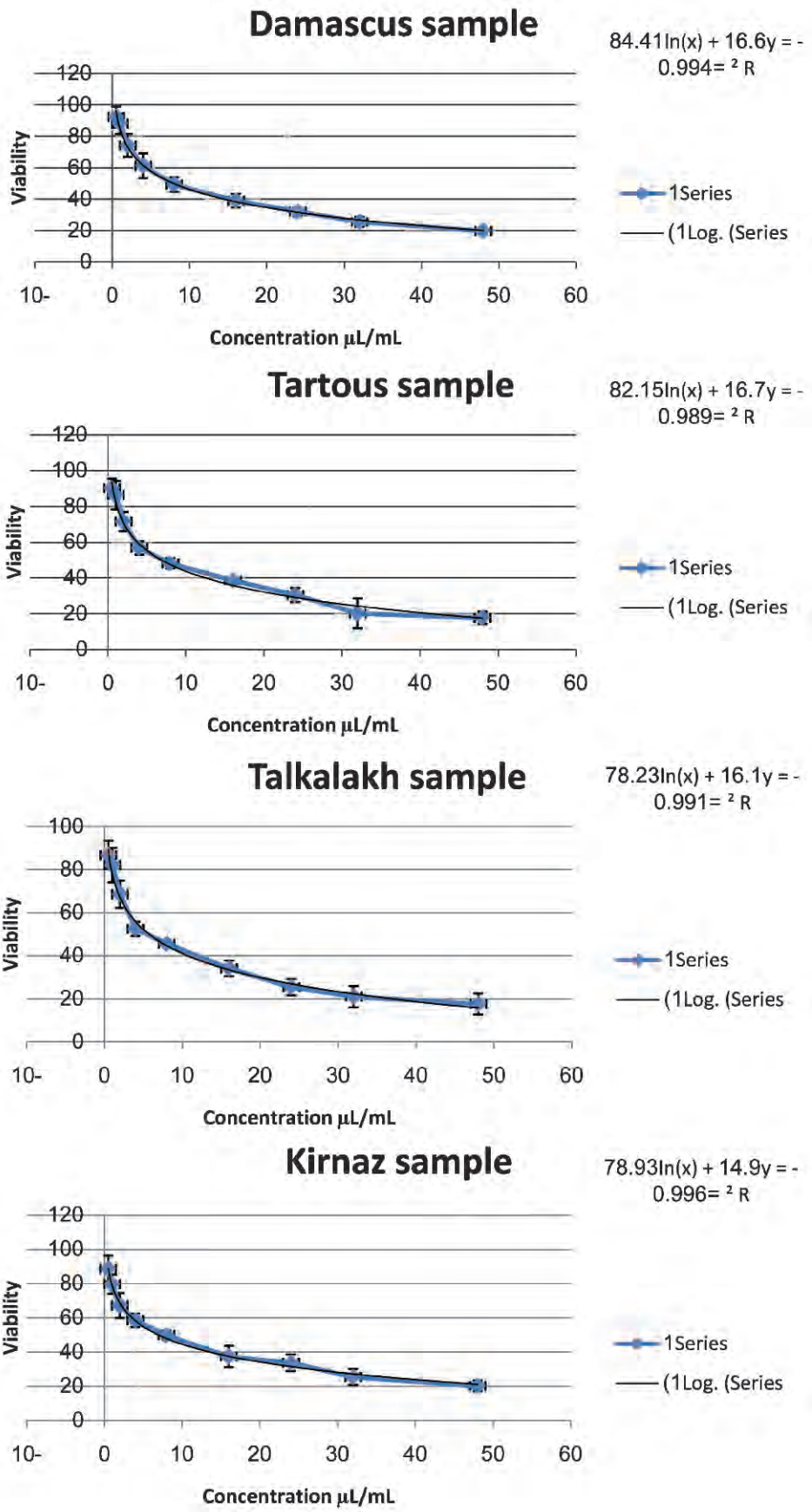

Figure 3. Effects of *Citrus limon* EOs on LIM1863 carcinoma cell line.

yl)-2, 5-diphenyl tetrazolium bromide (MTT). A 200 µl cell suspension (5×10^4 cells/ml) was seeded on to 96 microplates and incubated for 24 h (37°C, 5% CO_2, humidified air), after which 20 µl each of various EO concentrations was added. Microplates that contained cells and EOs were incubated for an additional 72 h under the same conditions. We have used doxorubicin (20µg/ml) as the positive control since this is the only oncologic drug currently available in Syria. The first row of each microplate was assumed to be the negative control that contained neither EOs nor doxorubicin. To evaluate cell survival, 20 µl of the MTT solution (5 mg/ml) was added to each well and incubated for 3 h. A total of 150 µl of a medium that contained MTT was replaced by DMSO and pipetted to dissolve any formazon crystals that formed. We determined absorbance at 540 nm by an enzyme-linked immunosorbent assay (ELISA) plate reader (SCO diagnostic, RS-232 Germany).

Each oil concentration was repeated eight times. Standard curves for each sample (absorbance value against cell number) were also plotted. The percentage of cell survival was determined by assuming 100% survival for the negative control. IC_{50} 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 with Microsoft Office Excel. Spearman correlation coefficient and Tukey test were performed with SPSS 17.0. $P<0.01$ was considered statistically significant.

Results

Chemical composition of the EOs

Hydrodistillation of the peels from four *Citrus limon* samples gave the following amounts of EOs (V/W): Kirnaz (1.68%), Damascus (1.9%), Tartous(2.43%), and Talkalakh (2.5%). According to the results there were 18 components that represented 98.81% of the total oil. The major components were *limon*ene (61.8%-73.8%), γ-terpinene (9.4%-10.4%), β-pinene (3.7%-6.9%), O-cymene (1%-2.4%), and citral (0.8%-5.4%). Figure 1 shows the GC-MS chromatogram results of the EOs from *Citruslimon* sample 1.

Cytotoxic effect of EOs

A preliminary study showed significant positive correlation between living cell count and absorbance value for MTT at the 540 nm wave length (Figure 2). LIM cells showed high resistance to doxorubicin (20 µg/ml), the positive control. Cell viability was 92%. Ethanol used to dilute EOs had no cytotoxicity. EOs decreased the viability of cancer cells (Lim) and this effect was observed with the lowest EOs concentration (Figure 3). Our study revealed that the inhibitory concentration (IC_{50}) of the Damascus sample was the highest at 7.92 µg/ml, while the IC_{50} was 6.82 µg/ml for the Tartous sample and 6.96 µg/ml for the Kirnaz sample. The Talkalakh sample had the lowest IC_{50} of 5.75 µg/ml (Figure 2).

Discussion

EOs composition

Our study revealed that hydrodistillation of the samples gave different amounts of EOs (V/W). The Talkalakh sample had the highest EO(2.5%) in accordance with results from Trease et al., whereas the Kirnaz EO was only 1.68%.[15] In a study of Venezuelan *Citruslimon* EOs, it was noted that the most abundant component of EOs from the peel was *limon*ene (65.65%).Other components present were β-pinene (11%), γ-terpinene (9.01%), α-pinene (1.88%), and β-cymene (0.1%).[16] In an investigation by Monajemi on *Citruslimon*, the EOs contained *limon*ene at a concentration of 98.4%, followed by myrcene 0.8%, citronellal 0.4%, and α-pinene 0.2%.[17] Our investigation agreed with some of the previous reports. The slight differences between our samples and other study samples can be explained by differing environmental cultivation conditions and/or differences in time of sample collection. The effect of environmental conditions and time of collection on EOs composition and

yield has been investigated. Reports from other plants have shown an increase in EOs yield and decrease in *limon*ene content at higher humidity levels.[18, 19]

Cytotoxic effect of EOs

LIM1863 showed high resistance to doxorubicin as demonstrated by a cell viability of 92%. This result confirmed results by Serpe et al.[14] Cell viability decreases of over 50% are usually cytotoxic. EOs from the *Citrus limon* peels decreased viability of the colorectal carcinoma cell line by over 80% at a rather low concentration (IC_{50}=5.75-7.92 µg/ml). Many reports have investigated the cytotoxic effect of different *Citrus* EOs on human carcinoma cell lines, but there was no report that investigated the cytotoxic effects of *Citrus limon* peel EOs against the LIM1863 cell line.

In an investigation on Iranian *Citrus limon*, the Eos were reported to have a cytotoxic effect against carcinoma cell lines such as MCF-7 (breast cancer cell line) and HeLa (cervical cancer cell line). Obtained values were an IC_{50} of 10 µg/ml for MCF-7 and 17 µg/ml for HeLa.[17] Another investigation reported the cytotoxicity of *Citrus limon* EOs on the Ehrlich as cites carcinoma cell line (EACC) at an IC_{50} of 120 µM/ml.[20]

The observed cytotoxic effect of *Citrus limon* peel EOs may be attributed to either a specific component or several components present in the complex mix of the EOs. Of particular interest are components such as *limon*ene and γ-terpinene. We know that *limon*ene is one of the most abundant naturally occurring monocyclic monoterpenes found in the oil of *Citrus* fruit peels. This compound reportedly has a chemoprotective effect against rodent and human tumors.[8] *Limon*ene has been found to induce apoptosis in addition to its ability induce phase 1 and 2 carcinogen-metabolizing enzymes (cytochrome p450). The latter enzymes metabolize carcinogens to less toxic forms and prevent the interaction of chemical carcinogens with DNA. Interestingly, *Limon*ene has demonstrated low toxicity in a clinical study on normal humans after single and repeated dosing for up to one year.[21]

In contrast to *limon*ene, γ-terpinene is a monoterpene of which few studies have reported its cytotoxic activity. The EO obtained from the dried leaves of Marjorana hortensist hat have a content of 15.0% γ-terpinene have shown cytotoxic effects against leukemia HL-60 and NB4 cells.[22] γ-terpinene was also investigated by Bourgou et al., who studied its cytotoxic activity against human lung carcinoma A-549 and colon adenocarcinoma DLD-1 cells. These researchers obtained IC_{50} values of≥100 µM (13.62 µg/mL) for both cell lines.[23]

Conclusion

This study revealed that the main components of *Citruslimon* Eos from samples collected in Syria are *limon*ene, γ-terpinene, β-pinene, O-cymene and citral. *Citruslimon* EO has a cytotoxic effect on the cancer cell line LIM1856 *in vitro*. It is also suggested that *Citrus limon* EOs should be considered for further studies, both *in vitro* and *in vivo*, to establish possible alternative cancer treatments.

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References

1. Hadjiakhoondi A, Baligh N. Practical guidance of medicinal plants. Tehran: Islamic Azad University Scientific Publication Center, 2005:5-20.
2. Fleming T. *Citrus* aurantium. In: PDR for herbal medicines, 2nd ed. Montvale, NJ: Medical Economics Company, 2000: 86-7.
3. Fisher K, Phillips C. Potential antimicrobial uses of essential oils in food. Is *citrus* the answer? *Trends Food Sci Technol* 2008;19:156-64.

4. Patil JR, Jayaprakasha GK, Chidambara KN, Tichy S, Chetti MB, Patil BS. Apoptosis-mediated proliferation inhibition of human colon cancer cells by volatile principles of *Citrus aurantifolia*. *J Food Chem* 2009;114:1351-8.
5. Chutia M, Deka P, Pathak MG, Sarma TC, Boruah P. Antifungal activity and chemical composition of *Citrus* reticulate Blanco essential oil against phytopathogens from North East India. *Food Sci Technol-Leb* 2009;42:777-80.
6. Komori T, Fujiwara R, Tanida M, Nomura J. Potential antidepressant effects of *limon* odor in rats. *Eur Neurol Psychol Pharmacol* 1995;5:477-80.
7. Hongratanaworakit T, Buchbauer G. Chemical composition and stimulating effect of *Citrus hystrix* oil on humans. *J Flavour Fragr* 2007;22:443-9.
8. Crowel P. Prevention and therapy of cancer by dietary monoterpenes. *Nutr* 1999;129:775-8.
9. Hata T., Sakaguchi I., Mori M. Induction of apoptosis by *Citrus* paradise essential oil in human leukemic (HL-60) cells. *in vivo* 2003;17:553-9.
10. Reddy BS, Wang CX, Samaha H, Lubert R, Steel VE. Chemoprevention of colon carcinogenesis by dietary perillyl alcohol. *J Cancer Res* 1997;57:420-5.
11. Bardon S, Picard K, Martel P. Monoterpenes inhibit cell growth, cell cycle progression and cyclin D1 gene expression in human breast cancer cell lines. *Nutr Cancer* 1998;32:1-7.
12. Carnesecchi Y, Schneidir J, Ceraline B, Raul F. Geraniol. A component of plant essential oils, inhibits growth and polyamine biosynthsis in human colon cancer cells. *J Pharmacol Exp Ther* 2001;298:197-200.
13. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *J Immunol Meth* 1983;65:55-63.
14. Serpe L., Catalano M., Cavalli R. Cytotoxicity of anticancer drugs incorporated in solid lipid nanoparticles on HT-29 colorectal cancer cell line. *Eur J Pharm Biopharm* 2004;58:673-80.
15. Trease GE, Evans WC, Evans D. Volatile oils and resins. In: Pharmacognosy, 15th ed. Edinburgh, NY: WB Saunders, 2002:253,88.
16. Ojeda G, Morales V, González N, Cabrera L, Sulbaran B. Composition of Venezuelan *limon* essential oil *Citrus limon* (L.) Burm .f., Rev. Fac. Agron. *LUZ* 1998;15:343-9.
17. Monajemi R., Oryan S, Haeri-Roohani A, Ghannadi A, Jafarian A. Cytotoxic effects of essential oils of some Iranian *citrus* peels. *Iranian J of Pharm Res* 2005;3:183-7.
18. Hassiotis C, Lazari D, Vlachonasios K. The effects of habitat type and diurnal harvest on essential oil yield and composition of *Lavandula Angustifolia*. *MillFres Environ Bull* 2010;19:1491-8.
19. Sangwan N, Farooqi A, Shabih F, Sangwan R. Regulation of essential oil production in plants. *Plant Growth Regu* 2001;34:3-21.
20. Mohammad A, El-Emary G, Ali H. Influence of some *citrus* essential oils on cell viability, glutathione-s-transferase and lipid peroxidation in Ehrlich as cites carcinoma cells. *J Am Sci* 2010;6(10):820-6.
21. Sun J. D-*limon*ene: Safety and clinical application. *Alternat Med Rev* 2007;12:259-64.
22. Romeilah RM. Anticancer and antioxidant activities of *Matricaria chamomilla L* and *Marjorana hortensis* essential oils. *Res J Med Sci* 2009;4:332-9.
23. Bourgou S, Pichette A, Marzouk B, Legault J. Bioactivities of black cumin essential oil and its main terpenes from Tunisia. *S Afr J Bot* 2010;76:210-6.