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Original Article

Cytotoxic Effects of Essential Oils of Some Iranian *Citrus* Peels

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Abstract

There have been efforts to overcome the problem in treatment of cancer using medicinal plants. It has been shown that *Citrus* essential oil of contains different terpens with antitumor activities. In this study we sought to determine the cytotoxicity of essential oils of Iranian *Citrus limon* (L.), *C. medica* (L.), *C. sinsensis* (L.) peels on cancer cell lines. Essential oils were prepared by hydrodistilation and characterized by GC-MS. The effects of *C. limon* (5-40 µg/ml), *C. medica* and *C. sinensis* (0.25-10 µg/ml) on two human tumor cell lines (MCF-7 and Hela) were determined. Different concentrations of essential oils were added to cultured cells and incubated for 72 h. Cell survival was evaluated using the MTT-based cytotoxicity assay. While limonene comprise about 98.4% and 98.8% of content of *C. limon* and *C. sinensis* essential oils respectively, its' percentage in *C. medica* was only 56.6%. In *C. medica* there was a considerable amount of β-pinene, γ-terpinene, α-terpinolene and trans-α-bergamotene. IC_{50} of essential oil for MCF-7 cell line was: *C. limon* ≈ 10 µg/ml, *C. medica* ≈ 1 µg/ml and *C. sinensis* ≈ 0.5 µg/ml. For Hela cell line IC_{50} was: *C. limon* ≈ 17 µg/ml, *C. medica* ≈ 1 µg/ml and *C. sinensis* ≈ 3 µg/ml. Our findings revealed that *C. limon* and *C. sinensis* had a greater cytotoxic effect on MCF-7 than that on Hela cells. Also, comparing IC_{50}, our findings indicated that *C. medica* and *C. sinensis* were more cytotoxic than *C. limon.* Comparison of the essential oil component of C. *limon* with *C. medica,* shows the presence of β-pinene (16.3%), α-terpineol (11.3%), γ-terpinene (4.4%), and trans- α-bergamotene (3.4%), which were not found in C. *limon.* Hence, it could be concluded that these components may have greater cytotoxic effects or they may also have synergistic effects with limonene.

Keywords: *C. limon*; *C. medica*; *C. sinensis*; cytotoxicity.

Introduction

The medicinal value of plants appears in all early records of human activity from 5000 years ago, to herbalists, pharmacists and physicians of all succeeding generations, to modern use of herbs, their extracts and synthetic products to treat minor ailments and diseases today. It is not surprising that the taxonomic family to which *Citrus* belongs, the Rutaceae, which include approximately 160 genera and 1700

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species, has been used in herbal medicine (1). In addition to various food products from pulp, *Citrus* peels are candied, fed to livestock, used to scent perfumes and soap products. Also it has been shown that limonene oil from peel has an insecticidal property. *Citrus* seeds are used to drive a cooking oil, and oils for plastic and soaps. Their flowers and foliage are used in perfume manufacturing. *Citrus* species essential oil contains: terpens, aliphatic sesquiterpene, oxygenated derivatives and aromatic hydrocarbons. The composition of the terpenic mix varies depending on the typology of examined *Citrus* fruit of the species to which it owns. Anyway, the mix of each typology is in different proportion made of: limonene, α–pinene, β-pinene, myrcene, linalol, and terpinen. Monoterpenes are important constituents of essential oil of *Citrus* fruits and other plants. A number of these monoterpenes have an antitumor activity. For example, d-limonene which comprises >90% of the orange peel oil has chemopreventive activity against rodents mammary, skin, liver, lung and forestomach cancer (2) and has been reported to induce apoptosis on tumor cells (3). Similarly, perillyl alcohol, a hydroxylated limonene analog, exhibits chemopreventive activity against liver, mammary gland, pancreas and colon cancer in rodent (4).

There are about 10 species of the Iranian *Citrus*, which are mostly found in northern and southern parts of the country. Although many studies have shown cytotoxic effect of limonene (5), there are few studies on cytotoxicity of other components of *Citrus* essential oils (6). Also to the best of our knowledge there has been no prior study on the cytotoxicity of essential oils of Iranian *Citrus* peels against human tumor cell lines. In this study we sought to determine the cytotoxicity of essential oil of Iranian *Citrus medica* L.*, C. limon* (L.) and *C. sinensis* (L.) peels on cancer cell lines.

Experimental

Plant material(s)

Fresh *Citrus* fruits were purchased from local markets of Isfahan during winter 2002 and spring 2003. These fruits were harvested in orchards of southern provinces of Iran. Herbarium department of Shiraz School of Pharmacy, Shiraz, Iran, confirmed the plants identities.

Essential oil isolation

The peels of the fresh fruits of each *Citrus* species (100 g) were chopped and hydrodistilled separately for 3 h using a Clevenger-type apparatus (7). The oils were dried over anhydrous sodium sulfate and stored in a refrigerator (4°C).

Essential oil analysis

The oils were analyzed by GC/MS using a Hewlett Packard 6890 mass selective detector coupled with a Hewlett Packard 6890 gas chromatograph, equipped with a cross-linked 5% PH ME siloxane HP-5MS capillary column (30 m×0.25 mm, film thickness 0.25 μm). Operating conditions were as follows: carrier gas, helium with a flow rate of 2 ml/min; column temperature, 60-275ºC at a rate of 4ºC/min; injector temperature, 280ºC; injected volume, 0.1 μl of the oil; and split ratio, 1:50. The MS operating parameters were as follows: ionization potential, 70 eV; ion source temperature, 200ºC; and resolution, 1000.

Identification of components present in the oil was based on computer matching with the WILEY275.L library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature (8-10).

Cell lines

Human cervix carcinoma (Hela) and Human breast adenocarcinoma (MCF-7) cell lines were obtained from Pasture Institute, Tehran, Iran. They were maintained in RPMI-1640 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% CO_2.

Maintenance of the human cell lines

Cell lines were maintained and grown in RPMI-1640 to 15 subcultures. A sample of each cell lines was frozen and kept under liquid nitrogen for future studies.

MTT-based cytotoxicity assay,

Assessment of cell viability was carried out by a modified method of Mosmann (11) using 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable tumor cells, into an insoluble colored formazon product, which can be measured spectrophotometrically after dissolving in dimethylsulfoxide (DMSO) (12). Briefly, 200 µl samples of cell suspension (5×10^4 cell/ml) were seeded in 96 microplates and incubated for 24 h (37°C, 5% CO_2, air humidified), and then 20 µl (essential oil + medium) was added. To make an adequate dilution of the essential oil, ethanol was used. Microplates containing cells and essential oils were incubated for another 72 h, under the same condition. Doxorubicin (20 µg/ml) was used as the positive control. The first row of each microplate was assumed as the negative control (containing no essential oil or doxorubicin). To evaluate cell survival, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 3 h. Then 150 µl of an old medium containing MTT was replaced by DMSO and pipetted to dissolve any formazon crystals formed. Then, absorbance was determined at 540 nm by an Enzyme-Linked Immunosorbent Assay (ELISA) plate reader. Each experiment was carried out in triplicate and repeated three times. Standard curves (absorbance against cell number) for each of the cell lines were also plotted. Intra-day and inter-day variations were also determined. Percentage of cell survival was determined, assuming 100% survival for the negative control. Compounds were considered as cytotoxic when they decreased viability of cells to less than 50%.

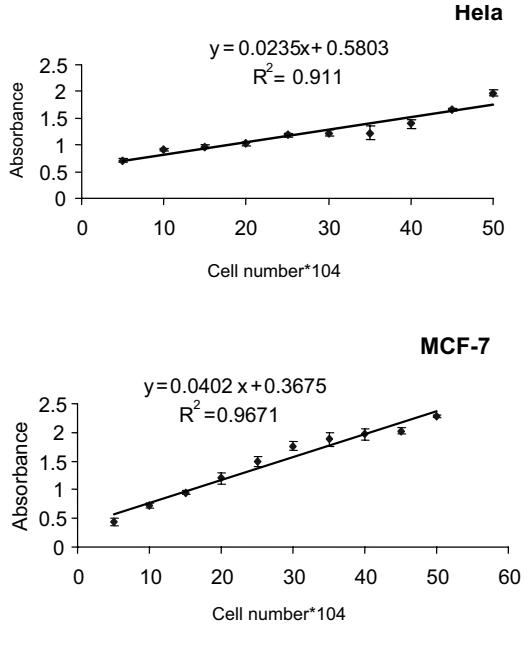

Figure 1. Relationship between absorbance and the number of viable cells. Number of viable cells was assessed using the MTT assay. Absorbance was determined at 540 nm by ELISA, (n = 18).

Statistical analysis

SIGMASTAT™ (Jandel Software, San Raphael, CA) was used to perform statistical tests. One way analysis of variance, followed by Tukey test, was used to distinguish the differences among groups. Significance was a assumed at 5% levels.

Results and Discussion

Composition of the volatile oils of Iranian *C. limon*, *C. medica* and *C. sinensis* (13) were determined by GC-MS (Tables 1-3). While limonene comprise about 98.4% and 98.8% of content of *C. limon* and *C. sinensis* essential oils respectively, its' percentage in *C. medica* was only 56.6%. In *C. medica* there was considerable amount of β-pinene, γ-terpinene, α-terpinolene and trans-α-bergamotene.

To evaluate cytotoxicity of the essential oils, MTT-based cytotoxity assay was performed. For Hela and MCF-7 cell lines, good relationships between the number of cells and absorbance were observed (Fig. 1). Intraday and interday variations for standard curves were in the acceptable range (CV%<20). Doxorubicin, a known cytotoxic agent (14), as a positive control significantly decreased viability of Hela and MCF-7 cell lines, indicating the accuracy of the method employed in this experiment ($P < 0.05$). Ethanol, as a solvent for essential oils, in the range used in this study showed no cytotoxicity.

The effect of different concentrations of essential oils of *C. limon* (5-40 µg/ml), *C. medica* and *C. sinensis* (0.25-10 µg/ml) on cell survival were studied. They significantly decreased the

Table 1. Composition of the volatile oil of Iranian *C. limon* L Burm. f.

Compound	Percentage	Compound	Percentage
α-pinene	0.2	Limonene	98.4
β-pinene	Trace	Citronellal	0.4
Myrcene	0.8		

Table 2. Composition of the volatile oil of Iranian *C. medica* L. var. *Cedrate*

Compound	Percentage	Compound	Percentage
α-pinene	1.5	4-terpineol	2.2
β-pinene	16.3	α-terpineol	11.3
Myrcene	0.2	Nerol	0.1
Limonene	56.6	Geranyl formate	0.1
γ-terpinene	4.4	Neryl acetate	0.1
Terpinolene	0.5	Geranyl acetate	0.1
Linalool	0.6	Cis-α bergamotene	1.3
Exo-fenchol	0.1	Trans-α bergamotene	3.4

viability of MCF-7 and Hela in a dose dependent manner (Figs.2 and 3). IC_{50} of the essential oil for MCF-7 cell line was: *C. limon* ≈ 10 µg/ml, *C. medica* ≈ 1 µg/ml and *C. sinensis* ≈ 0.5 µg/ml. For Hela cell IC_{50} was: *C. limon* ≈ 17 µg/ml, *C. medica* ≈ 1 µg/ml and *C. sinensis* ≈ 3 µg/ml. These results revealed that *C. limon* and *C. sinensis* had a greater cytotoxic effect on MCF-7 than that noted on Hela cells. Also by comparing the IC_{50}, it is clear from our findings that *C. medica* and *C. sinensis* were more cytotoxic than *C. limon.* It has been show that in murine B16 (F10) melanoma cells, the IC_{50} values for d-limonene and perillyl alcohol were 450 and 250 µM, respectively (15). Also, in another study the IC_{50} value for perillyl alcohol was found to be 290 and 480 µM for the human and hamster pancreatic tumor cell lines, respectively (16). We know that limonene is one of the most abundant naturally occurring monocyclic monoterpenes found in the oil of *Citrus* fruit peels and has chemoprotective effects against rodent and human tumor (17). This activity is observed both at initiation and promotion (18). Based on these findings it has been expected that essential oils with a higher percentage of

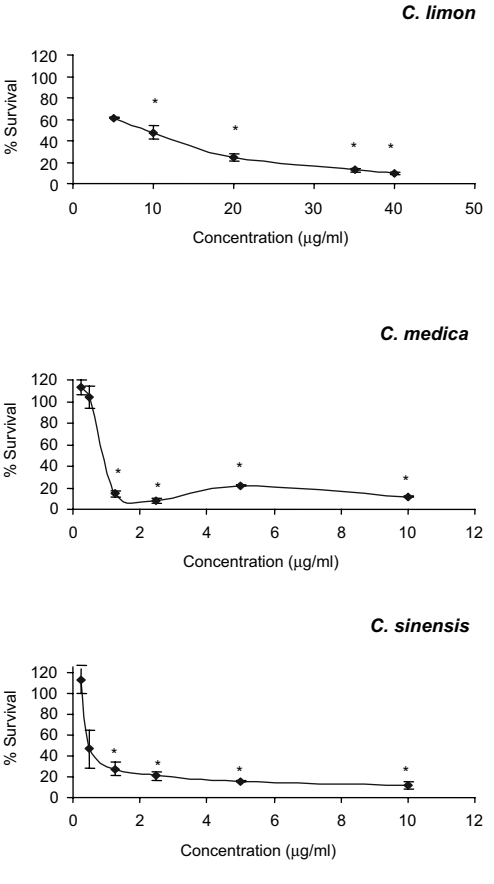

Figure 2. The effects of *C. limon, C. medica* and *C. sinensis* on MCF-7 cells. Cell viability was assessed using the MTT assay. Percentage of survival in the control group was assumed 100 (* = $p < 0.05$, n = 9).

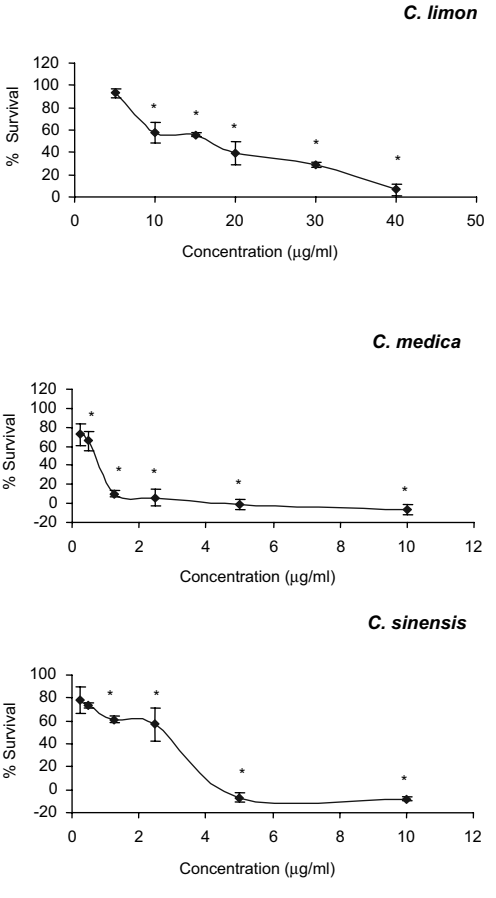

Figure 3. The effects of *C. limon, C. medica* and *C. sinensis* on Hela cells. Cell viability was assessed using the MTTassay. Percentage survival in the control group was assumed 100, (* = $p < 0.05$, n = 9).

Table 3. Composition of the volatile oil of Iranian *C. sinensis* (L.) Osbeck

Compound	Percentage	Compound	Percentage
α-pinene	0.2	Limonene	98.8
Sabinene	0.1	Linalool	0.3
β-pinene	Trace	Cis-limonene oxide	trace
Myrcene	0.4		

limonene, show greater cytotoxicity. Although *C. limon* had a large amount of limonene (98.4%), it was less effective than *C. medica* (with 56.6% limonene) on both cell lines. A number of mechanisms for limonene action have been suggested, including the induction of carcinogen metabolizing enzymes, growth factor receptor expression, inhibition of 3-hydroxy-3-methyl glutraryl coA reductase and inhibition of Ras protein farnesylation (19). Investigators have suggested the presence of several limonene metabolites with greater *in-vitro* antiproliferation activity than limonene (5, 17). Comparing the essential oil components of C. *limon* with *C. medica,* the presence of β-pinene (16.3%), α-terpineol (11.3%), γ-terpine (4.4%), and trans-α-bergamotene (3.4%), which were not found in C. *limon,* have been noted. Hence, so that it can be concluded that these components may have great cytotoxic effects or they may also have synergistic effects with limonene. To confirm this conclusion, investigation of the cytotoxic effects of pure components of *C. medica* essential oils is suggested.

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