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Full Length Research Paper

Chemical composition, cytotoxicity and antioxidant activities of the essential oil from the leaves of *Citrus aurantium* L.

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The species of the genus *Citrus* (Rutaceae) have been widely used in traditional medicine. In this study, the essential oil was extracted from the leaves of *Citrus aurantium* and its cytotoxicity effect on six tumor cell lines and a normal cell line was studied. Furthermore, antioxidant potential of the oil was tested by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, hydrogen peroxide scavenging and reducing antioxidant power methods. The composition of the essential oil was also analyzed by GC–MS. Results indicate 41 components that represented 97.81% of the total oil. The major components were limonene, linalool and *trans*-beta-ocimene. In addition, the essential oil also exhibited strong antioxidant activity. The IC_{50} of the oil in DPPH assay, H_2O_2 scavenging and reducing antioxidant power were 1040 ± 0.9, 140 ± 1.5 and 1580 ± 1.03 µg /ml, respectively. The essential oil also had marked cytotoxicity against the all tumor cell lines, with the highest activity on Jurkat and HL60.

Key words: *Citrus aurantium*, essential oil, chemical composition, cytotoxicity, antioxidant effect.

INTRODUCTION

The Citrus species (Rutaceae) have been used in traditional medicine for their interesting bioactivities such as skin care and effectively acting as sedatives, analgesics, anti-arrhythmic, stomachic, anti-inflammation of skin, anti-tachycardia and anti-rheumatism (Hadjiakhoondi and Baligh, 2005; Fleming, 2001; Zargari, 1997). Presently, the extract and the essential oil of *Citrus* genus are widely used in the cosmetic, food and pharmaceutical industries (Fisher and Phillips, 2008). Recently, the essential oils of *Citrus* species have been reported to have anti-microbial, antioxidant, anti-cancer, anti-fungal, anti-depressant effects and to reduce blood 2007; Patil et al., 2009; Chutia et al., 2009; Razzaghi-Abyaneh et al., 2009; Komori et al., 1995; Hongratanaworakit and Buchbauer, 2007).

C. aurantium L. grows in different parts of the world including tropical and temperate regions such as the Southern Iran (Parsa, 1995; Towsend, 1981). In some experimental studies, the anti-microbial, antioxidant, anti-dermatophyte, cytotoxicity, anxiolytic, sedative and gastro-protective effects reported for the essential oil from *C. aurantium* peel were ascribed to limonene (JongSeok et al., 2008; Sanguinetti et al., 2007; Carvalho-Freitas and Costa, 2002; Pultrini et al., 2006; Moraes et al., 2009). To date, cytotoxic and antioxidant activities of the essential oil of *C. aurantium* have not been reported. In the present study, the essential oil of *C.*

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aurantium leafs were obtained, then its cytotoxicity on six tumor cell lines (HL60, K562, Jurkat, PC3, HT29 and HeLa) and on a normal cell line (HUVEC) was studied using LDH assay. In addition, the antioxidant capacity of the oil was assessed by three different methods (DPPH assay, hydrogen peroxide (H_2O_2) scavenging and reducing antioxidant power). Moreover, the composition of the essential oil of *C. aurantium* leaves was also analyzed by GC-MS.

MATERIALS AND METHODS

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) (Gibco, New York, USA), penicillin, streptomycin, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH, 95%), butylated hydroxyl anisole (BHA) were purchased from Sigma–Aldrich (Steinheim, Germany). Ethanol, methanol, sodium acetate, sodium phosphate, potassium ferricyanide, anhydrous sodium sulphate, hydrogen peroxide (H_2O_2), triton X-100, trichloroacetic acid (TCA) and ferric chloride were purchased from Merck (Darmstadt, Germany). In addition, Lactate dehydrogenase (LDH) assay kit was bought from Roche Diagnostics Corporation (Indianapolis, IN, USA)

Plant material

The leaves of *C. aurantium* were collected in Shiraz (south of Iran) in September 2008, and the voucher specimen was deposited at the herbarium of the School of Pharmacy (011C), University of Medical Sciences, Kermanshah, Iran.

Isolation of the essential oil

The leaves of *C. aurantium* were air-dried and then the powdered leaves were separately submitted to hydrodistillation using a Clevenger-type apparatus for 8 h. To absorb the residual, anhydrous sodium sulphate was used. The oil was then stored at -20°C until tested.

GC–MS analyses

An HP 6890N GC system, coupled with a HP MSD5973N quadrupole mass spectrometer, was used. The oil was separated on an HP-5MS capillary column (30 m length × 0.25 mm I.D., 0.25 mm film thickness), then split injection was employed for distillation samples with a ratio of 50:1. The column oven temperature was programmed from 40 to 150°C at 4°C /min, then to 240 at 10°C /min. The injection temperature and ion source temperature were 240°C. Finally, Helium was applied as the carrier gas with a flow rate of 1.2 ml/min. The ionizing energy was 70 eV. All the data were obtained by collecting the full-scan mass spectra within the scan range 50 to 550 amu, using the Wiley 7 n.L Mass Spectral Library (Wiley, New York, NY, USA). The relative content of each compound was calculated by the ratio of the peak area to the total peak area of the volatile oil compounds

Cell lines and culture

The cell lines including human prostate adenocarcinoma (PC3), human colon adenocarcinoma (HT29), human cervix carcinoma (HeLa), human chronic myelogenous leukemia (K562), Jurkat (Adult T cell leukemia), Human promyelocytic leukemia (HL60) and human umbilical vein endothelial cell (HUVEC) were purchased from Pasteur Institute of Iran. These cell lines were grown and maintained in a humidified incubator at 37°C and 5% CO_2 atmosphere in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.

LDH assay

After incubation of the cell lines with different concentration of the oil (10, 25, 50, 100, 250, 500 and 1000 µg/ml) for 24 and 48 h, the medium was removed carefully for LDH assay (Schmitt et al., 2005). Briefly, 100 µL of the media from each well was transferred to a new microplate, and 100 µL of LDH reagent and catalyst (1:45) was added. Then, the mixture was incubated for 30 min in a dark place at room temperature and absorbance was read at 490 nm with background subtraction at 630 nm using the Synergy HT multi detection Micro Plate Reader. For untreated cells, 20 µL of 10% triton X-100 and the LDH reagent were added, and its results were collected as positive controls. The collected media without cells were considered as negative controls. The results were presented as percentage (%).

Antioxidant activity

DPPH assay

The antioxidant activity of the essential oil was evaluated by measuring the bleaching of the purple-colored ethanol solution of DPPH according to the method of Blois (1958). Briefly, 1 ml of DPPH (0.5 mM in ethanol) was mixed with 1.0 ml of different concentrations of the essential oil (25 to 2000 µg/ml) and 2 ml of 0.1 M sodium acetate buffer (pH 5.5). Then, the mixtures were well shaken and kept at room temperature in dark for 30 min, after which the absorbance was measured at 517 nm using a UV–Vis spectrophotometer (Agilent). The ascorbic acid and ethanol were used as a positive and negative control, respectively. The percentage of inhibition (% Inhibition) of DPPH radical was calculated using the equation:

$$\% \text{ Inhibition} = \frac{(A_o - A_s)}{A_o} \times 100$$

In this formula, A_o is the absorbance of the control (containing all reagents except the test compound), and A_s is the absorbance of the test compound. The IC_{50} value represents the concentration of the essential oil causing 50% inhibition.

Reducing power

The reducing antioxidant power of the essential oil was assayed by the method of Sfahlan et al. (2009). Different concentrations of the essential oil (25 to 2000 µg/ml) in 2.5 ml of methanol were mixed with sodium phosphate buffer (2.5 ml, 200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 min, then 2.5 ml of 10% TCA was added to the mixture and centrifuged for 10 min at 200 × g. Finally, the upper layer (5 ml) was mixed with 5 ml of distilled water and ferric chloride (1 ml, 0.1%), and the absorbance was measured at 700 nm using a UV–Vis spectrophotometer (Agilent). A higher absorbance indicates a higher reducing antioxidant power. EC_{50} value (the effective concentration at which the absorbance is 0.5 for reducing antioxidant power) was obtained by extrapolation from linear regression analysis (in concentration of mg/ml). BHA was used as a control.

Hydrogen peroxide (H_2O_2) scavenging activity

H_2O_2 scavenging activity was performed according to established spectrophotometry method (Ruch et al., 1989). First, a solution of H_2O_2 (40 mM) was prepared in a phosphate buffer (pH 7.4). The concentration of H_2O_2 was determined spectrophotometrically at 230 nm, then different concentrations (25 to 2000 µg/ml) of the oil were added to 0.6 ml of H_2O_2 (40 mM) and absorbance of the solution was read at 230 nm after 10 min against blank containing all regents without H_2O_2. The percent scavenging of H_2O_2 by the essential oil was calculated using the following formula:

$$\% \text{ Scavenging of } H_2O_2 = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The essential oil was obtained by hydrodistillation of *C. aurantium* leaves with the yield of 1.1% (v/w), based on dry weight of the sample. 41 compounds that represented 97.81% of total oil (Table 1) were obtained. In total sum, the major components were limonene (57.57%), linalool (8.01%), *trans*-beta-ocimene (4.66%), caryophyllene (2.6%), myrcene (2.37%), carvacrol (1.4%) and alpha-bergamotene (1.3%). Some other compounds were germacrene D (1.11%), (E)-anethole (1.05%), spathulenol (1%) and cadinol isomer (0.97%).

In an investigation reported by Lota et al. (2001) on sour orange, 47 components were identified. The most abundant compounds of the essential oil from leaves were linalool (1.0 to 37.7%), linalyl acetate (0 to 36.8%), sabinene (0.3 to 52.6%), limonene (0.6 to 44.0%), beta-pinene (2.1 to 36.7%) and *trans*-beta-ocimene (2.5 to 15.1%). There are many reports on the chemical composition of the essential oils extracted from the *Citrus* species indicating that the main components are limonene, myrcene and linalool (Abeysinghe et al., 2007; JongSeok et al., 2008; Carvalho-Freitas and Costa, 2002). Our investigation is therefore in agreement with these previous reports.

Antioxidant activity

Antioxidant activity of the essential oil of *C. aurantium* was assessed by three different test systems namely DPPH free radical scavenging, H_2O_2 scavenging and reducing antioxidant power, and the results are presented in Table 2. DPPH was used to evaluate antioxidant capacity and bleaches purple color when accepting electrons/hydrogens, thus indicating the scavenging activity (Oke, 2009). In the present study, the essential oil showed lower scavenging ability on DPPH radical (IC_{50}=1040 ± 0.9 µg/ml) than the BHA (IC_{50} = 160 ± 1.13 µg/ml). Another method for evaluation of the antioxidant capacity is reducing antioxidant power. Antioxidant substances reduce free radical molecules and in this way, protect the body against dangerous effects of the free radicals (Hsu et al., 2006). The reducing antioxidant power of the essential oil was lower (IC_{50} = 1580 ± 1.03 µg/ml) than BHA (IC_{50} = 250 ± 1.8 µg/ml). On the other hand, H_2O_2 molecules are highly damaging species in free radical pathology, and antioxidant agents have the ability to scavenge it (Pardini, 1995). The essential oil of *C. aurantium* also scavenged the H_2O_2 with IC_{50} = 1500 ± 1.7 µg/ml, which is higher in comparison with BHA that scavenged H_2O_2 with IC_{50} = 140 ± 1.5 µg/ml.

The essential oil is mainly composed of monoterpenes hydrocarbons such as limonene (57.57%) and linalool (8.01%) whose antioxidant properties have been previously reported (Malhotra et al., 2009; Ijaz Hussain et al., 2008). The IV_{50} DPPH scavenging and NO radical inhibitions of *C. aurantium* peel's oil are 06.7 and 1.8 µL/ml, respectively (JongSeok et al., 2008). In addition, the antioxidant activity of the essential oil extracted from peel of different *Citrus* species including *C. sinensis*, *C. erythrosa* and *C. grandis* has been determined in previous studies - this activity is related to high concentration of terpenes of limonene in these oils (Jong Seok et al., 2008; Malhotra et al., 2009; Ijaz Hussain et al., 2008). These results are significant because they are the first reports regarding antioxidant activity of the essential oil of *C. aurantium* leaves measured via three different methods.

Cytotoxicity activity

The measurement of Lactate dehydrogenase (LDH) content in culture medium is an indicator of cell cytotoxicity. LDH is a stable cytoplasmic enzyme present in all cells and rapidly released from cells upon cell membrane damage, which indicate cell death due to either necrosis or apoptosis. In this assay, $NADH/H^+$ reacted with tetrazolium salt in the presence of a catalyst to form red color formazan that could be measured at 490 nm (Johnson and Mukhtar, 2007). The results of cytotoxic effects of the essential oil obtained from *C. aurantium* on six human tumor cell lines and a normal cell line are shown in Table 3 and Figure 1. The essential oil had considerable cytotoxicity effects on all tumor cell lines with the highest activity against Jurkat and HL60 (IC_{50} = 17 ± 0.1 and 19 ± 0.15 µg /ml, respectively). Furthermore, lower effects were observed on PC3 and HUVEC with IC_{50} value of 35 ± 2.2 and 32 ± 1.2 µg /ml, respectively. The less sensitivity of HUVEC as a normal cell line is important because it has been shown that the essential oil has low toxicity against normal cell lines and probably against the body. The cytotoxic activity of the essential oil may be attributed to limonene and linalool that are present in the essential oil. Limonene has high cytotoxicity activity, and it is supposed that the isoprenylation and apoptosis would be among the possible mechanisms of its cytotoxicity (Sun., 2007).

Figure 1 .IC50 values (µg\ml) of cytotoxic effects of the essential oil from Citrus aurantium L.on different cell lines by LDH assay. Each column represents mean±S.E. of the three independent experiments. $p < 0.01$, compared with the control group.

Compounds	[a] RI	Percentage (%)	Identification methods*
alpha-pinene	933	0.5	RI, MS
sabinene	975	0.10	RI, MS, CoI
myrcene	993	2.37	RI, MS, CoI
limonene	1033	57.57	RI, MS
beta-ocimene	1039	0.25	RI, MS
trans-beta-ocimene	1057	4.66	RI, MS
gama-terpinene	1060	0.11	RI, MS
cis linalool oxide	1071	0.13	RI, MS
terpinolene	1086	0.13	RI, MS
hotrienol	1101	0.46	RI, MS, CoI
linalool	1103	8.01	RI, MS
trans-limonene oxide	1139	0.71	RI, MS
isopulegol	1143	0.12	RI, MS
citronellal	1154	0.89	RI, MS
terpinen-4-ol	1174	0.16	RI, MS
alpha-terpineol	1190	0.65	RI, MS, CoI
trans-carveol	1214	0.51	RI, MS
neral	1227	0.22	RI, MS
citronellol	1230	0.69	RI, MS
carvone	1242	0.4	RI, MS
geraniol	1254	0.57	RI, MS
e-anethole	1283	1.05	RI, MS
thymol	1291	0.59	RI, MS
carvacrol	1300	1.4	RI, MS
caryophyllene	1415	2.6	RI, MS
alpha-bergamothene	1430	1.3	RI, MS, CoI
humulene	1447	0.3	RI, MS
germacrene D	1478	1.11	RI, MS
bicyclogermacrene	1490	0.44	RI, MS
alpha-muurolene	1495	0.19	RI, MS
alpha-bisabolene	1504	2.1	RI, MS
delta-cadinene	1516	0.65	RI, MS
trans-nerolidol	1560	0.67	RI, MS
spathulenol	1564	1.0	RI, MS
caryophyllene oxide	1567	0.67	RI, MS
carotol	1594	0.34	RI, MS
widdrol	1598	0.09	RI, MS
delta-cadinol	1608	0.39	RI, MS
cadinol isomer	1630	0.97	RI, MS
cadinol	1650	0.68	RI, MS
beta-bisabolol	1671	0.47	RI, MS

*RI, retention indices relative to C6–C24 n-alkanes on the DB-5 column; MS, mass spectrum; CoI, coinjection with an authentic sample.

Table 2. Antioxidant activities of the essential oil from the leaves of *Citrus aurantium* L.

Sample	DPPH IC_{50} value (mg/ml)	EC_{50} values of reducing power assay (mg/ml)	H_2O_2 IC_{50} value (mg/ml)
Oils	1.04 ± 0.9	1.58 ± 1.03	1.5 ± 1.7
BHA	0.16 ± 1.13	0.25 ± 1.8	0 .14 ± 1.5

Table 3. Cytotoxicity of the essential oil from the leave of *Citrus aurantium* L.on six human cancer cell lines.

Cell line	IC_{50} (µg/ml)
Hela	25 ± 1.6
HT29	27 ± 1.1
PC3	35 ± 2.2
K562	22 ± 0.1
HL60	19 ± 0.15
Jurkat	17 ± 0.1
HUVEC	32 ± 1.2

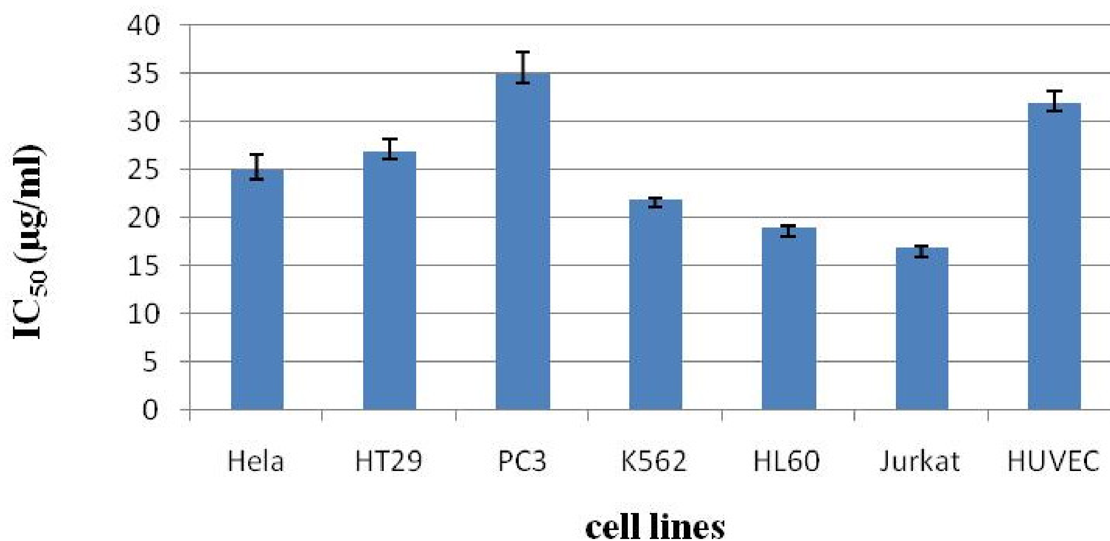

Figure 1.

In experimental studies on animals, the evidence to support the anti-cancer activity of limonene on the liver, mammary gland and gastric cancers was provided (Crowell et al., 1994). Moreover, it has been reported that linalool has stronger cytotoxicity in low concentration, inhibits mitochondrial complexes II and I, and decreases ATP- one of the possible targets for its action (Usta, 2009). Myrcene and caryophyllene which are found in the essential oil are also known to possess cytotoxic activity(Usta, 2009; Sibanda et al., 2004). Some previous studies reported that the essential oil of *C. aurantium* peel has cytotoxic activity against HeLa cell line with IC_{50} = 14 µg/ml (Bakkali, 2008) and human dermal fibroblasts with cell viability of approximately 60% at a concentration of 10 µg/ml. Other *Citrus* species such as *C. medica*, *C. sinensis* and *C. aurantifolia* have also been reported to exhibit cytotoxic activity against various cell lines (JongSeok et al., 2008; Patil et al., 2009; Ao et al., 2008). The cytotoxic activity of the essential oil on the tumor cell lines maybe ascribed to its antioxidant activity because antioxidants are believed to be anti-mutagenic and anti-carcinogenic due to their radical scavenging properties (Bakkali, 2008).

Conclusion

The results of this study suggest the possibility of using the essential oil of *C. aurantium* as a potential source of antioxidant ingredients for the food, cosmetic and pharmaceutical industry. Furthermore, our results indicate that the essential oils of these species may warrant further investigations into their potential capacity for cancer therapy. These results can be considered as the first report on *in vitro* cytotoxicity and antioxidant activity of the essential oil of *C. aurantium*.

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