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Research Article

Essential Oil Composition of *Citrus volkameriana* with Anticancer and Antimicrobial Potentials

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ABSTRACT

The present study was carried out to determine the chemical composition and to evaluate anticancer and antimicrobial activities of essential oil extracted from *Citrus volkameriana* leaves. GC-MS analysis of the essential oil obtained from *C. volkameriana* leaves revealed that the major compounds were limonene (21.26%), sabinene (14.14%), linalool (9.64%), Citronellal (7.08%) and Terpinene-4-ol (6.23%). The growth inhibitory effect of the essential oil were tested in the 3-cell lines panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using Sulforhodamine B (SRB) assay. Antimicrobial activity was tested against four gram positive bacteria, four gram negative bacteria strains, gram stain resistant microbe *Mycobacterium tuberculosis* and against four fungal yeasts. The oil showed a significant activity on MCF7 Breast cancer cell line and antimicrobial activity especially on *Micrococcus luteus* B-287 with MIC (31.25 µg/ml).

INTRODUCTION

Plants are one of the best and main sources of bioactive materials. Recent reports indicated that medicinal herbs are used by majority of the people living in rural areas as primary healthcare system [1]. For centuries genus Citrus was well known for its use in traditional medicine to cure many types of diseases from minor aliment to serious diseases [2]. Recent studies proved that Citrus extracts exerted many activities such as anticancer, anti-inflammatory, antioxidant [3], analgesic, antibacterial, antifungal [4] and anxiolytic [5]. These benefits were attributed to the accumulation of chemical compounds in citrus including flavonoids, alkaloids, terpenoids and coumarins [6]. Citrus essential oil is regarded as one of the most important citrus byproducts. It is mainly used as flavoring agents, air fresheners, in household products and cosmetics [7]. Recently the use of citrus essential oils in medicine has become of great interest.

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Several biological activities of different citrus oils have been reported including anticancer [8], antifungal[9], anti-inflammatory, antiaflatoxigenic and antioxidant [10]. *Citrus volkameriana Ten. & Pasq*. (*Citrus limonia* Osbeck.), known as volkamer lemon, belongs to family *Rutacea*. Previous studies of the plant proved that it contained volatile constituent with limonine is the predominant [11]. Cancer is a disease characterized by spread of abnormal cells [12]. The high death rate among cancer patients is an evidence of the limited efficiency of the current therapies including radiation, chemotherapy and surgery [13]. Research has the attention on the use of medicinal plants and natural products as crude plant extracts or a combination of different phytochemicals for cancer therapy; this trend is based upon: first, the synergistic effect of the different plant metabolites in the crude extract, second, is the multiple points of intervention of such extracts [14]. Plants represent a good source of antimicrobial agents since they are natural, have manageable side effects and available at affordable prices [15]. The main objective of this study is to investigate the chemical composition of the oil of *C. volkameriana* leaves and to evaluate biological effects of the oil as anticancer and antimicrobial effects.

MATERIAL AND METHODS

Plant Material

Fresh leaves of *Citrus volkameriana* were collected from the horticulture research institute, Giza, Egypt, during flowering in April 2011. The plant was kindly authenticated by senior botanist Dr. Mohammed El- Gibali. A dried specimen was placed at the museum of the pharmacognosy department, faculty of pharmacy, Cairo University with voucher number 130501.

Essential Oil Isolation

The essential oil was obtained by hydro-distillation of fresh leaves (500 g) for 6–8 h in Clevenger's apparatus. The prepared oil was dehydrated over sodium sulfate and stored in air-tight sealed glass vial at 4°C for further use. The yield of oil was recorded (0.4 ml/100 g Leaves). The major constituents were analyzed by GC-MS.

GC-MS Analysis

GC-Ms analysis was carried out using gas chromatography – mass spectrometry instrument stands at the Laboratory of Medicinal and Aromatic plants, National Research Center with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-WAX MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness). Analysis was carried out using helium as carrier gas at a flow rate of 1.0 mL/min at a split ratio of 1:10 and the following temperature program: 40 C for 1 min; rising at 4.0 C/min to 150 C and held for 6 min; rising at 4 C/min to 210 C and held for 1min. The injector and detector were held at 210 and 200 C, respectively. Diluted sample (1:10 hexane, v/v) of 0.2 μL of the mixture was injected. Mass spectrum was obtained by electron ionization (EI) at 70eV, using a spectral range of m/z 40-450. Most of the compounds were identified using two different analytical methods: (a) KI, Kovats indices in reference to n-alkanes (C9-C22) (National Institute of Standards and Technology 2009); and (b) mass spectra (authentic chemicals and Wiley spectral library collection) and comparison with previously published data [11].

Anticancer Assay

The human cell lines TK10, UACC62 and MCF7 were previously obtained from National Cancer Institute (NCI) inthe framework of a collaborative research program between CSIR and NCI. Cell lines were routinely maintained as monolayer cell cultures in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50µg/ml gentamicin.

For the screening experiment, the cells (3-19 passages) were inoculated in 96-well microtiter plates at plating densities of 7-10 000 cells/well and were incubated for 24 h. After 24 h one plate was fixed with TCA to represent a measurement of the cell population for each cell line at the time of drug addition (T0). The other plate with cells were treated with the oil which was previously dissolved in DMSO as 10000µg/ml stocks and diluted in medium to a final concentration 100 µg/ml. Cells without the oil served as controls. Blank wells contained complete medium without cells. Emetine was used as a reference standard. The plates were incubated for 48 h after addition of the oil. At the end of the incubation period, the cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed with SRB. Unbound dye was removed and protein-bound dye was extracted with 10mM Tris base for optical density determination at a wavelength 540 nm using a multiwall spectrophotometer [16]. Optical density measurements were used to calculate the net percentage cell growth.

The optical density of the test wells after 48-h period of exposure to the oil is Ti, the optical density at time zero is T0, and the control (untreated cells) optical density is C.4

Percentage cell growth is calculated as:

$$\mathbf{[(Ti-T0)/(C-T0)] \times 100}$$

For concentrations at which Ti≥T0

$$\mathbf{[(Ti-T0)/T0] \times 100}$$

For concentrations at which Ti<T0.

Antimicrobial Assay

The antimicrobial activity was measured using p-iodonitrotetrazolim violet (INT) assay to determine the minimum inhibitory concentration (MIC) of the oil according to method described by Eloff [17]. The MIC was defined as lowest concentration of the test sample that inhibited microbial growth. The INT tetrazolium salt is frequently used to indicate biological activity because it is colour less compound acts as an electron acceptor and

reduced to a purple formazan product by biologically active organisms, while the microbial inhibition were recorded as solution after incubation with INT, i.e. the MIC values were read as those concentrations where a marked reduction in colour formation was noted [17]. The use of INT assay has many of advantage, like higher sensitivity for lower active concentrations than the old agar diffusion methods, the assay can be detected visually with avoiding the problems associated with using microplat reader of chlorophyll interference and the clumped microbial cells at the bottom of the well, as the formazan product is insoluble in water. Also, because of the stability of the colour of reduction product of INT, the assay plates could be kept for reference purposes. In general, the INT method is robust, not expensive, gives reproducible results, requires a small quantity of sample, can be used for large numbers of samples, leaves a permanent record, and requires little time [17].

Procedure

Sample was prepared by dissolving 1 mg of the oil in 1 ml of DMSO, and then diluted with deionised water to prepare stock solutions of 10% DMSO.

Antimicrobial activity tests were carried out against four gram positive bacteria, Bacillus subtilis NRRL-B-4219, Micrococcus luteus B-287, Staphylococcus aureus ATCC 29213, Streptococcus faecalis ATCC 19433, four gram negative bacteria, Alcaligenes faecalis B-170, Escherichia coli ATCC 25922, Klebsiella pneumonia ATCC 10131, Pseudomonas aeruginosa ATCC 27953, gram stain resistant microbe Mycobacterium tuberculosis (clinical isolate; identified and obtained from Chemistry of Natural and Microbial Product Department, National Research Centre, Egypt).

The assay was performed according to Buwa and van Staden [18] with some modifications. The bacterial cultures were refreshed on nutrient agar (NA) media, and the fungal yeasts were refreshed on potato dextrose agar (PDA) media for 24 hours at 37°C. After incubation period, the microbial colonies were scrapped off the ager and transferred to nutrient and potato dextrose broth solutions for bacteria and yeast respectively, to prepare 0.5 McFarland of microbial cultures with turbidity standard 107 CFU/ml (colony-forming units). All the extracts were initially tested at 250 µg/ml by addition of 100 µl of plant extracts to the first wells of 96-well microplates and were serially diluted down (50%) with sterile water to 1.95 µg/ml. After that, 100 µl of inoculated broths were added to each well. The inoculated 96-well plates were incubated at 37 °C for 24 hours. The currently used antibiotic drug dipenacid (CID pharmaceutical company), composed of two bactericidal agents ampicillin and dicloxacillin, was included as positive control reference in each assay. Oil-free solution was used as a negative control, and 10% DMSO solution was used as blank control. As an indicator of microbial growth, 40 µl of p-iodonitrotetrazolium violet salt (INT, Sigma) (0.2 mg/ml) dissolved in water were added to the wells. The plates were incubated again at 37°C for 1 hour until the development of purple colour of formazan. The MIC values are recorded as the lowest concentration of the oil that completely inhibited bacterial growth, i.e. a clear well.

RESULTS

Our result showed that *Citrus volkameriana* leaves essential oil had a complex mixture that consists in its majority of monoterpene hydrocarbons, as indicated in Table 1. Anticancer and antimicrobial activities are included in Tables 2 and 3, respectively.

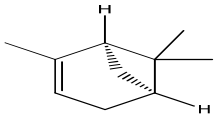
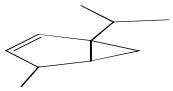
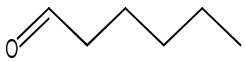
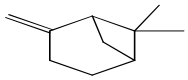
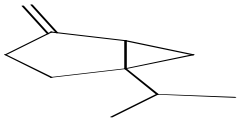
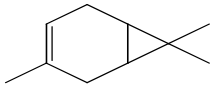
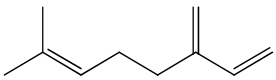
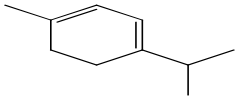
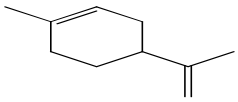
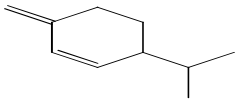
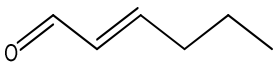
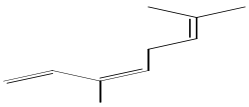
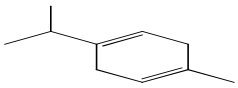
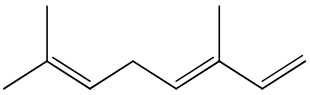
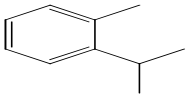
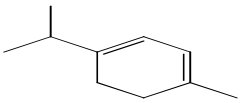
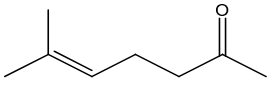
DISCUSSION

GC-MS profile of the prepared essential oil was shown in Fig. 1. The analysis led to the identification of 35 different compounds, representing 99.22% of total essential oil from leaves table (37). The major compounds detected were D-limonene (21.26%), sabinene (14.14%), L-linalool (9.72%), citronellal (7.14%) and terpinene-4-ol (6.28%). The percent of oxygenated compounds were 34.40% including alcohols (20.36%), aldehydes (11.29 %), ketones (1.13%) and esters (1.62%).

The oil also demonstrated selectivity on MCF-7 breast cancer cell line. Table 2 and Fig. 2 are showing the activity of the oil on the three different cancer cell lines.

Certain Citrus essential oils have proven to be cytotoxic on MCF7 cells [19]. The activity may be attributed to the chemical components of the oil especially limonene. 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 [20].

Table 1: The GC-MS Analysis of the Essential Oil of *Citrus volkameriana* Leaves.

Compounds	Formula	Structure	Mwt.	Rt	RRt	A %
Unidentified	--------	----------		3.27	0.43	0.27
α-Pinene	$C_{10}H_{16}$		136	3.97	0.52	2.57
β-Thujene	$C_{10}H_{16}$		136	4.06	0.53	0.92
Hexanal	$C_6H_{12}O$		116	5.18	0.68	0.15
β-Pinene	$C_{10}H_{16}$		136	5.39	0.71	2.54
Sabinene	$C_{10}H_{16}$		136	5.77	0.76	14.14
Δ-3- carene	$C_{10}H_{16}$		136	6.32	0.83	3.15
β-Myrcene	$C_{10}H_{16}$		136	6.82	0.90	3.96
α-Terpinene	$C_{10}H_{16}$		136	7.08	0.93	2.00
D-Limonene	$C_{10}H_{16}$		136	7.62	1.00	21.26
β-Phellandrene	$C_{10}H_{16}$		136	7.91	1.04	0.62
2-Hexenal	$C_6H_{10}O$		114	8.51	1.12	0.61
cis-Ocimene	$C_{10}H_{16}$		136	8.71	1.14	1.34
γ-Terpinene	$C_{10}H_{16}$		136	8.87	1.16	2.94
β-Ocimene	$C_{10}H_{16}$		136	9.22	1.21	6.62
o-Cymene	$C_{10}H_{14}$		134	9.61	1.26	0.76
α-terpinolene	$C_{10}H_{16}$		136	9.85	1.29	1.35
6-Methyl-5-heptene-2-one	$C_8H_{14}O$		142	11.97	1.57	1.13
Unidentified	----------	----------		13.52	1.77	0.14

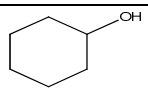
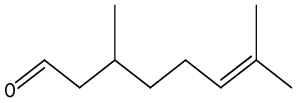
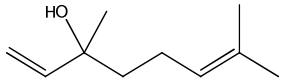
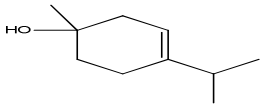
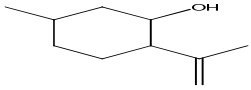
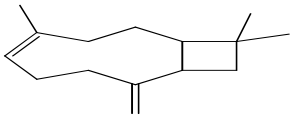
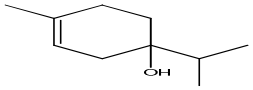
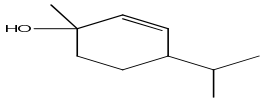
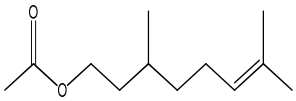
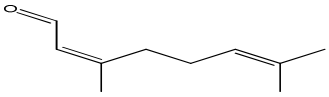
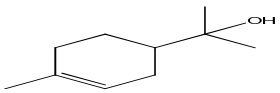
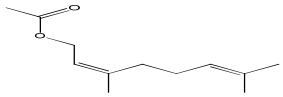
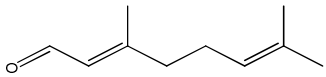
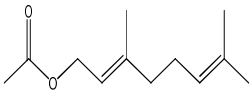
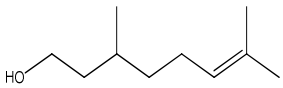
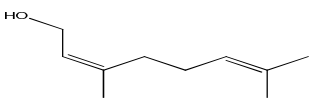
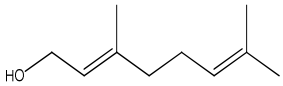
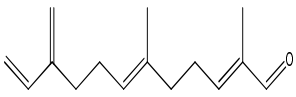
Cyclohexanol	$C_6H_{12}O$		116	15.85	2.08	0.22
Citronellal	$C_{10}H_{18}O$		170	16.42	2.15	7.14
L-Linalool	$C_{10}H_{18}O$		170	18.55	2.43	9.72
Terpinene-1-OL	$C_{10}H_{18}O$		170	18.86	2.48	0.42
Isopulegol	$C_{10}H_{18}O$		170	19.07	2.50	0.70
β-Caryophyllene	$C_{15}H_{24}$		204	19.26	2.53	0.53
Terpinene-4-ol	$C_{10}H_{18}O$		170	20.09	2.64	6.28
p-menth-2-en-1-ol	$C_{10}H_{18}O$		170	20.84	2.73	0.26
Citronellyl Acetate	$C_{12}H_{22}O_2$		230	21.77	2.86	0.35
Neral	$C_{10}H_{16}O$		168	22.58	2.96	1.38
α terpineol	$C_{10}H_{18}O$		170	22.88	3.00	1.41
Unidentified	----------	----------		23.22	3.05	0.16
Neryl acetate	$C_{12}H_{20}O_2$		228	23.66	3.10	0.68
Geranial	$C_{10}H_{16}O$		168	24.06	3.16	1.88
Geranyl acetate	$C_{12}H_{20}O_2$		228	24.51	3.22	0.59
β-Citronellol	$C_{10}H_{20}O$		172	24.8	3.25	0.76
Nerol	$C_{10}H_{18}O$		170	25.8	3.39	0.39
Geraniol	$C_{10}H_{18}O$		170	27.11	3.56	0.20
Beta sinensal	$C_{15}H_{22}O$		234	40.33	5.29	0.12
Unidentified	----------	----------		41.29	5.42	0.20

Table 2: Anticancer Activity of *Citrus volkameriana* Essential Oil

Sample	Growth TK10, %	SD	Growth UACC62, %	SD	Growth MCF7, %	SD
Essential oil	-25.54		-42.17		-62.91	
Emetin	-61.35	0.037	-86.66	0.059	-46.41	0.08

Table 3: The Antimicrobial Activity of the Essential Oil

	Microorganisms	MIC(µg/ml)	Dipenacid
Gram positive	*Bacillus subtilis NRRL-B-4219*	125	<3.9
	Micrococcus luteus B-287	31.25	
	Staphylococcus aureus ATCC 29213	250	
	Streptococcus faecalis ATCC 19433	-	
Gram negative	*Alcaligenes faecalis B-170*	250	
	Escherichia coli ATCC 25922	250	
	Klebsiella pneumoniae ATCC 10131	125	
	Pseudomonas aeruginosa ATCC 7953	125	
Gram resistant	*Mycobacterium tuberculosis*	125	
Yeast	*Candida albicans ATCC 10231*	-	
	Candida parapsilosis ATCC 22019	125	
	Candida tropicalis ATCC 750	250	
	Saccharomyces cerevisiae ATCC2180-1A	125	

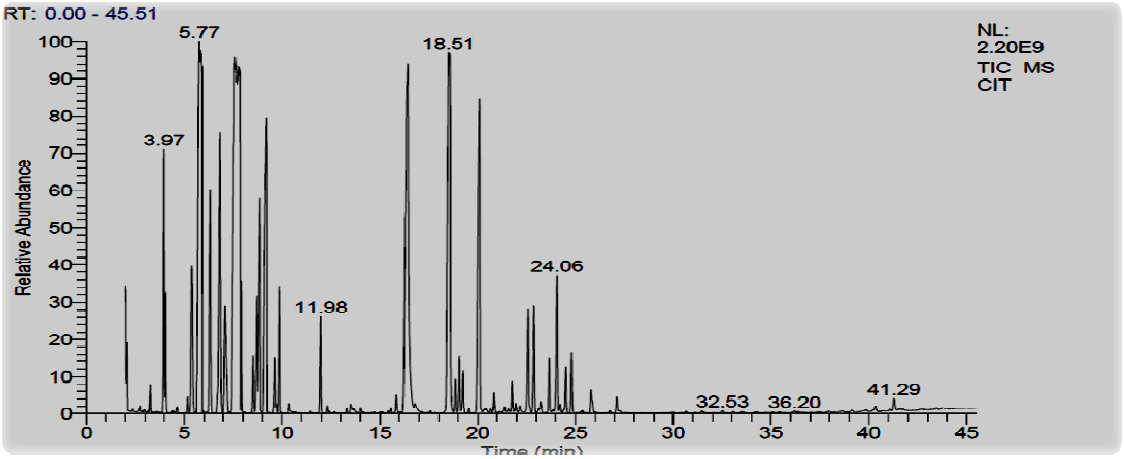


Figure 1: GC-MS Analysis of Essential Oil from *Citrus volkameriana* Leaves

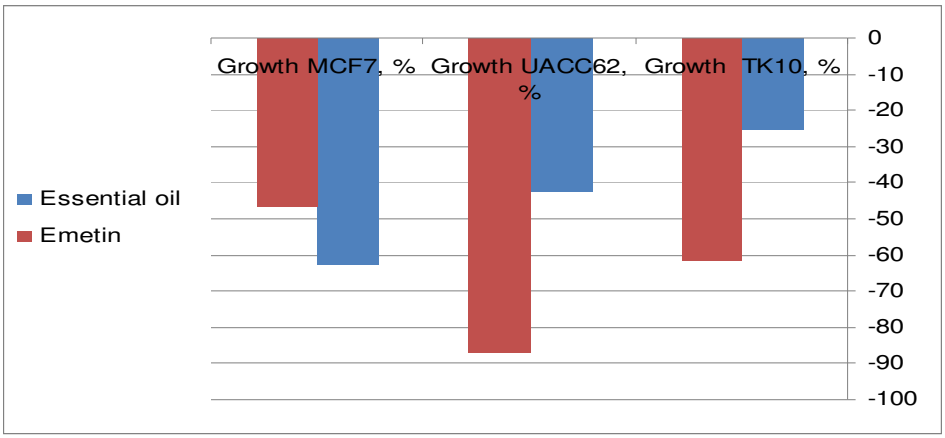


Figure 2: Anticancer Activity of *Citrus volkameriana* Leaves Essential Oil

Antimicrobial assay results also proved that the essential oil has antimicrobial effect especially on the gram positive bacteria Micrococcus luteus B-287 with MIC (31.25 µg/ml). The data of the antimicrobial activity are sumerized in Table 3. The activity of the oil may be attributed to limonene which was reported to increase the permeability of the bacterial cell membrane [21]. We cannot also discount the possibility that other minor compounds in the extracts function as bioactive agents or the bioactivity is the result of combination or synergistic effects of some undetermined compounds in the oil.

CONCLUSION

From the previous investigation it can be concluded that *Citrus volkameriana* leaves essential oil can be considered as a resource for potential anticancer and antimicrobial agents and it is necessary to do extensive *in vitro* and *in vivo* tests to assure the selection of active and nontoxic anticancer and antimicrobial phytochemicals.

CONFLICT OF INTEREST

There is no conflict of interest associated with the authors of this paper.

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