Journal of Applied Pharmaceutical Science Vol. 5 (01), pp. 006-012, January, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.50102
ISSN 2231-3354

Chemical Composition, Antiviral against avian Influenza (H5N1) Virus and Antimicrobial activities of the Essential Oils of the Leaves and Fruits of *Fortunella margarita,* Lour. Swingle, Growing in Egypt

Nabaweya A. Ibrahim[1], Seham S. El-Hawary[2], Magdy M. D. Mohammed[1,*], Mohamed A. Farid[3], Nayera A. M. Abdel-Wahed[3], Mohamed A. Ali[4], Eman A. W. El-Abd[1]

[1] Pharmacognosy Department, Pharmaceutical and Drug Industries Research Division, National Research Center, Dokki-12311, Cairo, Egypt.
[2] Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza, Egypt. [3]Chemistry of Natural and Microbial Products Department, Pharmaceutical and Drug Industries Research Division, National Research Center, Dokki-12311, Cairo, Egypt. [4]Center of Scientific Excellence for Influenza Viruses, National Research Center, Dokki-12311, Cairo, Egypt.

ARTICLE INFO

Article history:
Received on: 28/10/2014
Revised on: 10/11/2014
Accepted on: 05/12/2014
Available online: 30/01/2015

Key words:
Fortunella margarita Lour. Swingle; Rutaceae; essential oil; antiviral; antimicrobial.

ABSTRACT

Essential oils of the fresh leaves and fruits of *Fortunella margarita* Lour. Swingle (Family: Rutaceae) were prepared by hydrodistillation method, which resulted with 0.27 and 0.30% respectively. The resulted oils of both organs were analyzed by GC/MS which revealed the presence of twenty compounds in the leaves oil representing 86.96% of the oil, from which gurjunene, eudesmol and muurolene were identified as major compounds. The fruit's oil was found to contain fourteen compounds representing 77.77% of the oil, of which terpineol, *t*-carveol, limonene, muurolene and cadinene represented the major compounds. The antiviral activity of the essential oils of both leaves and fruits was tested against avian influenza-A virus (H5N1), and the results revealed higher potency of fruits oil. Moreover, the essential oils of the leaves and fruits were investigated for their antimicrobial and antifungal activities. The oil of the leaves showed antimicrobial activity higher than that of the fruits at dilution (1:50 v/v) against *Bacillus subtilis, Staphylococcus aureus, Sarcina luta* and *Streptococcus faecalis*, also it has a moderate activity against *Escherichia coli, Klebsilla pneumonia* and *Pseudomonas aeroginosa*. On the other hand, the antifungal activity of the leaves and fruits revealed that the fruits exhibited higher activity than that of the leaves against *Aspergillus niger* and *Candida albicans*.

INTRODUCTION

Kumquats (*Fortunella* spp.) belong to the *Citrus* genus; their fruits are usually eaten raw as a whole fruit together with the peel, excluding the seeds. The peel is sweet and edible with a typical aroma due to the presence of flavonoids and terpenoids (Koyasako and Bernhard, 1983). *Fortunella margarita* Lour. Swingle (Rutaceae) is an evergreen tree native to Southeastern Asia and more precisely to China. It is also known as the oval or nagami kumquat. The genus *Fortunella* has been used in folk medicine to treat fevers, gallstones, indigestion, hernial pain, stomachache, hepatitis, high blood pressure, prolapse of the uterus and anus, asthma, catarrhal cough, pneumonia, respiratory congestion and whooping cough (Khaleel *et al*., 2001). Certain *Fortunella* species were reported to be used as haemostatic, antiasthmatic and in treatment of diarrhea, *Fortunella margarita* Lour. Swingle is cultivated in Egypt for ornamental purposes (Khaleel *et al*., 2001). Kumquats are also an excellent source of nutrients and phytochemicals, including ascorbic acid, carotenoids, flavonoids and essential oils (Wang *et al*., 2012) *i.e.*, 3´,5´-di-*C*-*β*-glucopyranosyl phloretin, which is a characteristic flavonoid in *F. margarita* and all other *Fortunella* species (Ogawa *et al.*, 2001). Some *Fortunella* species *i.e., F. japonica* (round), *F. margarita* (oval or nagami) and *F. crassifolia* (jingdan or meiwa) are commonly cultivated in the Southern region of China. Most of *Fortunella* species can be used for the preparation of marmalade, fruit salad and as food preservative.

** Corresponding Author*
Dr. Magdy Mostafa Desoky Mohammed, Ph.D.
Department of Pharmacognosy, National Research Center, Dokki-12311, Cairo, Egypt. Tel.: +202-33371718; Fax: +202-33370931
E-mail: melhenawy111@gmail.com (Magdy M. D. Mohammed).

As a result of inadequate use of antiviral drugs, influenza viruses are mutating and mutant variants are evolving. To control the spread of these viruses as well as the expected and unexpected mutant variants, we have a great challenge to find out more potent antiviral agents. Plant and marine extracts, synthetic compound and target directed compounds are the main sources of these antiviral agents.

Antiviral and antimicrobial drugs are subject to microbial resistance, and this has become a growing public problem all over the world. Therefore, ample research to discover potent new antibiotics is compulsory. Since many essential oils have been reported to possess strong antimicrobial effects (Nakatsu *et al*., 2000, Rios and Recio, 2005, Koch *et al*., 2008), the aims of the present study were conducted to investigate the chemical composition of the essential oils of both leaves and fruits of *Fortunella margarita*, then the antiviral activity against pathogenic avian influenza virus (H5N1), and also the antimicrobial, and antifungal activities were performed.

MATERIALS AND METHODS

Plant materials

Fortunella margarita fruits and leaves were collected from Egypt green farm at Cairo-Alexandria agriculture road. Leaves were collected at the flowering stage while fruits were collected in February 2007. The plant was kindly identified by Mrs. Theresa Labib, consultant of taxonomy at the ministry of agriculture and the former director of El-Orman botanical garden. Voucher specimen of the whole plant (000120FC 04-09-06-25) was kept at the garden.

Essential oil isolation

The essential oils were extracted from fresh fruits and leaves of *F. margarita* by hydrodistillation method (ShunZhen *et al*., 2012). The oil content of each sample was determined as mean of triplicate. The collected oils were subjected to GC/MS analysis. Qualitative and quantitative identification of the oil constituents were carried out by comparing the retention times and mass fragmentation pattern with the previously published data (Adams, 1989, Walter and Takayuki, 1980).

Analysis of the essential oil

GC/MS conditions; the compounds were separated on an HP-5-MS fused silica capillary column (30 m x 0.25 mm ID x 0.25 μm (film thickness), Agilent, Palo Alto, CA, USA). Helium 5.0 was used as a carrier gas at a constant flow rate of 1.5 mL/min. The GC was operated in split less injection mode and the PTV injector was programmed from 60 to 285 ºC (1.1 min) at 14.5 ºC/s at an injection volume of 1 micro Litter (Ligon *et al*., 2008). The Trace MS Plus detector was operated in selected ion monitoring (SIM) mode at the ionization energy of 70 eV. The transfer line between GC and MS was kept at 250 ºC and the ion-source temperature was kept at 200 ºC. The MS calibration was done by auto-tuning.

Antiviral Activity

Virus and cells

Reasserted avian influenza A virus (H5N1) previously isolated from Egypt in 2006 (rgA/chicken/Egypt/1/2006), was used in this study to evaluate antiviral activity of the studied extracts. Madin-Darby canine kidney (MDCK) cells used for virus propagation were friendly obtained from St. Jude Children's Research Hospital. The MDCK cells were routinely passaged in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic mixture (penicillin- streptomycin-amphotericin B).

MTT assay (Cytotoxicity assay)

The stock samples were diluted with Dulbecco's Modified Eagle's Medium (DMEM) to desired concentrations. Stock solutions of the test compounds were prepared in DMSO at a concentration of 10% in dH_2O (distilled H_2O). The cytotoxic activity of the extracts were tested in MDCK cell line by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Hayden *et al*., 1980, Mossman, 1983) with minor modification. Briefly, the cells were seeded in 96-well plates (100 μL/well at a density of 3×10^5 cells/mL) and treated with various concentrations of the sample solutions. After 24 h, cells were washed with sterile phosphate buffer (PBS) 3 times and the supernatant was discarded. MTT solution (20 μL of 5 mg/mL) was added to each well and incubated at 37 ºC for 4 h.

$$\% \text{ Cytotoxicity} = \frac{(\text{Absorbance of cell without treatment} - \text{Absorbance of cell with treatment}) \times 100}{\text{Absorbance of cell without treatment}}$$

Then the medium was aspirated. In each well, the formed formazan crystals were dissolved with 200 μL of acidified isopropanol (0.04 M HCl in absolute isopropanol). An absorbance of formazan was detected by a dual wavelength UV spectrometer at 540 nm with 620 nm reference wavelength. The percentage of cytotoxicity compared to the untreated cells was determined with the equation given above. The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (TC_{50}).

Microorganisms

Bacterial strains

Five Gram-positive bacteria strains viz; (*Bacillus subtilis* NRRL 543, *Staphylococcus aureus* NRRL B-313, *Sarcina luta* NRRL B-1018, *Streptococcus faecalis* NRRL 537, *Arthobacter citreus* NRRL B-1258) and three Gram-negative bacteria strains viz; (*Escherichia coli* NRRL B-210, *Klebsilla pneumonia* NRRL B-117 *and Pseudomonas aeroginosa* NRRL B-23) were obtained from both of the Northern Utilization Research and Development Division, United State Department of Agriculture, Peoria, Illinois, USA and the Department of Microbiology, Faculty of pharmacy Cairo university, Egypt. The bacterial strains were revived for bioassay by sup-culturing in fresh nutrient broth medium for 24 hours before test.

Fungal strains

The tested fungi including *Aspergillus niger* NRRL-599 and *Candida albicans* NRRL Y-477 were cultured on potato dextrose agar (PDA) (2.5% agar) for 7 days at 28 °C before the experiment was carried out.

Preparation of plates

The antibacterial and antifungal tests of the essential oils were tested using agar diffusion method (Linday, 1962), the ready-made nutrient agar medium (for bacterial strains) and PDA (for fungal strains) were suspended in distilled water and autoclaved at pressure of 1.5 atm. For bacterial strains a suspension of tested microorganisms (0.1 mL of about 10^6 cells/mL) was seeded on solid media plates (9 cm diameter) and uniformly spread with a sterile spreader. Six to eight wells (8 mm) were made on the solid medium with a sterile cork borer. To each well, fixed volume (0.1 mL) of diluted essential oil and carefully was placed in each well. For fungal strains seven days old cultures of test organisms (0.1 mL) were used. Controls were maintained with paraffin oil only.

Antimicrobial activity of the oils

The antibacterial and antifungal tests of the essential oils were tested using the agar diffusion method (Linday, 1962). The treated and the controls were kept in an incubator at 37 °C for bacterial strains and at 28 °C for 24 to 72 hours. The zones of inhibition for each well were measured. Nystatin, erythromycin, oxacillin, methcilin and bacitracin were included in the test as references. At the end of incubation period, the diameter (mm) of the inhibition zones was measured. Plates were done in triplicate and an average + SD was recorded.

The minimum inhibitory concentration (MIC)

The stock solutions of the oils were diluted and transferred into the first tube, and serial dilutions were performed so that concentrations in the range of 0.001-0.02 μL/mL were obtained. A 10 μL spore suspension of each test strain was inoculated in the test tubes in nutrient medium and incubated for 24-72 hours at 37 °C. The control tubes containing the same medium were inoculated only with bacterial strains suspension. The minimal concentrations at which no visible growth was observed were defined as the MICs which were expressed in (v/v %).

Spore germination assay

Spore germination assay was carried out according to (Rana *et al*., 1997). Three concentrations (4, 2, and 1% v/v) of each oil sample, together with two controls (one sterile distilled water and other of 0.1 % (v/v) methanol in sterile distilled water) were tested for spore germination of *Aspergillus niger* and *Fusarium oxysporum*.

Aliquots of 0.1 mL from each sample were mixed with fungal spores obtained from 10 days old cultures of the tested fungi and placed on separate glass slides in triplicate. Slides containing the spores were incubated in a moist chamber at 28 °C for 24h. Each slide was then fixed in lactophenol-cotton blue and observed under the microscope for spore germination.

RESULTS AND DISCUSSION

Essential oils of the fresh leaves and fruits of *Fortunella margarita* Lour. Swingle (Family: Rutaceae) were prepared by hydrodistillation method, and resulted with a mean percentage of three replicates of 0.27 and 0.30 % respectively (Table 1). The oils of both organs were analyzed by GC/MS using the previously mentioned conditions, which revealed the presence of twenty compounds in the leaves representing 86.96% of the total leaves oil, from which the major compounds were eudesmol (36.66%), muurolene (10.26%), and gurjunene (9.98%).

The oil of the fruits were found to contain fourteen compounds representing 77.77% of the total fruits oil, terpineol (55.47%), *t*-carveol (5.51%), limonene (1.67%), muurolene (5.51%) and cadinene (2%) represented the major compounds. Terpineol is a naturally occurring monoterpene alcohol that has been isolated from a variety of sources such as cajuput oil, pine oil, and petitgrain oil (Merck Index). There are three isomers, *alpha*-, *beta*-, and *gamma*-terpineol, the last two differing only by the location of the double bond. Terpineol is usually a mixture of these isomers with *alpha*-terpineol as the major constituent. It has a pleasant odor similar to lilac and is a common ingredient in perfumes, cosmetics, and flavors. α-Terpineol is one of the two most abundant aroma constituents of lapsang souchong tea; the α-terpineol originates in the pine smoke used to dry the tea (Yao *et* al., 2005).

Previous reports by ShunZhen *et al*., (2012) of the essential oil composition isolated from the leaves and fruit peels of *F. margarita* revealed the presence of 27 compounds in the leaves with 91.37%, while 34 compounds were identified from fruit peels sample with 96.23%. The main components of both organs were different, except only 13 components *i.e.,* α-pinene, linalool, β-caryophyllene etc., were the same but with different content.

On the other hand the volatile compositions of three varieties of kumquats; *F. crassifolia*, *F. margarita* and *F. japonica* were determined by using headspace solid phase micro-extraction (HS-SPME) coupled with GC-MS. Twenty-eight components in the three tested kumquat varieties were identified, with D-limonene is the major component with 50.40%, 55.43% and 51.47%, respectively.

Furthermore, α-pinene, D-limonene, *cis*-α-cadinene, δ-cadinene, *iso*-caryophyllene, β-elemene and acetic acid esters were common in all samples. In addition, each kumquat variety contains its peculiar components, *i.e., F. crassifolia* Swingle contains 4-carene, *F. margarita* Swingle contains D-germacrene, cedrene and linalool and *F. japonica* (Thunb.) Swingle contains limonene, ocimene, 2,6,10,14-tetramethyl-heptadecanoic, cuba ene, 2,6-di-tert-butyl-4-butyl phenol and isopropyl palmitate (Zhonghai *et al*., 2009).

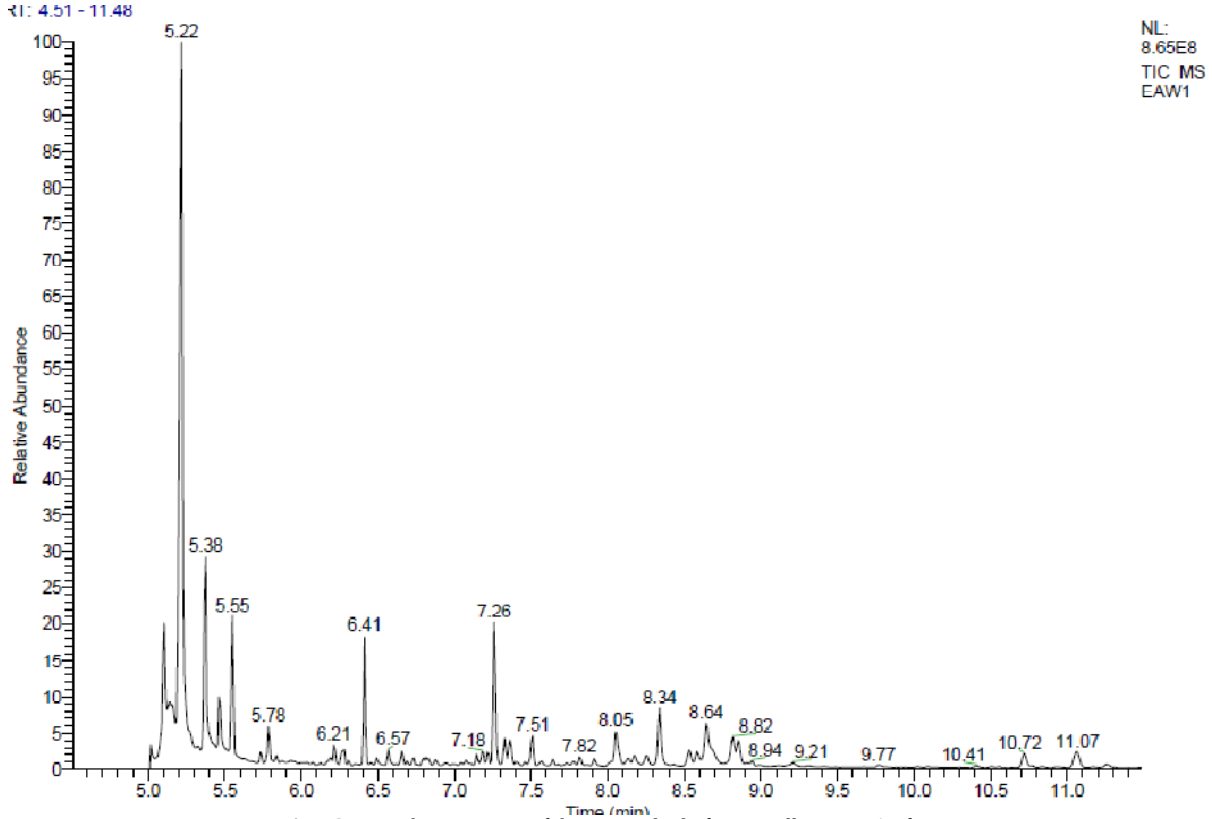

Fig.1 Gas ion chromatogram of the essential oil of *Fortunella margarita* fruits.

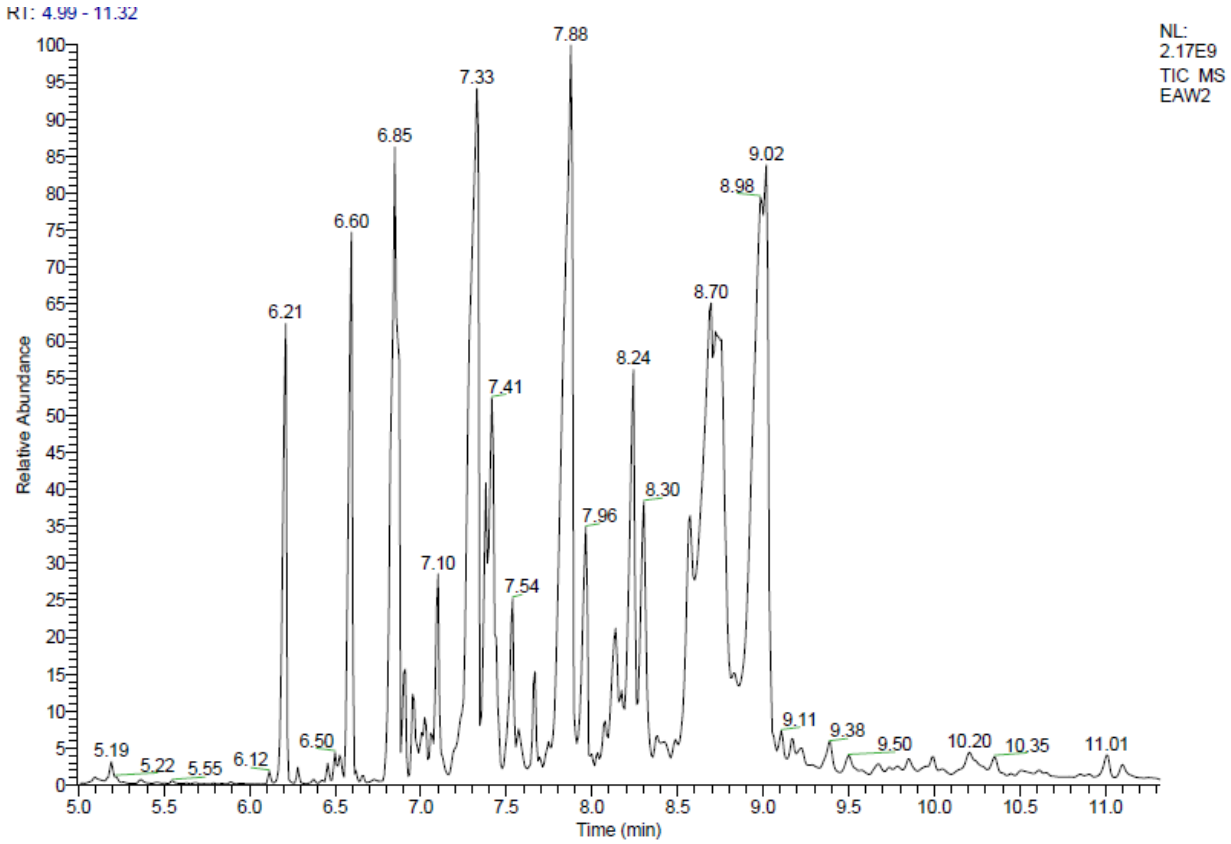

Fig. 2 Gas ion chromatogram of the essential oil of *Fortunella margarita* leaves

Table 1: GC/MS of the essential oil of *F. margarita* fruits and leaves

Peak no.	R_t	Components	RR_t (min.) of fruits	Conc.% of fruits	RR_t (min.) of leaves	Conc.% of leaves
Monoterpenes						
1	5.11	Linalool	0.98	0.84	-	-
2	5.17	Unknown	0.99	5.51	-	-
3	5.22	*α*-Terpineol	1	55.47	-	-
4	5.38	Carveol	1.03	5.51	-	-
5	5.55	Carvone	1.06	5.68	-	-
6	5.78	Limonene	1.1	1.67	-	-
7	6.21	Dihydrocarveol	-	-	0.69	2.4
8	6.41	Citronellal	1.23	5.01	-	-
Sesquiterpenes and others						
9	6.5	*α*-Cubebene	-	-	0.72	0.07
10	6.57	*δ*-Cadinene	1.25	0.67	-	-
11	6.6	*β*-Bourbonene	-	-	o.73	2.89
12	6.66	Bisabolene *trans*-gamma	1.28	0.67	-	-
13	6.85	*δ*-Elemene	-	-	0.76	5.32
14	6.91	*β*-Cubebene	-	-	0.77	0.6
15	6.95	*α*-Gurjunene	-	-	0.77	0.37
16	7.1	Bisabolene	-	-	0.79	0.89
17	7.26	*γ*-Muurolene	1.39	5.51	-	-
18	7.33	γ-Muurolene	-	-	0.82	6.53
19	7.33	Germacren B	1.4	1.75	-	-
20	7.38	*α*-Guaiene	-	-	0.82	1.26
21	7.41	*α*-Humulene	-	-	0.83	2.41
22	7.51	*α*-Cadinene	1.44	1.34	-	-
23	7.54	γ-Cadinene	-	-	0.84	1.55
24	7.67	γ-Eudesmol	-	-	0.85	0.6
25	7.88	Unknown	-	-	0.88	7.71
26	7.96	Unknown	-	-	0.89	1.88
27	8.05	Diethylphthalate	1.54	2.26	-	-
28	8.14	*δ*-Cadinene	-	-	0.9	1.29
29	8.24	Unknown	-	-	0.91	3.01
30	8.3	Cadinol	-	-	0.92	2.04
31	8.34	Unknown	1.6	2.34	-	-
32	8.38	*β*-Cedrene	-	-	0.94	1.89
33	8.64	Unknown	1.66	2.51	-	-
34	8.7	*β*-Gurjunene	-	-	0.97	9.98
35	8.72	*α*-Muurolene	-	-	0.97	10.26
36	8.82	Cedrol	1.69	1.5	-	-
37	8.89	*β*-Eudesmol	-	-	1	28.25
38	9.02	*γ*-Eudesmol	-	-	1.004	8.41
39	9.11	Germacren D-4-ol	-	-	1.02	0.42
40	10.72	Unknown	2.05	0.75	-	-
41	11.7	Galaxolide	1.24	1	-	-

RR_t: Relative Retention Time to *α*-Terpineol in Fruits and to *β*-Eudesmol in Leaves

Table 2: Antiviral activity of *F. margarita* fruits and leaves.

Oils	TC_{50} (*μ*g/mL)	H5N1			
		Initial Viral Count PFU/ml	Conc. Of Sample(*μ*g)	% of reduction	IC_{50} (*μ*g/mL)
F. margarita fruits	239.54	8.2×10^5	5	60.97	6.77
			10	70.73	
			20	78.04	
F. margarita leaves	185.47		5	39.02	38.89
			10	41.46	
			20	43.9	

TC_{50}: It is the Half Maximal (50%) Toxic Concentration of the Sample on Cell Line under Examination.
PFU: Plaque Forming unit: Refers to One Infectious Virion that Is Capable of Initiation of Infection in One Cell Rises into a Plaque after Incubation.
IC_{50}: It Is the Half Maximal (50%) Inhibitory Concentration (IC) of the Sample Reducing

Table 3: MICs of the essential oils from *F. margarita* fruits and leaves.

Test organism	MIC (% v/v) Oil of fruits	MIC (%v/v) Oil of leaves
1- *Bacillus subtilis*	2	0.01
2- Escherichia coli	1	ND
3- Aspergillus niger	0.01	0.01
4- Candida albicans	0.01	0.01

ND: Not Determined

Table 4: Antimicrobial activities of the essential oils from *F. margarita* fruits and leaves.

Inhibition zone diameter (mm) / Sample	Fruits oil[(a)]	Leaves oil[(a)]	Erythromycin 150 µg/mL	Methicillin 50 µg/mL	Oxacillin 10 µg/mL	Bacitracin 100 µg/mL	Nystatin 100 µg/mL
Gram +ve bacteria							
Bacillus subtilis	21	31	26	14	-ve	18	11
Staphylococcus aureus	22	28	16	-ve	-ve	12	12
Sarcina luta	20	32	25	24	24	26	-ve
Streptococcus faecalis	25	-ve	20	-ve	-ve	19	-ve
Anthrobacter	18	19	28	19	24	12	12
Gram -ve bacteria							
Escherichia coli	16	16	16	11	12	12	-ve
Klebsilla pneumonia	25	-ve	-ve	12	14	-ve	-ve
Pseudomonas aeroginosa	25	19	35	17	15	12	-ve
Yeast and Fungi							
Aspergillus niger	29	25	-ve	-ve	-ve	-ve	15
Candida albican	29	17	-ve	-ve	-ve	-ve	15

a: 0.1 mL of Diluted Essential Oil (1:50 v/v)

Table 5: Effect of essential oils from *F. margarita* fruits and leaves on spore germination of *A. niger* and *F. oxysporum*.

Essential oil	Conc. of oil (%v/v)	*Aspergillus niger*	*Fusarium oxysporum*
Oil of fruits	4	-ve	-ve
	2	-ve	-ve
	1	-ve	+ve
Oil of leaves	4	+ve	+ve
	2	+ve	+ve
	1	+ve	+ve

+ve Effect: Refers to Germination of the Fungal Strain after 24 and 48 Hours

Antiviral activity

Avian influenza (AI) is a highly contagious disease of poultry caused by influenza viruses type-A of the family Orthomyxoviridae. The rapid rate of spread and the high potential for genetic alterations of the virus has raised the spectra of wide spread human infection and the possibility of a pandemic. With emergence and multi-regional spread of AI especially highly pathogenic avian influenza (HPAI) of H5 and H7 subtypes in poultry, the epizootic has altered the world to the prospects of a potentially devastating human health challenge. The HPAI (H5N1) subtype virus has caused disease outbreaks in poultry in several countries in Asia including India. The role of antivirals is considered critical in preparedness for avian flu originated pandemic. Several at-risk nations and WHO have stored strategic stockpiles of antivirals especially oseltamivir to be used at the face of influenza pandemic. However, resistance to oseltamivir in the H5N1 subtype in Vietnam and other human influenza A viruses has become a cause for worry as far as pandemic preparedness is concerned. Therefore, searching for alternatives for antivirals that can effectively inhibit H5N1 or other influenza A viruses, and/or act in synergy with available antivirals, is an urgent need of the hour. Several novel antiviral agents that may be effective against influenza virus especially H5N1 avian flu virus, are currently under development. Among these, are plant-derived extracts, which have become the focus of many studies due to their proven beneficial health effects in several disease problems (Sood *et al*., 2012).

In the present study the essential oils of both organs (leaves and fruits) of *F. margarita* were tested for the first time for their antiviral activity against avian influenza (H5N1**)** virus, the obtained results (Table 2) revealed that the fruits essential oil was more effective (80% virus inhibition) that of the leaves, this was attributed to the presence of *α*-terpineol as a major component in fruits oil. Our obtained results comes in accordance with the previous study by (YaDong *et al*., 2009) which investigated the antiviral effect of *Curcuma zedoaria* volatile oil and *Hypericin perforatum* liquid extract on H5N1 avian influenza virus (AIV) in MDCK cell line and non-AIV-immunized chickens, and attributed the virucidal activity to the presence of curcumenol and hypericin.

Antimicrobial and Antifungal activity

The essential oils of both organs (fruits and leaves) showed antimicrobial properties (Table 3) with regard to their inhibition zone (IZ) ranging from 16 to 31 mm. Fruits oil was active against Gram +ve, Gram -ve, yeast and fungi. The diameter of inhibition zone was ≥25mm at 100 µL oil concentration against *Streptococcus faecalis*, *Klebsilla pneumonia*, *Pseudomonans aeroginosa*, *Asperigillus niger*, and *Candida albicans.*

Asperigillus niger, and *Candida albicans* were the most susceptible microorganisms inhibited by fruits oil, followed by the Gram +ve bacteria *Streptococcus faecalis*, Gram -ve bacteria *Klebsilla pneumonia*, *Pseudomonans aeroginosa*. However, the fruits oil being less active (IZ < 25) against *Bacillus Subtilis*, *Staphylococcus aureus, Sarcina luta*, *Anthrobacter* and *Escherishia coli*. Moreover, the leaves oil showed significant activity against all tested microorganisms except *Streptococcus faecalis* and *Klebsilla pneumonia*, with inhibition zone ≥ 25 observed for *Bacillus subtilis*, *Sarcina luta, Staphylococcus aureus*. This can be attributed to the presence of eudesmol as a major component of leaves oil which was reported to have a strong

antimicrobial and free radical scavenging activities (Amezouar *et al*., 2012). To the best of our knowledge the IZ for both oils were higher than that of the reference drug.

The minimum inhibitory concentrations of both oils of *Fortunella margarita* obtained by the agar diffusion method are shown in (Table 4). Both oils inhibited *Asperigillus niger* and *Candida albicans* at 0.01% v/v. Fruits oil inhibited *Bacillus Subtilis and Escherishia coli* at 2 and 1% respectively, while leaves oil inhibited Gram +ve bacteria *Bacillus Subtilis* at 0.01 %v/v.

The results in (Table 5) revealed that the fruits oil completely inhibited the germination of *Aspergillus niger* and *Fusarium oxysporum* spores at all concentrations tested except for *Fusarium oxysorum* at 1% (v/v). On the other hand, leaves oil has no inhibitory effect on seed germination of the tested fungal strains. A separate two control run simultaneously in the present study showed that it did not inhibit spore germination.

The mode of action of antimicrobial action of essential oil may be due to inhibition of respiration and disrupting the permeability barriers of the cell membrane structures (Cox *et al*., 2000).

CONCLUSION

The biological activity of these essential oils is due to the presence of synergistic effect of mixture of volatile compounds or their major constituents. The strong antiviral activity against pathogenic avian influenza (H5N1), and the broad spectrum of antimicrobial and antifungal activities of the essential oils against variety of bacterial and fungal strains so we can recommend that the essential oil of *F. margarita* can be incorporated in different pharmaceutical preparations for the first time.

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How to cite this article:

Nabaweya A. Ibrahim, Seham S. El-Hawary, Magdy M. D. Mohammed, Mohamed A. Farid, Nayera A. M. Abdel-Wahed, Mohamed A. Ali, Eman A. W. El-Abd. Chemical Composition, Antiviral against avian Influenza (H5N1) Virus and Antimicrobial activities of the Essential Oils of the Leaves and Fruits of *Fortunella margarita*, Lour. Swingle, Growing in Egypt. J App Pharm Sci, 2015; 5 (01): 006-012.