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A Comparative study of Chemical Compositions, Antimicrobial and Antiviral Activities of Essential Oils for *Citrus* medica **var.** *sarcodactylis* **Swingle Fruits and Leaves along with** *Limonia acidissima* **L. Leaves.**

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Abstract

The chemical composition of fruits and leaves derived oil of *Citrus medica* var. *sarcodactylis* Swingle (CMF&CML) and leaves of *Limonia acidissima* L. (LAL), Rutaceae were analyzed by gas chromatography-mass spectroscopy (GC-MS). Twenty-one components accounting for **(**98.51%**)** were identified in CMF oil. While in leaves oil, fourteen components were identified accounting for **(**92.69%**)** of the total oil content and the major component is limonene for both CMF and CML oil representing 60.31% and 32.11% respectively. Oil derived of LAL prevailed eight components were representing **(**97.90%**)** of its total oil and the major identified component is carvacrol (71.15%) Oils derived of fruits, leaves of *C. medica* and leaves of *L. acidissima* were investigated for their antimicrobial and antifungal activities. Fruits derived oil exhibited potent antimicrobial activity than the leaves of both plants. Antifungal activity was moderate for fruits essential oil against *Aspergillus niger*. Oil derived of *C. medica* var. *Sarcodactylis* and *L. acidissima* leaves at concentration 0.5 µg/µl showed comparable antiviral activity against avian influenza A H5N1 virus. It was the first comparative study for essential oils of *C. medica* var. Sarcodactylis fruits, leaves and *L. acidissima* L. leaves and their antiviral activity against H5N1 virus.

Key words: *Citrus medica* var. *sarcodactylis*; *Limonia acidissima;* antimicrobial; antiviral; H5N1; GC/MS

1.Introduction

Citrus species, are widely famous of their edible fruits such as lemons, oranges, mandarins, tangerines, limes, and kumquats, and their essential oils are utilized in the perfumery business. *Citrus* fruits are a valuable source of phytochemicals that are beneficial to the human body, such as vitamin C, vitamin B, potassium, phosphorous, and other elements. *Citrus* fruits also contain a number of active compounds that are used to treat heart disease and hypertension, as well as anticancer, inflammation, antiviral, antibacterial, and antifungal activity **[1].** *Citrus medica* var. *sarcodactylis* Swingle and *Limonia acidissima* L., both belonging to the Rutaceae family, are the plants under inquiry. *Citrus* is the most economically and medicinally significant member of the Rutaceae family, with a wide range of biological and pharmacological properties, including antibacterial activity **[2]**. Furthermore, several bioactive coumarins, flavonoids, tetranortriterpenoids, monoterpenoids, and acridone alkaloids have been discovered in *Citrus* species **[3].** *Citrus medica* var. *sarcodactylis* Swingle is known as fingered citron (Buddha's hand)in China and used in folk medicine as tonic, antispasmodic, antiemetic,

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expectorant and inhaler. Coumarins, steroids and triterpenoids were reported as well **[4].**

Limonia acidissima L., often known as elephant apple or wood apple, is a tiny tree that has long been utilized in herbal medicines as digestive, stimulant, astringent, carminative and as antidiarrheal **[5].** Phytochemicals as coumarins, limonoids and alkaloids were previously isolated **[6].** In Egypt, the highly pathogenic avian influenza subtype H5N1 virus was first detected in poultry in 2006 and confirmed enzootic in 2008. The majority of confirmed human A(H5N1) cases in Egypt before 2009 occurred in female patients aged 18 years old, according to epidemiological data. The rate of case fatality increased with age and delayed hospitalisation, and it was higher among females. The majority of cases recorded since 2009 have impacted backyard poultry breeders. Egypt's case-fatality rate (36%) is roughly half of the sum of case-fatality rates in all other countries affected **[7]**. During the 2014–15 winter season, Egypt recorded an extraordinary surge in the incidence of human infections with the highly dangerous avian influenza A(H5N1) virus. As other perspective of the management plan was overlooked, vaccination eventually became the only weapon utilized to control the H5N1 virus in Egypt. Given that outbreaks in poultry continued, this technique failed to prevent the spread of the H5N1 virus **[8].** Egypt accounted for two-thirds of all human cases reported worldwide by 2019**[9].**

As a result, it's critical to look for antiviral alternatives that may effectively suppress H5N1 or other influenza A viruses, as well as function in combination with currently available antivirals. Several novel antiviral medicines are now being developed that may be effective against influenza viruses, particularly the H5N1 avian flu virus. Plant-derived extracts are one of these, and they've been the subject of a lot of research because of their proven health benefits in a variety of diseases [10-11].

This research study targets the investigation of chemical composition of the oils of both leaves and fruits of *C.medica* (CMF&CML) and leaves of *L. acidissima* (LAL), then the antiviral activity against pathogenic avian influenza A virus (H5N1), as well as antibacterial and antifungal activities in order to use it safely as a supplement and to be incorporated in other pharmaceutical preparations to prevent huge economic loss in the poultry industry and decrease fatality rate in human due to pathogenic avian influenza virus.

1. 2.Materials and Methods

2.1. Plant materials

The fresh leaves and fruits of *Citrus medica* var. *sarcodactylis* Swingle were collected from Mareii farm Gamgara, Banha, Qalyuobia (Error! Hyperlink reference not valid.)in March 2016. While the fresh leaves of *Limonia acidissima* L. were collected from Zoo Garden Giza (https://goo.gl/maps/m87JstQpN1efnteM9)in March 2016.The voucher specimens were kept at faculty of pharmacy, Cairo university under no.29-12-2016 I, II. Mrs. Theresa Labib, former head of El-Orman botanical garden and taxonomy consultant at the Ministry of Agriculture, recognized the specimens.

All chemicals were purchased from Merck of high analytical grades.

2.2. Essential oils extraction

Essential oil samples (CMF, CML& LAL) were prepared by hydro distillation method using Clevenger-type apparatus for three hrs. as mentioned in Egyptian pharmacopeia **[12].** and each sample was determined by mean of triplicates. The collected oils which were dehydrated separately with anhydrous sodium sulphate and kept in freezer until subjected to GC/MS analysis.

2.3. Essential oils analysis

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m,1.25mm, 0.25µm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 5ml/min. The injector and MS transfer line temperature were set at 280ºC. For essential oil analysis the oven temperature was programmed at an initial temperature 40ºC (hold 3 min.) to 280ºC as a final temperature at an increasing rate of 5ºC/min. (hold 5 min.). Identification of the essential oil constituents was achieved by library search on Wiley275LGC/MS database, observed kovates index and by comparing retention time and mass fragmentation patterns to those of the available references as well as the published data **[13].**

2.4.1. Microbial test strains

Gram-positive bacterial strains (*Bacillus subtilis* NRRL 543, *Bacillus cereus* NRRL 469, *Staphylococcus aureus* ATCC 6538), Gram-negative bacterial strains (*Klebsiella pneumonia* ATCC10031), fungus (*Aspergillus niger* NRRL 599) and yeast (*Candida albicans* NRRL Y-477) were employed in this investigation. The American Type Culture Collection (ATCC) and the Northern Utilization Research and Development Division, United States Department of Agriculture, Peoria, Illinois, USA provided these microorganisms (NRRL). Before the bioassay, the bacteria were revived by subculturing in fresh nutrient broth medium for 24 hrs at 37°C. Fungi were cultivated on

2.5 % potato dextrose agar (PDA) for 7 days at 28°C before the experiment was carried out.

2.4.2. Agar well diffusion method

The antibacterial and antifungal activities of the essential oils were determined by the well diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) **[14].** 100 µl suspension of 24 hrs bacterial cultures containing 1 ×106 CFU/ml of pathological tested bacteria in sterile distilled water was added to 40 ml sterile nutrient agar media. For fungal and yeast strains, 100 µl suspension of seven days cultures containing 1 × 104CFU/ml was added to 40 ml sterile Sabourand dextrose agar (SDA), and potato dextrose agar (PDA) medium, respectively at 28°C. The mixture was transferred to sterile Petri dishes and allowed to solidify. Holes of 6 mm in diameter were made using a cork borer. Amounts of 50 µl of the diluted extract with dimethyl sulfoxide (DMSO) were poured inside the holes. A hole filled with DMSO only was also used as the negative control. The plates were left for 1 hr at 4°C as a period of pre-incubation diffusion. The inoculated plates were then incubated for 24 hrs at 37°C for bacteria and 48hrs at 28°C for yeast and fungi.

2.4.3. Estimation of the Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) of the most potent sample [*Citrus medica* var. *Sarcodactylis* fruits (CMF) oil] was determined. Two-fold serial dilutions of the tested compounds solutions were prepared using DMSO solution to obtain 50 mg/ml concentration. Subsequently, the concentration was diluted to obtain 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 mg/ml for the determination of the minimum inhibitory concentration (MIC) at the dose levels. Agar well diffusion method as described by Perez **[15]** was employed for the assay. The test organisms, the clinical isolates, were prepared with 0.5 McFarland standard and subcultured at 37ºC and maintained on nutrient agar media for bacteria and Sabouraud agar media for *Candida albicans*. Petri plates containing 20 ml of respective medium were seeded with selected microbial strains and incubated at 37ºC for 24 hrsfor bacteria and 48hrs at 28°C for yeast. Standard antimicrobial agents used as positive controls were chloramphenicol, erythromycin (Bio Basic, Canada INC.) and bifonazole (Bayer, Germany). After 24 hrs. the inhibition zone diameters (IZD) were recorded, and the mean calculated. The minimum inhibitory concentrations were then determined at various dilutions. The MIC is the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot.

2.5. Antiviral activity

2.5.1. Virus and cells

Avian influenza A virus (H5N1) previously isolated from Egypt in 2013 A/Chicken/M7217B/1/2013 (H5N1) and changed to be low pathogenic by reverse genetics. The Madin Darby Canine kidney cells (MDCK)were routinely passaged in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic mixture (penicillin-streptomycin-amphotericin B).

2.5.2. MTT assay (cytotoxicity assay) TC50

Samples were diluted with Dulbecco's Modified Eagle's Medium (DMEM). Stock solutions of the test compounds were prepared in 10 % DMSO in ddH_2O **(**distilled deionized water). The cytotoxic activity of the extracts was tested in Madin Darby Canine kidney (MDCK) cells by using the 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method **[16]** 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 incubated for 24 hrs at 37ºC in 5%CO_2. After 24 hrs, cells were treated with various concentrations of the tested samples in triplicates. After further 24 hrs, the supernatant was discarded, and cell monolayers were washed with sterile phosphate buffer saline (PBS) 3 times and MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37°C for 4 hrs followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCL in 50 ml isopropanol). Absorbance of formazan solutions were measured at λmax 540 nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation.

%cytotoxicity= (Absorbance of cell without treatment-Absorbance of cell with treatment) ×100/Absorbance of cell without treatment.

The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (LD50).

2.5.3. Antiviral assay (Plaque reduction assay)

Plaque reduction assay was carried out according to Hayden *et al*. **[17].** Briefly, in a six well plate,MDCK cells (10^5 cells / ml) were cultivated for 24 hrs at 37ºC. A/Chicken/M7217B/1/2013 (H5N1) virus was diluted to give 10^4PFU/well and mixed with the safe concentration of the tested extracts and incubated for 1 hour at 37ºC before being added to

the cells. Growth medium was removed from the cell culture plates and the cells were inoculated with (100 µl / well) virus with the tested extracts, after 1 hour contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose was added to the cell monolayer, plates were left to solidify and incubated at 37ºC till formation of viral plaques (3 to 4 days). Formalin (10%) was added for two hours then plates were stained with 0.1 **%**crystal violet in distilled water. Control wells were included where untreated virus was incubated with MDCK cells and finally plaques were counted and percentage reduction in plaques formation in comparison to control wells was recorded as following

% inhibition= viral count (untreated) - viral count (treated) × 100/ Viral count (untreated)

3. Result:

3.1. GC/MS analysis

The prepared oil of *C. medica* fruits, leaves, and *L. acidissima* leaves which were yellow and aromatic resulted in yield of 0.25, 0.5 and 3.33% v/w respectively as a mean of triplicate. GC/MS analysis of the resulted oils leads to identification of twenty-one components accounting for 98.51% in fruits oil of *C. medica*. While its leaves oil prevailed fourteen components only accounting for 92.69% of the total oil. Limonene was the major identified component for CMF and CML oil representing 60.31% and 32.11%, respectively. Moreover eight compounds were detected in the oil of LAL representing 97.90% of its total oil. Carvacrol was the major identified component 71.15% in LAL. Monoterpenes were detected as a major constituent in all investigated samples. On the other hand, sesquiterpenes component were minor. *Citrus medica* fruits oil contained high percentage of non-oxygenated compounds (97.90%) in contrast that of its leaves that included higher content of the oxygenated compounds (57.67%) than non-oxygenated (35.02%). Nevertheless, *Limonia acidissima* leaves oil proved to have the highest percentage of oxygenated compounds (97.77%) of them all. Table (1) figure (1)

3.2. The antimicrobial activity:

Table (2) showed that *Citrus medica* var. *Sarcodactylis* fruits (CMF) oil was active against all tested organisms and most potent against *Candida albicans* while(CML) oil had higher activity against gram-positive bacteria than gram-negative bacteria, fungi and had no activity on *Candida albicans.*In contrast *Limonia acidissima* L leaves (LAL) oil had higher activity on gram- negative than gram -positive bacteria and moderate activity against *Candida albicans*.Table (3) showed the minimal inhibitory concentration

(MIC) of the most active oil in comparison to bifonazole, erythromycin and chloramphenicol as standard drugs.

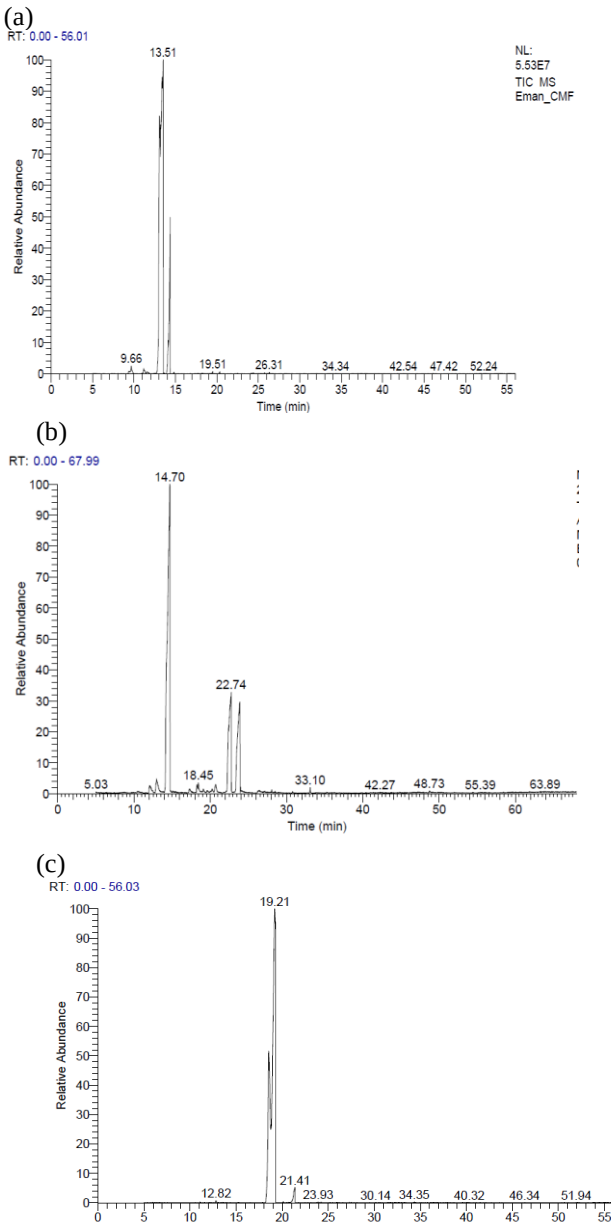

Figure (1): GC/MS chromatograms of essential oil of *Citrus medica* var. *sarcodactylis* Swingle fruits (a) and leaves (b) and *Limonia acidissima* L. leaves (c)

Table (1): Identified constituents in *Citrus medica* var. *sarcodactylis* Swingle fruits and leaves versus *Limonia acidissima* L. leaves essential oils

No.	RRt.	RI.	M.wt.	Compound	CMF Area%	CML Area%	LAL Area%
1	0.58	924	136	α-Thujene	0.09	-	0.05
2	0.69	930	136	Camphene	0.46	-	-
3	0.71	933	136	Sabinene	1.27	1.14	-
4	0.83	945	136	α-Pinene	1.08	0.1	-
5	0.85	950	136	β- Pinene	0.19	-	-
6	0.86	951	136	2- Carene	0.16	-	-
7	0.87	980	136	Myrecene	0.36	1.45	0.07
8	0.97	1013	136	α-Terpinene	14.06	-	-
9	0.98	1017	148	Estragol	-	-	10.58
10	0.99	1020	148	2-Methyl, 3- phenyl propanol	-	-	0.02
11	1	1021	136	Limonene	60.31	32.11	-
12	1	1303	150	Carvacrol	-	-	71.15
13	1.01	1037	136	Phellanderene	0.05	-	-
14	1.01	1044	148	5-Methoxyindane	-	-	15.98
15	1.02	1050	136	Ocimene	0.03	0.13	-
16	1.06	1088	136	α-Terpinolene	19.73	-	-
17	1.24	1203	152	Carveol	-	0.49	-
18	1.25	1407	178	Methyl eugenol	-	-	0.04
19	1.25	1168	154	Terpineol	-	0.57	-
20	1.3	1170	154	Citronellal	-	0.27	-
21	1.31	1181	154	l-4-Terpineol	0.03	-	-
22	1.44	1200	152	z-Citral	0.18	-	-
23	1.51	1205	152	Citral	0.34	28	-
24	1.62	1206	152	Nerol	-	27.8	-
25	1.63	1212	152	Neral	-	0.11	-
26	1.71	1340	154	Geraniol	0.01	0.12	-
27	1.79	1422	204	Caryophyllene	0.02	0.09	0.01
28	1.81	1516	204	Bergamotene	0.05	-	-
29	1.91	1565	192	β-Ionone	0.03	-	-
30	1.95	1581	204	α-Himachalene	0.06	-	-
31	2.25	1585	220	Caryophyllene oxide	-	0.31	-
% Total Identified compounds					98.51	92.69	97.9
% Unidentified compounds					1.49	7.31	2.1
%Monoterpenes					98.35	92.29	97.89
%Sesquiterpenes					0.13	0.4	0.01
% Non-oxygenated compounds					97.9	35.02	0.13
%Oxygenated compounds					0.59	57.67	97.77

RRt.: Relative retention time to limonene for citrus medica fruits and leaves and to carvacrol for *Limonia acidissima* leaves, RI.: retention index, M.wt: molecular weight, CMF: *Citrus medica* fruits, CML*: Citrus medica* leaves, LAL: *Limonia acidissima* leaves

Table (3): Minimal Inhibitory Concentration (MIC) of *Citrus medica* var. Sarcodactylis Swingle fruits oil.

Microorganism	CMF oil µg/ml	Bifonazole	Erythromycin	Chloramphenicol
C. albicans	3	10	N.d	N.d.
B. subtilis	25	125	2	15.6
K. pneumonia	25	78	2	7.8

N.d.: not detected

3.3. The antiviral activity

In table (4) we demonstrated the cytotoxicity of essential oils of *C. medica* var. *Sarcodactylis*f fruits, leaves and *L. acidissima* leaves which showed that *C. medica* var. *Sarcodactylis* fruits oil has high toxicity on the tested cells. Consequently, the antiviral activity is shown in table (5) which revealed potency of *C. medica* var. *Sarcodactylis* leaves oil (95% inhibition) then *L. acidissima* leaves oil (92% inhibition) and finally *C. medica* var. *Sarcodactylis* fruits oil (50% inhibition) at concentration 0.5 µg/µl.

Table (2): Antimicrobial activities of fruits and leaves derived oil of *C.medica* var. sarcodactylis Swingle versus leaves of *L. acidissima* L.

Sample	Gram-positive bacteria			Gram-negative bacteria	Yeast	Fungi
	Bacillus subtilis NRRL 543	*Bacillus cereus* NRRL469	*Staph. aureus* ATCC 6538	*Klebsiella pneumonia*ATCC10031	*Candida alibicans*NRRL Y-477	*Aspergillus niger* NRRL 599
	Inhibition zone (mm)					
CMF oil	17	15	17	15	27	11
CML oil	13	21	12	10	-	13
LAL oil	12	-	-	18	15	-
Tetracycline 30ug	18	18	28	18	18	ND
Erythromycin 10 µg	25	25	25	20	ND	ND
Chloramphincol 30 µg	22	22	22	18	ND	ND
Bifonazole 125 µg	11	11	13	18	18	18

All samples were tested at 10mg/ml of fruits and leaves derived oil of *Citrus medica* var. sarcodactylis Swingle (CMF&CML) and leaves of *Limonia acidissima* L. (LAL)

Table (4): *In-vitro* cytotoxic assay for Essential oil of *C. medica* var. *Sarcodactylis* fruits, leaves and *L. acidissima* leaves.

Sample Cytotoxicity	CMF oil Conc. µg/µl	% Inhibition	CML oil Conc. µg/µl	% Inhibition	LAL oil Conc. µg/µl	% Inhibition
	4.037	89.92	0.2	98.25	0.2	97.96
	2.018	88.79	0.1	27.36	0.1	96.94
	1.0093	88.95	0.05	0	0.05	1.45
	0.504	87.81	0.025	0	0.025	0
			0.012	0	0.012	0
			0.006	0	0.006	0
TC50 µg/µl	High toxicity		0.122		0.094	

Table (5): *In-vitro* Antiviral assay for Essential oil of *C. medica* var. *Sarcodactylis* fruits, leaves and *L. acidissima* leaves.

Sample	CMF oil		CML oil		LAL oil	
Antiviral activity	Conc. µg/µl	% Inhibition	Conc. µg/µl	% Inhibition	Conc. µg/µl	% Inhibition
	0.504	50	0.5	95	0.5	92
	0.252	20.8	0.25	90	0.25	88

4.Discussion:

The chemical content of CMF oil was previously investigated separately, and limonene (45.36%), terpinene (21.23 %), and dodecanoic acid (7.52 %) were found to be the most prominent compounds. Harvest time, geographic origin, and regional agro-climatic variables could all have an impact on concentrations. CMF's antimicrobial action was also tested against food-borne bacteria, which supports our existing findings that CMF oil has greater effect on gram-positive than gram-negative bacteria **[18-19]**. While CML oil was found to contain limonene (49.19%), geranial (25.93%) and neral (16.41%) and showed strong inhibitory activity against gram-positive and weak activity against gram-negative which was in direct proportion with oil concentration **(2)**. In addition, methyl chavicol (27.2 %), t-anethol (10.94 %), thymol (24.4 %), and *p*-cymen-7-ol (7.3 %) were shown to be substantial components of LAL oil **[20]**. Other studies revealed that the principal chemical compounds detected were Eudesma-4 (14).11-dine (46.3%), carvacrol (29.6%) and 1,5-cyclodecandine (13.4%).The antibacterial activity of the essential oil was tested against a variety of clinically isolated Gram positive and Gram-negative bacterial strains, and it was stated that the essential oil had modest antibacterial activity against all the tested strains [**21].**The activity of the CMF and CML oils was mainly due to the presence of limonene which represents the major component in the oils and exhibited antibacterial activity against *Staphylococcus aureus* and that is due to its mode of action in damaging bacterial cell membranes **[22-23].** The antibacterial mode of action of limonene in the essential oil might be improved when compared to when it was used alone. It suggested that the oil might have a synergistic impact, amplifying its biological activity. The phenolic monoterpene carvacrol is the major component of the essential oils of *Limonia acidissima* L leaves possessed antibacterial effect, which is greater against Gram-positive bacteria than Gram-negative bacteria, is based on bacterial membrane lysis **[24].**

The antiviral activity of *Citrus medica* var. Sarcodactylis fruits and leaves essential oil probably was attributed to the high content of limonene (60.31%, 32.11%) respectively which comes in accordance with previous study of antiviral activity against H5N1 of *Citrus reshni* hort. ex ripe and unripe fruits peel essential oil with limonene as a major constituent (91.68% and 82.41%) respectively **[25].** Additionally, limonene was discovered to be the predominant component in essential oils extracted from fruit peels and leaves of four cultivars of *Citrus deliciosa* var. *tangarina* that showed anti-H5N1 action. **[26]**. While the activity of *Limonia acidissima* leaves essential oil is due to presence of carvacrol at high concentration (71.15%) which studied before in the essential oil of Mexican oregano against different human and animal viruses and showed high antiviral activity against human Rota virus (RV) and bovine herpesvirus type 2 (BoHV-2) **[27].**

Conclusion

The essential oil (EO) isolated from *Citrus medica* var. *sarcodactylis* Swingle fruits and leaves and *Limonia acidissima* L. leaves (CMF, CML &LAL) exhibited significant antimicrobial and potent antiviral activity against avian influenza A/H5N1 virus. In *Citrus medica* var. *sarcodactylis* fruits and leaves oils Limonene was identified as the predominant compound. While, *Limonia acidissima* L. leaves oil carvacrol was the major compound detected. For the first time, we can recommend that the essential oils of both plants be used in various therapeutic compositions.

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References

[1] Abobatta W.F. Nutritional Benefits of Citrus Fruits. (2019). Am J Biomed Sci & Res. 3(4): 303-306.

[2] Theanphong O., Songask T. and Mingvanish W. (2008). Chemical composition and antimicrobial activity of the essential oil from *Citrus medica* L. var. Sarcodactylis (Sieber) Swingle leaves. *Mahidol university journal of pharmaceutical sciences*, 35: 57-61.

[3] Shende S., Ingle A.P., Gade A., Rai M. (2015). Green synthesis of copper nanoparticles by Citrus medica Linn. (Idilimbu) juice and its antimicrobial activity. World J. Microbiol. Biotechnol. (31): 865-873.

[4] Chan, Y. Y., Hwang, T. L., Kuo, P. C., Hung, H. Y., & Wu, T. S. (2017). Constituents of the Fruits of Citrus medica L. var. sarcodactylis and the Effect of 6,7-Dimethoxy-coumarin on Superoxide Anion Formation and Elastase Release. Molecules (Basel, Switzerland), 22(9), 1454.

[5] Intkehab J., Aslam M. (2009). Isolation of a flavonoid from Feronia *Limonia*. *Journal of Saudi Chemical Society*, 13: 295-298.

[6] Musa H. Abu Zarga. (1986) Three new simple indole alkaloids from *Limonia Acidissima*. *Journal of natural product*,49: 901-904.

[7] Gomaa MR, Kayed AS, Elabd MA, Zeid DA, Zaki SA, El Rifay AS. (2014) Avian influenza A(H5N1) and A(H9N2) seroprevalence and risk factors for infection among Egyptians: a prospective, controlled seroepidemiological study. *J Infect Dis*, 211: 1399–407.

[8] Kayali, G., Kandeil, A., El-Shesheny, R., Kayed, A. S., Maatouq, A. M., Cai, Z., McKenzie, P. P., Webby, R. J., El Refaey, S., Kandeel, A., & Ali, M. A. (2016). Avian Influenza A(H5N1) Virus in Egypt. Emerging infectious diseases, 22(3), 379–388.

[9] World Health Organization [WHO] (2019). *Cumulative Number of Confirmed Human Cases of Avian Influenza A(H5N1)*.

[10] Sood R., Swarup D., Bhatia S., Kulkarni D.D., Day S., Saini M., Dubey S.C. (2012). Antiviral activity of crude extracts of *Eugenia jambolana* Lam. against highly pathogenic avian influenza (H5N1) virus. *Indian J. Exp. Biol.,* 50: 179-186.

[11] Sornpet B, Potha T, Tragoolpua Y, Pringproa K. (2017) Antiviral activity of five Asian medicinal pant crude extracts against highly pathogenic H5N1 avian influenza virus. Asian Pac J Trop Med. ;10(9):871-876.

[12] Egyptian Pharmacopoeia General Organization for Governmental Printing Office, Ministry of Health, Cairo, Egypt. (1984) 31-33.

[13] Adams R. P. (2001). Identification of Essential Oil by Gas Chromatography Quadrupole Mass Spectrometry, Allured Publishing Corporation, Carol Stream, Illinois, USA. P. 455.

[14] National Committee for Clinical Laboratory Standards NCCLS (1993). Performance standards for antimicrobial susceptibility tests. Approved standards NCCLS Publication M2-A5, Villanova, PA, USA.

[15] Perez C, Paul M, Bazerque P. (1990). An antibiotic assay by the agar-well diffusion method. *Acta Biol Med Exp* 15: 113-115.

[16] Mossman T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, 65: 55-63.

[17] Hayden, F.G., Cote, K.M., Douglas, R.G. (1980). Plaque inhibition assay for drug susceptibility testing of influenza viruses.*Antimicrob.Agents Chemother*,17, 865–870.

[18] Li Z.H., Cai M., Liu Y.S., Sun P.L., and Luo S.L. (2019). Antibacterial Activity and Mechanisms of Essential Oil from Citrus medica L. var. sarcodactylis. *Molecules*, 24,1577,1:10.

[19] AL-Kalifawi E.J. (2015). The Antimicrobial Activity of Essential Oils of Al-Abbas′s (AS) Hand Fruit Peel *(*Citrus Medica) var. Sarcodactylis Swingle. *Journal of Natural Sciences Research*, 5: 19-28.

[20] Ahmad A., Misra L.N., and Thakur R.S. (1989). Composition of the Volatile Oil from Feronia limonia Leaves. *Planta medica*, 55: 199-200.

[21] Kumar A.S., Venkatesalu V, Kannathasan K., Chandrasekaran M. (2010). Chemical constituents and antibacterial activity of the leaf essential oil of *Feronia limonia*. *Indian J Microbiol*, 50: S70–S73.

[22] Han Y., Chen W., Sun Z. (2021). Antimicrobial activity and mechanism of limonene against *Staphylococcus aureus. J Food Saf., 41: e12918, 1-14.*

[23] Van Vuuren, S.F.; Viljoen, A.M. (2007). Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and incombination. *Flavour and Fragrance Journal*. 22: 540–544.

[24] Nostro A., Papalia T. (2012).Antimicrobial activity of carvacrol: current progress and future prospective.*Recent Patents on Anti-Infective Drug Discovery*. 8: 28-35.

[25] Nagy M.M., Al-Mahdy D.A., Abd El Aziz O.M., Kandil A.M., Tantawy M.A. & El Alfy T.S.M. (2018). Chemical Composition and Antiviral Activity ofEssential Oils from Citrus reshni hort. ex Tanaka (Cleopatra mandarin) Cultivated in Egypt. *Journal of Essential Oil-Bearing Plants*. 21: 264-272.

[26] El-hawary, S.S., Taha, K.F., Abdel-Monem, A.R., Kirillos, F.N. and Mohamed, A.A. (2013). Chemical composition and biological activities of peels and leaves essential oils of fourcultivars of *Citrus deliciosa* var. tangarina. American *Journal of Essential Oils and NaturalProducts.* 1: 1-6.

[27] Pilau M.R., Alves S.H., Weiblen R., Arenhart S., CuetoA.P., Lovato L.T. (2011). Antiviral activity of the Lippia graveolens (Mexican Organo) essential oil and its main compound carvacrol against human and animal viruses. *Brazilian Journal of Microbiology*. 42: 1616-1624