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Antimicrobial, Antiviral Activity and GC-MS Analysis of Essential Oil Extracted from *Achillea fragrantissima* Plant Growing In Sinai Peninsula, Egypt

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Abstract

Medicinal plants are considered new resources for producing agents that could act as alternatives to antibiotics in treatment of antibiotic-resistant bacteria. The present study aimed to evaluate the efficacy of essential oil extracted from *Achillea fragrantissima* plant growing in Egypt for antimicrobial, antiviral activities and chemical composition analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). We also performed determination of essential oil antimicrobial activity by agar desk diffusion method and Minimal Inhibitory Concentration (MIC). Also, antiviral activity on ORF virus by pock reduction test was performed. It was reduced virus titer from 5.9 to 1.00 at 180 minutes. Detection of beta lactams resistant bacteria (Gram-positive bacteria S*taphylococcus aureus, Staphylococcus epidermidis* (MRSA) and Gram-negative bacteria *Escherichia coli*) by PCR with primers of mecA gene and Bela gene. The essential oil obtained by hydrodistillation was analyzed by GC-MS. The major components were found to be *Santolina triene* (1.97%), 2,5,5-trimethyl-3,6-heptadien-2-ol (8.23%) Eucalyptol 8.17, trans-2,7-Dimethyl-4,6octadien-2-ol (24.40%), 1,5-Heptadien-4-one-3,6-trimethyl (7.65%), Artemisia alcohol (3.49%), à Thujone (33.97%), Cissabinol (1.92%), Lavandulol (0.71%), 2-Octen-4-ol, 2-methyl (2.02%), 3-Cyclohexen1ol,4-methyl1 (1 methylethyl) (CAS) (2.15%), à terpineol (0.05%), Estragole (0.71%) Lavandulyl acetate (0.49%), Sabinyl acetate (2.12 %), Germacrened (0.94%). Finally, our study proved that the essential oil has bactericidal effect on some bacterial resistant antibiotic (Gram-positive bacteria *Staphylococcus aureus, Staphylococcus epidermidis* (MRSA) and Gram-negative bacteria *Escherichia coli*) as well as antiviral activity on ORF virus but it is still need further extensive studies for safety and drug interaction.

Keywords: *Achillea fragrantissima*; Essential oil; Antimicrobial activity; GC/MS; Antiviral; PCR

Introduction

Medicinal plants still constitute one of the major source of drugs in modern as well as traditional medicine throughout the world [1,2]. Since a long period of time, plants have been a valuable source of natural products for maintaining animals and human health [2,3]. The development of multidrug-resistant(MDR) bacteria takes place because of the accumulation of different antibiotic resistance mechanisms inside the same strain which able to live in the presence of antibiotics drugs so that standard treatments become ineffective [4,14-16]. Although, the pharmacological companies have produced a number of new antibiotics recently, but even then microorganism resistance to antibiotics increased throughout the world [5,7]. This situation has attract attention of researchers towards herbal products for developing and improve the antibacterial drugs quality [6,7].

Natural products of higher plants may possess a new source of antimicrobial agents with possibly novel mechanisms for treatment of infectious diseases [8,9]. Medicinal plants contain active principles which can be used as alternative effective herbal drugs against common bacterial infections [10]. They would be the best source to obtain a variety of drugs and active compounds. Therefore, such plants should be investigated to better understand their properties, safety and efficiency [11]. Essential oils are commercially used in different industries such as pharmaceutical, agronomic, food, sanitary, cosmetic and perfume [12]. In medicine they are used antioxidant, antitumor and antifungal [13-16].

The genera Achillea belongs to Family Asteraceae are widely distributed in the Middle East countries [17]. Ancient people had been used Achillea species in traditional medicine to alley pain, spasms and inflammation [18,3,4]. In Arabian countries all Achillea species were referred to locally names as Qaysum, Gesoom or Bu`eithraan [20,42] Numerous studies describe the various pharmacological effects of the *Achillea fragrantissima* hydrodistilled volatile oils in management of several diseases, used topically and orally [18-21].

Researchers studied antibacterial properties of various plants against Gram-negative as well as Gram-positive bacterial strains but only few reports on drug resistant bacteria and antiviral activity. Our study was aimed to evaluate in vitro antibacterial of essential oil extracted from *Achillea fragrantissima* plant against Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis* (MRSA) and Gram-negative bacteria *Escherichia coli* isolated from bovine mastitis as well as antiviral activity against ORF virus isolated from skin lesion of human and animals.

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Materials and Methods

Plant material and extraction

The aerial parts with leaves and flowers of *Achillea fragrantissima* plant were collected from Saint Catherine, South Sinai, Egypt in November 2013. Authentication of the plant was performed by the group of Genetics and Breeding of Medicinal and Aromatic plants at the Department of Genetics and cytology, Genetic Engineering and Biotechnology Division, National Research Centre, Cairo, Egypt. These samples were air-dried in shade at room temperature until constant weight (about 20 days). The dried aerial parts of the plant materials were grounded with a blender. The aerial plant parts powder was stored at 4°C in a tightly covered bottle. One hundred grams of plant powder was subjected to hydrodistillation in a microscale (v/w) return flow distillation apparatus according to [25]. The essential oil was collected, dried over anhydrous sodium sulphate and stored at 4°C until used.

Gas chromatography and mass spectrometry (GC-MS) analysis

The essential oil was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (NRC-GC/El-MS Lab), according to method [25-27] column with 0.25 µm film thickness, Helium was used as carrier gas; the flow through the column was 1.4 ml/min. The column temperature was programmed from 40 to 85°C at 20°C/min, increased from 85 to 300°C at rate of 5°C/min and finally held for 10 min. The MS operating parameters were as follows: ionization potential, 70 V; ionization current, 2 A; ion source temperature, 200°C, resolution, 1000. Mass unit were monitored from 30 to 450 m/z. Determination and identification of each components in the oil was based on RI relatives to n-alkanes and computer matching with library search report, as well as by comparison of the fragmentation patterns of mass spectra [27].

Bacterial strain

Microbial strains: Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis* (MRSA) and Gram-negative bacteria *Escherichia coli* were isolated from bovine mastitis provided by Veterinary Research Division at National Research Center (NRC), Egypt. All bacteria strains were stored in broth containing 25% (v/v) glycerol (Sigma- Aldrich) at -20°C. Prior to use, the culture were propagated twice in the appropriate media with shaking for 24 h at 37°C to physiologically active them.

Virus strain

The egg-adapted ORF virus was provided by Dr. G.S. Zeedan, NRC, Cairo, Egypt. Virus was propagated in the Chorio-Allantoic Membranes (CAM) of Specific Pathogen Free (SPF) embryonated chicken eggs (11 days age) as previously described by [28]. Both CAM and AF were harvested and ground in 0.1 M sterile PBS. The homogenate was frozen at -20°C and thawed three times and then centrifuged at 3000 rpm/15 min. Then supernatant was titrated and stored at -20°C until used.

Animal used

Twenty five clinically healthy Albino rats (130-150 g body weight) were obtained from Animal house of National Research Center Cairo Egypt, and randomly divided into 5 groups for experiment. The Animals were housed in a well-ventilated animal room under standardized conditions of 24°C; relative humidity 50 ± 5% and 12 hours light/dark cycle at the Animal House, National Research Center, Giza, Egypt. Feed and water were supplied adlibitum to meet the requirements of the NRC [29]. Rats were acclimatized for 15 days before the start of the experiment.

Toxicity of essential oil

Essential oil was diluted as the following: 100, 200, 300, 400 and 800 µl/ml then 1ml for each rat was injected S.C/I.P twice dose one week interval. Behavioral alterations, inflammatory effects, illness, and weight changes were recorded for 2 weeks post-treatment. Control animals (Rats). The toxicity of essential oil in ECE was examined as follows: inculcated 0.2 ml from each concentration of essential oil into Chorio-Allantoic Membranes (CAM) and Chorio-Allantoic Sac (CAS) incubated at 37°C for 7 days daily examination. The maximum non-lethal dose was taken as the maximum non-toxic concentration.

Antiviral activity of essential oil determined by Pock Reduction Assay

Virus titers were estimated from cytopathogenicity by tenfold dilution method 0.02 ml of each dilution was inoculated in five CAM of ECE for each dilution and expressed as 50 % Egg Infectious Dose per ml (EID50/ml). The calculation Calculated for each tested were was performed according to [30].

Antimicrobial activity

Essential oils were individually tested against Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis* (MRSA) and Gram-negative bacteria *Escherichia coli* were previously isolated from bovine mastitis by Agar diffusion methods and Minimal Inhibitory Concentration (MIC).

Agar diffusion method: These methods were performed according to Forbes et al. [31]. Bacteria cultures were diluted with sterile physiological saline solution with reference to the MC Farland 0.5 standard to achieve inoculums of approximately 10^6 CFU/ml. A suspension was swab in three directions on 4 mm thick Mueller Hinton Agar (MHA) (Oxoid) with a cotton swap. Modified discs of 6 mm were prepared using a Whitman filter paper. 100 discs were obtained by punching and putting in vials-bottles and sterilizing in an oven at 170°C for 30 min. The discs were impregnating with 20 µl of essential oil. Prepared discs containing the various essential oil were carefully placed on the inoculated plates using a sterilized forceps in each case [31]. The disc with solvent alone used as negative control and antibiotic discs as control positive. The plates were then turned upside-down and incubated at 37°C for 24 h in an incubator by the same manner used rosette with 6 well puncture the agar and 50 µl each well by essential oil, Tween 20 with saline and saline only as control negative. The results were taken by measuring the Diameter of the Inhibition Zone (DIZ) around the disc or well. This was repeated thrice and mean ± SD was calculated.

Minimal inhibitory concentration (MIC) using microdilution method

It was done according to [32,33] guidelines for determination of Minimum Inhibitory Concentration (MIC). The MIC was defined as lowest concentration of essential oils that inhibiting visible bacterial growth after incubation for 20 h at 37°C. Into each well of microplate 100 µl of Mueller-Hinton Broth (Oxoid) inoculated with the bacteria strain. An aliquot (100 µl) of the essential oil was added in first well serial dilutions of the essential oils were prepared in a 96 well micro titer plate, including one growth control and one sterility control. The contents of the wells were mixed and micro plates were incubated

at 37°C for 24 h. Micro titer plates were incubated at 37°C for 24 h. The activity was measured as a function of turbidity. Lack of turbidity was further confirmed by pouring suspension aliquot of 0.1 ml into pre-sterilized Petri dishes with nutrient agar medium. The tests were conducted in triplicate.

Estimation of the number of the viable bacterial cells

The time that taken by essential oil to kill staphylococcus and *E. coli* were evaluated by the time-kill assay method. The Time-kill curves were performed in tube containing nutrient or Muller Hinton broth, using inoculums density of approximately (10^6 CFU/mL) in the presence of essential oil. The tubes were continuously shaken and incubated at 37°C. Samples were obtained at 0, 2, 4, 8 and 16. At each sample time, they were taken and serially diluted log 2 2, 4, 8, 16, 32. 100 µl of undiluted and diluted samples were inoculated on nutrient agar or Muller Hinton agar. The plates were incubated at 37°C for 24 h at the appropriate temperatures, the number of colonies on each plate was counted, and the number of the viable cells in the cultures was calculated.

Polymerase chain reaction (PCR)

Detection of the mec A and Bela A Genes for *Staphylococcus epidermidis* strains and *E. coli* were accomplished using PCR amplification. Cells were suspended in a lysis buffer containing 1 M Tris HCl, 5 M NaCl, and 0.1 M EDTA, which was incubated at 95°C for 10 minutes. After incubation, the suspension was centrifugated at 23 000 rpm for 5 min. The supernatant was used as a template in PCR. PCR assay was carried out as described by [34]. Using primers Beta-lactams gene resistant MRSA Mec A F (GTGAAGATATACCAAGTGATT) and R (GTGAAGATATACCAAGTGATT) gave 147 bp PCR product of mecA gene from strains *Staphylococcus epidermidis* and/also using primer Beta-lactams gene resistant for *E. coli* bla C (F) TGGCCAGAACTGACAGGCAAA (R) TTTCTCCTGAACGTGGCTGGC 462 [35,34]. The final PCR products were visualized using UV-trans-illuminator after electrophoresis on 1.5% agarose gel containing 50 mg/mL EtBr.

Statistical analysis

All data were subjected to statistical analysis including the calculation of the mean and standard Deviation. Significance between data of control and infected groups was evaluated by the Student t-test at level $P<0.05$ according to [36] using SPSS for windows version 15 computer program.

Results

The volatile oil or (essential oil) was extracted from aerial parts, leaves and flowers of *A. fragrantissima* by conventional hydro distillation, which gave liquid oil, ranged pale yellow oil to yellow with a strong penetrating pleasant odor characteristic of each plant. The identified compounds, together with the Retention Indices (RI) of the compounds are shown in Table 1. The main chemical compounds detected in the oil were *Santolina triene* (1.97%), 2,5,5-trimethyl-3,6-heptadien-2-ol (8.23%) Eucalyptol 8.17, trans-2,7-Dimethyl-4,6-octadien-2-ol (24.40%), 1,5-Heptadien-4-one-3,6-trimethyl (7.65%), Artemisia alcohol (3.49%), à Thujone (33.97%), Cissabinol (1.92%),-Lavandulol (0.71%), 2-Octen-4-ol, 2-methyl (2.02%), 3 Cyclohexen1ol,4methyl1 (1methylethyl) (CAS) (2.15%), à terpineol (0.05%), Estragole (0.71%) Lavandulyl acetate (0.49%), Sabinyl acetate (2.12%), Germacrened (0.94%) as in (Table 1 and Figures 1 and 2).

No.	RT	Compounds	RRT	Area %
1	9.1	Santolina triene	0.57	1.97
2	12.5	2,5,5trimethyl3,6he ptadien2ol	0.75	8.23
3	13.16	Eucalyptol	0.83	8.17
4	13.67	trans2,7Dimethyl4, 6octadien2ol	0.86	24.4
5	14.23	1,5Heptadien4one, 3,3,6trimethyl	0.89	7.65
6	15.18	Artemisia alcohol	0.95	3.49
7	15.76	àThujone	1	33.97
8	16.62	Cissabinol	1.04	1.92
9	17.38	- Lavandulol	1.09	0.71
10	17.47	2 Octen4ol, 2methyl	1.1	2.02
11	17.42	3Cyclohexen1ol,4methyl1(1methylethyl)(CAS)	1.11	2.15
12	18.08	à Terpineol	1.13	1.05
13	18.24	Estragole	1.15	0.71
14	20.77	Lavandulyl acetate	1.3	0.49
15	20.87	Sabinyl acetate	1.31	2.12
16	25.84	Germacrened	1.62	0.94
		Total area		99.99

RT: Retention time

RRT: Relative retention time

Table 1: Chemical composition and relative content of the essential oil extracted from *A. fragrantissima* by Gas Chromatography–Mass Spectrometry (GC/MS) .

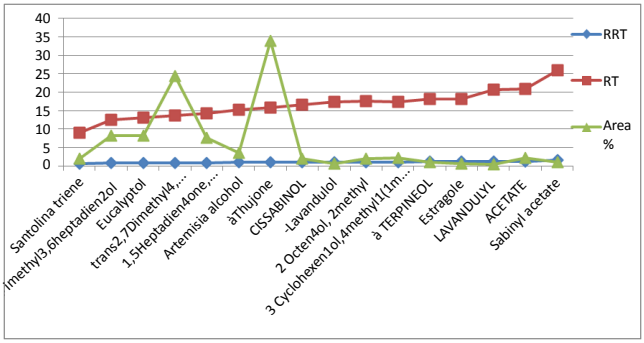

Figure 1: Determination of volatile oil fraction area by GC/MS analysis.

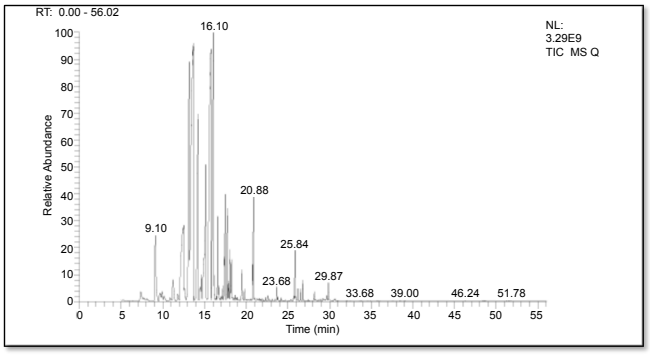

Figure 2: Achillea fragrantissima essential oil analyzed by GC-MS.

Antimicrobial activity by the agar diffusion method

The essential oil extracted from *A. fragrantissima* inhibited the growth of the microorganisms tested. It was especially active against *Staphylococcus aureus*, MRSA and *E. coli*. The *Staphylococcus aureus* showed the highest sensitivity to essential oil effect ranged from (18-22 mm–24-26 mm) and MRSA from (14-16 mm), (16-18 mm) and followed by *E. coli* from (8-11 mm) and (12-16 mm) inhibition zone

Microorganisms		Antibacterial disc diffusion methods DIZ : mm				Antibacterial well diffusion methods DIZ : mm				MIC (mg/ml)				MBC (mg/ml)			
		Essential oil	DMSO	Gentamicin	Tetracycline	Essential oil	DMSO	Gentamicin	Tetracycline	Essential oil	DMSO	Gentamicin	Tetracycline	Essential oil	DMSO	Gentamicin	Tetracycline
Gram positive bacteria	*S. aureus*	22	1	18	12	26	0	20		6.25	0	0.01	12.5	50		50	50
	S.epidermdis	16	1	R	R	18	0	R	R	12.5	0	≥ 4	R	25		R	R
	MRSA																
Gram negative bacteria	*E. coli*	11	0	20	R	12	0	22	R	0.01	0	0.01	R	0.01		50	50

Table 2: Determination of the of essential oil antibacterial activity by agar diffusion methods and Minimal Inhibitory Concentration (MIC). MIC: Minimal Inhibitory Concentration; DIZ: Diameter of Inhibition Zone: mm; MBC: Minimal Bactericidal Concentration; DMSO: Dimethylsulphoxide as negative control.

Time	Log_{10} ORFV titer kept at 37°C	Log_{10} ORFV titer kept at 4°C	Log_{10} ORFV titer was treated with essential oil (v/v).	
			Log_{10} titer	Reduction index
0 min	5.9	5.4-5.9	3.5	2.4
15 min	5.6		2	3.6
30 min	5.4		1.8	3.6
45 min	5.2		1.6	3.6
60 min	4.8		1.2	4.2
75 min	4.2		0	4.2
90 min	3.9		0	3.9
105 min	3.5		0	3.5

Table 3: Antiviral effect of *A. fragrantissima* essential oil on ORF virus titer by pock reduction test.

diameter by desk and well diffusion method s respectively .

Discussion

Achillea fragrantissima plant has been used for many years in traditional medicine in Middle East countries for the treatment of some diseases [37]. Several Achillea plants have been found to possess antiseptic and infection preventing properties [38,39]. They are being used as ingredients in medicinal and cosmetic preparations [40]. Recently, it was documented that the essential oil has anti-inflammatory, antioxidant and anti-proliferative capacities [41]. The present study showed that the essential oil has wide varieties of volatile oil terpene hydrocarbons (aliphatic and cyclic) and their corresponding isoprenoid derivatives and analogues, mixtures of these substances, terpinen-4-01 demonstrated fraction that strongly antimicrobial effect by acting only in eukaryotic bacteria cell as in Table 1 and Figures 1 and 2 and this finding is in agreement with [42].

The essential oil extracted from *A. fragrantissima* plant inhibits growth of microorganism to various degree can determined by disc and well diffusion methods according to diameter of inhibition zone. The inhibition zone of *Staphylococcus aureus* from 18 mm to 22 mm, followed by MRSA from 12 to 16 and followed by Gram negative bacteria *Escherichia coli* nearly smaller to MRSA inhibition zones, from 7.0 mm to 10.4 mm *Staphylococcus aureus* was the most sensitive organism to *A. fragrantissima* essential oil than MRSA and *E. coli*. However, the sensitivity of *Escherichia coli* not only similar to that of the MRSA but also required a higher concentration from essential oil to induce the desirable bactericidal effect as in Table 2 and Figures 3 A and 3B this results is in agreement with [43].

Surprising results that was no difference between inhibition zones of methicillin-resistant and methicillin-susceptible strain may be due to the some component of essential oil caused bacterial cell damaged this result is in agreement with [44-46]. They found that essential oil caused gross membrane damaged and provoke whole cell lysis of eukaryotic. The mechanism of spread antibiotic resistance from food animals to humans remains controversial and resistance to the same drugs [47]. The most of the bacteria isolated from bovine mastitis showed a multi-drug resistant for antibiotics due to the presences of drug resistant gene as beta lactimase which easily detected and evaluated by PCR as in Figure 4 this result is in agreement with [48]. The explanation of antibacterial activity of essential oil on bacterial cell depend on the action of terpenes fraction that involving their action by causing membrane perturbation and marked leakage of cytoplasmic material lead to induce irreversible damage to the cytoplasmic membran but fully action of terpenes still unknown and this explanation is agreement with [48,49].

The mecA and Bel gene coding for methicillin resistance via penicillin binding protein 2a (PBP2a) are well established and is considered as "gold standard" for detection of methicillin resistance in comparison with phenotypic methods [46,47]. Our results revealed that the inhibitory effect of essential oils on Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis* (MRSA) and Gram-negative bacteria *Escherichia coli* bacteria may be due to reduction or modified of β-lactamse (methicillin) resistance but the actual mechanism is unknown. However, is likely explained by some structural change in the resistant bacteria. Firstly, volatile oils fraction can be connected with the perturbation of cell membrane, the second mechanism may be inhibited the production of protein binding protein (PBP2) by inhibition of mecA and bel genes expression and final stage involved to subset synthesis of peptidoglycan of cell wall (transpeptidation reaction), which present outside the cell membrane this explanation is agreement with [50].

The essential oil was observed safe, no effect in the nature and color of embryo fluid comparing with control as well as experimental animals this result is in agreement with [51,52]. It was reduced infectivity titer

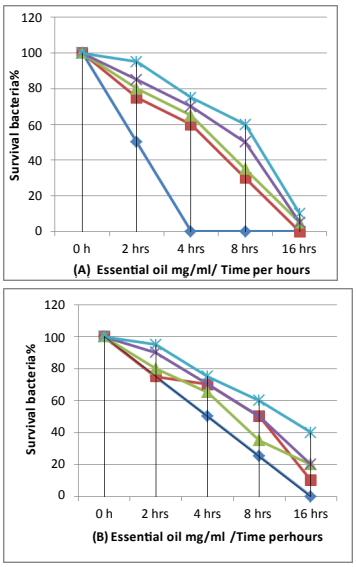

Figure 3: Effect of A Achillea fragrantissima essential oil on the viability of various microorganisms. The bacterial cells were grown broth media incubated with essential oil at 37°C with different time ranged from 0 to 16 hours and the number of viable cells was estimated as previously described Figure 3 (A) *Staphylococcus aureus* and Figure 3 (B) *E. coli.*

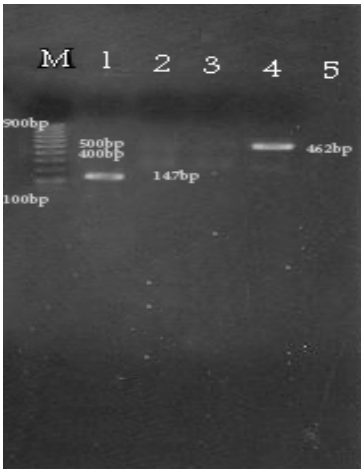

Figure 4: Electrophoretic pattern of PCR product (147 pb for mec A gene and 462 bp for bat gene) in 1.5% agarose gel stained with ethedium bromide lane1: amplified 147 bp for MRSA mec A gene, lane2, 3 and 5: negative PCR product amplified and Lane 4 amplified 462 bp for bat gene for *E. coli.* M: DNA marker (100pb).

of ORF virus from $\log_{10}$ 5.9 to 0.00 within 180 minutes. Antiviral effect of essential oil may be due to block the viral infection by blocking the cell membrane receptor for ORF virus or induce internal changes in the host cells, which in turn affect the virus replication cycle or due to production cytokines which blocked viral infection other health cells as in Table 3 and Figure 5. This result was in agreement with [53]. They found the essential oil caused sharp reduction in small pox viruses infectivity titer. Also, they observed that the concentration of viruses were reduce $\log_{10}$ 5-1 and they infectivity titer were reduced 21 and 25 time more than control group. We found that the essential oil reduced plaque size and number when mixing with ORF virus before inoculation on CAM, Finally, Our results proved that the *Achillea*

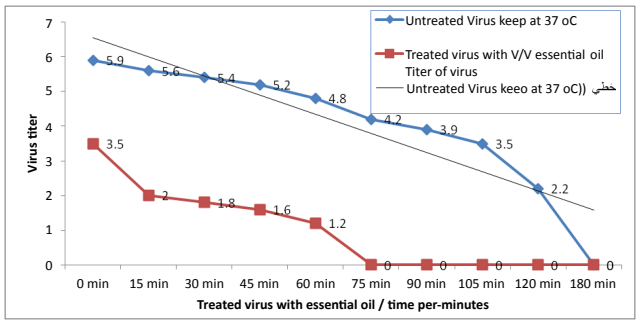

Figure 5 : Reduction of ORF virus titer by treated with *A. fragrantissima* essential oil and kept at 37°C.

fragrantissima essential oil has antiviral and antibacterial activities on B-lactmase resistant

Conclusion

The composition of the essential oil of *Achillea fragrantissima* growing in Sinai, Egypt has been analyzed by GC-MS and its antimicrobial activity against Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis* (MRSA) and Gram-negative bacteria *Escherichia coli* as well as antiviral activity against ORF virus reduced titer from 5.9 to 1. Detection of B-lactam antibiotic resistance bacteria was done by PCR with primers mec A and Bela genes. The results proved that the essential oil can be used in the treatment of diseases caused by the drug resistant microorganism. But, still need further extensive researches are required to prove safety, drugs interaction and clinical studies are required to prove the safety of the oil as a medicine.

Authors' Contributions

Gamil S.G. Zeedan and Abeer M. Abdalhamed authors': conception of the research idea, study design, data collection, main part of laboratory work and interpret the data and reviewed the manuscript; Mohmoud *E. Ottai* and Sobhy Abdelshafy authors': study design, collection of plant from Sinai, Egypt, Identification of plant with group of Genetics and Breeding of Medicinal and Aromatic plants, data analysis of GC-MS and drafting the manuscript; Eman Abdeen author: study design and part of laboratory work. All authors have read and approved the final revised manuscript

Conflict of Interests

The authors do not have any conflict of interests regarding the content of the paper.

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