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Antioxidant and Antiviral Activities of Essential Oils from *Callistemon viminalis* and *Schinus molle* L.

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ABSTRACT

The essential oils of *Callistemon viminalis* and *Schinus molle* leaves from plants grown in Egypt, obtained by hydrodistillation in a Clevenger type apparatus were investigated by GC/MS. The main components of *C. viminalis* oil were 1,8-cineole (65.92%), *α*-pinene (12.34%) while the results showed that the major components of *S. molle* oil were *α*-phellandrene (25.81%), elemol (11.02%). Both *C. viminalis* and *S. molle* oils exhibited strong DPPH scavenging activity, with IC_{50} values of 72.98 µg/mL and 172.41µg/mL respectively. The antiviral assays were performed with herpes simplex virus type 1 (HSV-1) and using RC-37 cells as a host cell. TC_{50} (50% cytotoxic concentration) of *C. viminalis* oil (676.35 µg/mL) demonstrated significantly lower toxicities towards the RC-37 cells than the *S. molle* oil (476.48 µg/mL). IC_{50} (inhibitory concentration for 50% of plaques) for HSV-1 of *C. viminalis* and *S. molle* oils were 63.73 and 48.06 µg/mL respectively, while *SI* (*Selectivity index* = TC_{50}/IC_{50}) of *C. viminalis* oil (10.61) was higher than *S. molle* oil (9.91). Both essential oils exhibited high anti-HSV-1 activity by direct interaction with free virus particles. To conclude, *C. viminalis* and *S. molle* oils could be a promising source of natural antioxidants, and antiviral agents.

Keywords: *Callistemon viminalis, Schinus molle*, essential oils, antioxidant, anticancer, antiviral.

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INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases, because they contain chemical components of therapeutic values [1]. Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. They are usually obtained by steam or hydro-distillation first developed in the Middle Ages by Arabs [2]. In ancient Rome, Greece and Egypt, the essential oils have been used as perfumes, food flavors, deodorants and pharmaceuticals [3]. Natural products and their derivatives are important sources of novel therapeutic molecules [4]. Researchers from all over the world are trying to characterize arrange of biological properties of essential oils which includes antimicrobial, antiviral, antimutagenic, anticancer, antioxidant activities [2]. Inhibition of viral replication is believed to be due to the presence of monoterpene, sesquiterpene and phenylpropanoid constituents of essential oils [5].

Callistemon is a genus of 34 species of shrubs belonging to family Myrtaceae and it has a great medicinal importance. Some species of *Callistemon*, especially *Callistemon viminalis* being an important source of chemical compounds with insecticidal, fungicidal and antimicrobial properties [6]. Chemical studies of the essential oils of *C. viminalis* from Australia, Egypt, India have been previously reported. 1,8-Cineole (47.9-82.0%) was the predominant constituent of the oils. Other significant components included α-pinene, β-pinene, myrcene, limonene, linalool and menthyl acetate [7-9]. *C. viminalis* essential oil is a great potential source of antibacterial and antioxidant compounds useful for new antimicrobial drugs from the natural basis [10]. Also, *C. viminalis* oil showed highly antimicrobial against two bacteria and one fungus strain [11] and it can be used as fumigant agent against insects of *A. obtectus* and *C. maculatus* [12].

Schinus molle L., commonly known as pink pepper or American pepper is a tree belonging to the Anacardiaceae family, which is native to subtropical regions of South America. It was introduced and naturalized in Southern Europe, including Portugal, as an ornamental plant [13,14]. In folk medicine, *Schinus molle* has been used due to its antibacterial, antiviral, topical antiseptic, antifungal, antioxidant, anti-inflammatory, anti-tumoural, anti-spasmodic, analgesic properties, as well as a stimulant and an antidepressant [15-19]. The results of some studies have revealed the antimicrobial and antioxidant properties of essential oil and extracts of *Schinus molle* [20-23].

The aim of the present study was to evaluate the essential oils from dried leaves of *Callistemon viminalis* and *Schinus molle* as antioxidant agents by using DPPH radical scavenging assay, also as antiviral agents against herpes simplex virus type-1 (HSV-1).

MATERIALS AND METHODS

Plant material

The dried leaves of *Callistemon viminalis* belonging to the family Myrtaceae and the dried leaves of *Schinus molle L.* belonging to the family Anacardiaceae were collected from Faculty of Agriculture, Cairo University, Egypt. The plant samples were kindly identified by Dr. Mohamed Osama El-Segaee, Professor of Taxonomy, Faculty of Agriculture, Cairo University. A voucher specimen has been deposited in the Department of Botany for further reference.

Essential oil extraction

One hundred grams of dried leaves of *C. viminalis* and dried leaves of *S. molle* were hydro-distilled in a Clevenger type apparatus [24]. The essential oils were dried over anhydrous sodium sulphate, stored in a dark glass bottle, and kept at 4 ^{o}C until analysis. The amount of oil obtained from plant material was calculated as:

$$\text{Oil (\% v/w)} = \text{observed volume of oil (mL)/weight of sample (g)} \times 100$$

GC/MS analysis of essential oils

The essential oils were analyzed by GC-MS [25]. GC/MS analysis was performed on a Thermoquest-Finnigan Trace GC-MS equipped with a DB-5 (5% phenyl) methylpolysiloxane column (60 m \ 0.25 mm i.d., film thickness 0.25 μm). The injection temperature was 220 ^{o}C and the oven temperature was raised from 40 ^{o}C (3 min hold) to 250 ^{o}C at a rate of 5 ^{O}C/min, then held at 250 ^{O}C for 2 min; transfer line temperature was 250 ^{O}C. 1 µL of sample was injected and helium was used as the carrier gas at a flow rate of 1.0 ml/min. The mass spectrometer was scanned over the 40 to 500 m/z with an ionizing voltage of 70 eV and identification was based on standard mass library that National Institute of Standards and Technology (NIST Version 2.0) to detect the possibilities of essential oil components.

Antioxidant activity of essential oils using DPPH radical scavenging assay

Radical scavenging activity of plant essential oils against the stable DPPH radical was determined spectrophotometrically [26]. The colorimetric changes (from deep-violet to light-yellow), when DPPH$^{\bullet}$ is reduced, were measured at 517 nm on a UV/visible light spectrophotometer. The antioxidant activity of essential oils were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH. Fifty microliters of various concentrations (25, 50, 75, 100 and 200 µg/mL) of the essential oils in dimethyl sulphoxide (DMSO) as well as ascorbic acid (as standard antioxidant compound) were put into appropriate tubes, and 5 mL of 0.004% methanolic solution of DPPH$^{\bullet}$ was added to each tube to give final concentrations (25, 50, 75, 100, 200 µg/mL). Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined after 1 h for all samples. Methanol was used to zero the spectrophotometer. Absorbance of the DPPH radical without antioxidant, *i.e.* the control, was measured. Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution. The DPPH radical by the samples was calculated according to the following formula [27]:

$$\% \text{ inhibition} = ((A_{C(o)} - A_{s(t)}) / A_{C(o)}) \times 100$$

Where $A_{C(o)}$ is the absorbance of the control at t = 0 min and $A_{s(t)}$ is the absorbance of the antioxidant at t = 1 h.

The percentage of scavenging activity was plotted against the essential oil concentrations to obtain the inhibitory concentration (IC_{50}), defined as the essential oil concentration necessary to cause 50% scavenging. Tests were carried out in triplicate.

***In vitro* assay for antiviral activity of essential oils**

Cell culture and herpes simplex virus type 1 (HSV-1)

RC-37 cells (African green monkey kidney cells) were grown in monolayer culture with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 U/mL penicillin and 100 µg/mL streptomycin. The monolayers were removed from their plastic surfaces and serially passaged when-ever they became confluent. The cells were plated out onto 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and propagated at 37 °C in an atmosphere of 5% CO2. Herpes simplex virus type 1 (HSV-1) strain KOS was used for all experiments. Viruses were routinely grown on RC-37 cells and virus stock cultures were prepared from supernatants of infected cells and stored at -80 °C. Infectivity titers were determined by a standard plaque assay on confluent RC-37 cells [28].

Cytotoxicity assay

For cytotoxicity assays, the cells were seeded into 96-well plates and incubated for 24 h at 37 °C. The medium was removed and fresh DMEM containing the appropriate dilution of the essential oils was added onto sub confluent cells in five replicates for each concentration of the essential oils (25, 50, 75, 100, 200, 500 and 1000 µg/mL). Wells containing medium with 1% ethanol but no essential oil were also included on each plate as controls. After 3 days of incubation, the growth medium was removed and the viability of the essential oil treated cells was determined in a standard neutral red assay [29]. Neutral red dye uptake was determined by measuring the optical density (OD) of the eluted neutral red at 540 nm in a spectrophotometer. The mean

OD of the cell-control wells was assigned a value of 100%. The cytotoxic concentration of the essential oils which reduced the viable cell number by 50% (TC_{50}) was determined from dose-response curves. Additionally the maximum non-cytotoxic concentration (MNTC) of each essential oil was determined.

Dose-response assays

Inhibition of HSV-1 replication was measured by a plaque reduction assay. Usually 2 x 10^3 plaque forming units (pfu) were incubated with different concentrations of essential oils for 1 h at room temperature, then the virus was allowed to adsorb to the cells for 1 h at 37 °C. The residual inoculum was discarded and the infected cells were overlaid with medium containing 0.5% methylcellulose. Each concentration was performed in three replicates, virus-infected cells in wells containing medium with 1% ethanol but no essential oil were also included on each plate as controls. After incubation for 3 days at 37 °C, the mono-layers were fixed with 10% formalin. The cultures were stained with 1% crystal violet and subsequently plaques were counted. By reference to the number of plaques observed in virus control monolayers (untreated cultures), the concentration of essential oil, which inhibited plaque numbers by 50% (IC_{50}) was determined from dose-response curves. Also, the selectivity index (SI) was calculated according to the ratio TC_{50}/IC_{50} [30].

Time of addition assay

In order to determine the mode of antiviral action for essential oils, viruses were incubated with essential oils before infection, the cells were pretreated with essential oils before viral infection or infected cells were incubated immediately after penetration of the virus into cells. Essential oils were always used at MNTC [30].

Pretreatment of viruses with essential oils (virucidal assay)

For pretreatment of herpes simplex virus with essential oils, about 2 × 10^3 pfu of HSV were incubated in medium containing the MNTC of the essential oils for 1 h at room temperature prior to infection of RC-37 cells.

Pretreatment of cells with essential oils

Cell monolayers were pretreated with essential oils prior to inoculation with virus by adding the components to the culture medium followed by incubation for 1 h at 37^{o}C. The essential oils were aspirated and cells were washed before the HSV inoculum was added.

Post treatment (Addition of essential oils during viral intracellular replication)

The effect of essential oils against HSV was also tested during the replication period by addition of essential oils after cell infection to the overlay medium, as typical performed in antiviral susceptibility studies. Each assay was run in three replicates. Plaque reduction assays were carried out as described above and number of plaques of essential oil-treated cells and viruses were compared to untreated controls. Wells containing medium with 1% ethanol but no essential oil were also included on each plate as controls.

Statistical analysis

Statistical analyses (standard deviation "SD" and standard error "SE") was carried out [31]. LSD (Least significant difference) test was used to compare the significant differences between means of treatment [32]. The statistical package for social science S.P.S.S. [33] program version was used for all analysis.

RESULTS

The yields of the oils obtained from the hydro-distillation of *C. viminalis and S. molle* leaves were 0.76% and 2.02% (v/w), respectively. Table (1) lists the components identified in each essential oil composition with their percentages by using GC/MS analysis.

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Table 1: Chemical composition of dried leaves of *C. viminalis* and *S. molle* essential oils.

No.	Compound name	% Peak area	
		C. viminalis	*S. molle*
1	α-Thujene	0.08	0.81
2	α-Pinene	12.34	4.77
3	Camphene	-	0.60
4	Sabinene	-	0.93
5	β-Pinene	1.01	1.05
6	β-Myrcene	0.05	3.09
7	α-Phellandrene	0.77	25.81
8	α-Terpinene	0.61	1.12
9	p-Cymene	2.51	0.26
10	Limonene	0.07	6.35
11	1,8-Cineol	65.92	-
12	*cis*-β-Ocimene	-	0.46
13	γ-Terpinene	0.25	0.81
14	β-Phellandrene	-	10.83
15	Terpinolene	-	0.40
16	*trans*-Sabinene hydrate	-	0.05
17	Linalool	0.43	0.48
18	Camphor	-	1.01
19	*endo*-Fenchol	0.22	-
20	*trans*-Pinocarveol	0.09	-
21	Pinocarvone	1.02	-
22	Terpinen-4-ol	1.12	0.31
23	*cis*-Sabinol	0.26	-
24	α-Terpineol	6.56	0.45
25	Carvone	0.62	-
26	Geraniol	0.49	-
27	Eugenol	0.40	-
28	Bronyl acetate	-	0.39
29	Terpenyl acetate	-	0.93
30	Eugenol acetate	0.09	-
31	α-Copaene	-	0.42
32	β-Elemene	-	0.57
33	β-Caryophyllene	-	3.56
34	*trans*-Caryophyllene	-	0.03
35	α-Gurjunene	-	0.99
36	α-Humulene	-	0.79
37	Aromadendrene	-	0.52
38	Bicyclogermacrene	-	0.81
39	Germacrene D	-	5.68
40	α-Muurolene	-	0.53
41	Spathulenol	0.56	1.02
42	Caryophyllene oxide	1.71	0.71
43	γ-Cadinene	-	0.54
44	Germacrene A	-	0.83
45	δ-Cadinene	-	2.99
46	Elemol	-	11.02
47	Germacrene B	-	0.82
48	α-Cadinene	-	0.55
49	α-Eudesmol	-	2.14
50	β-Eudesmol	-	1.51
51	δ-Cadinol	-	0.88
52	α-Cadinol	-	0.45
Monoterpene hydrocarbons		17.69	57.29
Oxygenated monoterpenes		77.22	3.62
Sesquiterpene hydrocarbons		-	19.63
Oxygenated sesquiterpenes		2.27	17.73
% Identified compounds		97.18	98.27
% Oil yield (v/w)		0.76	2.01

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GC/MS analysis of essential oils

Twenty-three constituents were identified and quantified in the oil of *C. viminalis,* representing 97.18% of the total oil, while forty-three compounds of *S. molle* essential oil were identified having the total area of 98.27%. The *C. viminalis* oil is characterized by the dominance of 1,8-cineole (65.92%), *α*-pinene (12.34%) and *α*-terpineol (6.56%), while the results show that the major components of *S. molle* oil were *α*-phellandrene (25.81%), elemol (11.02%), *β*-phellandrene (10.83) and limonene (6.35%). Minor components of *C. viminalis* are β-myrcene (0.05%), limonene (0.07%), *α*-thujene (0.08%), *trans*-pinocarveol (0.09%) and eugenol acetate (0.09%), while in *S. molle* oil the minor components were *trans*-caryophyllene (0.03%) and *trans*-sabinene hydrate (0.05%). The most abundant chemical structure within components of *C. viminalis* essential oil were oxygenated monoterpenes (77.22%) followed by monoterpene hydrocarbons (17.69%) and low quantity of oxygenated sesquiterpenes (2.27%). Monoterpene hydrocarbons were the main compounds group (57.29%) of *S. molle* oil followed by sesquiterpene hydrocarbons (19.63%), oxygenated sesquiterpenes (17.73%) and low quantity of oxygenated monoterpenes (3.62%). The structures of the main constituents of the essential oils are reported in Figure 1.

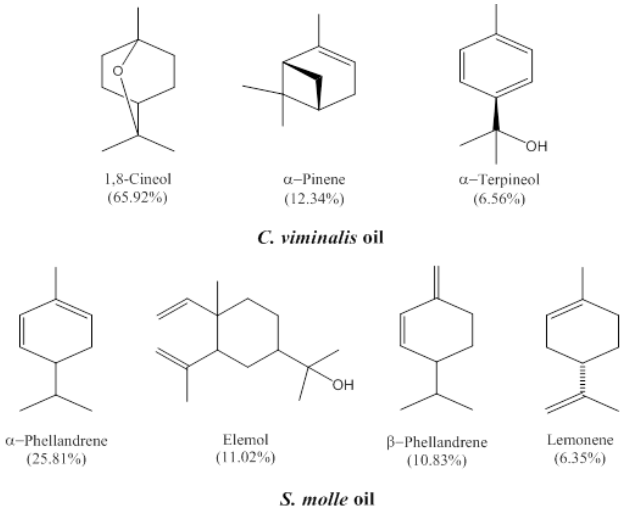

Figure 1: Chemical structures of the main constituents of *C. viminalis and S. molle* essential oils.

Antioxidant activity of essential oils

Antioxidant activities of essential oils from aromatic plants are mainly attributed to the active compounds present in them. This can be due to the high percentages of main constituents, but also to the presence of other constituents in small quantities or to synergy among them. In this study, the antioxidant activities of essential oils of two plants belonging to different plant families compared with ascorbic acid as a reference anti-oxidant compound were determined by the method of DPPH$^{\bullet}$ radical scavenging assay and the results are summarized in table (2). It was found that the essential oils of two analyzed plants showed good antioxidant capacities compared with ascorbic acid. The results indicate that the radical scavenging activity (% inhibition) of the essential oil from *C. viminalis* was measured as 30.57, 37.26, 50.67, 71.17 and 90.08% with different concentrations of the essential oil 25, 50, 75, 100, 200 µg/mL respectively, whereas treated with the same concentrations of *S. molle* oil reached the percentages of DPPH$^{\bullet}$ inhibition of 8.78, 20.44, 30.03, 39.11 and 52.88% respectively. On the other hand, the radical scavenging activity of ascorbic acid was determined as 34.20, 61.66, 81.58, 91.70 and 96.58% with the same previous concentrations.

It was observed that the scavenging activity of the essential oils were significantly increased with the increased of the essential oils concentrations. All essential oils had lower antioxidant activities than ascorbic acid. It is clear from the data that the concentration of 200 µg/mL of *C. viminalis* essential oil gave the highest percentage inhibition of DPPH$^{\bullet}$ (90.08%) which was high significant inhibition value compared with other treatments, while the same concentration of ascorbic acid gave 96.58% inhibition. On the other hand, DPPH$^{\bullet}$ scavenging activity of the *S. molle* essential oil was 52.88% at 200 µg/mL concentration. Lower IC_{50} value indicated higher antioxidant activity. Essential oils reduction with IC_{50} values are reported as follows: IC_{50} (*C. viminalis*) = 72.98 µg/mL; IC_{50} (*S. molle*) = 172.41 µg/mL and IC_{50} (ascorbic acid) = 34.61 µg/mL. The quantity of

C. viminalis and *S. molle* essential oils required were about 2.11 and 4.98 fold, respectively, when compared with the standard antioxidant ascorbic acid.

Table 2: Percentage of scavenging activity of DPPH radicals induced by *C. viminalis, S. molle* essential oils and ascorbic acid (standard antioxidant compound).

Treatment	Concentration of essential oil (µg/mL)	% Inhibition of DPPH$^{\bullet}$	IC_{50} (µg/mL)
***C. viminalis* oil**	25	$30.57^{h} \pm 1.37$	72.98
	50	$37.26^{g} \pm 2.28$	
	75	$50.67^{f} \pm 2.18$	
	100	$71.17^{d} \pm 1.70$	
	200	$90.08^{b} \pm 0.59$	
***S. molle* oil**	25	$8.78^{j} \pm 1.07$	172.41
	50	$20.44^{i} \pm 1.92$	
	75	$30.03^{h} \pm 1.15$	
	100	$39.11^{g} \pm 1.22$	
	200	$52.88^{f} \pm 1.74$	
Ascorbic acid	25	$34.20^{gh} \pm 1.33$	34.61
	50	$61.66^{e} \pm 1.25$	
	75	$81.58^{c} \pm 1.05$	
	100	$91.70^{b} \pm 1.04$	
	200	$96.58^{a} \pm 1.25$	
$LSD_{0.05}$		4.28	

- The values are means ± SE. The mean values with different small letters within a column indicate significant differences ($P < 0.05$).

Antiviral activities of essential oils

Cytotoxicity effects of essential oils

The essential oils represent complex mixtures of different chemical substances and are not water-soluble. When the lipophilic essential oil is mixed directly to the aqueous cell culture medium, it floats on top and is not in solution. The insolubility of essential oils in water rendered these agents unsuitable for cytotoxicity tests with standard cultured cell lines. Lipophilic solvents such as ethanol are able to dissolve essential oils, consequently different concentrations (25 to 1000 µg/mL) of each essential oils were prepared by dilute the hydro-distilled essential oils with ethanol and add to cell culture medium. The viability percentages of RC-37 cells after incubation for 3 days were determined by using the neutral red assay and data are given in table (3). No significant changes in RC-37 cells viability percentages when treated with *C. viminalis* and *S. molle* essential oils at concentrations range from 25 to 200 µg/mL compared with untreated control (1 mL medium with 1% ethanol). The concentrations of 500 and 1000 µg/mL of the two essential oils significantly decrease RC-37 cells viability compared with other treatments as well as untreated control. On the other hand, it was found non-significant differences in the viability cells between *C. viminalis* and *S. molle* essential oils at concentrations of 1000 µg/mL. The concentration of each essential oil, which reduced viable cells number by 50% (TC_{50}), was determined from dose-response curves. TC_{50} of *C. viminalis* essential oil (676.35 µg/mL) demonstrated significantly lower toxicities towards the RC-37 cells than the essential oil of *S. molle* (476.48 µg/mL). The maximum noncytotoxic concentrations (MNTC) of *C. viminalis* and *S. molle* essential oils were 220.08 and 119.83 µg/mL respectively (Table 5).

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Table 3: Cytotoxic effect of *C. viminalis* and *S. molle* essential oils with different concentrations on RC-37 cells.

Treatment	Concentration (µg/mL)	RC-37 Cell viability (%)
Untreated control	0	99.80^{a} ± 0.20
***C. viminalis* oil**	25	99.09^{a} ± 2.01
	50	98.52^{a} ± 0.92
	75	93.84^{a} ± 2.92
	100	95.23^{a} ± 1.08
	200	98.89^{a} ± 0.50
	500	74.27^{b} ± 1.01
	1000	12.72^{d} ± 1.21
***S. molle* oil**	25	99.27^{a} ± 1.89
	50	99.76^{a} ± 2.45
	75	96.28^{a} ± 2.41
	100	99.60^{a} ± 1.69
	200	93.16^{a} ± 1.02
	500	44.60^{c} ± 3.60
	1000	9.66^{d} ± 3.66
LSD $_{0.05}$		5.76

- The values are means ± SE. The mean values with different small letters within a column indicate significant differences (P < 0.05).

Table 4: Antiviral effect of different concentrations of *C. viminalis* and *S. molle* essential oils against HSV-1.

Concentration (µg/mL)	5	10	25	50	75	100	200
	Remaining infectivity (% of control)						
***C. viminalis* oil**	96.53^{a} ± 1.42	86.82^{b} ± 3.08	74.01^{d} ± 2.25	53.92^{f} ± 1.76	29.38^{h} ± 2.18	0.00^{i} ± 0.00	0.00^{i} ± 0.00
***S. molle* oil**	95.76^{a} ± 1.26	80.61^{c} ± 1.48	64.55^{e} ± 2.33	49.00^{g} ± 1.79	26.78^{h} ± 1.98	4.28^{i} ± 1.39	0.00^{i} ± 0.00
LSD $_{0.05}$	4.77						

- The values are means ± SE. The mean values with different small letters indicate significant differences (P < 0.05).

Table 5: Selectivity indices of *C. viminalis* and *S. molle* essential oils against HSV-1.

Treatment	MNTC (µg/mL)	TC_{50} (µg/mL)	IC_{50} (µg/mL)	Selectivity index (SI)
***C. viminalis* oil**	220.08^{a} ± 6.05	676.35^{a} ± 8.05	63.73^{a} ± 2.00	10.61
***S. molle* oil**	119.83^{b} ± 4.86	476.48^{b} ± 21.48	48.06^{b} ± 1.17	9.91
LSD $_{0.05}$	17.89	52.24	5.35	

- The values are means ± SE. The mean values with different small letters within a column indicate significant differences (P < 0.05).

The antiviral effects of essential oils

The antiviral activity of *C. viminalis* and *S. molle* essential oils with different concentrations (5 to 200 µg/mL) against herpes simplex virus type 1 (HSV-1) was evaluated *in vitro* by using a plaque reduction assay as

described above and the plaque numbers of essential oils treated cells and viruses were compared with untreated controls. Wells containing medium with 1% ethanol but no oil were also included on each plate as controls. Results of remaining HSV-1 infectivity (%) related to untreated control are given in Table (4). It is clear that increasing of essential oils concentrations, significantly reduced infectivity (%). Also significantly decrease was noticed in remaining virus infectivity (%) when treated with 100 and 200 µg/mL of essential oils concentrations. The highest values of infectivity reduction were zero % when infected cells treated with 100 and 200 µg/mL of *C. viminalis* and *S. molle* essential oils respectively. The IC_{50} for HSV-1 were determined 63.73 µg/mL for *C. viminalis* oil which was significant decrease compared with 48.06 µg/mL for *S. molle* oil. Selectivity indices for compounds were calculated as the TC_{50}/IC_{50} ratio (Table 5). The selectivity index (SI) of *C. viminalis* oil (10.61) was higher than *S. molle* oil (9.91).

Mode of antiviral action

The effect of *C. viminalis* and *S. molle* essential oils against HSV-1 with different time of addition assay (pretreatment of virus, pretreatment of cells and post-treatment or replication) were studied and results are shown in Table (6). The infectivity of the HSV-1 was significantly reduced by the treatment of the virus with the essential oils for 1 h prior to inoculation. At maximum non-toxic concentrations (MNTC), the observed plaque reductions (%) were 100% for the *C. viminalis* oil and 98% for *S. molle* oil. When host cells were pretreated with essential oils prior to infection, both tested essential oils showed minor effects on viral infection where the results of the plaque reductions (%) were 12.78 % for *C. viminalis* oil and 17.57 for *S. molle* oil. In contrast, when the essential oils were added to the overlay medium after penetration of the viruses into the host cells (post-treatment), plaque reduction was slightly increased (11.36% and 10.94% for *C. viminalis* and *S. molle* essential oils respectively) compared with untreated control.

Table 6: Antiviral activity of *C. viminalis* and *S. molle* essential oils against HSV-1 in time of addition assays.

Treatment	Plaque forming unit (PFU)	Plaque reduction (%)
Untreated control	$50.09^{a} \pm 1.55$	0.00 ± 0.00
C. viminalis		
Pretreatment of viruses (virucidal assay)	$0.00^{c} \pm 0.00$	100.00 ± 0.00
Pretreatment of cells	$43.69^{b} \pm 1.75$	12.78 ± 3.49
Post-treatment (Replication)	$44.40^{b} \pm 1.03$	11.36 ± 2.06
S. molle		
Pretreatment of viruses (virucidal assay)	$1.00^{c} \pm 0.16$	98.00 ± 0.32
Pretreatment of cells	$41.29^{b} \pm 1.56$	17.57 ± 3.12
Post-treatment (Replication)	$44.61^{b} \pm 1.40$	10.94 ± 2.79
$LSD_{0.05}$	3.62	

The values are means ± SE. The mean values with different small letters within a column indicate significant differences ($P < 0.05$).

DISCUSSION

Our results are in agreement with author [34] who reported that the essential oil of *C. viminalis* was characterized by a high amount of 1,8-cineole (65.0%). Chemical studies of the essential oils of *C. viminalis* from Australia, Egypt, India have been previously reported. 1,8-Cineole (47.9-82.0%) was the predominant constituent of the oils [7-9]. The *C. viminalis* oil was characterized by the dominance of 1,8-Cineole (66.36%), *α*-pinene (20.43%) and *α*-terpineol (6.65%) [11]. Although, the essential oil compositions of *C. viminalis* from different countries have been studied, there are differences in the yield and profile of the oil constituents, which could be attributed to many environmental factors viz. latitude, geographical distribution etc. On the other hand, The main components were α-phellandrene (26.5%), β-phellandrene (12.4%), elemol (10.8%), limonene (8.6%) as described by [35]. Our results disagree with author [36] who indicated that essential oil of *S. molle* leaf contained 24 components; mainly delta-cadinene (11.28%) and alpha-cadinol (10.77%). Delta

cadinene as major component of leaf oil [37]. Several components of *S. molle* L. oil were identified, among which sabinene was the main component, followed by (α, β)-pinene and terpinen-4-ol [38]. The composition of *S. molle* exhibit significant differences. The composition may differ by season and the region that the plant material was collected [39].

The antiradical scavenging activity of oils might be attributed to the replacement of hydroxyl groups in the aromatic ring systems of the phenolic compounds as a result of their hydrogen donating ability [26]. The different of antioxidant activity between the two essential oils may be due to the differences of chemical composition specially a high amount of oxygenated monoterpenes in *C. viminalis* oil.

Leaf essential oil from *C. viminalis* exhibited the highest antioxidant activity of 88.60% comparable to gallic acid, a standard compound (80.00%) [10]. DPPH radical scavenging IC_{50} of *C. viminalis* oil were found to be in the range of 28.4–56.2 µg/mL [40]. The antioxidant activity of *S. molle* essential oil against DPPH free radicals showed an IC_{50} of 36.3 µg/mL [41]. On the other hand, essential oil of *S. molle* showed a weak free radical scavenging activity in the DPPH assay (IC_{50} = 3697.6 µg/mL). The major components of *S. molle* essential oil (*α*- and *β*-phellandrene) seems to have a low DPPH free radical scavenging [42]. The sesquiterpene elemol that is present in the *S. molle* essential oil in about 13% was near in our sample percentage (11.02%) [43]. In our study antioxidant activity of *S. molle* oil against DPPH free radical may be due to the presence of elemol and limonene and also, minor constituents with strong antioxidant activity, such as *γ* –terpinene, sabinene, cadinol and terpinolene.

The parameters for the definition of a compound as a good antiviral candidate are not well defined, showing a great variation among values of IC_{50} and SI for different viruses and products. There are numerous variables that influence the final results of a susceptibility test, including cell culture, virus titer, incubation times, concentration of the antiviral compound, reference strains, assay methods, calculations and interpretation criteria [44]. However, the basis of our discussion will be a suggestion from author [45] applied to herpes simplex viruses (HSV), which propose that a selectivity index (SI) greater than four would be appropriate to consider that a compound has a potential antiviral activity. Following the guidelines cited above, it could generally be concluded that the essential oils examined showed antiviral activities towards HSV-1. In particular, the *C. viminalis* oil (SI = 10.61) and *S. molle* oil (SI = 9.91) presented high SI values in the virucidal assay. On the other hand, IC_{50} values for promising natural products against infectious diseases, e.g. for extracts below 100 µg/mL and below 25 µM for pure compounds [46]. The essential oils in our study revealed IC_{50} values of 48.06 µg/mL and 63.73 µg/mL for *S. molle* oil and *C. viminalis* oil, respectively, and are below the recommended cut off and present a promising anti-infective agents according to this suggestion.

From previous data, it is clear that the pre-treatment of cells with essential oils or the incubation of these essential oils with the cells after virus inoculation (post treatment) did not result in antiviral activities at the same level as the direct treatment of the virus. These results suggest that the investigated essential oils acted directly on the viral particle and interfered with viral infectivity. The antimicrobial actions of the essential oils have previously been explained based on their effects on the structure and functions of the bacterial membrane. The activities of these compounds were attributed to their lipophilic characteristics that would allow their interaction with the lipidic membrane of the bacteria [47,48]. A similar effect could be expected with the lipidic viral envelope [49]. In fact, several essential oils and their major compounds have demonstrated antiviral activities against viruses with a lipidic envelope, suggesting that these compounds inactivated the virus by directly interfering with the viral envelope structures or masking viral structures needed for the absorption and entry to the cell [30,49].

Our results suggested that the studied essential oils have a good potential as antiviral agents, which in the same trend with that reported by many authors. Manuka, a plant belonging to the family Myrtaceae essential oil exhibited high levels of virucidal activity against HSV-1 [50]. *C. viminalis* essential oil is a great potential source of antibacterial compounds useful for new antimicrobial drugs from the natural basis [10]. Also, the *C. viminalis* oil was highly active against *Escherichia coli* when compared with the standard antibacterial gentamicin [11]. At the same path, the volatile oil of different *Callistemon* species has been reported to possess antimicrobial [51,52]. On the other hand, the essential oil of *S. molle* exhibited antibacterial activity using the microdilution broth method against two Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) and one Gram-negative bacteria (*Escherichia coli*) [35]. The essential oil of *S. molle* growth in Tunisia possessed antimicrobial activity [21]. The essential oil of *S. molle* showed potential

antimicrobial activity [36]. The results showed that the crude extract essential oil has a potent antibacterial effect on *Staphylococcus aureus* [38].

CONCLUSION

In conclusion, the essential oils of *C. viminalis* and *S. molle* showed high scavenging activity against DPPH radicals. Also, These results suggest that the two essential oils exhibit antiherpetic activity and might be used as a potential antiviral agents against HSV-1. To conclude, *C. viminalis* and *S. molle* oils could be a promising source of natural antioxidants and antiviral agents. However, studies *in vivo* are needed to assess the true antioxidant and antiviral activities of these essential oils for human and to determine the metabolic pathways involved in their degradation.

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REFERENCES

[1] Derwich E, Benziane Z, Boukir A. Int J Agric Biol 2010; 12(2): 199-204.
[2] Bakkali F, Averbeck S, Averbeck D, Idaomar M. Food Chem Toxicol 2008; 46: 446-475.
[3] Baris O, Gulluce M, Sahin F, Ozer H, Kilic H, Ozkan H, et al. Turk J Biol 2006; 30: 65-73.
[4] Clardy J, Walsh C. Nature 2004; 432: 829-837.
[5] Astani A, Reichling J, Schnitzler P. Evid Based Compl Altern Med 2011; 8 pages, http://www.hindawi.com/journals/ecam/2011/253643/.
[6] Silva CJ, Barbosa LCA, Pinheiro AL, Andrade NJ. Química Nova 2010; 33(1): 104-108.
[7] Srivastava SK, Ahmad A, Jain N, Aggarwal KK, Syamasunder KV. Flavour Fragr J 2003; 18(5): 361-363.
[8] Mahmoud II, Marzouk MSA, Moharram J, Nolte J, Fobbe R, Saleh MI. Bull Fac Pharm Cairo Univ 2000; 40: 119-112.
[9] Brophy JJ, Forster PI, Goldsack RJ, Hibbert DB, Punruckvong A. Austral Syst Bot 1997; 10(1): 1-13.
[10] Salem MZM, Ali HM, El-Shanhorey NA, Abdel-Megeed A. Asian Pac J Trop 2013; 6(10): 785-791.
[11] Gohar AA, Maatooq GT, Gadara SR, Aboelmaaty WS. J Biotechnol Pharmaceut Res 2014; 5(1): 007-011.
[12] Ndomo AF, Tapondjou, LA, Ngamo, LT, Hance T. J Appl Entomol 2010; 134: 333–341.
[13] Bailey LA, Bailey EZ. Hortus Third a Concise Dictionary of Plants Cultivated in the United States and Canada. McMillan Publishing Co, New York; 1976.
[14] Taylor L. The Healing Power of Rainforest Herbs. A Guide to Understanding and Using Herbal Medicinals. Square One Publishers, New York; 2005.
[15] Duke J. Handbook of Medicinal Herbs. CRC Press, Boca Raton. Florida; 1985.
[16] Alanís-Garza BA, González-González GM, Salazar-Aranda R, Torres NWd, Rivas-Galindo VM. J Ethnopharmacol 2007; 114(3): 468-471.
[17] Machado DG, Kaster MP, Binfaré RW, Dias M, Santos ARS, Pizzolatti MG, et al. Prog Neuropsychopharmacol Biol Psychiatr 2007; 31(2): 421-428.
[18] Molina-Salinas GM, Pérez-López A, Becerril-Montes P, Salazar-Aranda R, Said-Fernández S, Torres NWd. J Ethnopharmacol 2007; 109(3): 435-441.
[19] Guala M, Elder H, Perez G, Chiesa A. Inf Tecnol 2009; 20(2): 83-88.
[20] Dikshit A, Naqvi AA, Husain A. Appl Environ Microbiol 1986; 51(5): 1085-1088.
[21] Hayouni EA, Chraief I, Abedrabba M, Bouix M, Leveau JY, Mohammed H, Hamdi M. Int J Food Microbiol 2008; 125(3): 242-251.
[22] Murray A, Gurovic M, Rodriguez S, Murray M, Ferrero A. Nat Prod Commun 2009; 4(6): 873-876.
[23] Salazar-Aranda R, Pérez-López LA, López-Arroyo J, Alanís-Garza BA, Torres NWd. Evid Based Complement Alternat Med 2011; p. 6.
[24] Council of Europe. Council of Europe, European Pharmacopoeia 3rd ed., Council of Europe, Strasbourg, France; 1997.
[25] Adams RP. Identification of essential oils by ion trap mass spectroscopy. Academic press, New York; 1989.
[26] Brand-Williams W, Cuvelier ME, Berset C. Lebensm.-Wiss Technol 1995; 28: 25-30.

[27] Yen GC, Duh PD. J Agric Food Chem 1994; 42: 629–632.
[28] Schnitzler P, Nolkemper S, Stintzing FC, Reichling J. Phytomed 2008; 15: 62-70.
[29] Söderberg T, Johannson A, Gref R. Toxicol 1996; 107: 99-109.
[30] Astani A, Reichling J, Schnitzler P. Phytother Res 2010; 24(5): 673-679.
[31] Fisher RA. Statistical method for research workers Edinburgh ed. 14, Oliver and Boyed; 1970, p.140.
[32] Waller RA, Duncan DB. An State Assoc J 1969; 65: 1485-1503.
[33] S.P.S.S. Statistical Package for the Social Science Inc. Chicago; 2014.
[34] Dewhirst FE. Prostaglandins 1980; 20(2): 209-222.
[35] Belhamel K, Abderrahim A, Ludwig R. Int J Essent Oil Ther 2008; 2: 175-177.
[36] Deveci O, Sukan A, Tuzun N, Kocabas EEH. J Med Plants Res 2010; 4: 2211–2216.
[37] Rossini C, Menendez P, Dellacassa E, Moyna P. J Essent Oil Res 1996; 8(1): 71-73.
[38] de Mendonça Rocha PM, Rodilla JM, Díez D, Elder H, Guala MS, Silva LA, et al. Molecules 2012; 17(10): 12023-36.
[39] Abdel-Sattar E, Zaitoun AA, Farag MA, Gayed SH, Harraz FMH. Nat Prod Res 2010; 24(3): 226-235.
[40] Zubair M, Hassan S, Rizwan K, Rasool N, Riaz M, Zia-Ul-Haq M, et al. The Scientific World Journal. 2013; 8 pages.
[41] Díaz C, Quesada S, Brenes O, Aguilar G, Cicció JF. Nat Prod Res 2008; 22(17): 1521-1534.
[42] Bendaoud H, Romdhane M, Souchard J, Cazaux S, Bouajila J. J Food Sci 2010; 75(6): C466-72.
[43] Maffei M, Chialva, F. J Flav Fragr 1990; 5(1): 49-52.
[44] Swierkosz EM, Hodinka RL. Antiviral agents and susceptibility tests. *I*n: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH (eds). Manual of Clinical Microbiology. American Society for Microbiology, Washington, DC; 1999; p.1624-1639.
[45] Amoros M, Simões CMO, Girre L, Sauvager F, Cormier M. J Nat Prod 1992; 55(12): 1732-1740.
[46] Cos P, Vlietinck AJ, Berghe DV, Maes L. J Ethnopharmacol 2006; 106(3): 290-302.
[47] Helander IM, Alakomi HL, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid EJ, et al. J Agr Food Chem 1998; 46(9): 3590-3595.
[48] Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmington JR, et al. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (Tea tree oil). J Appl Microbiol. 2000; 88(1): 170-175.
[49] Schnitzler P, Koch C, Reichling J. Antimicrob Agents Ch 2007; 51(5): 1859-1862.
[50] Schnitzler P, Wiesenhofer K, Reichling J. Pharmazie 2008; 63(11): 830–835.
[51] Sudhakar M, Raju, DB. Int J Chem Sci 2005; 3(3): 513-516.
[52] Abdelhady MI. Br Bull Faculty Pharm (Cairo University) 2009; 47(3): 139-146.