Osong Public Health and Research Perspectives

Journal homepage: http://www.kcdcphrp.org

Original Article

Chemical Constituents of Essential Oils Possessing Anti-Influenza A/WS/33 Virus Activity

Hwa-Jung Choi *

Department of Beauty Science, Kwangju Women's University, Gwangju, Korea

Article history:

Received: April 30, 2018
Revised: November 23, 2018
Accepted: November 27, 2018

Keywords:

essential oils,
influenza, linalool, virus

ABSTRACT

Objectives: This study was conducted to determine whether essential oils had anti-influenza A/WS/33 virus activity and whether there were specific compounds associated with this activity.
Methods: There were 63 essential oils evaluated for anti-influenza (A/WS/33 virus) activity using a cytopathic effect reduction method. The chemical composition of the anti-influenza essential oils was phytochemically analyzed by gas chromatography-mass spectrometry.
Results: The antiviral assays demonstrated that 11 of the 62 essential oils (100 μg/mL) possessed anti-influenza activity, reducing visible cytopathic effects of influenza A/WS/33 virus activity by > 30%. Furthermore, marjoram, clary sage and anise oils exhibited anti-influenza A/WS/33 virus activity of > 52.8%. However, oseltamivir (the anti-influenza A and B drug), showed cytotoxicity at the same concentration (100 μg/mL) as the essential oils. The chemical composition detected by GC–MS analysis, differed amongst the 3 most potent anti-viral essential oils (marjoram, clary sage and anise oils) except for linalool, which was detected in all 3 essential oils.
Conclusion: This study demonstrated anti-influenza activity in 11 essential oils tested, with marjoram, clary sage and anise essential oils being the most effective at reducing visible cytopathic effects of the A/WS/33 virus. All 3 oils contained linalool, suggesting that this may have anti-influenza activity. Further investigation is needed to characterize the antiviral activity of linalool against influenza A/WS/33 virus.

https://doi.org/10.24171/j.phrp.2018.9.6.09
pISSN 2210-9099 eISSN 2233-6052

Introduction

Influenza viruses are enveloped RNA viruses that infect humans and animals, and cause respiratory complications resulting in high morbidity rates [1]. The preferred treatment for influenza infection are neuraminidase inhibitors (oseltamivir and zanamivir) [2]. However, their use has been limited by side-effects, and the emergence of resistant viral strains [3,4].

Essential oils are known to possess multifunctional properties other than their traditional roles, as various biological agents have been shown to demonstrate anti-bacterial, anti-fungal, and anti-inflammatory activities [5,6]. Several studies have documented antiviral activity of essential oils [7-9]. Recent studies have demonstrated that eucalyptus essential oil showed inhibitory effects on adenovirus and mumps virus [10]. A previous study showed that the volatile oil from *Cynanchum stauntonii* possessed direct inhibitory activity against influenza virus [11].

In this present study, the potential anti-viral properties of 62 essential oils on influenza A/WS/33 virus have been analyzed using the cytopathic effect (CPE) reduction method. Marjoram (*Thymus mastichina* L.), clary sage (*Salvia sclarea* L.) and anise (*Pimpinella anisum* L.) oils showed anti-influenza A/WS/33 activity and were phytochemically examined by gas

*Corresponding author: Hwa-Jung Choi
Department of Beauty Science, Kwangju Women's University, Gwangju, South Korea
E-mail: rerived@kwu.ac.kr

chromatography-mass spectrometry (GC-MS) analysis, and their chemical compositions analyzed.

Materials and Methods

1. Materials and cell culture

Essential plant oils (n = 62) were purchased from UNIQ F&F Co., Ltd. (Seoul, Korea) and listed in Table 1. The samples were deposited in Seoul National University herbarium. To test the materials, the oils were solubilized in dimethylsulfoxide to give a final concentration of 10 mg/mL, and stored at -20°C until further use.

The Influenza A/WS/33 virus was provided by ATCC (American Type Culture Collection, Manassas, VA, USA) and propagated in Madin-Darby canine kidney (MDCK) cells at 37°C. MDCK cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and

Table 1. List of 62 plant essential oils tested for antiviral activity.

Family	Species	Oil	Part
Alliaceae	*Allium sativum* L.	Garlic	Root
Annonaceae	*Cananga odorata* Hook. f. et Thomson	Ylang ylang	Flower
Apiaceae	*Pimpinella anisum* L.	Anise	Fruit
Apiaceae	*Carum carvi* L.	Caraway seed	Seed
Apiaceae	*Coriandrum sativum* L.	Coriander	Flower
Apiaceae	*Coriandrum sativum* L.	Coriander herb	Leaf
Apiaceae	*Foeniculum vulgare* Mill.	Fennel	Seed
Asteraceae	*Artemesia vulgaris* L.	Armoise	Whole plant
Asteraceae	*Tagetes minuta* L.	Tagette	Leaf
Cupressaceae	*Juniperus irginiana* L.	Cedarwood	Bark
Cupressaceae	*Cupressus sempervirens* L.	Cypress	Twig
Cupressaceae	*Juniperus communis* L.	Juniperberry	Berry
Ericaceae	*Gaultheria procumbens* L.	Wintergreen	Leaf
Fabaceae	*Myroxylon balsamum* var. *pereirae* Royle	Balsam peru	Resin
Lamiaceae	*Ocimum basilicum* L.	Basil	Flower
Lamiaceae	*Nepeta cataria* L.	Catnip	Leaf & flower
Lamiaceae	*Salvia sclarea* L.	Clary sage	Flower
Lamiaceae	*Hyssopus officinalis* L.	Hyssop	Leaf
Lamiaceae	*Thymus mastichina* L.	Marjoram	Leaf
Lamiaceae	*Origanum vulgare* L.	Oregano	Leaf
Lamiaceae	*Pogostemon cablin* (Blanco) Benth.	Patchouly	Leaf
Lamiaceae	*Mentha piperita* L.	Peppermint	Flower
Lamiaceae	*Mentha pulegium* L.	Pennyroyal	Leaf
Lamiaceae	*Rosmarinus officinalis* L.	Rosemary	Flower
Lamiaceae	*Satureja hortensis* L.	Savory	Leaf
Lamiaceae	*Mentha spicata* L.	Spearmint	Flower
Lamiaceae	*Thymus vulgaris* L.	Thyme	Leaf
Lamiaceae	*Thymus vulgaris* L.	Thyme white	Leaf
Lauraceae	*Cinnamomum cassia* Bl.	Cassia pure	Bark
Lauraceae	*Cinnamomum zeylanicum* Garc. Ex Blume Nees	Cinnamon bark	Bark
Lauraceae	*Cinnamomum zeylanicum* Blume	Cinnamon leaf	Leaf
Lauraceae	*Aniba roseadora var. amazonica* Ducke	Rosewood	Wood
Myrtaceae	*Eucalyptus globulus* Labill.	Eucalyptus	Leaf

Table 1. (Continued).

Family	Species	Oil	Part
Myrtaceae	*Pimenta dioica* (L.) Merr.	Pimento berry	Flower
Myrtaceae	*Melaleuca alternifolia* (Maid. & Bet.) Cheel	Tea tree	Leaf
Oleaceae	*Eugenia caryophyllata* Thumb.	Clove bud	Bud
Oleaceae	*Eugenia caryophyllata* Thumb.	Clove leaf	Leaf
Pinaceae	*Pinus sylvestris* L.	Pine	Needle
Poaceae	*Cymbopogon nardus* L.	Citronella java	Leaf
Poaceae	*Pelargonium graveolens* L.	Geranium	Flower
Poaceae	*Cymbopogon martinii* Stapf.	Palmarosa	Grass
Rutaceae	*Citrus bergamia* Risso	Bergamot	Peel
Rutaceae	*Citrus paradisi* Macfadyen	Grapefruit	Fruit
Rutaceae	*Citrus sinensis* (L .) Osbeck	Orange	Peel
Rutaceae	*Citrus limonum* L.	Lemon	Peel
Rutaceae	*Citrus aurantifolia* Swing.	Lime	Peel
Rutaceae	*Citrus reticulata* Blanco	Mandarine	Peel
Rutaceae	*Citrus aurantium* L.	Neroli	Flower
Santalaceae	*Santalum album* L.	Sandalwood	Wood
Rosaceae	*Rosa damascene* Mill.	Rose	Flower
Asteraceae	*Chamomilla recutita* (L.) Rauschert	Chamomile blue	Flower
Asteraceae	*Artemisia dracunculus* L.	Estragon	Leaf
Poaceae	*Cymbopogon citratus* (DC) Stapf.	Lemongrass	Whole plant
Myrtaceae	*Pimenta racemosa* (Mill.) J.W.Moore	Bay	Leaf
Lauraceae	Litsea cubeba L.	Litsea cubeba	Fruit
Clusiaceae	*Calophyllum inophyllum* L.	Tamanu	Fruit
Rutaceae	Zanthoxylum armatum	Xanthoxylum	Seed
Myrtaceae	Eucalyptus citriodora	Eucalyptus (citriodora)	Leaf
Zingiberaceae	*Zingiber officinale* Roscoe	Ginger	Rhizome

0.01% antibiotic-antimycotic solution. Antibiotic-antimycotic solution, trypsin- ethylenediamine tetra-acetic acid (EDTA), fetal bovine serum and MEM were supplied by Gibco BRL (Grand Island, NY). Tissue culture plates were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ). Sulforhodamine B (SRB) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of reagent grade. Oseltamivir (F. Hofmann-La Roche Ltd, Switzerland) was purchased from a pharmacy in Korea as prescribed by a traditional Korean doctor.

2. Antiviral and cytotoxicity assay

Anti-influenza (A/WS/33 virus) activity of essential oils was determined by the CPE reduction method [12]. There were 2×10^4 MDCK cells/well seeded into a 96-well culture plate in MEM supplemented with trypsin-EDTA containing 0.01% antibiotic–antimycotic solution and incubated at 37°C in 5% CO_2 for 24 hours. Thereafter, the medium was aspirated and cells were washed with phosphate buffered saline (PBS). Subsequently, the diluted virus suspension (0.09 mL) containing 50% tissue culture infective dose ($TCID_{50}$) of the virus, was added to MDCK cells to produce an appropriate CPE within 48 hours after infection. Thereafter, MEM containing essential oils in 100 μg/mL were added to each well. The culture plates were incubated at 37°C in 5% CO_2 for 2 days until 50% CPE was achieved. Subsequently, the 96-well plates were washed once with PBS (100 mL). Ice-cold 70% acetone in water (100 mL) was added to each well and incubated for 30 minutes at –20°C. After 70% acetone had evaporated, the plates were dried in an oven at 55°C for 30 minutes. The plate was developed using 0.4% (w/v) SRB in 1% acetic acid solution (100 mL) which was added to each well and left to stand for 30 minutes at room temperature. The excess SRB solution was removed by washing the plates 5 times with 1% acetic acid in water, then the plate was dried at 55°C. Bound SRB was then solubilized with 10

mM unbuffered Tris-base (SigmaAldrich) solution (100 mL). After 30 minutes, the absorbance was read at 524 nm with a VERSAmax microplate reader (Molecular Devices, Palo Alto, CA, USA) with a reference absorbance determined at 650 nm. The percent protection achieved by the test compound in the influenza A/WS/33 virus-infected cells was calculated using the equation below. Oseltamivir was used as the positive control and dimethylsulfoxide as a negative control. The morphology of influenza A/WS/33 virus-infected cells was observed using a light microscope at 32 × 10 magnification (AXIOVERT10; ZEISS, Oberkochen, Germany), and images were recorded.

Antiviral activity index = [(ODt) virus – (ODc)virus]/[(ODt) mock – (ODc)virus] × 100%

To evaluate cytotoxicity, MDCK cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells per well. The next day, the medium was replaced with medium containing serially diluted compounds. After 2 days of incubation at 37°C in 5% CO_2, cytotoxicity was evaluated using the SRB assay. The culture medium was aspirated and cells were washed with PBS. The next step was performed per the antiviral activity assay described above. Results were expressed as the percentage of the controls.

3. Effect of essential oils on morphological changes of influenza virus-induced MDCK cells

The effect of essential oils on influenza virus-induced CPE was observed. Briefly, MDCK cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells per well. The next day, the culture medium was removed and the cells were washed with PBS. Diluted virus suspension (0.09 mL) and 0.01 mL of medium supplemented with trypsin-EDTA containing essential oils at 100 µg/mL were added to each well. After incubation at 37°C in 5% CO_2 for 2 days, the morphology of cells was observed under the microscope at 32 × 10 magnifications (AXIOVERT10, ZEISS, Germany), and images were recorded.

4. Gas chromatography

Gas chromatography analysis was performed on an Agilent 6890N equipped with a DB-1MS column (30 mm × 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA). The oven temperature was programmed as: isothermal at 40°C for 1 minute, then raised to 250°C (6°C/minute) and held at this temperature for 4 minutes. Helium was used as the carrier gas at the rate of 1.5 mL/minute in split mode (50:1 ratio). The constituents of the plant essential oil were identified by comparing their GC retention indices (RI). The RI of the constituents for each plant essential oil were identified by co-injection of essential oil and a mixture of aliphatic hydrocarbons (C8-C20; Sigma-Aldrich, St. Louis, USA). RI was calculated using the equation proposed by van Den Dool and Kratz (1963) [13].

5. Gas chromatography-mass spectrometry

The oils (marjoram, clary sage and anise) were analyzed further on a gas chromatograph (Agilent 6890N)-mass spectrometer (Agilent 5973N MSD) equipped with a DB-5MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness, J & W Scientific, Folsom, CA). The oven temperature was programmed as described previously. Helium was used as the carrier gas at the rate of 1.0 mL/minute. The effluent of the GC column was introduced directly into the source of the MS via a transfer line (250°C). Ionization voltage was 70 eV and the ion source temperature was 230°C. Scan range was 41-450 amu. Compounds were identified by comparison of mass spectra of each peak with those of authentic samples in the NIST MS library.

Results

1. Marjoram, clary sage and anise oils possess anti-influenza A/WS/33 virus activity

Anti-influenza (A/WS/33 virus) activity of all essential oils were investigated. Eleven essential oils amongst the 62 essential oils tested possessed antiviral activity of > 30% and did not show cytotoxicity at a concentration of 100 µg/mL (Figure 1A). There were 3 oils (marjoram, clary sage and anise oils) that exhibited a higher anti-influenza activity (> 52%) than oseltamivir, with no cytotoxicity at a concentration of 100 µg/mL. However, oseltamivir showed cytotoxicity at this concentration (Figure 1B).

2. Marjoram, clary sage and anise oils reduce anti-influenza A/WS/33 virus-induced morphological changes

The effects of essential oils on influenza A/WS/33 virus-induced CPE were investigated (Figure 2). After a 2-day infection of MDCK cells with influenza A/WS/33 virus, mock cells (Figure 2A) or cells treated with 100 µg/mL essential oils (Figures 2E, 2G and 2I) showed typical morphology. At this concentration (100 µg/mL), there were no signs of cytotoxicity induced by the essential oils. However, oseltamivir was weakly toxic to MDCK cells at concentration of 100 µg/mL (Figure 2C). Infection with influenza A/WS/33 virus, in the absence of essential oils, resulted in a severe CPE (Figure 2B). Addition of the essential oils to the influenza infected MDCK cells, inhibited the formation of a visible CPE (Figures 2F, 2H and 2J). However, the addition of oseltamivir to influenza A/WS/33 virus-infected MDCK cell resulted in a small reduction in CPE

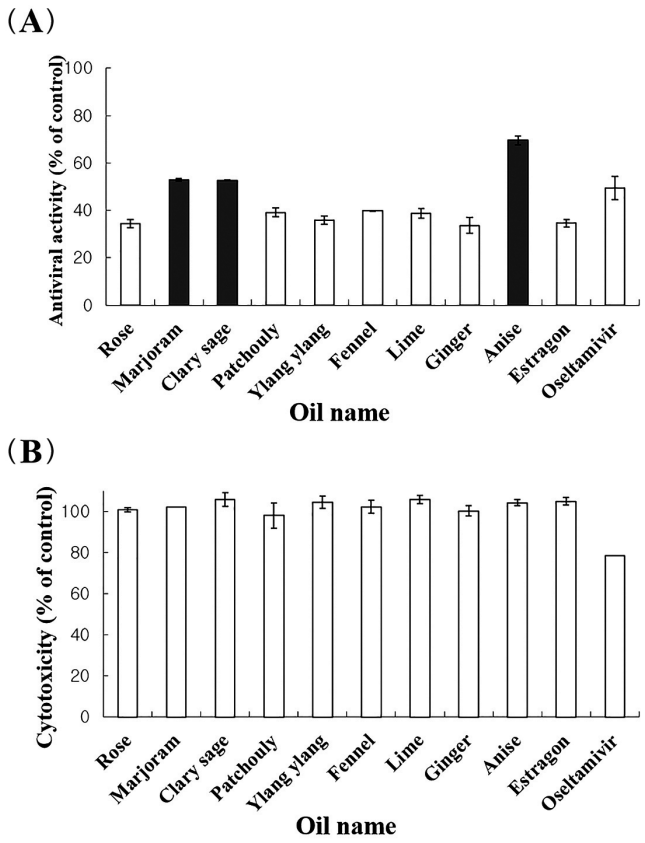

Figure 1. Antiviral activity of essential oils on influenza A/WS/33 virus.
Virus suspension and media containing essential oil (100 μg/mL) were added to the cells. After incubation 2 days, antiviral activity was investigated using the CPE reduction assay.
Results are presented as the mean percentage values obtained from 3 independent experiments carried out in triplicate ± SD.

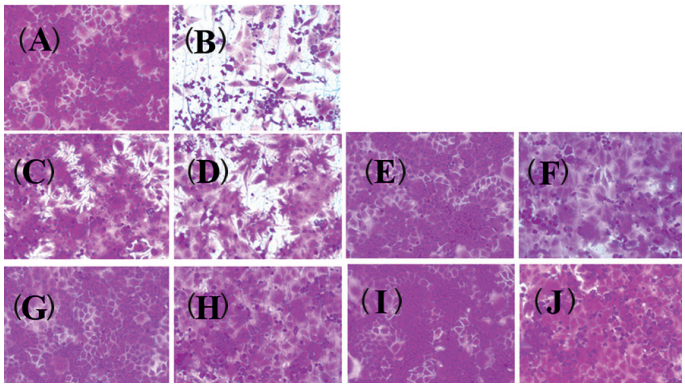

Figure 2. The effect of essential oils on influenza B/Lee/40 virus-induced CPE. The virus-infected cells were treated with essential oils (100 μg/mL). After incubation 2 days, the cells was stained by SRB and the morphology was examined.
Non-infected cells; (B) virus-infected cells without oils; (C) non-infected cells with oseltamivir; (D) virus-infected cells with oseltamivir; (E) non-infected cells with marjoram oil; (F) virus-infected cells with marjoram oil; (G) non-infected cells with clary sage oil; (H) virus-infected cells with clary sage oil; (I) non-infected cells with anise oil; (J) virus-infected cells with anise oil.
CPE = cytopathic effect; SRB = sulforhodamine B.

Table 2. Chemical composition of marjoram oil.

No.	Compound	Retention time (min)	Relative composition ratio (%)
1	α -pinene	928	4.21
2	Sabinene	962	1.82
3	β-pinene	967	5.81
4	β-myrcene	981	1.02
5	*p*-cymene	1,010	0.94
6	1,8-cineole	1,018	64.61
7	Linalool	1,084	15.28
8	Terpinen-4-ol *	1,159	1.29
9	α-terpineol *	1,170	2.40
10	Bornylacetate *	1,435	2.61
Total			100.0

* Identified by mass library.

Table 3. Chemical composition of clary sage oil.

No.	Compound	Retention time (min)	Relative composition ratio (%)
1	β-myrcene	981	1.53
2	Linalool	1,084	22.06
3	α-Terpineol	1,170	4.21
4	Cinnamaldehyde *	1,234	1.81
5	Linalayl acetate *	1,240	61.16
6	Geranyl acetate	1,360	2.40
Total			93.17

* Identified by mass library.

(Figure 2D). Thus, the CPE of the virus infection was shown to be prevented by the presence of the essential oils.

3. Chemical compositions of the marjoram, clary sage and anise oils

The chemical compositions of marjoram, clary sage and anise essential oils are shown in Tables 2 to 4. A total of 10 compounds were identified in marjoram oil by GC and GC-MS analysis (Table 2). Among the identified compounds, 1,8-cineole (64.61%) was the most abundant compound followed by linalool (15.28%) and β-pinene (5.81%; Table 2). The chemical compositions of clary sage oil showed linalayl acetate (61.16%) to be the highest concentration, followed by linalool (22.06%)

Table 4. Chemical composition of anise oil.

No.	Compound	Retention time (min)	Relative composition ratio (%)
1	α –pinene	928	0.85
2	Limonene	1,019	2.55
3	Linalool	1,084	2.74
4	Estragole	1,173	8.21
5	4-Allylanisole *	1,211	1.21
6	*trans*-Anethole	1,259	82.78
7	Charvicol *	1,650	1.08
Total			99.41

* Identified by mass library.

and α–terpineol (4.21%; Table 3). The chemical compositions of anise oil were *trans*-anethole (82.78%), estragole (8.21%) and linalool (2.74%; Table 4).

Discussion

Many antiviral compounds have been developed against the influenza virus, the long-term efficacy of which is often limited due to toxicity or the emergence of drug-resistant virus mutants [14]. Hence, new approaches for the control of highly pathogenic influenza viruses must be explored.

Previous studies showed antiviral activity of essential oils against DNA viruses such as herpes simplex virus [7,8]. *Ocimum basilicum* (OB), also known as sweet basil, showed antiviral activities against DNA viruses (herpes viruses (HSV), adenoviruses (ADV) and hepatitis B virus and RNA viruses (coxsackievirus B1 (CVB1) and enterovirus 71 (EV71). Apigenin, linalool and ursolic acid isolated from crude aqueous and ethanolic extracts of OB, exhibited a broad spectrum of antiviral activity against these viruses [15].

In this work, marjoram, clary sage and anise oils exhibited strong anti-influenza A/WS/33 virus activity. Linalool was a common constituent in the chemical compositions of marjoram, clary sage and anise oils. Therefore, the anti-influenza A/WS/33 activity of marjoram, clary sage and anise oils appeared to be associated with linalool. However, further studies will be required to explore the anti-influenza A/WS/33 virus effects of linalool.

In conclusion, marjoram, clary sage and anise oils showed interesting anti-influenza A/WS/33 activity. A common constituent of the 3 oils was linalool. Therefore, further studies are required to understand whether or not linalool possesses antiviral activity against influenza A/WS/33 virus.

Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

Acknowledgments

This paper was supported (in part) by Research Funds of Kwangju Women's University in 2018 (KWUI18-035).

References

[1] Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56 (1):152–79.
[2] Garman E, Laver G. Controlling influenza by inhibiting the virus's neuraminidase. Curr Drug Targets 2004;5(2):119–36.
[3] Kiso M, Mitamura K, Sakai-Tagawa Y, et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 2004;364(9436):759–65.
[4] Le QM, Kiso M, Someya K, et al. Avian flu: isolation of drug-resistant H5N1 virus. Nature 2005;437(7062):1108.
[5] Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol 1999;86(6):985–90.
[6] Meepagala KM, Sturtz G, Wedge DE. Antifungal constituents of the essential oil fraction of *Artemisia dracunculus* L. var. dracunculus. J Agric Food Chem 2002;50(24):6989–92.
[7] Tragoolpua Y, Jatisatienr A. Anti-herpes Simplex Virus Activities of *Eugenia caryophyllus* (Spreng). Bullock & S. G. Harrison and Essential Oil, Eugenol Phytother Res 2007:21 (12):1153–8.
[8] Koch C, Reichling J, Schneele J, et al. Inhibitory effect of essential oils against herpes simplex virus type 2. Phytomed 2008;15(1-2):71–8.
[9] Schnitzler P, Schuhmachera A, Astania A, et al. *Melissa officinalis* oil affects infectivity of enveloped herpesviruses. Phytomed 2008;15(9):734–40.
[10] Cermelli C, Fabio A, Fabio G, et al. Effect of eucalyptus essential oil on respiratory bacteria and viruses. Curr Microbiol 2008;56(1):89-92.
[11] Yang ZC, Wang BC, Yang XS, et al. Chemical composition of the volatile oil from *Cynanchum stauntonii* and its activities of anti-influenza virus. Colloids and Surfaces B: Biointerfaces 2005;43(3-4):198–202.
[12] Choi HJ, Kim JH, Lee CH, et al. Antiviral activity of quercetin 7-rhamnoside against porcine epidemic diarrhea virus. Antiviral Res 2009;81(1):77–81.
[13] van Den Dool H, Kratz PD. Generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr 1963;11:463–71.
[14] Hayden FG. Antivirals for influenza: historical perspectives and lessons learned. Antivir Res 2006;71(1-2):372–8.
[15] Chiang LC, Ng LT, Cheng PW, et al. Antiviral activities of extracts and selected pure constituents of *Ocimum basilicum*. Clin Exp Pharmacol Physiol 2005;32(10):811-6.