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Contributed Paper

## Inhibitory Effect of Peppermint Extracts and Menthol against Herpes Simplex Virus Infection

Raenu Yucharoen [a,b], Puttinan Meepowpan[c] and Yingmanee Tragoolpua\*[b]

[a] Biotechnology, Graduate School, Chiang Mai University, Chiang Mai, 50200, Thailand.

[b] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand.

[c] Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand.

\*Author for correspondence; e-mail: [yingmanee.t@chiangmai.ac.th](mailto:yingmanee.t@chiangmai.ac.th)

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### ABSTRACT

Herpes simplex virus (HSV) is pathogenic to humans. This study demonstrated anti-herpes simplex virus activity of dichloromethane and methanol extracts of peppermint, and pure compound, menthol on green monkey kidney cells. The cells were prevented from HSV-1 infection when the cells were treated before viral infection by the dichloromethane and methanol extracts of peppermint with therapeutic indices (TI) of 1.79 and 1.09, respectively. Dichloromethane extract of peppermint demonstrated higher inhibitory effects during HSV-1 and HSV-2 adsorption with TI of 4.21 and 7.63 while TI of 5.41 and 3.15 were observed after HSV-1 and HSV-2 adsorption. HSV-1 and HSV-2 were also inhibited by menthol with TI of 1.79 and 2.12 after viral adsorption. HSV-1 and HSV-2 viral particles were directly inhibited and viral yield was reduced when treatment with peppermint extracts and menthol. The inhibition of approximately 40 kDa HSV proteins, which might involve HSV capsid assembly, was observed after treatment with the dichloromethane extract. Therefore, peppermint extracts and menthol showed anti-HSV activities at various stages of the viral multiplication cycle.

**Keywords:** peppermint, menthol, herpes simplex virus.

### 1. INTRODUCTION

Herpes simplex viruses (HSV) are endemic in all human populations and pathogenic to human. They are DNA viruses and classified into 2 types. HSV-1 and HSV-2 are members of a subfamily of the *Alphaherpesvirinae*, with common biological activities, but they are different in many aspects. Herpesviruses are large, enveloped

viruses that contain linear DNA. The virus particle consists of a deoxyribonucleic acid genome packaged in icosahedral capsid containing 162 capsomers. The nucleocapsid is surrounded by an envelope that is derived from the nuclear membrane of the infected cell and contains viral glycoprotein spikes. The envelope of the mature virion is acquired in

the cytoplasm by budding into vesicles derived from the golgi complex. The space between the envelope and the nucleocapsid is called the tegument and it contains viral proteins and enzymes that facilitate the viral infection and replication [1].

Herpesvirus genome is double-stranded DNA molecule that consists of nucleotide composition ranged from 32 to 75% G + C, depending on the virus species [2]. The HSV genome encodes 80-100 polypeptides for protein regulation and virus-specific enzymes, including a DNA-dependent DNA polymerase and enzymes such as deoxyribonuclease, thymidine kinase and ribonucleotide reductase. Thus, HSV-1 and HSV-2 genomes are most similar in their Unique long ( $U_L$ ) components, and least similar in the inverted repeats that bound  $U_L$  termed as long component terminal repeat (TRL) and long component internal repeat (IRL). HSV genome has two covalently joined sections of the unique long and unique short regions, both of which are flanked by a pair of inverted repeat regions (TRL-IRL) and (IRS-TRS). The overall organisation of the genome can be represented as TRL-UL-IRL-US-TRS [3].

HSV-1 is the primary agent of orolabial disease or fever blister. The primary symptoms of HSV-1 infection include a prodromal flu-like syndrome with fever, headache, malaise, and diffuse myalgias, followed by local symptoms consisting of itching and appearance of painful papules. In many instances, the infections are asymptomatic, but they may be present as acute gingivostomatitis, eczema herpes, keratoconjunctivitis, genital herpes, meningitis and encephalitis. Genital herpes involves HSV-2 and it is an important sexually transmitted disease, contributing to risk of HIV infection. However in immunocompromised patients and neonates, herpetic infections can cause

serious systemic illnesses [4-7].

After primary infection, HSV ascends peripheral sensory nerves and remains latent within neurons that innervate mucosal and squamous epithelial surfaces in the oral cavity and genital regions. Recurrent infection is characterized by episodic reactivation with milder symptoms compared to the original acute infection. Recurrent herpes labialis is the most frequent clinical manifestation of reactivated HSV-1 infection. Recurrent genital disease is due to reactivation of the initial strain of virus from recently infected sacral nerve root ganglia [8-10].

Since viruses are intracellular parasites and utilize organelles within host cells, it is difficult to completely eliminate viruses. However, the effective chemically synthesized antiviral drugs have been used to treat HSV infection. They are mostly used therapeutically and administered as soon as possible after the first sign of infection. Antiviral agents for treatment of HSV infections include nucleoside derivatives such as acyclovir, penciclovir, famciclovir, valaciclovir and ganciclovir that they have been approved for treatment of HSV infections worldwide but some such antiviral agents may produce toxic side-effects. Acyclovir (ACV) has been widely used for treatment, prevention of HSV infection and being the first of the nucleoside analogues. Once ACV enters HSV infected cells, it is phosphorylated using HSV-encoded thymidine kinase and then it is further phosphorylated to ACV-diphosphate and ACV-triphosphate, which achieved by cellular kinase. ACV-triphosphate specifically inhibits the herpes simplex virus DNA polymerase [11]. However, ACV is expensive, and a major problem associated with ACV therapy is the development of drug resistant strains of HSV. Mutations in the HSV thymidine kinase or DNA polymerase

genes of HSV may occur after long term treatment and these mutants are particularly important as opportunistic infectious agents in immunocompromised patients [12-15].

Antiviral agents from medicinal plants with new effective compounds exhibiting different modes of action against viral infections are urgently needed. Plant extracts offer a potential alternative agent since they are widely used in folklore medicine and they consist of many chemicals for treatment of infectious diseases. Peppermint (Lamiaceae) is widely used in complementary medicine as constituents of medical products, cosmetic, food industry, flavouring, additives, beverage and pharmaceutical industries. The major constituents of peppermint from different sources were found such as menthone, menthofuran, menthol, pulegone, 1,8-cineole, isomenthone, limonene, menthyl acetate, terpenes, isomenthone and carvone [16-20]. Moreover, essential oils of peppermint showed antimicrobial and antiplasmid activities and they have been used to treat symptoms of irritable bowel syndrome, tension headache and colonic spasm [21, 22]. The action of peppermint oil as spasmolytic effect on intestinal smooth musculature via calcium channel antagonistic has been demonstrated [23]. The oil of peppermint was also used as phytoremediator in soils contaminated with heavy metals [19]. Previous study showed that peppermint oil demonstrated anti-HSV activity [24]. Therefore, peppermint crude extracts and pure compound, menthol were selected for investigation of mode of action on various stages of HSV multiplication cycle.

## 2. MATERIALS AND METHODS

### 2.1 Plant extracts and acyclovir

Peppermint was purchased from the Thai Royal Project. Dried plant materials were cut into small pieces, ground and soaked

in dichloromethane and methanol at room temperature. The extracts were filtered, concentrated and lyophilized to form dried extracts. The dried extracts were reconstituted in dimethylsulfoxide (DMSO) and further diluted in medium for determination of cytotoxicity and anti-HSV activity. Pure menthol was purchased from Sigma-Aldrich and prepared as a stock solution of 160 mg/ml in DMSO

Acyclovir (ACV), a commonly used anti-HSV synthetic drug, was used as a positive control (Sigma Aldrich, USA). ACV was dissolved in sterile distilled water and was diluted with MEM before determination of anti-HSV activity. 50% inhibition concentration ( $IC_{50}$ ) of ACV was calculated.

### 2.2 Cell lines and viruses

Green monkey kidney (GMK) cells were grown in monolayers with Eagle's minimum essential medium (MEM) (Hyclone, UK) supplement with 10% heat inactivated fetal calf serum (Starrate, Australia) and 40  $\mu$ g/ml gentamycin. Cells were incubated at 37°C in an atmosphere of 5%  $CO_2$  incubator. HSV-1 strain F and HSV-2 strain G were propagated and grown on GMK cells. The virus stock was prepared from supernatants of infected cells and stored at -85°C until use. The viral infectivity titers were determined by plaque titration assay on confluent GMK cells and were expressed as plaque forming units (PFU) per ml.

### 2.3 Cytotoxicity assay

For the cytotoxicity assay, crude peppermint extracts and pure compound, menthol (Sigma-Aldrich, USA) were prepared in DMSO and serial two-fold diluted with MEM. Nontoxic concentrations of dichloromethane extract of peppermint used in the assay ranged from 0.009-0.078 mg/ml whereas methanol extract of peppermint

ranged from 0.019-0.156 mg/ml. Each dilution of the plant preparations were added in quadruplicated wells of 96-well tissue culture plate. Then, the GMK cell suspensions containing  $1 \times 10^6$  cells/ml were seeded into the culture plate. After 4 days of incubation, the cells were stained with 0.1% crystal violet in 1% ethanol for 15 minutes. The cytotoxicity was expressed as the 50% cytotoxic dose ( $CD_{50}$ ) and calculated according to modified protocol of Reed and Muench [25].

#### 2.4 Plaque titration assay

The cells were seeded into 24-well tissue culture plates and incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator for 2 days. The GMK cells were grown to 70-80% confluence. Viral stocks were serially ten-fold diluted in MEM and each dilution was added to the cell monolayer. After 1 hour adsorption, the infected cells were then overlaid with overlay medium containing 1.5% carboxymethylcellulose and incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator for 4 days before staining with 0.1% crystal violet in 1% ethanol for 15 minutes. Virus plaques were counted and expressed as plaque forming units per milliliter (PFU/ml).

#### 2.5 Plaque reduction assay

Confluent cell monolayer in 24-well tissue culture plates were infected with 100-200 PFU/ 0.1 ml of HSV for 1 hour at room temperature. The infected cells were incubated with the various concentrations of crude peppermint extracts, menthol and ACV. The infected cells were then overlaid with medium, containing 1.5% carboxymethylcellulose. After incubation for 3 days at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  incubator, the infected cells were stained with 0.1% crystal violet, in 1% ethanol, for 15 minutes. The percentage of viral inhibition after treatment with the extracts was calculated as percentage inhibition

compared with the untreated viral infected cells control from triplicate experiments.

#### 2.6 Effect of plant extracts on pretreated cells

Cell monolayers were treated with various non toxic concentrations of crude peppermint extract and menthol for 1 hour. The extracts were removed before adding HSV inoculums. After incubation of the cells with HSV at room temperature for 1 hour, overlay medium was added. The infected cells were incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator for 4 days. The number of plaques was counted and compared with controls.

#### 2.7 Effect of plant extracts on HSV during viral adsorption

Confluent cell monolayers cultivated in 24-well tissue culture plate were infected with 200 PFU/ 0.1 ml of HSV. Then, non toxic concentrations of crude peppermint extracts and menthol were added onto cell monolayers and incubated for 1 hour at room temperature for virus adsorption. After that, the residual inoculum was removed and replaced by overlay medium containing 1.5% carboxymethylcellulose, and incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator for 4 days. After incubation, the virus plaques were stained with 0.1% crystal violet in 1% ethanol for 15 minutes. The number of plaques was counted and the 50% effective doses ( $ED_{50}$ ) were determined from dose-response curves.

#### 2.8 Effect of plant extracts on HSV after viral adsorption

Confluent cell monolayers cultivated in 24-well tissue culture plates were infected with 200 PFU/ 0.1 ml of HSV and incubated for 1 hour at room temperature for virus adsorption. After viral adsorption, non toxic concentrations of crude peppermint extracts and menthol were added onto the infected

cells. Then, the cells were overlaid with overlay medium containing 1.5% carboxymethylcellulose and incubated for 4 days at 37°C in a 5% CO<sub>2</sub> incubator. The number of plaques was counted and the 50% effective doses (ED<sub>50</sub>) were also determined.

### 2.9 Effect of plant extracts on viral replication

Cells were grown in 25 cm<sup>2</sup> flasks until confluence, after which the cells were infected with 1x10<sup>6</sup> PFU/ml of HSV for 1 hour. After that, the infected cells were washed twice with PBS and treated with the highest non toxic concentrations of crude peppermint extracts, menthol, and compared with ACV at IC<sub>50</sub>. Virus-infected cells in flasks containing medium with 2% fetal calf serum were also included as a drug negative control. The infected cells were further incubated at 37°C in a 5% CO<sub>2</sub> incubator and the cells were collected at 18, 24 and 30 hours after viral infection. The infected cells were frozen and thawed twice before determination of virus titers using a plaque titration assay.

### 2.10 Direct inactivation of viral particles

For investigation of the effects of crude peppermint extracts on viral particles, the highest non-toxic concentrations of each extract and menthol were mixed with HSV and incubated for 1, 2, 3 and 4 hours at room temperature. After incubation, the inactivated viral particles were adsorbed onto cells for 1 hour at room temperature. The infected cells were overlaid with overlay medium containing 1.5% carboxymethylcellulose and incubated for 4 days at 37°C in a 5% CO<sub>2</sub> incubator. Then, infected cells were stained with 0.1% crystal violet. The percent of viral plaque inhibition by the extracts were calculated and compared with those of untreated controls.

### 2.11 Statistical analysis

Data were given as mean ± S.D. of three independent experiments. Statistical comparison between groups was analyzed by one way analysis of variance (ANOVA) and Post hoc Tukey's-b test. The *p* values less than 0.05 (*p* < 0.05) were considered significance.

### 2.12 Viral protein determination

Inhibitory effect of peppermint extracts on HSV proteins was determined. The cells were infected with 1x10<sup>6</sup> PFU/ml of HSV-1 or HSV-2 for 1 hour at room temperature. Then, the infected cells were treated with the peppermint extracts at highest non toxic concentration or media without extract for 24 hours. After that, the cells were observed for cytopathic effect by an inverted microscope and harvested after treatment with the extracts by scraping with sterile cell scrapers (Nunc, USA). The pellets were centrifuged in 15 ml centrifuge tube at 2,000 revolutions per minute (rpm) for 5 minutes. The supernatants were discarded and pellets were kept in 1.5 ml microcentrifuge tubes and washed again with cold 1X PBS. HSV protein was extracted by using NucleoSpin<sup>®</sup> kit (MACHEREY-NAGEL, Germany) and separated on polyacrylamide gels using 12% sodium dodecyl sulfate polyacrylamide gels electrophoresis. Each sample of 20 µl was loaded into wells. The gel electrophoresis was performed at 100-125 volts until the dye front moved into gels bottom. The proteins were transferred to one rectangle of nitrocellulose membrane using semi-dry blotting method at 15 volts 4°C for 4 hours. The membranes were washed in saline and incubated overnight at 4°C with 3% bovine serum albumin. After treatment, the membranes were rinsed with saline and then incubated with goat anti herpes simplex virus 1&2:HRP (AbD serotec, USA) with ratio

of 1:100 by dilution of 4  $\mu$ l of antibody in 400  $\mu$ l of 3% BSA. The incubation was performed overnight at 4°C according to modified protocol of Harlow and Lane [26]. Then, the membranes were incubated with 0.06% 4-chloro-1-naphthol and 0.01% H<sub>2</sub>O in 1X PBS for 20 minutes. The membrane was detected through the colorimetric substrate that was converted by the enzyme to colored product and was visible on the membrane as blue-black color. The protein bands were scanned, analyzed and quantified by Gene Tools Match (LAB Focus, Co.ltd).

### 2.13 Gas chromatography/Mass spectrometry assay (GC/MS)

Crude peppermint extracts were analyzed at Department of Chemistry, Faculty of Science, Chiang Mai University. The relative peak area for individual constituents was determined. The quantification was computed as the percentage contribution of

each compound to the total amount present. The percentage composition of extracts was computed by the normalization method from GC peak areas. The Gas chromatography/Mass spectrometry experiments were performed on AgcMethod PN0904 model.

### 3. RESULTS

Yield of dichloromethane and methanol extracts of peppermint were 7.09 and 6.22 % after extraction. Both dichloromethane and methanol extracts of peppermint were tested for toxicity on GMK cells and CD<sub>50</sub> values were calculated according to modified protocol of Reed and Muench [25]. The results showed that CD<sub>50</sub> values of both dichloromethane and methanol extracts of peppermint were 112.29 and 158.87  $\mu$ g/ml. The CD<sub>50</sub> value of menthol was 224.4  $\mu$ g/ml.

The potential inhibitory effects of the

**Table 1** Inhibitory effects of dichloromethane and methanol extracts of peppermint on pretreated cells during and after viral adsorption.

Test	ED <sub>50</sub> ( $\mu$ g/ml)					
	Pretreated cells		During viral adsorption		After viral adsorption	
	HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2
<b>Methanol extract</b>						
Peppermint	145.64 $\pm$ 4.19 (1.09 $\pm$ 0.03) <sup>A</sup>	-	53.19 $\pm$ 8.48 (2.99 $\pm$ 0.53) <sup>A</sup>	85.89 $\pm$ 1.75 (1.85 $\pm$ 0.04) <sup>A</sup>	36.24 $\pm$ 3.24 (4.38 $\pm$ 0.38) <sup>B</sup>	80.77 $\pm$ 1.14 (1.97 $\pm$ 0.03) <sup>A</sup>
<b>Dichloromethane extract</b>						
Peppermint	62.70 $\pm$ 2.92 (1.79 $\pm$ 0.09) <sup>B</sup>	-	26.65 $\pm$ 3.31 (4.21 $\pm$ 0.52) <sup>B</sup>	14.72 $\pm$ 0.30 (7.63 $\pm$ 0.15) <sup>B</sup>	20.74 $\pm$ 0.98 (5.41 $\pm$ 0.26) <sup>C</sup>	35.63 $\pm$ 1.03 (3.15 $\pm$ 0.09) <sup>C</sup>
<b>Menthol</b>	-	-	-	-	125.11 $\pm$ 3.67 (1.79 $\pm$ 0.05) <sup>A</sup>	105.77 $\pm$ 1.56 (2.12 $\pm$ 0.03) <sup>B</sup>

$$() = (TI = CD_{50} / ED_{50})$$

Data in table are given as mean  $\pm$  standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using Post hoc Tukey's-b test. Values with the different alphabets within each column are significantly different ( $P < 0.05$ ).

peppermint extracts and menthol, which is pure compound found in peppermint, against both HSV-1 and HSV-2 were investigated to clarify antiviral activity on pretreated cells, during viral adsorption and after viral adsorption period using plaque reduction assay. After pretreatment of the cells with the extracts, but before their infection with HSV, both dichloromethane and methanol extract of peppermint were effective against only HSV-1 with  $ED_{50}$  values of 62.70 and 145.64  $\mu\text{g/ml}$  and therapeutic indices ( $TI = CD_{50}/ED_{50}$ ) of 1.79 and 1.09. Thus, higher TI reflected higher therapeutic potential of the extracts. However, the cells were not protected from HSV infection by menthol (Table 1).

During viral adsorption to the cells,  $ED_{50}$  values of the dichloromethane extract of peppermint on both HSV-1 and HSV-2 were 26.65 and 14.72  $\mu\text{g/ml}$ , respectively. Thus, the dichloromethane extract of peppermint showed the highest TI of 4.21 and 7.63 during HSV-1 and HSV-2 adsorption to the cells. While,  $ED_{50}$  values of the methanol

extract of peppermint on both HSV-1F and HSV-2G were 53.19 and 85.89  $\mu\text{g/ml}$ , and the TI of 2.99 and 1.85, respectively. Both HSV1 and HSV-2 were not inhibited by menthol during viral adsorption (Table 1).

Anti-HSV activities of the peppermint extracts and menthol were also determined after viral adsorption. The dichloromethane extract of peppermint showed  $ED_{50}$  values of 20.74 and 35.63  $\mu\text{g/ml}$ , which resulted in the highest TI of 5.41 and 3.15 when tested with HSV-1 and HSV-2. The Methanol extract of peppermint showed inhibitory effects on both of HSV-1 and HSV-2 with  $ED_{50}$  values of 36.24 and 80.77  $\mu\text{g/ml}$ , and TI of 4.38 and 1.97, respectively. HSV-1 and HSV-2 were also inhibited by menthol with  $ED_{50}$  values of 125.11 and 105.77  $\mu\text{g/ml}$  and TI of 1.79 and 2.12, respectively.

Direct inactivation of HSV-1 and HSV-2 by the peppermint extracts was evaluated by plaque titration assay at 1, 2, 3 and 4 hours after viral inactivation and the results were compared with the untreated virus control.

**Table 2.** Direct inactivation of HSV by dichloromethane and methanol extracts of peppermint

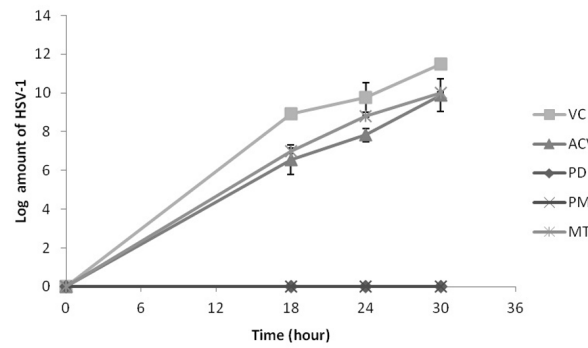
Test	Log amount of viruses (PFU/ml) at one hour interval							
	1		2		3		4	
	HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2
<b>Methanol extract</b>								
Peppermint	4.17±0.38 <sup>B</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>
<b>Dichloromethane extract</b>								
Peppermint	3.53±0.44 <sup>A</sup>	<1 <sup>A</sup>	1.48±0.76 <sup>B</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>	<1 <sup>A</sup>
Menthol	6.01±0.54 <sup>C</sup>	5.84±0.03 <sup>B</sup>	5.73±0.90 <sup>C</sup>	5.99±0.23 <sup>B</sup>	5.74±0.14 <sup>B</sup>	5.49±0.08 <sup>B</sup>	5.36±0.09 <sup>B</sup>	5.00±0.06 <sup>B</sup>
Virus control	6.38±0.07 <sup>D</sup>	6.47±1.20 <sup>C</sup>	6.12±0.85 <sup>D</sup>	6.77±0.20 <sup>C</sup>	6.18±1.50 <sup>C</sup>	6.73±0.60 <sup>C</sup>	6.3±0.19 <sup>C</sup>	6.56±0.80 <sup>C</sup>

Data in table are given as mean  $\pm$  standard deviation (SD) from triplicate experiments. Values with the different alphabets within each column are significantly different ( $P < 0.05$ ).

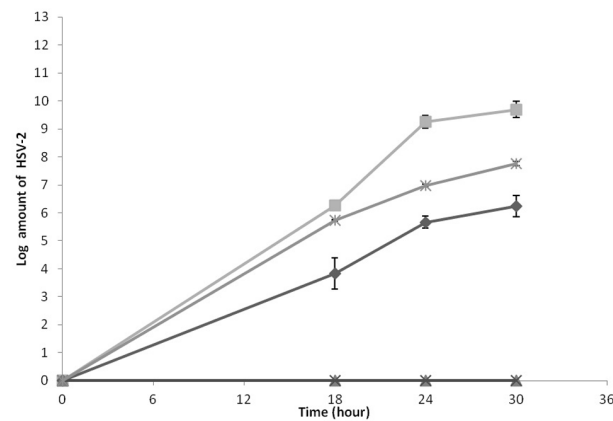
Both dichloromethane and methanol extracts of peppermint completely inactivated HSV-2 within 1 hour. The methanol extract of peppermint was also active against HSV-1 as the viral particles were completely inactivated within 2 hours whereas the viruses were completely inactivated within 3 hours after treatment with dichloromethane extract of peppermint. HSV-1 and HSV-2 were inactivated by reduction of log virus titer (PFU/ml) approximately 1 and 1.5 when treatment with menthol (Table 2).

The efficiency of extracts on viral growth

kinetics was also observed at 18, 24 and 30 hours after viral infection, and compared with the inhibition of viral replication by ACV, and the virus control. ACV was used at  $ED_{50}$  concentrations, which were 1.5 and 1.3  $\mu\text{g}/\text{ml}$  after treatment with HSV-1 and HSV-2, respectively. After 30 hours of viral infection, HSV-1 yield was inhibited, as observed by reduction of log virus titer (PFU/ml) by 9.17, 8.41, 1.49 and 1.61 after treatment with methanol and dichloromethane extract of peppermint, menthol and ACV, respectively (Figure 1), whereas the reductions



**Figure 1.** Titer of HSV-1 at 0, 18, 24 and 30 hours after treatment with dichloromethane extract of peppermint (PD) 78  $\mu\text{g}/\text{ml}$ , methanol extract of peppermint (PM) 156  $\mu\text{g}/\text{ml}$ , and menthol (MT) 156  $\mu\text{g}/\text{ml}$  compared with virus control and ACV at  $ED_{50}$  concentration, 1.5  $\mu\text{g}/\text{ml}$ .



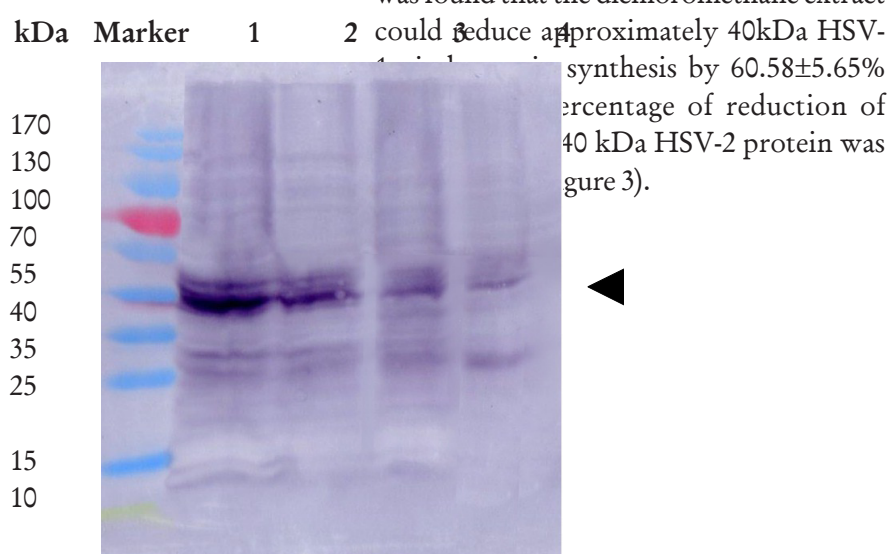
**Figure 2.** Titer of HSV-2 at 0, 18, 24 and 30 hours after treatment with dichloromethane extract of peppermint (PD) 78  $\mu\text{g}/\text{ml}$ , methanol extract of peppermint (PM) 156  $\mu\text{g}/\text{ml}$ , and menthol (MT) 156  $\mu\text{g}/\text{ml}$  compared with virus control and ACV at  $ED_{50}$  concentration, 1.3  $\mu\text{g}/\text{ml}$ .



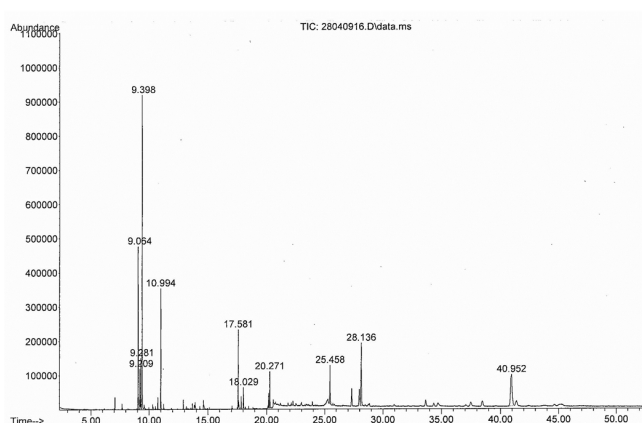
in log HSV-2 titer (PFU/ml) were 9.70, 9.70, 1.94 and 3.46 after treatment with methanol and dichloromethane extract of peppermint, menthol and ACV, respectively (Figure 2).

Moreover, viral proteins of both standard HSV-1 and HSV-2 were

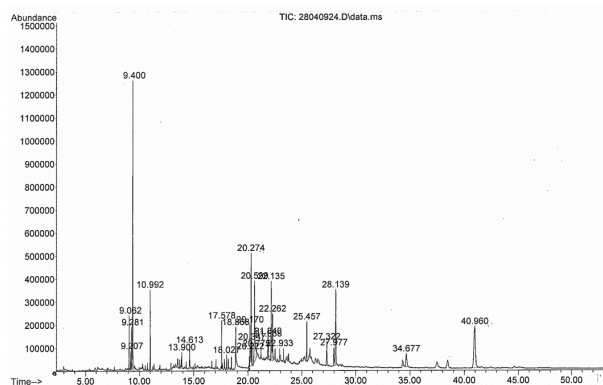
determined in the presence or absence of dichloromethane of peppermint by SDS-PAGE. The HSV proteins were detected with goat anti-herpes virus 1 and 2 conjugated with horseradish peroxidase enzyme. The density of protein bands were analyzed by Gene Tools Match (USA). It was found that the dichloromethane extract



**Figure 3.** Western Blots of HSV-1 proteins control (lane 1), HSV-1 proteins in the presence of dichloromethane extract of peppermint (lane 2), HSV-2 proteins control (lane 3), and HSV-2 proteins in the presence of dichloromethane extract of peppermint (lane 4) after detection by horseradish peroxidase-conjugated IgG against HSV compared with protein marker. Arrow heads indicate the reduction of viral proteins after treatment with the extracts.



**Figure 4.** Chromatograph of dichloromethane extract of peppermint using GC-MS analysis.



**Figure 5.** Chromatograph of methanol extract of peppermint using GC-MS analysis.

The major constituents of dichloromethane extract of peppermint were identified by GC/MS. These compounds were cyclohexanol,5-methyl-2-(1-methylethyl)-(CAS), 3-p-menthanol, menthol, p-Menthan-3-olL(-)-Menthol, Cyclohexanol, 5-methyl-2-(1-methylethyl)-[1R-(1.alpha.,2.beta.,5.alpha.)](CAS), (-)-Menthol, l-Menthol Cyclohexanol, 5-methyl-2-(1-methylethyl)-(CAS) and 3-p-Menthanol (Figure 4). While, the major constituents of methanol extract of peppermint were cyclohexanol,5-methyl-2-(1-methylethyl)-[1s-(1.alpha.,2.beta.,5.beta.)]L(-)-Menthol, Cyclohexanol, 5-methyl-2-(1-methylethyl)-[1R-(1.alpha.,2.beta.,5.alpha.)](CAS), (-)-Menthol, l-Menthol, (R)-(-)-Menthol Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.beta.,5.alpha.)-(+/-)- and Menthol (Figure 5). The different chromatographs of dichloromethane and methanol extracts of peppermint after GC-MS analysis were shown in Figure 4 and 5.

#### 4. DISCUSSION

Many aromatic plants used in phytotherapy are considered to be important sources for the production of raw materials or preparations containing phytochemicals that have significant activity against microorganisms. Thus, plant extracts and essential oils have been widely used in

traditional medicine for treatment of many diseases.

In this study, the dichloromethane and methanol extracts of peppermint were able to prevent pretreated cells from HSV-1 infection. Although the extracts and menthol inhibited HSV-2 by less than 50% and  $ED_{50}$  concentrations could not calculate, cytopathic effects of the infected cells observed under inverted microscope after treatment with the extracts were reduced. Small plaque sizes were determined by measurement the diameter of plaques comparing with controls. The small plaque might result from reduction of viral infectivity to the neighboring cells.

The highest inhibition during HSV-2 adsorption was observed when treatment with the dichloromethane extract of peppermint. Moreover, the most effective extracts against HSV-1 and HSV-2 after viral adsorption were the dichloromethane extract of peppermint. However, inhibitory effect of menthol on HSV was observed only when menthol was applied after HSV-1 and HSV-2 adsorption.

Time dependent virucidal effects of the peppermint extracts on HSV-1 and HSV-2 particles were shown by reduction of amount of viral plaque by 100%. The virucidal activity of the crude peppermint extracts was better than menthol. The similar results were observed since essential oils of peppermint

showed high level of virucidal activity against HSV-1 and HSV-2 and were effective against HSV before viral adsorption [24]. Moreover, anti-herpes simplex virus effects of berberine from *Coptidis rhizoma*, a major component of a Chinese herbal medicine was suggested to interfere with the viral replication cycle after virus penetration and no later than the viral DNA synthesis step [27].

After 30 hour after infection, the methanol and dichloromethane extracts of peppermint showed the highest inhibitory activities on HSV-1 and HSV-2 by reduction of logarithm PFU/ml of viral titer for 8.41-9.70 compared with menthol and ACV at  $ED_{50}$ . Moreover, the dichloromethane extract of peppermint could inhibit approximately 40 kDa HSV-1F protein by 60.58%.

*UL16 gene* of HSV-1 is an essential gene that encodes protein with predicted molecular weight of 40 kDa and other homologs have similar molecule weight. HSV-1 *UL16* gene product colocalizes with intranuclear capsid proteins [28-31]. 40 kDa and 41kDa proteins expressed in *UL16* fusion protein of HSV-1 and HSV-2 in late phase of HSV infection [31]. 40 kDa protein from HSV-1 infected cells was also associated with *UL11* protein, which is important for nucleocapsid development and egress. Thus, 40kDa protein, which is the product of *UL16* gene involved nucleocapsid assemble [32]. Moreover, molecular weight of *VP21 (UL26* gene product) was also around 40-47 kDa and the molecular weight of different forms of HSV-1 *VP22a (UL26.5* gene product) from purified virion and infected cells on Western blots ranged from 36 to 52kDa. These proteins are required as scaffold for assembly of viral capsid [33, 34]. Thus, the observations from this study showed that the peppermint extracts might interfere with herpesvirus capsid assembly on approximately 40 kDa of HSV-1 viral protein.

Many compounds in the extracts were identified by GC/MS. Although, menthol was observed mainly in the extract, inhibitory activity on HSV was not high so that menthol might be not only the compound that affected HSV. The combination of this compound with other compounds in the extract might be necessary to form higher activity of extract. The utilization of crude plant extracts, which composed of many compounds for inhibition of HSV infection, should be performed since the crude extracts were more effective than each compound alone. Each compound might affect different mode of action on HSV inhibition and increased the therapeutic treatment, which resulted from additive or synergistic action of many compounds. Several studies on anti-HSV after combination of many extracts were also reported [35, 36].

## 5. CONCLUSION

Our findings demonstrated that the activity of methanol and dichloromethane extracts of peppermint showed higher anti-HSV activity during and after viral adsorption. Thus, the extracts were able to interfere during the attachment stage of the viruses by directly inhibition of viral particles and also during the viral replication and viral protein expression as observed from the reduction of viral yield and viral proteins. Menthol, which is the main compound in the peppermint extracts showed anti-HSV activity after viral adsorption. Thus, activity of menthol against HSV occurred during viral replication. The results from this study demonstrated the antiviral activity of the peppermint extracts and indicated the potential for development of an alternative therapeutic antiherpetic agent.

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