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Full Length Research Paper

Anti-herpes simplex virus activity of extracts from the culinary herbs *Ocimum sanctum* L., *Ocimum basilicum* L. and *Ocimum americanum* L.

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This study demonstrates anti-herpes simplex virus activity of dichloromethane and methanol extracts of *Ocimum sanctum* L., *Ocimum basilicum* L. and *Ocimum americanum* L. Green monkey kidney cells were protected from HSV-2 infection by the dichloromethane extract of *O. americanum* L. and the methanol extract of *O. sanctum*, with therapeutic indexes (TI) of 1.865 and 1.644, respectively, when the cells were treated before viral infection. Herpes simplex virus-2 (HSV-2) infection was inhibited during viral adsorption when the cells were treated with methanol extracts of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L. with TI of 2.345, 2.473 and 1.563, respectively, whereas dichloromethane extracts of *O. americanum* L. and *O. basilicum* L. resulted in TI of 2.623 and 1.835, respectively. The methanol extract of *O. americanum* L. and the dichloromethane extract of *O. basilicum* L. inhibited HSV-1F with TI of 1.63 and 2.215, respectively, after viral adsorption. The inhibitory effects of extracts on HSV-2G, after viral adsorption, were quite high, for the dichloromethane extract of *O. sanctum* L. and the methanol extract of *O. sanctum* L. with TI of 10.003 and 29.395, respectively. The inhibitory effect of the *O. americanum* L. extract on HSV-1F and HSV-2 yield, after viral replication, was highest 30 h after treatment. The reduction of viral titers by 8.0 and 10.8 folds was observed when cells were treated with dichloromethane and methanol extracts of *O. americanum* L. Moreover, time-dependent virucidal effects of the extract on viral particles were demonstrated, since direct inhibition of both HSV-1F and HSV-2G was shown by a reduction in the amount of plaques by 100%, after treatment with the dichloromethane and methanol extracts of *O. americanum* L. Therefore, dichloromethane and methanol extracts of *O. sanctum* L., *O. basilicum* L. and *O. americanum* L. showed anti-HSV activities at various steps of the viral multiplication cycle.

Key words: Medicinal plant, herpes simplex virus, *Ocimum sanctum* L., *Ocimum basilicum* L., *Ocimum americanum* L.

INTRODUCTION

Herpes simplex virus (HSV) type 1 and herpes simplex virus type 2 are members of a subfamily of the alpha herpes viruses, with common biological activities, but they are different in many aspects. They can infect and establish latency in the neurons of the sensory ganglia.

Moreover, HSV can infect the central nervous system, causing meningitis and encephalitis. Viral latency is a problem in the management of HSV treatment. Lethal infections have also been reported in immunocompromised patients (Whitley, 1990; Morfin and Thouvenot, 2003). Acyclovir is nucleoside analogue, which specifically inhibits DNA polymerase of HSV (Elion, 1993). Consequently, it has been used to treat HSV infection. However, the high cost of this synthetic drug means that

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Many patients can not afford to use it. Furthermore, development of HSV resistant variants may occur after long-term treatment (Crumpacker et al., 1982; Christopher et al., 1998). Plant extracts offer a potential alternative since they are widely used in folklore medicine and they consist of many chemicals for treating disorders or infectious diseases.

The herbs, *Ocimum sanctum* L., *Ocimum basilicum* L. and *Ocimum americanum* L. belong to the vascular plant family Lamiaceae and are cultivated as edible herbs in many countries. Leaves from *Ocimum* species are used as culinary condiments. The plants and their essential oils have been used extensively in food, dental and oral products and perfumery for many years. Moreover, antioxidant activity of *Ocimum* species has also been reported (Politeo et al., 2007; Klem et al., 2000). *O. basilicum* L. (Sweet basil) extracts have been used to treat headaches, cough, diarrhea, warts, constipation, kidney mal-functions and microbial infections (Simon et al., 1999; Wannissorn et al., 2005). Aglycones found in *O. basilicum* L. have high antioxidant capacity (Politeo et al., 2007), whereas antimutagenic properties of linalool, which induce DNA strand breaks have also been demonstrated (Berić et al., 2008). Ethanol extracts of *O. basilicum* L. decrease cholesterol and lipid accumulation in human macrophages (Bravo et al., 2008). Moreover, an extract of *O. sanctum* (Holy basil) showed chemopreventive activity against 7, 12-dimethylbenz (a) anthracene (DMBA)-induced hamster buccal pouch carcinogenesis (Karthikeyan et al., 1999).

In this study, we investigated the potential anti-HSV activity of *O. sanctum* L., *O. basilicum* L. and *O. americanum* L. extracts.

MATERIALS AND METHODS

Cell lines and viruses

African green monkey (GMK) cells were cultivated as monolayers in Eagle's minimum essential medium; MEM (Hyclone, UK) containing 10% heat inactivated fetal bovine calf serum (Starrate, Australia) and 40 µg/ml gentamycin. Cells were incubated at 37°C in 5% CO_2 in a humidified atmosphere incubator. HSV-1 strain F and HSV-2 strain G were propagated on GMK cells. The virus stocks were prepared from supernatants of infected cells and stored at -85°C until use. Viral titers were determined by the plaque titration assay and were expressed as plaque forming units per mililiter (PFU/ml).

Plant extracts and acyclovir

O. americanum L., *O. sanctum* L. and *O. basilicum* L. were purchased from the Thai Royal Project. Voucher specimens were deposited in the herbarium at Queen Sirikit Botanic Garden herbarium, Chiang Mai Province, Thailand. Voucher specimens of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L. are QBG 7430, 39227, and 39244, respectively. Dried plant leaves were milled and soaked in dichloromethane and methanol at room temperature for 3 days. The ratio of plant material to solvent is 50 g/ 5 l. The extracts were filtered, concentrated and lyophilized to form dried powder extracts and then reconstituted in dimethylsulfoxide (DMSO) to be a stock concentration of 160 mg/ml before determination of anti-HSV activity.

Acyclovir (ACV) was purchased in powder from Sigma Aldrich chemical Company, USA and dissolved in sterile water before use. The stock concentration of ACV is 1 mg/ml. ACV was diluted with MEM before testing for its anti-HSV activity. Different concentrations of ACV ranging from 0.39 to 100 µg/ml were applied to culture wells in triplicate. The 50% inhibition concentration (IC_{50}) of ACV was calculated.

Cytotoxicity assay

For evaluation of cytotoxicity, the extracts were reconstituted by dilution of two-fold with MEM. Serial two-fold dilutions of the plant preparations ranging from 1 to 125 µg/ml were added in quadruplicated wells of 96-well tissue culture plate. Thus, final concentrations of DMSO in plant preparations after dilution were less than 2.5% v/v, which did not affect the cells. Then, the GMK cell suspension, containing 1 x 10^6cells/ml, was seeded into the culture plate and after 4 days of incubation, the cells were stained with 0.1% crystal violet in 1% ethanol for 15 min. The cytotoxicity was expressed as the 50% cytotoxic dose (CD_{50}) and calculated according to modified protocol of Reed and Muench (1938).

Plaque titration assay

GMK cells were seeded into 24-well culture plates and incubated at 37°C in 5% CO_2 in an incubator. Serial dilutions of the treated virus were adsorbed on to cell monolayers. Viral stocks were serially diluted in MEM and each dilution was added onto a cell monolayer. After 1 h adsorption, the infected cells were then overlaid with 1.5% carboxymethylcellulose medium and incubated at 37°C in 5% CO_2 in an incubator for 3 days, before staining with 0.1% crystal violet in 1% ethanol for 15 min. Then virus plaques were counted and expressed as plaque forming units per milliliter (PFU/ml).

Plaque reduction assay

Confluent cell monolayers in 24-well tissue culture plates were adsorbed with 100-200 PFU of HSV for 1 h at room temperature. Then, the infected cells were incubated with the extracts at concentrations ranging from 9 to 78 µg/ml and ACV at concentrations range of 0.39 to 100 µg/ml. Infected cells were then overlaid with medium, containing 1.5% carboxymethylcellulose. After incubation for 3 days at 37°C in 5% CO_2, infected cells were stained with 0.1% crystal violet, in 1% ethanol, for 15 min. Percentage of viral inhibition after treatment with the extracts was calculated as percentage inhibition compared with untreated viral infected cells control from triplicate experiments.

Effects of the herb extracts on pretreated cells

Cell monolayers were treated with various non toxic concentrations of herb extracts ranged from 9 to 78 µg/ml for 1 h. The extracts were removed, before adding the HSV inoculum. After incubation at room temperature for 1 h, overlay medium was added. The infected cells were incubated at 37°C in 5% CO_2 for 3 days. The numbers of plaques were counted and compared with the untreated viral infected cell controls.

Effects of the herb extracts on viral particles

HSVs were incubated at room temperature for 1, 2, 3 and 4 h with

various non-toxic concentrations of herb extracts ranging from 39 to 78 µg/ml. After incubation, titers of the virus were determined by plaque titration assay compared with the untreated viral infected cell controls.

Effects of the herb extracts on HSV during viral adsorption

Confluent cell monolayers, cultivated in 24-well plates, were infected with 100 PFU of HSV. Herb extracts at concentrations with range from 9 to 78 µg/ml were added into the cell monolayers and incubated for 1 h at room temperature during virus adsorption. After that, the inoculum was removed and infected cells were overlaid with overlay medium, and incubated at 37°C in 5% CO_2 for 3 days. The virus plaques were stained with 0.1% crystal violet in 1% ethanol for 15 min. The 50% effective dose (ED_{50}) was calculated and compared with the untreated viral infected cell controls.

Effects of the herb extracts on HSV after viral adsorption

Confluent cell monolayers cultivated in 24-well plate were infected with 100 PFU of HSV and incubated for 1 h at room temperature for virus adsorption. After viral adsorption, various non toxic concentrations of the extracts ranging from 9 to 78 µg/ml were added onto the infected cells. Then, the cells were overlaid with overlay medium and incubated for 72 h at 37°C in 5% CO_2 incubator. The virus plaques were stained with 0.1% crystal violet in 1% ethanol for 15 min. The 50% effective dose (ED_{50}) was calculated and compared with the untreated viral infected cell controls.

Effects of the herb extracts on viral replication

Cells were grown in 25 cm^2 flask until confluence, and then infected with 1 x 10^6 PFU/ml of HSV. After incubation, the infected cells were washed twice with phosphate buffered saline (PBS) and treated with non-toxic concentrations of the plant extracts range from 39 to 78 µg/ml. Then, the infected cells were incubated at 37°C in 5% CO_2 and collected 18, 24 and 30 h after viral infection. The infected cells were frozen and thawed twice before determination of virus titers, using plaque titration assay compared with the untreated viral infected cell controls.

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 Turkey's b test. The p values less than 0.05 ($p < 0.05$) were considered significant.

RESULTS

Evaluation of cytotoxicity is an important part of the assessment of a potential antiviral agent since the beneficial extracts should be selective for virus-specific processes with little or no effects on the metabolisms of host cells. Thus, both dichloromethane and methanol extracts of *O. basilicum* L., *O. sanctum* L. and *O. americanum* L. were tested on GMK cells. Cytotoxic doses at 50% were calculated using the method of Reed and Muench (1938). The CD_{50} value for both dichloromethane and methanol extracts of *O. sanctum* L. and *O. americanum* L. was 110 µg/ml, whereas the value for both dichloromethane and methanol extracts of *O. basilicum* L. was 56 µg/ml.

The potential inhibitory effects of the extracts, against both HSV-1F and HSV-2G, were investigated to clarify antiviral activity on pretreated cells, during the adsorption and after adsorption period using plaque reduction assay. After treatment of the cells with the extracts, before their infection with HSV, only the effective doses at 50% (ED_{50}) of the methanol extract of *O. sanctum* L. and the dichloromethane extract of *O. americanum* L. were determined, against HSV-2G, which were 66.96 and 59.1 µg/ml and therapeutic indexes (TI = CD_{50}/ ED_{50}) of 1.644 and 1.865. Other extracts inhibited both HSV-1F and HSV-2G less than 50%. Therefore, ED_{50} could not be calculated (Table 1).

Antiviral activity of these extracts was also determined by adding the extracts both during and after HSV adsorption. Both dichloromethane and methanol extracts of *O. americanum* L. and *O. basilicum* L. affected HSV-2G during adsorption. The ED_{50} of the methanol extract of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L. on HSV-2G were 46.95, 44.49 and 35.83 µg/ml, respectively, and the TI were 2.345, 2.473 and 1.563, respectively. The ED_{50} of the dichloromethane extracts of *O. americanum* L. and *O. basilicum* L. on HSV-2G were 41.95 and 30.58 µg/ml, respectively and the TI were 2.623 and 1.835, respectively (Table 1). In contrast, HSV-1F was inhibited less than 50% during adsorption by all extracts.

In order to determine anti-HSV activity of the plant extracts after viral adsorption, only the ED_{50} of the methanol extracts of *O. americanum* L. and dichloromethane extracts of *O. basilicum* L. were shown against HSV-1F, which were 67.51 and 25.29 µg/ml, respectively, and TI were 1.63 and 2.215, respectively. After HSV-2G adsorption, the ED_{50} of the methanol extracts of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L. were 4.02, 3.95 and 25.73 µg/ml, respectively, and the TI were 27.357, 29.395 and 2.176, respectively. Whereas, the ED_{50} of dichloromethane extract of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L. were 15.64, 11.24 and 30.83 µg/ml and TIs were 7.04, 10.003 and 1.817, respectively.

Direct inactivation of HSV-1F and HSV-2G by the plant extracts was evaluated by plaque titration assay 1, 2, 3 and 4 h after viral inactivation, compared with the untreated virus control. HSV-1F and HSV-2G particles were not found after treatment with the dichloromethane and methanol extracts of *O. americanum* L. 1, 2, 3 and 4 h inactivation.

Moreover, the efficiency of extracts on viral replication was also observed 18, 24 and 30 h after viral infection, and compared with ACV and the virus control. ACV was used at ED_{50} concentrations, which were 1.5 and 1.3 µg/ml when tested with HSV-1F and HSV-2G, respectively.

Both dichloromethane and methanol extracts of *O.*

Table 1. Inhibitory effects of dichloromethane and methanol extracts of *Ocimum sanctum* L., *O. basilicum* L. and *O. americanum* L. on pretreated cells, during and after viral adsorption.

Test	ED_{50} (µg/ml)					
	Pretreated cells		During viral adsorption		After viral adsorption	
	1F	2G	1F	2G	1F	2G
Methanol extracts						
O. americanum L.	-	-	-	46.95 ± 1.80 ^{d} (2.345 ± 0.092) ^{C}	67.51 ± 2.00 ^{b} (1.630 ± 0.048) ^{A}	4.02 ± 0.05 ^{a} (27.357 ± 0.321) ^{B}
O. sanctum L.	-	66.96 ± 2.62 ^{a} (1.644 ± 0.064) ^{A}	-	44.49 ± 0.77 ^{cd} (2.473 ± 0.043) ^{CD}	-	3.95 ± 1.20 ^{a} (29.395 ± 7.655) ^{B}
O. basilicum L.	-	-	-	35.83 ± 0.43 ^{b} (1.563 ± 0.019) ^{A}	-	25.73 ± 0.06 ^{d} (2.176 ± 0.005) ^{A}
Dichloromethane extracts						
O. americanum L.	-	59.10 ± 2.62 ^{a} (1.865 ± 0.107) ^{A}	-	41.95 ± 0.90 ^{c} (2.623 ± 0.056) ^{D}	-	15.64 ± 0.53 ^{c} (7.040 ± 0.246) ^{A}
O. sanctum L.	-	-	-		-	11.24 ± 1.95 ^{b} (10.003 ± 1.883) ^{A}
O. basilicum L.	-	-	-	30.58 ± 1.67 ^{a} (1.835 ± 0.100) ^{B}	25.29 ± 0.46 ^{a} (2.215 ± 0.040) ^{B}	30.83 ± 0.59 ^{e} (1.817 ± 0.035) ^{A}

( ) = (TI = CD_{50}/ED_{50}); Data in table are given as mean ± 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 ($P < 0.05$).

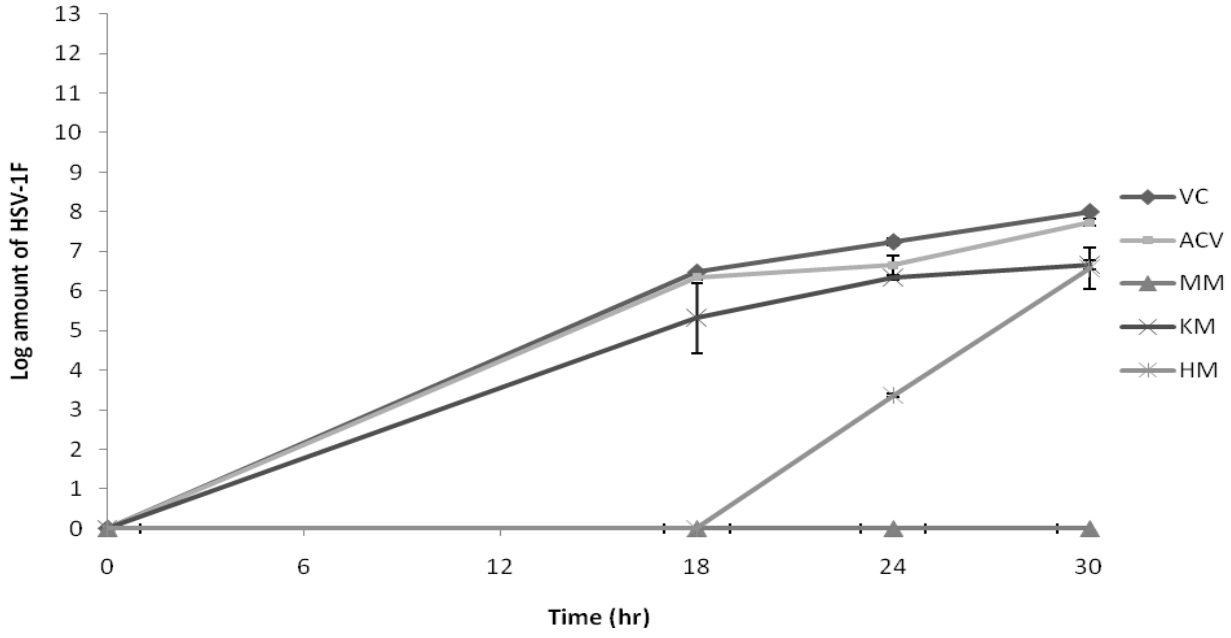

Figure 1. Titer of HSV-1F at 0, 18, 24 and 30 h after treatment with methanol extract of *O. americanum* L. (MM) 78 µg/ml, *O. sanctum* L. (KM) 78 µg/ml and *O. basilicum* L. (HM) 39 µg/ml compared with untreated virus control and ACV at ED_{50} concentration, 1.5 µg/ml.

americanum L., *O. sanctum* L. and *O. basilicum* L. were used at maximum non toxic concentrations of 80, 80 and 40 µg /ml, respectively. After 30 h of viral infection, HSV-1F yield was inhibited, as observed by reduction of log virus titer (PFU/ml) by 8.0, 1.4 and 1.4 after treatment with methanol extract of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L., respectively (Figure 1), whereas the reduction of log virus titer (PFU/ml) by 8.0, 0.8 and

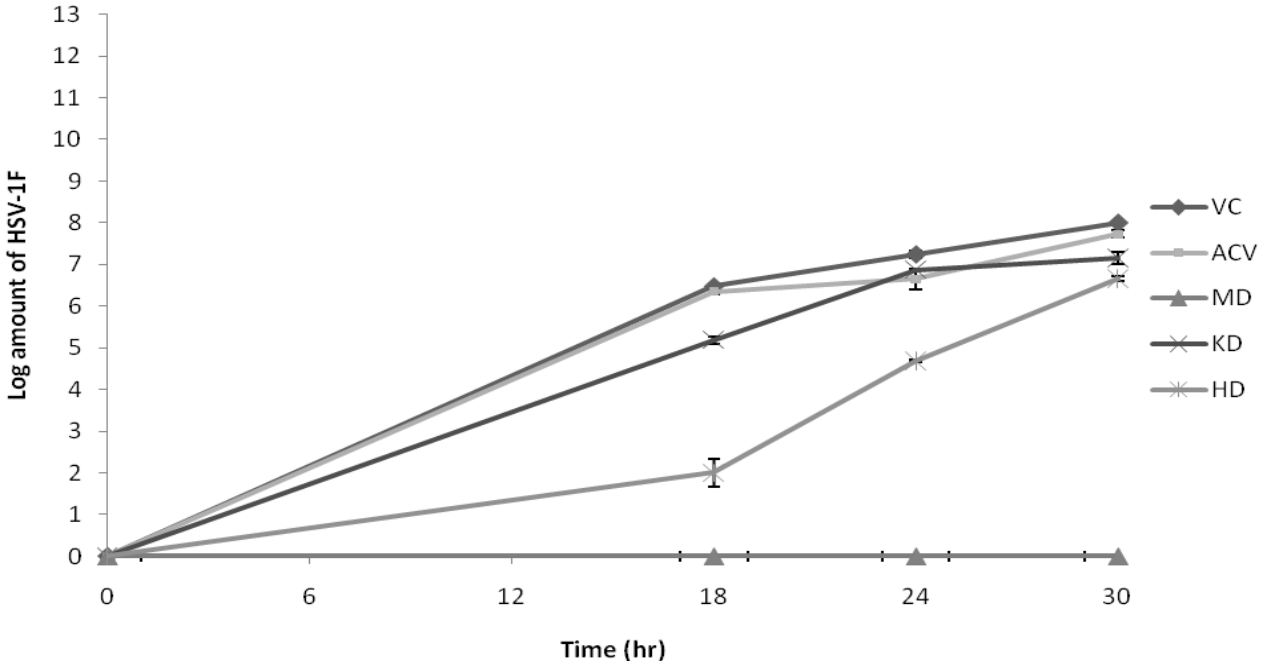

Figure 2. Titer of HSV-1F at 0, 18, 24 and 30 h after treatment with dichloromethane extract of *O. americanum* L. (MD) 78 µg/ml, *O. sanctum* L. (KD) 78 µg/ml and *O. basilicum* L. (HD) 39 µg/ml compared with untreated virus control and ACV at ED_{50} concentration, 1.5 µg/ml.

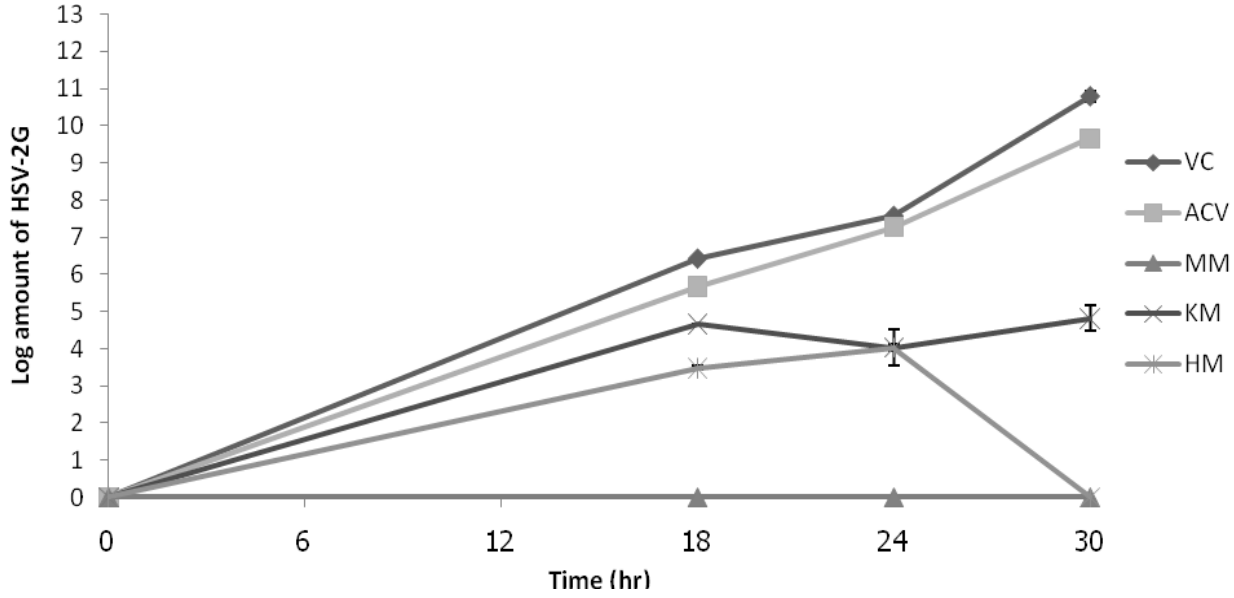

Figure 3. Titer of HSV-2G at 0, 18, 24 and 30 h after treatment with methanol extract of *O. americanum* L. (MM) 78 µg/ml, *O. sanctum* L. (KM) 78 µg/ml and *O. basilicum* L. (HM) 39 µg/ml compared with untreated virus control and ACV at ED_{50} concentration, 1.3 µg/ml.

1.3 was determined after treatment with dichloromethane extract of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L., respectively (Figure 2).

Moreover, HSV-2G titer was reduced after treatment for 30 h. The reduction of log virus titer (PFU/ml) was 10.8, 6.0 and 10.8 after treatment with methanol extract of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L. respectively (Figure 3), whereas the reduction of log virus titer (PFU/ml) was 10.8, 6.7 and 10.8 after treatment with dichloromethane extract of *O. americanum* L., *O.*

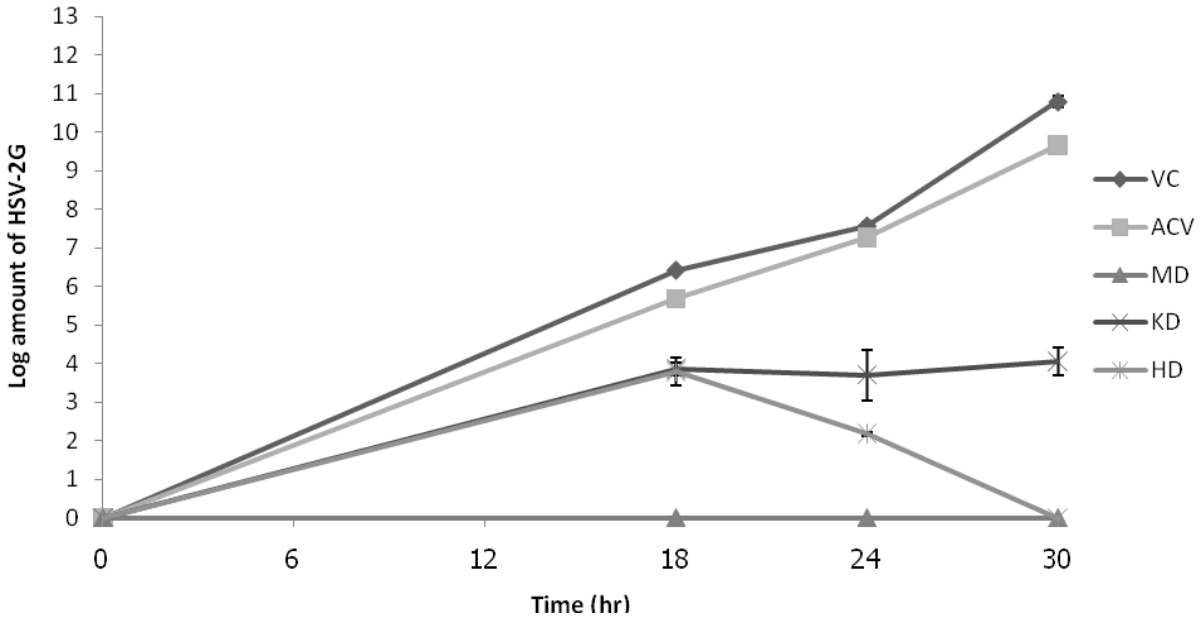

Figure 4. Titer of HSV-2G at 0, 18, 24 and 30 h after treatment with dichloromethane extract of *O. americanum* L. (MD) 78 µg/ml, *O. sanctum* L. (KD) 78 µg/ml and *O. basilicum* L. (HD) 39 µg/ml compared with untreated virus control and ACV at ED_{50} concentration, 1.3 µg/ml.

sanctum L. and *O. basilicum* L. respectively (Figure 4).

DISCUSSION

Nowadays, various medicinal plants and culinary herbs are attracting interest for the development of new, more effective and specific-antimicrobial agents, because they may be used for the production of raw materials or preparations containing phytochemicals with significant activity against microorganisms. Plant extracts and essential oils have been widely used in traditional medicine for treatment of many diseases. Recently, the anti-HSV activity of some plant essential oils, as well as various constituents of essential oils, have been reported (Schuhmacher et al., 2003).

In this study, we observed that dichloromethane and methanol extracts of *O. sanctum* L., *O. basilicum* L. and *O. americanum* L. showed anti-HSV activity at different modes of action. Cell infection was prevented by the dichloromethane extract of *O. americanum* L. and the methanol extract of *O. sanctum* L., since inhibitory activity on HSV-2G was observed with TI of 1.865 and 1.644, when the cells were treated before viral infection.

Moreover, interference with viral adsorption was evident as HSV-2 infection was reduced when cells were treated with methanol extract of *O. americanum* L., *O. sanctum* L. and *O. basilicum* L. with TI of 2.345, 2.473 and 1.563, respectively, whereas the dichloromethane extract of *O. americanum* L. and *O. basilicum* L. showed TIs of 2.623 and 1.835, respectively. Essential oils from other culinary herbs have also been reported to affect the virus before adsorption e.g. *Melissa officinalis* (lemon balm), *Mentha piperita* (peppermint), *Matricaria vecutita* (chamomile), *Hyssopus officinalis* (hyssop), *Thymus vulgaris* (thyme) and *Zingiber officinale* (ginger) (Schnitzler et al., 2008; Schuhmacher et al., 2003; Koch et al., 2008).

The methanol extract of *O. basilicum* L. showed, significantly inhibited HSV-1F higher than the dichloromethane extract of *O. americanum* L. with TI of 2.215 and 1.630, respectively, after viral adsorption. The efficiency of extracts on HSV-2G, after viral adsorption, was quite high, since treatment with dichloromethane extract of *O. sanctum* L. showed TI of 10.003 and treatment with methanol extract of *O. americanum* L. and *O. sanctum* L. showed significantly highest TI of 27.357 and 29.345, respectively.

The efficiency of extracts on viral replication was also demonstrated, since the highest inhibition of HSV-1F yield was observed 30 h after treatment with both dichloromethane and methanol extracts of *O. americanum* L., resulting in a reduction of logarithm PFU/ml of viral titer of 8.0. The highest inhibition of HSV-2G yield was also expressed as a reduction of logarithm PFU/ml of viral titer by 10.8, when treating with both dichloromethane and methanol extracts of *O. americanum* L.. Therefore, extracts of *O. americanum* L. showed the highest antiviral activities on both HSV-1F and HSV-2G, 30 h after viral infection compared with the ACV at ED_{50}.

Ocimum species have also been reported to show activities against a wide variety of disease, antioxidant and antibacterial activities (Politeo et al., 2007; Klem et al., 2000; Simon et al., 1999; Wannissorn et al., 2005).

The chemical composition of these plants were reported such as eugenol, 1,8-cineole (Murbach Freire et al., 2006) and ursolic acid (Chiang et al., 2005). Several studies on anti-HSV activity have been reported. Pure constituent, ursolic acid isolated from crude extract of *O. basilicum* L. was shown to have anti-HSV activity with TI of 15.2; apigenin, linalool and ursolic acid isolated from the aqueous extract of *O. basilicum* L. were active against adenovirus and enterovirus (Chiang et al., 2005).

Moreover, time dependent virucidal effects of the extract on viral particles were demonstrated. The direct inhibition of both HSV-1F and HSV-2G was shown by a reduction of the amount of plaques by 100%, after treatment with the dichloromethane and methanol extracts of *O. americanum* L. The results from this study will be useful to clarify the antiviral activity of extracts of the culinary herbs; *O. sanctum* L., *O. basilicum* L. and *O. americanum* L. and indicate potential for the development of therapeutic antiherpetic drugs.

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Abbreviations

HSV, Herpes simplex virus; **DMBA ,** 7, 12-dimethylbenz (a) anthracene; **GMK,** African green monkey; **MEM,** minimum essential medium; **DMSO,** dimethylsulfoxide; **ACV,** Acyclovir; **PBS,** phosphate buffered saline.

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