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Contributed Paper

Efficacy of *Houttuynia cordata* Thunb. Extracts Against Herpes Simplex Virus Infection

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ABSTRACT

The efficacy of the aqueous and ethanolic extracts of *Houttuynia cordata* Thunb. in inhibiting herpes simplex virus (HSV) is demonstrated in this present study. The inhibitory effect of the plant extracts in the various stages of the HSV multiplication cycle was investigated using plaque reduction assay. The result showed that the CD_{50} values of the aqueous and ethanolic extracts of *H. cordata* were 2845.3 μg/ml and 1426.0 μg/ml, respectively. The highest anti-HSV-1 and HSV-2 of the aqueous extract of *H. cordata* was observed when the extract was treated during viral attachment with therapeutic index (TI) values of 3.51±0.0 and 4.48±0.11. However, the ethanolic extract of *H. cordata* showed the highest direct inactivation of both the types of HSV at 20 minutes of treatment. Furthermore, it was observed that viral multiplication was inhibited with the highest reduction of log HSV-1 and HSV-2 titers at 30 hours after treatment with the ethanolic extract of *H. cordata*. The ethanolic extract of *H. cordata* also exerted potent inhibitory effect against viral DNA and protein synthesis. Therefore, the *H. cordata* plant extract should be used as a potential medicinal plant for the treatment of herpes simplex virus infection.

Keywords: herpes simplex virus, *Houttuynia cordata*, medicinal plant extract, anti-viral activity

1. INTRODUCTION

Herpes simplex virus (HSV) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*. The viruses are classified into two types: HSV-1 and HSV-2. HSV can infect several localized skin areas, such as the cutaneous membrane, mucous membrane, lips, oral cavity, facial area, pharynx, and genital area [1-3]. Both the types of HSV are characterized by physiochemical property, serological test, and clinical epidemiology, as well as the site of latency in sensory neural ganglia. Normally, the sites of HSV-1 and HSV-2 latency are the trigeminal ganglion and sacral ganglion. Thus, evidently, HSV infection is an important viral disease worldwide since the disease is easily transmitted from one person to the other [4].

Nowadays, HSV infection is not entirely

cured by antiviral drugs because the virus remains for a lifetime in the body, and the latent virus is frequently reactivated from nerve ganglia, leading to the major difficulty in the treatment of this disease. Synthetic drugs for the treatment of HSV infection have been approved and are continuously being developed for more effective inhibitors of the viral metabolite pathways and for specific targets for drug action. The drugs are generally used to reduce the severity of infection and to expedite the wound-healing process. Thus, effective nucleoside analogue drugs, such as acyclovir (ACV), and other nucleoside derivatives, such as famciclovir, ganciclovir, penciclovir, and valaciclovir, have been used to treat HSV infection [5-6]. However, these chemical synthetic drugs are quite expensive, and these drugs may cause serious side effects such as the development of viral resistance against antiviral drugs after receiving long-term prophylactic treatment. As a result of these problems, the generally and widely followed therapies for curing HSV diseases have been rather ineffective [1-2, 7].

There are many health promotions and therapies which propose the use of medicinal plants as potential medicaments. Medicinal plants have exhibited many pharmacological activities and have been known to have low toxicity [8]. These plants have been widely used in primary healthcare for a long time for the prevention and treatment of a variety of infectious and non-infectious diseases. Thus, novel antiviral medicines have been sourced from natural products to treat HSV infection. *Houttuynia cordata* Thunb, or "Khao-tong," or "Plu-khao" in Thai, is a medicinal plant belonging to the family *Saururaceae*. This plant has been widely used for long in the eastern and southern regions of Asia. In Chinese medicaments, *H. cordata* has also been used as a therapeutic medicinal plant in folk medicine to treat various kinds of diseases [8-13]. *H. cordata* plants are edible plants that have thin stalks, heart-shaped leaves, and a fishy smell [11-12, 14]. Bioactive compounds such as flavonoids, methyl nonyl ketone, camophyllene, bornyl acetate, α-pinene, β-pinene, limonene, pectic-like substance, tannin, terpenoids, geraniol ester, camphene, sabinene, n-decanal contents, caryophyllene, aldedhyde, glycones, phenolic compounds, limonene, 4-terpineol, tetradecanoyleiter, methyl nonyl ketone, and tetradecanoyl-phorbol-acetate have been identified in *H. cordata* [8, 11]. Therefore, it is evident that *H. cordata* is a hub of pharmacological activities such as antimicrobial, antiviral, antiseptic, anti-inflammatory, and anticancer activities. The plant is used to increase mast cell activation, inhibit anaphylactic reaction, and treat many diseases such as chronic sinusitis and nasal polyps, besides being used as a diuretic [8, 11-12, 15-16].

Thus, *H. cordata* medicinal plant extracts are safe, have low toxicity, and are suitable for use as an alternative anti-HSV agent obtained from natural sources. In this study, the efficacy of the aqueous and ethanolic extracts of *H. cordata* was investigated for their inhibitory effect against both the types of HSV.

2. MATERIALS AND METHODS

2.1 Plant Material and Plant Extract

Dried aerial parts of *H. cordata* were purchased from Lampang Herb Conservation, Thailand. The dry plant powders were extracted using the powders and a solvent in the ratio of 1:10 (w/v). The extraction was performed twice with distilled water at 45°C for 3 hours or 95% ethanol at room temperature for 3 days. Then, the plant extracts were filtered through Whatman No.1 and concentrated using a rotary evaporator to remove the solvent that was used for extraction; thereafter, the

filtrate was lyophilized. The dry extract of *H. cordata* was reconstituted by dissolving in dimethylsulfoxide (DMSO), and then it was kept at 4°C in an amber glass bottle before investigating for anti-HSV activity.

2.2 Cell Line and Viruses

Standard herpes simplex virus type 1 (HSV-1; F strain) and type 2 (HSV-2; G strain) were propagated on Vero cells. The cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and 40 μg/ml gentamycin sulfate. The infected cells were maintained at 37°C in a humidified incubator having 5% CO_2 atmosphere, following which the quantitation of the virus titers was conducted using standard plaque titration assay and expressed as plaque forming unit/ml (PFU/ml).

2.3 Cytotoxicity of Plant Extracts Using Cell Viability Assay

The cytotoxicity of the *H. cordata* extracts was determined on Vero cells using cell viability assay. The extracts were serially two-fold diluted with MEM, and different concentrations of the extracts were added to quadruplet wells on a 96-well culture plate. Then, Vero cells at a concentration of 1×10^6 cells/ml were added to each well. After incubation for 96 hours, the cell viability was determined by staining with 0.1% crystal violet in 1% ethanol for 20 minutes. A total of 50% cytotoxicity dose (CD_{50}) of the plant extract was calculated [17].

2.4 Plaque Titration Assay

Titers of HSV were investigated using plaque titration assay. Vero cells were seeded into a 24-well plate until confluence as a monolayer prior to infection of each with a dilution of HSV and incubation for 1 hour at room temperature. Each well was overlaid with an overlay medium containing 1.5% sodium carboxymethyl cellulose and a growth medium (1:3), and further incubated at 37°C in a humidified 5% CO_2 atmosphere for 3-4 days. Plaque formation was observed after the media was removed, and the cells were fixed and strained with 0.1% crystal violet in 1% ethanol for 20 minutes. The numbers of plaque were counted and expressed as plaque forming unit/ml (PFU/ml).

2.5 Plaque Reduction Assay

Vero cells were infected with 100 plaques of HSV per well in a 24-well plate for 1 hour at room temperature. Plant extracts at non-toxic concentrations were added and overlaid with the overlay medium. After 3-4 days of incubation at 37°C in a humidified 5% CO_2 atmosphere, the media were discarded and the cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. Then, the numbers of plaque were counted and expressed as PFU/ml. A total of 50% effective dose (ED_{50}), which was the concentration of extracts that showed an inhibition of the plaque numbers by 50%, was calculated from the dose-response curves in comparison with that of ACV and virus control.

2.6 Effect of Plant Extracts on HSV Before Viral Attachment

For the HSV inhibition assay, a monolayer of Vero cells, which were grown in 24-well tissue culture plate, was treated with non-toxic concentrations of extracts for 30 minutes at room temperature. After that, the culture media was removed prior to adding the virus and the overlay medium. After incubation at 37°C in 5% CO_2 incubator for 3-4 days, plaque formation was observed after the removal of the inoculum; thereafter, the infected cells were stained with 0.1%

crystal violet in 1% ethanol for 20 minutes. The inhibition of HSV infectivity was calculated in comparison with that of antiviral agents, ACV, and virus control.

2.7 Effect of Plant Extracts on HSV During Viral Attachment

HSV (100 plaques/well) and non-toxic concentrations of plant extract were added on Vero cells in a 24-well tissue culture plate. After viral adsorption for 30 hours at room temperature, the inoculum was removed before adding overlay medium, and incubated at 37°C in a humidified incubator having 5% CO_2 for 3-4 days. After the incubation, plaque formation was observed once the inoculum was removed; thereafter, the infected cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. The inhibition of HSV infectivity was calculated in comparison with that of antiviral agents, ACV, and virus control.

2.8 Effect of Plant Extracts on HSV After Viral Attachment

Vero cells were added into 24-well tissue culture plate and incubated at 37°C in 5% CO_2 incubator. Then, the cell monolayer was infected with 100 plaques of HSV for 1 hour. After infection, plant extracts at non-toxic concentration were added to the infected cells. Then, the mixture was incubated at 37°C in a humidified incubator with 5% CO_2 for 3-4 days, and the infected cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. The inhibition of HSV infectivity was calculated in comparison with that of ACV and virus control.

2.9 Inactivation Kinetics

Direct inhibition of HSV was measured by way of incubation of HSV with non-toxic concentrations of aqueous plant extracts at room temperature for 20, 40, 60, 80, 100,and 120 minutes, while a mixture of virus and ethanolic extract was incubated for 5, 10, 15, and 20 minutes. At the end of the incubation period, the mixture was kept at -80°C. The HSV titer was determined using plaque titration assay, and compared to the untreated virus control.

2.10 Effect of Plant Extracts on HSV Replication

Vero cells were infected with HSV at room temperature. After 1 hour of adsorption, the inoculum was removed and the infected cells were washed twice using PBS 1X, prior to treatment with non-toxic concentrations of plant extracts. Then, the treated cells were incubated at 37°C in a humidified 5% CO_2 atmosphere, and the cells were collected at 0, 1, 2, 3, 4, 5, 6, 12, 24, and 30 hours after treatment. After that, the infected cells were frozen and thawed twice, and the virus was kept at 80°C. The virus titer was investigated using plaque reduction assay, and compared to the infected cells control.

2.11 Effect of Plant Extracts on HSV DNA Synthesis

Vero cells were infected with HSV-1 or HSV-2 with a multiplicity of infection (MOI) of 1 in the presence or absence of extracts. The infected cells were collected when the cytopathic effect was observed by 80-90%. Then, the cells were lysed with lysing solution. Thereafter, 5M sodium chloride, 10% SDS, 10 mg/ml of proteinase K, and 10 mg/ml of RNase A were added, and the mixture was incubated at 37°C for 1 hour and 30 minutes. After that, extraction was performed using the ratios phenol: chloroform: isoamyl alcohol (50:50:1) and chloroform:isoamylalcohol (50:1). The solution was precipitated by adding 3 M sodium acetate and cold absolute ethanol, and kept at -20°C overnight to obtain viral DNA.

The viral DNA was washed with 70% ethanol after centrifugation at 10000 rpm. The viral DNA was then dried and resuspended in 20 µl sterile distilled water. The amount of viral DNA after treatment with the extract was visualized by using agarose gel electrophoresis, and compared to viral DNA of untreated control.

2.12 Effect of Plant Extracts on HSV Protein Synthesis and Western Blot Analysis

Vero cells were infected with HSV-1 and HSV-2 with MOI of 1, and the cells were treated with the plant extracts. The infected cells were collected by scraping with sterile cell scrapers when the cytopathic effect was 80-90%. Then, NP-40 lysis buffer containing protease inhibitors cocktail No.3 (1:200) (Calbiochem) was added. After centrifugation, the supernatant of the cell lysate was collected and frozen at -80°C. The total viral proteins were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein was transferred to nitrocellulose membrane using Towbin transfer buffer, pH 8.3. Next, the membrane was rinsed with saline and incubated with 3% bovine serum albumin in 0.9% normal saline overnight, followed by incubation with horseradish peroxidase-conjugated IgG against HSV (AbDserotec) for 24 hours. Finally, the filter was washed in saline and incubated in 0.06% 4-chloro-1-naphthol (sigma)/0.01% H_2O_2 in phosphate buffer saline (PBS) to observe the HSV protein.

2.13 Statistical Analysis

Randomized complete blocks (RCB) and Post hoc Tukey's b statistical tests were used to determine the differences between the experimental and control groups.

3. RESULTS

The aerial part of *H. cordata* was extracted using water and ethanol. The percentages of yield of the aqueous and ethanolic extracts of *H. cordata* were 5.0% and 6.68%, respectively, after extraction. The toxicities of the aqueous and ethanolic *H. cordata* extracts were determined using the cell viability assay on Vero cells and expressed as 50% cytotoxicity dose (CD_{50}), with the calculations carried out according to the modified protocol of Reed and Muench (1938). The obtained result showed that the CD_{50} values of the aqueous and ethanolic extracts of *H. cordata* were 2845.30 µg/ml and 1426.00 µg/ml, respectively. Both the aqueous and the ethanolic extracts were selected at the non-toxic concentrations of 2000 µg/ml and 1000 µg/ml, respectively.

The efficacy of *H. cordata* extracts against HSV when used for treatment with the extracts before, during, and after viral attachment to the cells using plaque reduction assays was investigated. It was found that the aqueous and ethanolic extracts of *H. cordata* could not inhibit either of the types of HSV when the treatment was carried out before the virus attachment. The aqueous and ethanolic extracts of *H. cordata* could protect against HSV-1 infection when added during the HSV attachment, with therapeutic index (TI) values of 3.51±0.0 and 1.6±0.01, while HSV-2 infection was inhibited with TI values of 4.48±0.11 and 3.73±0.02, respectively (Table 1). Additionally, the efficacy of the *H. cordata* extracts was investigated also when the treatment was done after the HSV attachment to the cell culture. It was found that the aqueous and ethanolic extracts of *H. cordata* could inhibit HSV-1 after attachment, with TI values of 2.23±0.01 and 2.67±0.01, respectively. However, only the aqueous extract of *H. cordata* was found to be able to inhibit HSV-2, with a TI value of 1.58±0.01 (Table 1).

Table 1. Inhibitory Effect of Aqueous and Ethanolic Extracts of *H. cordata* on Herpes Simplex Virus.

Medicinal plant extracts	TI value*					
	Before viral attachment		During viral attachment		After viral attachment	
	HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2
Aqueous	-^{A}	-^{A}	3.51±0.00^{A}	4.48±0.11^{A}	2.23±0.01^{A}	1.58±0.01^{A}
Ethanolic	-^{B}	-^{B}	1.6±0.01^{B}	3.73±0.02^{B}	2.67±0.01^{B}	-^{B}

*TI = Therapeutic index (CD_{50}/ED_{50}); the data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical comparison between the groups in each column done using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group (A, B) within each column showed significantly different value ($P < 0.05$).

In addition, the ability for direct inactivation of the HSV particles by the *H. cordata* extracts was also determined. The result showed that the HSV-1 titers were inhibited by 87.12±0.15%, 98.70±0.00%, and 99.91±0.01% after treatment for 20 minutes with the ethanolic extract of *H. cordata* at 250 μg/ml, 500 μg/ml, and 1000 μg/ml, respectively. The HSV-2 titers were also found to have reduced by 85.77±0.93%, 99.58±0.03%, and 100±0.00% after treatment with the ethanolic extract of *H. cordata* at 250 μg/ml, 500 μg/ml, and 1000 μg/ml, respectively (Table 2). Significantly, the highest inhibition of HSV-1 and HSV-2 at 120 minutes was observed to be by 91.98±0.91% and 92.04±0.04% when the treatment was carried out with the aqueous extract of *H. cordata* at 2000 μg/ml (Table 3).

Table 2. Direct Inactivation of HSV-1 and HSV-2 by Ethanolic Extract of *H. cordata.*

Time (minute) / (μg/ml)	Inhibition of ethanolic extract of *H.cordata* against HSV infection (%)*			
	5	10	15	20
HSV-1				
1000	99.36±0.00^{B}	99.69±0.37^{B}	99.83±0.04^{B}	99.91±0.01^{B}
500	93.70±0.58^{B}	95.88±0.43^{B}	98.35±1.72^{B}	98.70±0.00^{B}
250	36.89±1.42^{A}	55.65±0.63^{A}	61.50±0.71^{A}	87.12±0.15^{A}
HSV-2				
1000	99.76±0.01^{b}	100.00±0.00^{b}	100.00±0.00^{b}	100.00±0.00^{b}
500	97.29±0.78^{b}	98.91±0.01^{b}	99.52±0.01^{b}	99.58±0.03^{b}
250	27.27±1.42^{a}	43.34±1.43^{a}	63.96±0.48^{a}	85.77±0.93^{a}

*The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group (A, B or a, b) within each column showed significantly different value ($P < 0.05$).

Table 3. Direct Inactivation of HSV-1 and HSV-2 by Aqueous Extract of *H. cordata.*

Time (minute) / (μg/ml)	Inhibition of aqueous extract of *H.cordata* against HSV infection (%)*					
	20	40	60	80	100	120
HSV-1 (2000)	23.34± 0.47^{A}	40.28± 0.67^{AB}	56.18± 0.57^{AB}	84.49± 0.84^{B}	88.44± 0.86^{B}	91.98± 0.91^{B}
HSV-2 (2000)	43.25± 0.64^{A}	79.31± 0.00^{AB}	85.69± 0.91^{AB}	88.08± 0.54^{B}	90.80± 0.21^{B}	92.04± 0.04^{B}

*The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets (A, B, AB) within each column showed significantly different value ($P < 0.05$).

The inhibition effect of the aqueous and ethanolic extracts of *H. cordata* against HSV replication was also examined and observed by way of yield reduction assay. HSV was inhibited by the aqueous extract of *H. cordata* at a concentration of 2000 μg/ml at 30 hours after incubation. HSV-1 and HSV-2 titers were observed to have reduced by 1.48±0.10 log PFU/ml and 1.52±0.34 log PFU/ml, compared to the controls. The ethanolic extract at concentrations of 250 μg/ml, 500 μg/ml, and 1000 μg/ml showed inhibition of HSV-1 replication at 30 hours by 3.52±0.30 log PFU/ml, 4.84±1.43 log PFU/ml, and 5.10±0.23 log PFU/ml, respectively, whereas HSV-2 replication was inhibited by 3.46±0.26 log PFU/ml, 4.72±1.23 log PFU/ml, and 5.01±1.43 log PFU/ml, respectively (Table 4, Figure 1, and Figure 2).

Table 4. Yield Reduction of Log HSV-1 and HSV-2 Titers after Treatment with Aqueous and Ethanolic Extracts of *H. cordata* at 30 hours after Viral Infection.

Medicinal plant extracts	Concentration (μg/ml)	Yield reduction ± SD*	
		Log PFU/ml of HSV-1	Log PFU/ml of HSV-2
Aqueous extract	2000	1.48±0.10^{A}	1.52±0.34^{A}
Ethanolic extract	1000	5.10±0.23^{D}	5.01±1.43^{D}
	500	4.84±1.43^{C}	4.72±1.23^{C}
	250	3.52±0.30^{B}	3.46±0.26^{B}

*The data were presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis was conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b statistical tests. The values with the different alphabets (A, B, C, D) within each column showed significantly different value ($P < 0.05$).

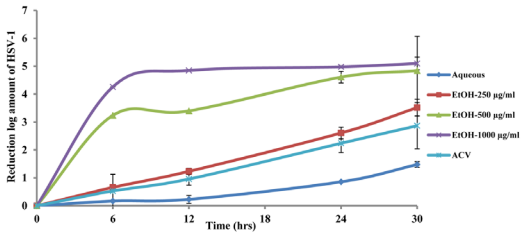

Figure 1. The reduction in log PFU/ml of HSV-1 at 0, 6, 12, 24, and 30 hours after treatment with the aqueous (2000 µg/ml) and ethanolic extracts (250 µg/ml, 500 µg/ml, and 1000 µg/ml) of *H. cordata* in comparison with the antiviral agent, acyclovir (ACV), at 1.5 µg/ml.

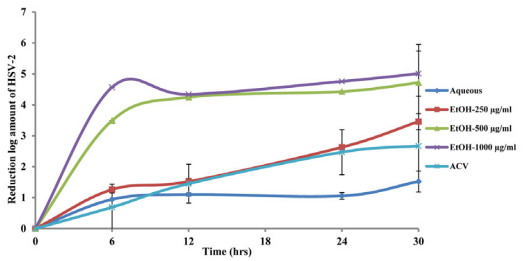

Figure 2. The reduction in log PFU/ml of HSV-2 at 0, 6, 12, 24, and 30 hours after treatment with the aqueous (2000 µg/ml) and ethanolic extracts (250 µg/ml, 500 µg/ml, and 1000 µg/ml) of *H. cordata* in comparison with the antiviral agent, acyclovir (ACV), at 3.1 µg/ml.

Furthermore, the inhibitory effect of the *H. cordata* extracts on viral DNA synthesis was also determined. It was found that both the aqueous and the ethanolic extracts could inhibit HSV-1 and HSV-2 DNA synthesis after 22 hours of treatment. The percentages of HSV-1 and HSV-2 DNA remaining after treatment with the aqueous extract of *H. cordata* were 58.81±7.43% and 54.48±4.56%, respectively. The percentages of HSV-1 and HSV-2 DNA remaining after treatment with the ethanolic extract were 13.64±2.78% and 28.39±0.85%, respectively (Figure 3).

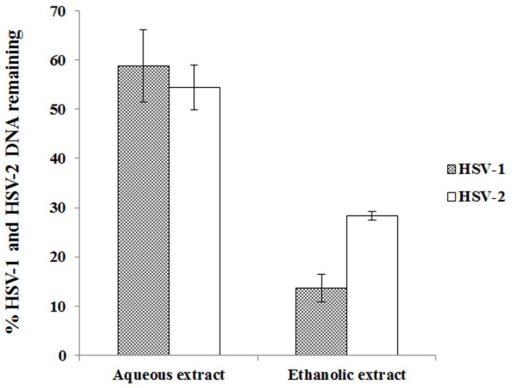

Figure 3. The percentage of HSV-1 and HSV-2 DNA remaining after treatment with the aqueous and ethanolic extracts of *H. cordata*.

The effects of the aqueous and ethanolic extracts of *H. cordata* on HSV protein synthesis were also investigated. HSV viral proteins were separated on SDS-polyarcrylamide gels and the activity against HSV detected using horseradish peroxidase-conjugated IgG, after transferring to a nitrocellulose membrane, using the Western blot analysis. The result showed that the HSV proteins had reduced after treatment with the aqueous and ethanolic extracts of *H. cordata* in comparison with the viral proteins in the absence of plant extracts and protein marker.

Table 5. Inhibition of Aqueous and Ethanolic Extracts of *H. cordata* on HSV-1 and HSV-2 Viral Protein Synthesis.

Protein molecular weight (kDa)	% Inhibition ± SD*	
	Aqueous extract	Ethanolic extract
HSV-1		
135.00	16.58±2.40^{A}	97.34±2.02^{A}
75.00	36.28±4.84^{D}	96.50±1.14^{D}
48.00	29.60±3.13^{C}	100.00±0.00^{C}
45.00	34.16±1.41^{B}	94.40±2.70^{B}
HSV-2		
75.00	22.21±8.68^{c}	100.00±0.00^{c}
48.00	17.86±8.17^{b}	100.00±0.00^{b}
45.00	14.95±6.54^{a}	87.09±4.48^{a}

*The data were presented as mean ± standard deviation (SD) of duplicate experiments. The statistical analysis conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group (A, B, C, D or a, b, c) within each column showed significantly different value ($P < 0.05$).

A quantitative analysis showed that the intensities of the HSV-1 and HSV-2 proteins at molecular weights of approximately 75kDa had significantly reduced, more than those of the other proteins, after treatment with the ethanolic extract of *H. cordata*, by 96.50±1.14% and 100±0.0%, respectively. Meanwhile, other HSV-1 proteins at molecular weights of approximately 45 kDa, 48 kDa, and 135 kDa were observed to have reduced by 94.40±2.7%, 100.00±0.00%, and 97.34±2.02% after treatment with the ethanolic extract, compared to viral control bands (Table 5, Figure 4). Moreover, HSV-2 proteins with molecular weights at 45 kDa and 48 kDa were also inhibited by the ethanolic extract of *H. cordata* by 87.09±4.48% and 100±0.0%, respectively (Table 5, Figure 4).

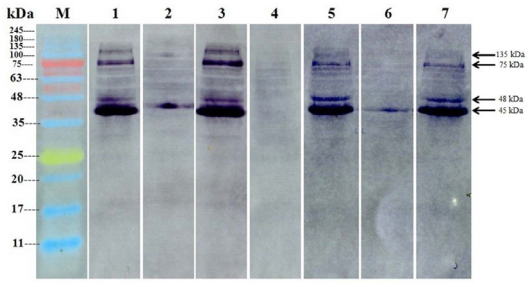

Figure 4. The effect of *H. cordata* extracts on HSV-1 protein synthesis in Vero cells detected by Western blotting analysis in the presence of the aqueous extract (lane 1), in the presence of the ethanolic extract (lane 2), in the absence of extracts (lane 3), and uninfected Vero cells (lane 4), as well as on HSV-2 protein synthesis in the presence of the aqueous extract (lane 5), in the presence of the ethanolic extract (lane 6), and in the absence of extracts (lane 7) after detection by horseradish peroxidase-conjugated IgG against HSV.

4. DISCUSSION

Traditional medicine provides unlimited plant materials for use as effective treatment for various diseases because of their health benefits. Thus, the efficacy of the aqueous and ethanolic extracts of *H. cordata* for inhibition during the various stages of HSV multiplication cycle was demonstrated in this present study. The aqueous and ethanolic extracts of *H. cordata* were tested for their toxicity using cell viability assay. This method is used for the determination of toxic concentration, and it is necessary for the selection of the concentration of plant extracts, which should be the least or which should not interfere with host cell activity. The toxicity of the plants should not affect the cell shape, the morphology, and the other cellular changes such as membrane permeability and ATP synthesis, while the efficacy of the plants should be specific to the HSV particle [18-19]. From the results obtained, it was found that the ethanolic extracts of *H. cordata* had higher toxicity than the aqueous extracts.

In this study, the aqueous and ethanolic extracts of *H. cordata* showed inhibitory effect more against HSV-2 than against HSV-1 at the time of treatment during the viral attachment. Although the ethanolic extract *H. cordata* could inhibit HSV-2 less than 50%, it was found that the plaque size of HSV-2 was smaller than that of viral control. Thus, evidently, it was possible that this plant extract was able to affect plaque formation and reduce or interfere with the efficacy as regards transmission

of progeny virion infectivity to spread to neighboring cells [13, 20]. Hence, this could suggest that the extracts might block or interfere with the transmission of HSV particles by interaction with viral glycoproteins that are responsible for specific attachment to cellular receptors on the surface of the host cell membrane. In addition, it could interfere with the process of fusion of the virion envelope with the cell membrane [21-23].

Furthermore, the efficacy of the ethanolic extract of *H. cordata* on direct inactivation of HSV-1 and HSV-2 particles was better than that of the aqueous extract at 20 minutes of incubation. However, the highest ability of the aqueous extract on direct inactivation of HSV particles was found at 120 minutes of infection. These results indicate that the compound in the plant extracts could directly inactivate HSV and might neutralize or interfere with virion envelope structures or cell receptors or viral structures that are necessary for entry into the host cells. The extract may lead to the formation of a modified chemical or receptor on the viral envelope and degradation of the essential viral protein on the envelope [13]. Thus, this experiment emphasized that the infectivity of the virus was destroyed at room temperature after treatment with *H. cordata* extracts when compared to viral control.

These results suggest that the aqueous and ethanolic extracts of *H. cordata* demonstrate significant capacity for inhibition during and after viral attachment. Thus, this extract might reduce the efficiency of virus infectivity by inactivating the viral particle or by interfering with the viral attachment stage or the entry to the cell [24]. Moreover, these extracts might be able to interfere with virus propagation and with the viral multiplication cycle.

The efficacy of the ethanolic extract of *H. cordata* in inhibiting HSV-1 and HSV-2 replication was observed to be significantly higher than that of the aqueous extract. The inhibition of HSV by the extract of *H. cordata* was selectively targeted on the HSV particle and replication. A similar result was reported where it was found that the hot water extract of *H. cordata* had considerable inhibitory efficacy against HSV replication [25]. Therefore, the extract can be utilized in both primary and recurrent infections to reduce viral shedding and to prevent viral infection.

The ethanolic extract of *H. cordata* showed inhibition of HSV-1 and HSV-2 DNA synthesis more than the aqueous extract. This could suggest that HSV DNA synthesis might have been interrupted by some compound in this extract, thus affecting HSV DNA replication and/or inactivating essential enzymes involved in virus DNA synthesis [4].

Thus, it can be concluded that both the extracts possess significantly potent activities for reducing HSV protein at approximately 75 kDa, and HSV protein is likely to be a protein generated from *UL6* gene [26]. The *UL6* gene is essential for the replication of viral DNA and DNA maturation [26-27]. The UL6 protein is required for the cleavage of viral DNA before packaging into capsid [28-29].

Other studies on the anti-viral activities of *H. cordata* were investigated. In 2009, Chou et al. [14] had reported that two new compounds, houttuynoside A and houttuynamide A, showed strong inhibitory effect against replication of HSV-1. The hot water extract of *H. cordata* was found to be capable of inhibiting the replication of HSV [25] and of inhibiting HSV-2 infection through the inhibition of NF-κB activation [30]. Hayashi et al. (1995)

[31] reported that essential oil extracted from *H. cordata* showed time- and dose-dependent inhibition of HSV by reducing HSV-1 titer. In 2011, Ren *et al.* [32] reported that *H. cordata* could inhibit pseudorabies herpesvirus (PrV) infection *in vitro*. Moreover, it was observed that the aqueous extract of *H. cordata* could inhibit SARS-CoV 3C-like protease and RNA-dependent RNA polymerase. The extract could also stimulate T-cell proliferation and could inhibit inflammation by reducing macrophage migration [15, 33]. Therefore, evidently, *H. cordata* is capable of inhibiting cytokines and the multiple steps of mast cell migration [9, 16, 34]. In addition, the water extract of *H. cordata* was found to show high antioxidation properties [35-36], and some isolated compounds of *H. cordata* were observed to demonstrate antityrosinase activity [14]. Moreover, alkaloids isolated from *H. cordata* showed antiplatelet aggregation activities, in addition to exhibiting cytotoxicity against human cancer cell lines [37-38].

5. CONCLUSION

The inhibitory effect of the aqueous and ethanolic extracts of *H. cordata* against HSV-1 and HSV-2 infection on Vero cells was examined during the various stages of the HSV multiplication cycle. The aqueous extract of *H. cordata* demonstrated the highest anti-HSV-1 and anti-HSV-2 activities when the extract was added during viral attachment. The ethanolic extract of *H. cordata* revealed higher antiviral activity against HSV particles and viral replication than the aqueous extract. Furthermore, the ethanolic extract of *H. cordata* exhibited potent inhibitory effect against HSV DNA and protein synthesis.

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