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Yin Chen Hao Tang, a Chinese prescription, inhibits both herpes simplex virus type-1 and type-2 infections *in vitro*

Hua-Yew Cheng [a], Liang-Tzung Lin [b], Hsin-Hsin Huang [c,d], Chien-Min Yang [e], Chun-Ching Lin [f,*]

[a] *Department of Cosmetic Applications & Management, Tung Fang Institute of Technology, 829 Kaohsiung County, Taiwan*
[b] *Department of Microbiology & Immunology, Dalhousie University, B3H 1X5 Halifax, Nova Scotia, Canada*
[c] *Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, 807 Kaohsiung City, Taiwan*
[d] *Department of Pharmacy, Shin-Kong Wu Ho-Su Memorial Hospital, 111 Taipei City, Taiwan*
[e] *Graduate Institute of Pharmaceutical Sciences, College of Pharmacy, Kaohsiung Medical University, 807 Kaohsiung City, Taiwan*
[f] *Faculty of Pharmacy, College of Pharmacy, Kaohsiung Medical University, 807 Kaohsiung City, Taiwan*

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Abstract

Yin Chen Hao Tang (YCHT) is one of the most frequently used prescriptions in the long history of traditional Chinese medicine practice. The prescription contains three Chinese herbs, namely *Artemisia capillaries* Thunb. (Compositae), *Rheum officinale* Baillon (Polygonaceae), and *Gardenia jasminoids* Ellis (Rubiaceae), and has been widely used to treat acute hepatitis with jaundice. In this study, the *in vitro* anti-HSV-1 and HSV-2 activities of the water extract of YCHT were investigated. Results showed that YCHT water extract inhibited both HSV-1 and HSV-2 infections. However, the inhibition was more effective against HSV-2 than against HSV-1. The IC_{50} and IC_{90} values of YCHT water extract against HSV-1 infection were in the range of 142.5–150.1 and 191.3–393.9 μg/ml, and against HSV-2 infection they were in the range of 19.6–29.4 and 42.2–97.7 μg/ml, respectively. The water extract of YCHT showed no cytotoxic effect at a concentration of 500 μg/ml or below, and had a CC_{50} value of 850.7 ± 1.7 μg/ml. The prescription was found to diminish HSV-2 infectivity in a dose-dependent manner, and the activity was influenced by the incubation periods and the incubation temperatures. Concurrent addition of virus with YCHT or pre-treatment of the virus with the prescription extract both protected the cells from infection. In summary, the water extract of YCHT was concluded to inhibit infections by HSV-1 and HSV-2 and this effect was likely mediated through direct inactivation of the virus infectivity.

Keywords: Yin Chen Hao Tang; Traditional Chinese medicine; Anti-HSV activity; Virus inactivation

1. Introduction

Yin Chen Hao Tang (YCHT) or In-Chern-Hau-Tang, and also known as Inchin-ko-to in Kampo medicine, is a traditional Chinese medicine (TCM) prescription that has been used for several hundred years by the Chinese community. This classical botanical formulation consists of three Chinese herbs that include the dried seedling of *Artemisia capillaries* Thunb. (Compositae), the dried rhizome of *Rheum officinale* Baillon (Polygonaceae), and the dried ripe fruit of *Gardenia jasminoids* Ellis (Rubiaceae). The prescription possesses several functions such as clearing away heat, promoting diuresis, removing the poisonous quality from substances, lessening the virulence of pathogenic organisms, and treating jaundice (Chen et al., 1993; Xie et al., 1997). In customary practices, the preparation is often applied for jaundice due to damp heat, mouth dryness without feeling of thirst, constipation, difficulty in urination or cloudy urine, and liver cirrhosis (Chen et al., 1993; Xie et al., 1997). Nowadays, it is widely used for the treatment of acute hepatitis with jaundice.

In literatures, a variety of biological activities and effects from YCHT have been described. YCHT has been reported to suppress liver injury in mice induced by Fas, or in rat hepatoma cells and primary cultured hepatocytes induced by transforming growth factor β-1, through prevention of apoptosis (Yamamoto et al., 1996, 2000). The prescription has also been observed to attenuate the development of hepatic fibrosis in rats induced by a choline-deficient L-amino acid-defined diet (Sakaida et al.,

* Corresponding author at: No. 100, Shin-Chuan 1'st Road, Kaohsiung Medical University, Kaohsiung, Taiwan. Tel.: +886 7 3121101x2122; fax: +886 7 3135125.
E-mail address: aalin@kmu.edu.tw (C.-C. Lin).

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2003), thioacetamide (Imanishi et al., 2004), or repeated injections of carbon tetrachloride or pig serum (Inao et al., 2004) through suppression of proliferation and fibrogenesis of hepatic stellate cells. YCHT can also decrease dental calculus formation by inhibiting the development of oral calcium phosphate precipitates (Hidaka et al., 1993), enhance choleresis in rat liver by stimulation of exocytosis and insertion of mutlidrug resistance-associated protein 2 in the bile canaliculi (Shoda et al., 2004), and induce apoptosis in cultured rat hepatic stellate cells at low concentration by increasing expression of p53 and decreasing expression of Bcl-2 and phosphorylation of Akt and Bad (Ikeda et al., 2006). In addition, this medicinal formulation showed beneficial effect on post-operative biliary atresia patients by improving markers of hepatic fibrosis such as hyaluronic acid, prolyl hydroxylase, procollagen III peptide, and type VI collagen (Kobayashi et al., 2001).

Herpes simplex virus (HSV) is a common human pathogen that causes a variety of illnesses ranging from asymptomatic infection to fulminant disseminated diseases, such as labials herpes, keratitis, genital herpes, encephalitis, etc. The major antiviral therapy for the treatment of HSV infection is the use nucleoside analogues, for example acyclovir. However, the increasing clinical application of this type of antiviral agents has been associated with the emergence of drug-resistant herpesvirus strains (Czartoski et al., 2006; Danve-Szatanek et al., 2004; Malvy et al., 2005; Morfin and Thouvenot, 2003). Therefore, the development of new anti-HSV agents with a different mode of action is highly warranted.

Chinese herbs are potential sources of useful medicinal plants. Recently, research in herbs prescribed in TCM has attracted great attention as many of them have been shown to exhibit numerous biological activities including anti-HSV abilities (Cheng and Lin, 2005; Chiang et al., 2003). In an effort to discover novel antiviral agents or antiviral formulations from TCM, YCHT, one of the most frequently used Chinese prescriptions in TCM clinical practice, was investigated for its *in vitro* anti-HSV-1 and HSV-2 activities.

2. Materials and methods

2.1. Plant materials

A. capillaries, *R. officinale*, and *G. jasminoids* were purchased by medicinal plant expert, Ming-Hong Yen, Ph.D. (Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan) from a local Chinese herb drug store located in Kaohsiung city in October, 2004. Their authenticity was further confirmed by Prof. Chun-Ching Lin.

2.2. Preparation of the YCHT water extract

1.8 liter of de-ionized distilled water (dd water) was added to 180 g of YCHT prescription, which comprises 108 g of *A. capillaries*, 36 g of *R. officinale*, and 36 g of *G. jasminoids*, and was heated until the preparation boiled. The boiling was continued for 1 h. The decoction was then percolated to obtain filtrate, and dregs were re-boiled with fresh 1.8 L of dd water. These procedures were repeated for another two times. Collected filtrates were then poured together and concentrated under reduced pressure. The concentrated liquid was further lyophilized to dryness. The yield of the YCHT water extract was 24.7% or 44.4 g in weight. It was stored at −20 °C until use.

Acyclovir (ACV) was purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). ACV and the water extract of YCHT were dissolved in dimethyl sulfoxide (DMSO) and then diluted with sterile de-ionized distilled water before use. The final concentration of DMSO was less than 0.1%, which was not toxic to Vero cells as shown previously (Cheng et al., 2004).

2.3. Cells and viruses

African green monkey kidney cells (Vero) (ATCC CCR-81) and HEp-2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were propagated in Dulbeco's modified eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS), 200 U/ml penicillin G sodium, 200 μg/ml streptomycin sulfate, and 0.5 μg/ml amphotericin B. The overlay medium for the plaque assay consisted of DMEM plus 2% FCS, 1% methylcellulose, and antibiotics as described above.

HSV-1 strain KOS, HSV-2 strain 196, and strain G were provided by Dr. Lien-Chai Chiang (Department of Microbiology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan). The virus titer was determined by plaque assay and was expressed as plaque-forming units (pfu) per ml; virus stocks were stored at −80 °C until use.

2.4. Titration of virus

Viral titer was determined by plaque assay as described by Burleson et al. (1992). Briefly, Vero cells were seeded onto 24-well culture plates (TPP, Trasadingen, Switzerland) at a density of 10^5 cells/well and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h until reaching at least 95% confluency. Serial dilution of the virus stock was prepared, and the cell monolayer was infected with the dilution of virus. After 1 h of inoculation, the medium was aspirated and replaced with overlay medium containing 1% methylcellulose. The infected cell monolayer was incubated for another 48 h. The overlay medium was then removed and the cell monolayer was fixed with 10% formalin in PBS for 1 h. After fixation, 1% crystal violet was used to stain the cell monolayer. The plaque number per well was recorded and the virus titer in plaque-forming units (pfu) was calculated.

2.5. Cytotoxic assay

The cytotoxic effect of YCHT extract on cells was evaluated by XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid) (Sigma–Aldrich Inc., St. Louis, MO, USA) assay as described previously (Cheng et al., 2005). Cells were seeded onto 96-well culture plates (Nunc, Roskilde, Denmark) at a number of 10^4 per well. After 4 h of incubation to allow seeding of the cells,

various concentrations of YCHT water extract were added into each well. The plate was then incubated at 37 °C with atmosphere of 5% of CO_2 for 72 h. Later, the medium was discarded and the XTT reagent was added. The plate was re-incubated at 37 °C for an additional 3 h to allow the development of formazan. The optical densities were then measured with an ELSIA reader (Lab Systems Multiskan EX, MA, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The cytotoxic effect of YCHT water extract was determined and the 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis of the dose-response curve generated from the data (Cheng et al., 2005).

2.6. Antiviral assay

2.6.1. Plaque reduction assay

Cells were seeded onto 24-well culture plates (TPP, Trasadingen, Switzerland) at a density of 10^5 cells per well and incubated for 48 h to reach at least 95% confluency. The medium was then discarded and the cell monolayer was infected with 100 pfu of HSV-1 or HSV-2 in the presence or absence of YCHT water extract. After 1 h incubation for virus adsorption, cells were overlaid with an overlay medium containing 1% of methylcellulose. The plate was further incubated for 48 h. Later, the overlay medium was removed and the infected cell monolayer was fixed with 10% formalin. The virus plaques formed on Vero cells were stained with 1% crystal violet. The fraction of percent inhibition in inhibiting HSV replication was determined and the minimal concentration of YCHT water extract required to inhibit the formation of virus plaque number by 50% (IC_{50}) and 90% (IC_{90}) was calculated by regression analysis of the dose-response curve generated from the data (Cheng et al., 2006). To investigate the time-of-addition effect of YCHT water extract on HSV-2 infection, YCHT was added at different periods of infection (pre-infection, concurrent to infection, and post-infection). The prescription was either washed out at indicted times or remained present for the entire 48 h period of infection.

2.6.2. Virus yield reduction assay

Cells were seeded onto 12-well culture plates (Nunc, Roskilde, Denmark) at a density of 2×10^5 cells per well and incubated for 48 h. Cells were then infected with 200 pfu of HSV-1 or HSV-2 in the presence or absence of YCHT water extract. After 48 h of infection, cells were scraped and viruses were released from cells by freeze–thawing for three times. Cell pellets were removed by centrifugation at $1100 \times g$ for 10 min. The virus titer was determined by plaque assay and the percent of inhibition in inhibiting HSV replication was calculated.

2.7. Virucidal assay

The direct effect of YCHT water extract on HSV-2 infectivity was evaluated according to the procedures as described previously with minor modifications (Yang et al., 2005). Various concentrations (0, 1.0, 12.5, and 25.0 μg/ml) of the extract were mixed thoroughly with 1×10^6 pfu HSV-2. The mixture was then incubated at different temperatures (26, 32, and 37 °C) for 0, 1, 2, 4, and 6 h. After incubation, the residual virus infectivity was determined by plaque assay. The effect of YCHT water extract on virus infectivity was calculated. The higher the percent in control signifies the lower the virucidal ability of the extract (Yang et al., 2005).

2.8. Statistical analysis

Data are presented as mean ± S.D. of three independent experiments. The IC_{50}, IC_{90}, and CC_{50} values were calculated by Microsoft Excel 2003. The significance between the test sample and the solvent control was analyzed by one-way ANOVA followed by Scheffe multiple comparison test. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Cytotoxic effect from the YCHT water extract on cell viability

As the purpose was to determine the drug concentrations that did not affect cell viability and to be used for subsequent assays, the cytotoxic effect of YCHT water extract towards Vero cells was evaluated using the XTT assay (Table 1). Results showed that YCHT displayed cytotoxic effect only at high concentration. The survival rate of the cells was 67.5% under the concentration using 750 μg/ml of YCHT water extract and decreased to 24.1% when treated by 1000 μg/ml. YCHT showed 50% cytotoxic effect toward cells (CC_{50}) at a concentration of 850.7 ± 1.7 μg/ml. The prescription, however, was found to be less toxic toward Vero cells at concentrations of 500 μg/ml or below with the survival rate from the cells approximating 100%. A concentration of 500 μg/ml of YCHT extract also did not affect the growth of the cells (data not shown). Moreover, it was also non-toxic towards HEp-2 cells at concentration of 500 μg/ml (data not shown). Therefore, only concentrations of 500 μg/ml or below were selected for the subsequent studies.

3.2. Anti-HSV-1 and HSV-2 activities of YCHT water extract

The inhibitory effect of YCHT water extract on HSV-1 and HSV-2 infections was investigated by plaque reduction and virus yield reduction assays. Results showed that the YCHT water extract could inhibit both HSV-1 and HSV-2 infections at different magnitudes of activity (Table 1).

YCHT inhibited HSV-1 infection in a dose-dependent manner. The percent inhibition in plaque reduction assay increased from 42.2 to 96.8% as the concentration of YCHT rose from 100 to 500 μg/ml. The concentrations that inhibited 50% (IC_{50}) and 90% (IC_{90}) of HSV-1 replication were in the range of 142.5–150.1 and 191.3–393.9 μg/ml, respectively.

YCHT also inhibited HSV-2 infection in a dose-dependent fashion as it did on HSV-1 replication. However, the inhibitory effect was more potent against HSV-2 than against HSV-1. For plaque reduction assays, the prescription at concentration of 100 μg/ml was found to inhibit approximately 91.6% HSV-2

Table 1
The *in vitro* cytotoxic effect and antiviral activity of the water extract of the Chinese prescription, Yin Chen Hao Tang (YCHT)[a]

	Concentration (μg/ml)							
	YCHT				Acyclovir			
	CC_{50}[b]	IC_{50}[c]	IC_{90}[c]	SI[d]	CC_{50}[b]	IC_{50}[c]	IC_{90}[c]	SI[d]
Cytotoxic effect								
Vero	850.7 ± 1.7	–[e]	–[e]	–[e]	>1000	–[e]	–[e]	–[e]
Antiviral activity								
HSV-1 KOS strain	–[e]	142.5 ± 1.7	393.9 ± 4.7	6.0	–[e]	0.05 ± 0.002	0.3 ± 0.001	>20,000
KOS strain[f]	–[e]	150.1 ± 14.2	191.3 ± 12.7	5.7	–[e]	0.06 ± 0.002	0.27 ± 0.003	>166666.6
HSV-2 196 strain	–[e]	29.4 ± 1.4	97.7 ± 4.7	28.9	–[e]	0.31 ± 0.01	3.8 ± 0.1	>3225.8
196 strain[f]	–[e]	19.6 ± 2.1	54.6 ± 3.3	43.4	–[e]	0.09 ± 0.01	0.8 ± 0.1	>11111.1
196 strain[g]	–[e]	23.1 ± 1.7	79.4 ± 9.3	–[e]	–[e]	2.1 ± 0.3	2.9 ± 0.2	–[e]
G strain	–[e]	21.5 ± 2.2	42.2 ± 18.5	39.6	–[e]	0.46 ± 0.1	1.4 ± 0.3	>2173.9

Data were mean ± S.D. of three independent experiments.
[a] The cytotoxic effect was determined by XTT assay, and the antiviral activity was determined in Vero cells by plaque reduction assay unless those indicated specifically.
[b] CC_{50} (50% cytotoxic concentration) was the concentration that showed 50% toxic effect on Vero cells.
[c] IC_{50} (50% inhibitory concentration) and IC_{90} (90% inhibitory concentration) were the concentrations that inhibited 50% and 90% of virus infection, respectively.
[d] SI (selectivity index) was the ratio of CC_{50} to IC_{50}.
[e] Not evaluated.
[f] The antiviral activity was evaluated by virus yield reduction assay.
[g] The antiviral activity was determined in HEp-2 cell.

infection versus the 42.2% in HSV-1 replication. The IC_{50} and IC_{90} of HSV-2 infection were in the range of 19.6–29.4 and 42.2–97.7 μg/ml, respectively.

With the CC_{50} and IC_{50} values, the selectivity index (SI) was calculated by dividing the CC_{50} with the IC_{50}. The SI values of the YCHT water extract were 5.7–6.0 for HSV-1 and 28.9–43.4 for HSV-2.

The time-of-addition effect of YCHT extract on HSV-2 infection was also investigated (Fig. 1). The results showed that YCHT added at 6 h prior to virus infection and then being washed out before the infection did not have antiviral activity. The concurrent addition of YCHT with virus and washing the extract out at 1 h post-infection also did not inhibit the HSV-2 infection. In contrast, adding YCHT concurrently to virus infection

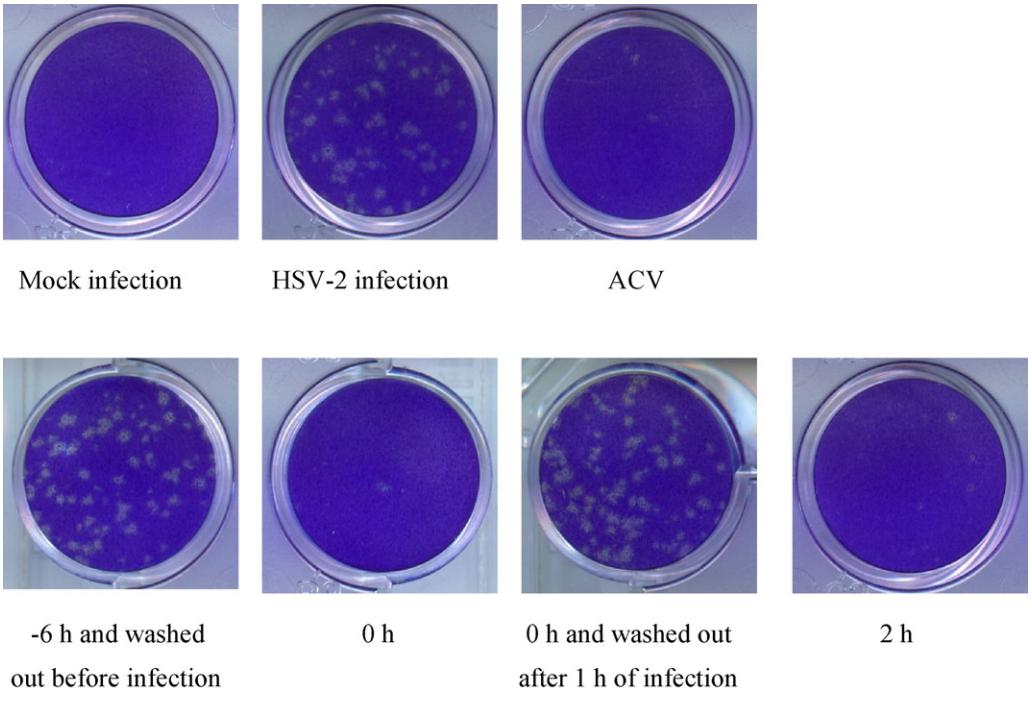

Fig. 1. The time-of-addition effect of the water extract of the Chinese prescription, Yin Chen Hao Tang (YCHT), on HSV-2 infection. One hundred microgram per millilitre of YCHT extract was added prior to infection (−6 h), during infection (0 h) or after infection (2 h). The prescription was either washed out at indicted times or continuously remained present for the entire 48 h period of infection. ACV was added concurrently with HSV-2 at concentration of 1 μg/ml.

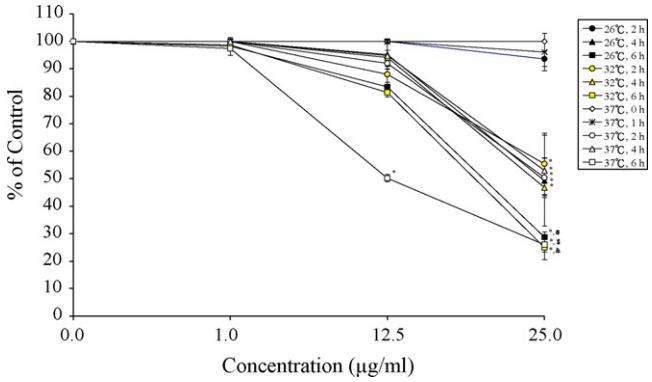

Fig. 2. The effect of the water extract of the Chinese prescription, Yin Chen Hao Tang (YCHT), on HSV-2 infectivity at various temperatures and different incubation periods. Data were presented as % of control in which the higher the percentage signifies the lower the virucidal ability of the YCHT extract. Data represent the mean ± S.D. of three independent experiments. * Indicates significant difference between test compound and solvent control ($P < 0.05$). #: indicates significant difference in YCHT extract's effects at temperature 26 °C of the same concentration but of different incubation time ($P < 0.05$). &: indicates significant difference in YCHT extract's effect at temperature 32 °C of the same concentration but of different incubation time ($P < 0.05$). $: indicates significant difference in YCHT extract's effect at temperature 37 °C of the same concentration but of different incubation time ($P < 0.05$).

or adding YCHT at 2 h post-infection, with continued presence of the water extract for the entire 48 h period of infection, did not yield any plaque formation.

3.3. Viral inactivation effect of the YCHT water extract on HSV-2 infectivity

Since the time-of-addition assays indicated possible viral inactivation activity from YCHT's inhibitory effect observed, the viral inactivation effect of YCHT was evaluated by incubating the prescription with HSV-2 at different temperatures (26, 32, and 37 °C) and various time periods (0, 1, 2, 4, and 6 h). The reason to investigate YCHT at temperatures 26, 32, and 37 °C was because 37 °C is the temperature at which cells are incubated and infected by viruses, 32 °C is the temperature of the human body epidermis, and 26 °C is the average room temperature under which the whole study was conducted. As shown in Fig. 2, YCHT was found to diminish HSV-2 infectivity depending on the dosage level. A concentration of 25 μg/ml of YCHT significantly reduced viral infectivity when incubated at 37 °C for 2, 4, and 6 h but not 1 h. At the concentration of 12.5 μg/ml, the prescription also caused HSV-2 to lose infectivity at 37 °C and 6 h of incubation. However, the low concentrations showed little effect. In addition, 25 μg/ml of YCHT was incubated with the virus at 37 °C for 2 h. The prescription was then diluted out and the virus was re-incubated at 37 °C for an additional 6 h. However, no discrepancies were observed between the tested samples and the mock infection control suggesting that HSV-2 did not regain the infectivity. This indicated that the pre-treatment of the virus with YCHT protected the cells from HSV-2 infection and that the inactivation of viral infectivity by YCHT was irreversible (data not shown).

When the incubation temperature was shifted to 32 °C, similar viral inactivation effect found at 37 °C was observed. The only exception was that HSV-2 did not lose infectivity at 12.5 μg/ml of YCHT.

The temperature was then further shifted to 26 °C. A concentration of 25 μg/ml of YCHT was found to diminish viral infectivity under this incubation temperature. However, HSV-2 did not lose its infectivity at 2 h of incubation time.

In contrast, ACV, a well-defined HSV-2 replication inhibitor, had not effect on virus infectivity (data not shown). Even when the incubation period was extended and also the incubation temperature was increased, the virus infectivity was unaffected as compared to the solvent control group.

4. Discussion

In this study, the water extract of YCHT was found to inhibit *in vitro* HSV-1 and HSV-2 infections at different magnitudes of activity. Although it could inhibit the replication of both viruses, the activity was more prominent against HSV-2 than against HSV-1. YCHT extract was only active in inhibiting HSV-2 infection when added concurrently with the virus and continuously remained present for the whole period of infection. In addition, the extract was also found to reduce HSV-2 infectivity when the virus was pre-treated with it, and the effect was observed to be irreversible, thus suggesting viral inactivation as a possible mechanism of action of YCHT.

In taxonomy, both HSV-1 and HSV-2 are members of the genera *Simplexvirus* from the subfamily *Alphaherpesvirinae* in *Herpesviridae* family (Roizman and Pellet, 2001). They have many similar characteristics but can be distinguished by clinical manifestations and biochemical and serological examinations. The currently available anti-HSV medicines are generally active for both HSV-1 and HSV-2 (De Clercq, 2004; Naesens and De Clercq, 2001). Our results revealed that the water extract of YCHT was more potent in inhibiting HSV-2 than HSV-1. This finding suggested that YCHT extract is specific in anti-HSV activity. Nevertheless, further studies are needed to verify the underlying reason(s) of differential inhibitory effect of YCHT extract in inhibiting HSV-1 and HSV-2 infections.

As the most common causative agents of genital ulcer disease worldwide, HSV has a pivotal role in human immunodeficiency virus (HIV) infection (Celum et al., 2005; Chen et al., 2000). The importance of HSV infection in the dynamics of HIV acquisition, infection, and transmission suggests that anti-HSV management is one of the ways to hamper the epidemic of HIV (Celum et al., 2004; Schacker, 2001; Schacker et al., 2002; Wald and Link, 2002). The prevention of HSV-2/HIV shedding and that of genital ulcer formation are direct approaches (Celum, 2004; Celum et al., 2004). Although further evidence and consideration are needed to conclude out the recommended management for this problem, the antiherpetic agents, which kill or inactivate the virus directly, may be beneficial (Celum, 2004; Celum et al., 2005).

In this study, the water extract of YCHT was also found to diminish virus infectivity significantly at concentration lower than that of the IC_{50} value (Fig. 2). The effectiveness was

affected by YCHT concentration, incubation period, and incubation temperature (Fig. 2). The most interesting aspect is that the lowering of the incubation temperature did not dramatically influence YCHT extract's viral inactivation activity. However, the prescription took at least 2 h to significantly reduce viral infectivity, which may limit its potential use as a direct virucidal or viral inactivation agent. Nevertheless, the herbal formulation could be further explored as a supportive prescription in treating HSV infections.

In summary, the water extract of YCHT was found to inhibit both HSV-1 and HSV-2 infections. The prescription was observed to be more potent in inhibiting HSV-2 than HSV-1. It was also found to reduce HSV-2 infectivity in dose-, time-, and temperature-dependent manners. Finally, our observations suggested that the anti-HSV activity of YCHT extract was likely mediated through direct inactivation of the virus infectivity.

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