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Acetone, ethanol and methanol extracts of *Phyllanthus urinaria* inhibit HSV-2 infection in vitro

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Abstract

Phyllanthus urinaria Linnea (Euphorbiaceae) is one of the traditional medicinal plants that are widely applied by oriental people, especially by Chinese and Indian, to ameliorate various kinds of ailments. Many biological activities, including anti-hepatitis B virus, anti-Epstein–Barr virus and anti-retroviral reverse transcriptase, of *P. urinaria* have been reported, but not against herpes simplex virus (HSV). In this study, the anti-HSV-1 and HSV-2 activities of different solvents extracted from *P. urinaria* were investigated in vitro by plaque reduction assay. Results showed that acetone, ethanol and methanol extracts of *P. urinaria* inhibited HSV-2 but not HSV-1 infection. The 50% inhibitory concentration against HSV-2 infection (IC_{50}) of acetone, ethanol and methanol extracts was 4.3 ± 0.5, 5.0 ± 0.4 and 4.0 ± 0.9 mcg/ml, respectively. All three extracts showed no cytotoxic effect against Vero cells at concentrations of 10.0 mcg/ml or below. The time-of-addition study demonstrated that these three extracts were only effective when added during the HSV-2 infection which, therefore, suggested that they disturb the initial stage of HSV-2 infection. Furthermore, they can diminish virus infectivity without significantly affecting incubation time and temperature. Therefore, the acetone, ethanol and methanol extracts of *P. urinaria* were concluded to likely inhibit HSV-2 infection through disturbing the early stage of virus infection and through diminishing the virus infectivity.

Keywords: Anti-HSV activity; *P. urinaria*; Plant; Early stage of infection; Virus infectivity

1. Introduction

Herpes simplex virus (HSV) is a single large double-stranded DNA enveloped virus that is extremely widespread in the human population. It is responsible for a broad range of diseases, ranging from gingivostomatitis to keratoconjunctivitis, genital disease, encephalitis, and also infection of newborn and immunocompromised patients (Whitley et al., 1998). After the primary infection, HSV tends to persist in the neuron of the ganglia (Baringer and Swoveland, 1973). Reactivation of latent HSV, which is very common during the deficiency of immunity, causes recurrent herpetic infection.

Acyclovir, valaciclovir, famciclovir and cidofovir have been used for the treatment of HSV infection and associated diseases (Cassady and Whitley, 1997). However, resistance of HSV to currently used antiviral agents (Englund et al., 1990) and the property of latency of HSV infection have limited the efficacy of existing antiviral management (Bean, 1992). Viral strains that are resistant to drugs commonly used in the therapy of HSV infection have been increasingly isolated (Nugier et al., 1992), particularly from immunocompromised patients (Erlich et al., 1989). Therefore, the search for new antiviral agent emerges as an imperative need.

Phyllanthus urinaria Linnea (Euphorbiaceae) is a widely used medicinal plant in Chinese community. The plant is traditionally believed that it can reduce heat, remove food stagnancy, improve eyesight, relieve inflammation, calm the

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liver, suppress *yang* hyperactivity of liver (subdue the excessive syndrome and heat syndrome caused by liver for example hypertension, neurasthenia etc), detoxify poison from body and increase the flow of urine (Committee on Chinese Medicine and Pharmacy, 2003). In Taiwan, the whole plant of *P. urinaria* is habitually used as the therapy for diarrhea, dysentery, contagious hepatitis, edema, infantile malnutrition, acute conjunctivitis, aphthae and unreasonable pyogenic infections (Committee on Chinese Medicine and Pharmacy, 2003).

Besides conventional usage, many pharmacological activities of *P. urinaria* have been reported in the scientific literature. These activities include protecting CCl_4-induced injuries of liver cells (Zhou et al., 1997), relaxing the contraction of guinea pig trachea (Paulino et al., 1996a,b), producing the pronounced systemic, spinal and supraspinal antinociceptive effect in mice (Santos et al., 1995, 1999), inducing the contractile response in the urinary bladder (Dias et al., 1995), decreasing the blood glucose level in streptozotocin-induced diabetic rats (Higashino et al., 1992), inducing apoptosis in cancer cells (Huang et al., 2003, 2004a,b; Giridharan et al., 2002), seroconverting hepatitis B e-antibody status in the patients from negative to positive (Wang et al., 1994, 1995), inhibiting the intracellular HbsAg formation in hepatoma cells (Ji et al., 1993) and the activity of retroviral reverse transcriptase (Suthienkul et al., 1993), and possessing anti-HBV (Chen et al., 1995; Zhong et al., 1998) and anti-EBV (Liu et al., 1999) activities.

The traditionally usage of *P. urinaria* as therapy for virus caused-hepatitis hinted that it is an antiviral agent. The reported anti-HBV (Chen et al., 1995; Zhong et al., 1998), anti-EBV (Liu et al., 1999) and anti-retroviral reverse transcriptase (Suthienkul et al., 1993) activities supported this hypothesis. However, the anti-HSV activity of *P. urinaria* has not been reported in literature. In this study, a series of experiments were conducted to investigate the anti-HSV-1 and HSV-2 activity of *P. urinaria*in vitro.

2. Materials and methods

2.1. Plant materials

P. urinaria was collected from southern area of Taiwan. Its authenticity was identified and confirmed using morphological and anatomical techniques by our co-author, T.C. Lin, Ph.D. (Department of Pharmacy, Tajen Institute of Technology, Ping-Tung, Taiwan). A voucher specimen was deposited at the Herbarium of the Graduate Institute of Natural Products of Kaohsiung Medical University, Taiwan.

2.2. Preparation of extracts

Fifty grams of fresh whole plants of *P. urinaria* were cut into small pieces and then immersed in 300 ml of related solvent (acetone, benzene, chloroform, ethanol, ethyl acetate, *n*-hexane and methanol) at room temperature (about 26 °C) for two days. The solvent was filtered by gauze and subsequently collected into a flask. The dregs were processed again according to the procedures as described above for another two times. The collected solvents were then poured together and re-filtered to remove residues. It was concentrated by evaporating the solvent under the reducing pressure. The concentrated liquid was finally lyophilized to dry. The weight of acetone, benzene, chloroform, ethanol, ethyl acetate, *n*-hexane and methanol extracts were 6.2, 5.8, 6.0, 6.0, 5.6, 5.7 and 5.9 g, respectively.

Acyclovir (ACV) was purchased from Sigma Company (St. Louis, USA). ACV and extracts were dissolved in dimethyl sulfoxide (DMSO) and then diluted with sterile deionized distilled water before use. The final concentration of DMSO was <0.1% which was no toxic to Vero cells as we have shown previously (Cheng et al., 2004a).

2.3. Cell and viruses

All reagents and medium for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). African green monkey kidney cells (Vero) (ATCC CCR-81) were obtained from the hospital of Kaohsiung Medical University (Kaohsiung, Taiwan). 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. Overlay medium for the plaque assay consisted of DMEM plus 2% FCS, 1% methylcellulose and antibiotics as described above.

HSV-1 strain KOS and HSV-2 strain 196 was provided by our co-author Dr. Lien-Chai Chiang (Department of Microbiology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan). Its 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. Cytotoxic assay

The cytotoxic effect of extracts of *P. urinaria* on Vero cell was determined by XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid) (Sigma, USA) assay as described previously (Lin et al., 2002). Vero cells were seeded onto 96-well culture plates (Falcon; BD Biosciences, NJ, USA) at number of 10^4 per well. After 4 h of incubation to allow seeding of the cells, the various concentrations of extracts 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 cells were subsequently rinsed with phosphate buffered saline (PBS). The XTT reagent was added and the plate was reincubated at 37 °C for an additional 2 h to allow the development of formazan. The optical densities (OD) were then measured with enzyme immunoassay (EIA) reader (Lab Systems; MTX Labs, VA, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The toxicity

effect of each extract was calculated and the 50% cytotoxic concentration (CC_{50}) of each extract was calculated by regression analysis of the dose–response curve generated from data (Cheng et al., 2002).

2.5. Antiviral assay

The inhibitory effect of extracts in suppressing HSV multiplication was investigated by plaque reduction assay (Cheng et al., 2004a). Vero cells were seeded onto 24-well culture plates (Falcon; BD Biosciences, NJ, USA) at density of 10^5 cells per well and incubated for 48 h to reach at least 95% confluency. The medium was discarded and cell monolayer was infected with 100 pfu of HSV-1 or HSV-2 in the absence or presence of extract. After 1 h incubation for virus adsorption, cells were overlaid with overlay medium containing 1% of methylcellulose. The plate was incubated at 37 °C with atmosphere of 5% of CO_2 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 extract required to suppressing the formation of virus plaque number by 50% (IC_{50}) was calculated by regression analysis of the dose–response curve generated from data (Cheng et al., 2002).

2.6. Time-of-addition study

The time-of-addition effect of acetone, ethanol and methanol extracts of *P. urinaria* was examined according to the previously described procedures with minor modifications (Cheng et al., 2004b). Vero cells were seeded onto 24-well culture plates (Falcon; BD Biosciences, NJ, USA) at density of 10^5 cells per well and incubated for 48 h to reach at least 95% confluency. Ten mcg/ml of extract was then added onto the cells at either before (−6 and −2 h), during (0 h) or after (2, 4 and 8 h) periods of HSV-2 infection. Later, the similar procedures from "antiviral assay" section was followed except that cells were washed thrice by PBS to eliminate extract prior to the inoculation of virus for pre-infection (−6 and −2 h) group, or the extracts were added at different time for during (0 h) and post-infection (2, 4 and 8 h) groups.

2.7. Virucidal assay

The direct effect of acetone, ethanol and methanol extracts of *P. urinaria* on HSV-2 infectivity was evaluated according to the procedures as described by Cheng et al. (2004b) with modifications. Briefly, different concentrations (2.5, 5.0 and 10.0 mcg/ml) of extract were mixed thoroughly with 1×10^5 pfu HSV-2. The mixture was then incubated at different conditions (26 °C for 0.5 h, 37 °C for 0.5 h and 37 °C for 6 h). After the incubation, the residual virus infectivity was determined by plaque assay. The effect of extracts on virus infectivity was calculated. The higher the percent in control signifies the lower the virucidal ability of the extracts.

2.8. Statistical analysis

Data are presented as mean ± S.D. of three independent experiments. The IC_{50} and CC_{50} values were calculated by Microsoft Excel 2000. The Student's unpaired *t*-test was used to test significance between the test sample and solvent control. A *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Cytotoxic effect of the extracts of P. urinaria on viability of Vero cells

The concentrations of extracts of *P. urinaria* that did not affect the viability of Vero cells were investigated by XTT assay. Result showed that each extract had different magnitude of toxicity on Vero cells. Benzene and *n*-hexane extracts had no cytotoxic effect up to the concentration of 125.0 mcg/ml. Ethyl acetate extract was less toxic at 50.0 mcg/ml and chloroform extract was not deadly at 25.0 mcg/ml. Acetone, ethanol and methanol extracts did not significantly affect the viability of cell at concentrations lower than 25.0 mcg/ml. The CC_{50} value of acetone, benzene, chloroform, ethanol, ethyl acetate, *n*-hexane and methanol extracts was 14.9 ± 1.4, >125.0, 40.2 ± 9.0, 19.9 ± 3.2, 120.6 ± 6.4, >125.0 and 16.2 ± 2.6 mcg/ml, respectively. We, therefore, examined the anti-HSV activity of acetone, ethanol and methanol extracts at concentrations of 10.0 mcg/ml or lower and of benzene, chloroform, ethyl acetate and *n*-hexane extracts at concentrations of 20.0 mcg/ml or lower.

3.2. Anti-HSV-1 and HSV-2 activities of the extracts of P. urinaria

Fig. 1 summarized the antiviral activity of 10.0 mcg/ml of acetone, ethanol and methanol extracts, and that of 20.0 mcg/ml of benzene, chloroform, ethyl acetate and *n*-hexane extracts as evaluated by plaque reduction assay. Results showed that acetone, ethanol and methanol extracts slightly inhibited HSV-1 multiplication with an inhibition rate of 31.4 ± 5.2%, 40.4 ± 4.2% and 41.3 ± 2.9%, respectively. Other four extracts had inhibition rate lower than 10%. For anti-HSV-2 activity, acetone, ethanol and methanol extracts exhibited significant inhibitory effect, whereas benzene, chloroform, ethyl acetate and *n*-hexane fractions possessed slight effect. The inhibition rate of 10.0 mcg/ml of acetone, ethanol and methanol extracts was higher than 90%.

Since 10.0 mcg/ml of acetone, ethanol and methanol extracts suppressed HSV-2 multiplication extensively, the antiviral activity was further examined by lower concentrations. Fig. 2 demonstrated that the three extracts had minor effect

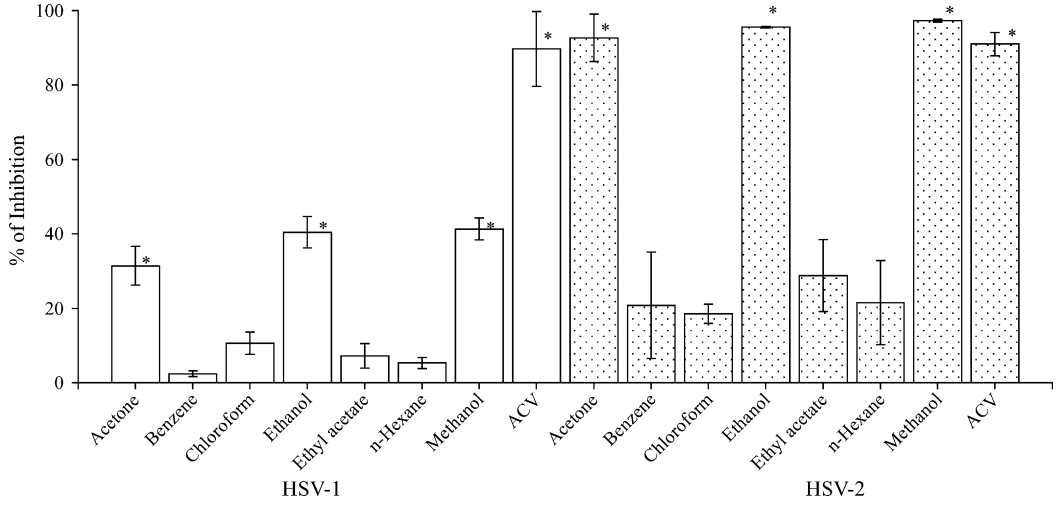

Fig. 1. Effect of extracts of *P. urinaria* on HSV replication in Vero cells determined by plaque reduction assay. The experimental concentration of acetone, ethanol and methanol extracts was 10.0 mcg/ml, whereas of benzene, chloroform, ethyl acetate and *n*-hexane extracts was 20.0 mcg/ml, and of ACV was 0.05 mcg/ml. Each bar represents the mean ± S.D. of three independent experiments. (*) Significant difference between test extract and solvent control ($p < 0.05$).

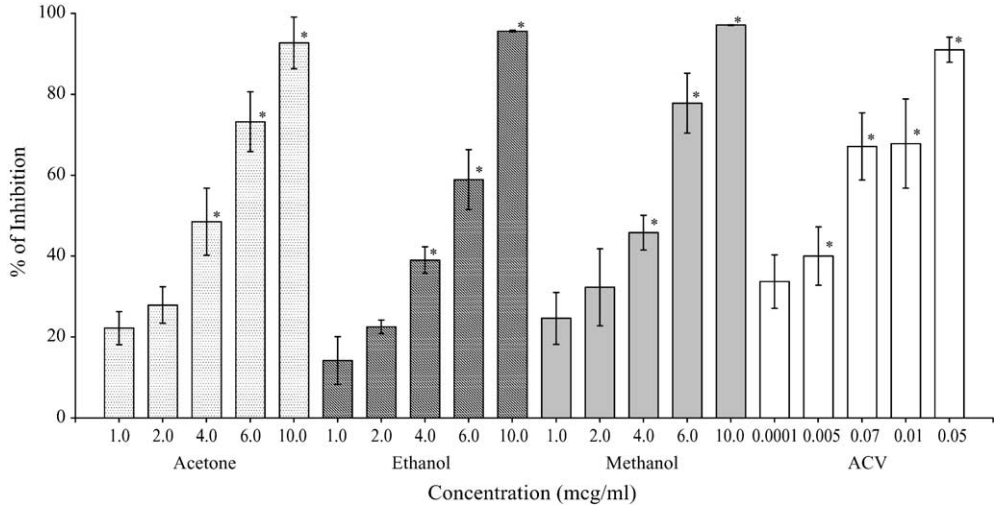

Fig. 2. Concentration effect of acetone, ethanol and methanol extracts of *P. urinaria* on HSV-2 replication in Vero cells. The 50% inhibitory concentration (IC_{50}) of each extract was calculated using regression line. Each bar represents the mean ± S.D. of three independent experiments. (*) Significant difference between test extract and solvent control ($p < 0.05$).

in suppressing HSV-2 replication at concentrations of 1.0 and 2.0 mcg/ml, with an inhibition rate lower than 30%. The IC_{50} value of acetone, ethanol and methanol extracts was 4.3 ± 0.5, 5.0 ± 0.4 and 4.0 ± 0.9 mcg/ml, respectively.

3.3. Effect of addition time on the antiviral activity of the extracts of P. urinaria

To study the inhibitory effect of acetone, ethanol and methanol extracts of *P. urinaria* on the stage of HSV-2 infection, the extracts were added at different periods (before, during, and after) of HSV-2 infection. Results showed that the three extracts suppressed HSV-2 infection when added just after the virus inoculation (0 h). The inhibitory rate was higher than 85% (Fig. 3). However, the inhibitory rate declined to 40% or less when added at either prior (−6 and −2 h) or post (2, 4 and 8 h) infection. This observation indicated that the extracts affect the initial stage of HSV-2 infection.

3.4. Effect of the extracts of P. urinaria on HSV-2 infectivity

Table 1 displays the virucidal ability of acetone, ethanol and methanol extracts of *P. urinaria* on HSV-2. The three extracts completely diminished HSV-2 infectivity in the condition of 37 °C, 6 h. They remained active even when low concentration (2.5 mcg/ml) was applied. When the incubation time was reduced from 6 to 0.5 h, the virucidal activity was mildly decreased at lower concentration, especially for 2.5 mcg/ml of acetone extract. But 2.5 mcg/ml of acetone ex-

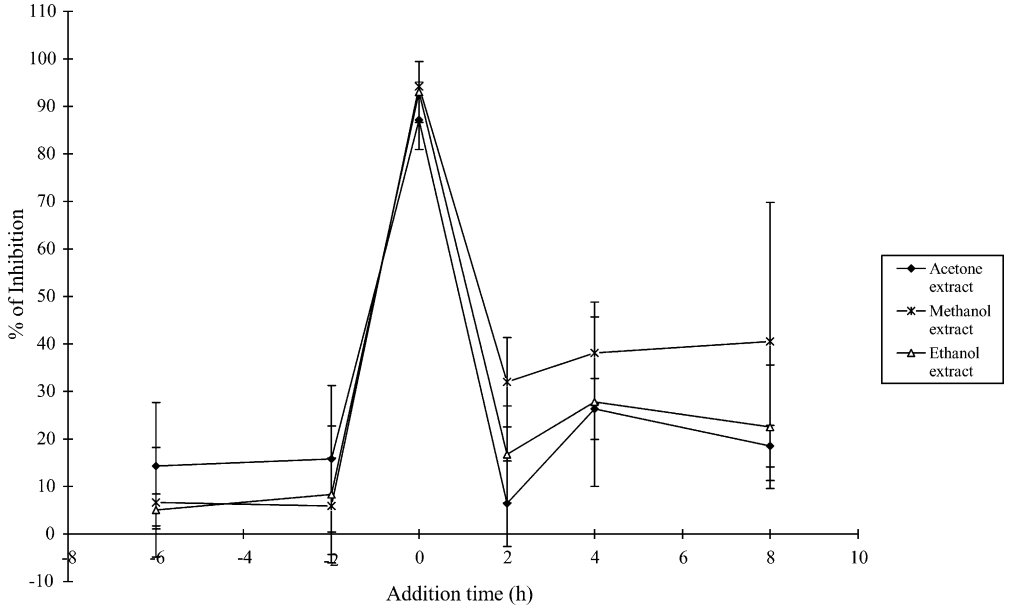

Fig. 3. Time-of-addition effect of acetone, ethanol and methanol fractions of *P. urinaria* on HSV-2 replication in Vero cells. Ten mcg/ml of acetone, ethanol and methanol extract of *P. urinaria* was added at either before (−6 and −2 h) or after (0, 2, 4 and 8 h) virus infection. The extracts that were added before virus infection were rinsed off prior to virus exposure. Each value is the result of mean ± S.D. of three independent experiments.

Table 1
Effect of acetone, ethanol and methanol extracts of *P. urinaria* on HSV-2 infectivity

Concentration (mcg/ml)	Percentage of control		
	26 °C, 0.5 h	37 °C, 0.5 h	37 °C, 6.0 h
Solvent control[a]			
0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Acetone extract			
2.5	31.7 ± 4.5*	28.2 ± 5.3*,#	0.0 ± 0.0*
5.0	28.6 ± 9.0*,$	4.2 ± 0.9*,#	0.0 ± 0.0*
10.0	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Ethanol extract			
2.5	5.7 ± 0.9*	9.1 ± 0.9*,#	0.0 ± 0.0*
5.0	0.3 ± 0.4*	0.7 ± 0.3*	0.0 ± 0.0*
10.0	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Methanol extract			
2.5	5.2 ± 0.7*	2.4 ± 0.9*	0.0 ± 0.0*
5.0	0.2 ± 0.2*	0.0 ± 0.0*	0.0 ± 0.0*
10.0	0.2 ± 0.2*	0.0 ± 0.0*	0.0 ± 0.0*

Values represent the mean ± S.D. of three independent experiments.
[a] Solvent control is sterile de-ionized distilled water which contains less than 0.1% of DMSO.
* $p < 0.05$ compared to the solvent control.
# $p < 0.05$ compared to the 37 °C, 6.0 h.
$ $p < 0.05$ compared to the 37 °C, 0.5 h.

tract was still considered to be active to reduce virus infectivity when compared to the solvent control group ($p = 0.0028$). Lowering the temperature from 37 to 26 °C did not drastically affect the virucidal activity of the extracts except for 5.0 mcg/ml of acetone extract. In general, all three extracts significantly reduced HSV-2 infectivity in different conditions (37 °C, 6 h; 37 °C, 0.5 h and 26 °C, 0.5 h). Nevertheless, they did not affect HSV-1 infectivity in the condition of 37 °C, 6 h (data not shown).

4. Discussion

In this study, the acetone, ethanol and methanol extracts of *P. urinaria* were found to inhibit HSV-2 but not HSV-1 infection appreciably. The three extracts were only effective when added concurrently with HSV-2 infection. Also, they can directly reduce virus infectivity.

The efficacy of acetone, ethanol and methanol extracts of *P. urinaria* to suppress HSV-2 but not HSV-1 infection hinted

a specific activity of these three extracts in their antiviral activity. In taxonomy, both HSV-1 and HSV-2 are the members of genera *Simplexvirus* of subfamily *Alphaherpesvirinae* of family *Herpesviridae* (Roizman and Pellet, 2001). They have many similar characteristics but can be distinguished by clinical manifestations, and biochemical and serological examinations. The available anti-HSV medicines are generally active for those of HSV-1 and HSV-2 (De Clercq, 2001; Naesens and De Clercq, 2001). Our results discovered that the acetone, ethanol and methanol extracts of *P. urinaria* suppressed HSV-2 multiplication. They were, however, less effective in inhibiting HSV-1 infection. Moreover, the extracts did not inactivate cell-free HSV-1 as they did for HSV-2 (data not shown). These findings suggest that the antiviral profile of the extract from *P. urinaria* is in someway different with currently offered anti-HSV drugs.

The antiviral activities in treating the Vero cells with acetone, ethanol and methanol extracts of *P. urinaria* for 0 h or 6 h before challenge by HSV-2 were similar (data not shown). Pre-incubation of the Vero cells with extracts of *P. urinaria* and then washing the extracts out did not protect the cells from HSV-2 infection (Fig. 3). Furthermore, the extracts of *P. urinaria* can inhibit HSV-2 infection only when it was added on during (0 h) but not after (2 h or later) the virus inoculation period (Fig. 3). These observations indicated that the extracts of *P. urinaria* inhibited the initial stage of HSV-2 infection. However, as the data suggested, there is no benefit to give the extract before infection. Therefore, the extracts of *P. urinaria* were concluded to be disturbing the initial stage of HSV-2 infection which included the attachment of the virus to the cell membrane, the penetration of the virus through the cell membrane and the transfer of viral DNA into the cell nucleus. Nevertheless, further studies are needed to verify the underlying mechanism of action of the extracts from *P. urinaria* in inhibiting HSV-2 infection.

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; Wald and Link, 2002). The prevention of HSV-2/HIV shedding and that of genital ulcer formation are the direct approaches (Celum, 2004). Although further evidence and consideration are needed to conclude out the recommended management for this problem, the antiherpetic agents which kill virus directly may be beneficial (Celum, 2004).

In this study, the acetone, ethanol and methanol extracts of *P. urinaria* were found to diminish virus infectivity significantly at concentration lower than that of IC_{50} value. The most interesting aspect is that the lowering of incubation temperature or time did not influence their virucidal activity dramatically. Those observations indicate that *P. urinaria* has potential to be further explored as a virucidal agent.

In summary, the acetone, ethanol and methanol extracts of *P. urinaria* were concluded to possess anti-HSV-2 activity specifically. They inhibit HSV-2 infection primarily through interfering with the early stage of HSV-2 multiplication and also through diminishing the HSV-2 infectivity. These findings merit further investigation on antiviral activity of *P. urinaria*. We are continuing our studies on the anti-HSV-2 profile of *P. urinaria* by purifying the pure compounds from the effective extracts, and also by examining their inhibitory effect on other RNA and/or non-enveloped viruses.

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