



***In vitro* antiviral activity of styrylpyrone derivative-incorporated formulations against Herpes simplex virus type-1**

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ABSTRACT

Aims: This study was aimed to evaluate *in vitro* antiviral activity of topical formulations incorporated with a styrylpyrone derivative (SPD) against Herpes Simplex Virus type 1 (HSV-1).

Methodology and results: Two types of SPD-incorporated formulations (ointment and gel) were tested for their antiviral activity against HSV-1 clinical strain using plaque reduction assay on Vero cells. The antiviral activity was determined based on the percentage of plaque reduction occurred between treatment and control (non-treated infected cells). In this study, 10% SPD-gel (SPD = 0.004 mg) and 20% SPD-ointment (SPD = 0.003 mg) showed plaque reduction percentage of 87% and 79% respectively. Further evaluation on the ointment base, gel base (formulation without SPD) demonstrated less than 10% of antiviral activity while pure SPD at 0.0025 mg showed 81% of plaque reduction. These results indicated that the antiviral activity observed in both SPD-incorporated ointment and gel was mainly due to SPD regardless of formulation components. Furthermore, the antiviral activities observed in both SPD-incorporated products were also in agreement with the antiviral activity observed in pure SPD.

Conclusion, significance and impact study: SPD-incorporated products retained the antiviral activity and can further be tested in animal model.

Keywords: HSV-1, antiviral, *Goniothalamus umbrosus*, styrylpyrone derivative

INTRODUCTION

Current strategy to fight HSV-1 infection is by using commercial drug such as acyclovir (ACV). The drug reacts as DNA chain terminator during DNA synthesis, where viral thymidine kinase (TK) followed by cell host kinases phosphorylate ACV into competitive inhibitor of viral DNA polymerase (De Clercq, 2004). Viral strains resistant to acyclovir have emerged due to mutation in TK or DNA polymerase (Hussin *et al.*, 2013). Other alternative such as foscarnet is also facing resistance issue (Saijo *et al.*, 2005). This scenario has encouraged scientists to develop alternative drugs which depend on different antiviral pathway.

Styrylpyrone derivative (SPD) is one of the bioactive compounds isolated from *Goniothalamus umbrosus*. Its potent antiviral property against HSV-1 was by inducing apoptosis and cell cycle arrest in infected cells (Md. Nor, 2015) and virucidal activity (Moses *et al.*, 2014). However, the effect of the compound *in vivo* has yet to be studied. Prior to *in vivo* study of this compound, the antiviral efficacy of topical formulation should be tested *in vitro*, since some components in the formulation might interfere with the biological activity of the active ingredient

(Georgetti *et al.*, 2006). Thus, the aim of this study is to evaluate the antiviral activity of SPD-incorporated formulations against HSV-1.

MATERIALS AND METHODS

Preparation of topical formulation

Two types of SPD-incorporated products were used in this study which were ointment-based and gel-based formulation. Preparation of SPD crystal isolated from root extract of *Goniothalamus umbrosus* was done through crystallization process based on Jewers *et al.* (1972). The purity of isolated SPD was evaluated using gas chromatography-mass spectrometry (GC-MS). For SPD-incorporated ointment, it consisted of 20% (w/w) SPD, 50% blackseed oil and 80% (w/w) white petroleum jelly (Vaseline™). For SPD-incorporated gel, it consisted of 10% (w/w) SPD, 1% (w/w) soy-lecithin (Shaklee®), 20% (v/w) methyl benzoate (Nacalai Tesque, Japan) 4% (v/w) Tween 80 (Sigma-Aldrich, USA), 2% (w/w) hydroxypropyl methylcellulose (Lotioncrafter®, USA) and deionized water. Both formulations were prepared based on Allen *et al.* (2011) with several modifications in terms of the

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volume and ratio of the components in the formulation. Both formulations were stored at room temperature.

Preparation of both formulations for cytotoxicity and antiviral activity was done based on Dorwal (2012) with modifications of ethanol being used as the solvent. Briefly, SPD was extracted from ointment or gel by mixing ~10 mg of respective formulation with absolute ethanol and vortexed for 15 min. Undissolved components were separated by centrifugation at 4000 rpm for 5 min (Microfuge 16, Beckman Coulter, USA). This step was repeated twice. The extracted SPD was diluted into several concentrations for cytotoxicity testing. Final concentration for ethanol was less than 1%.

Cytotoxicity screening

Screening on cytotoxicity of extracted SPD and respective formulation bases were conducted using 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) based on Mossman (1983). First, Vero cells at 2×10^5 cells/mL was seeded in 96-well flat bottom plate supplemented with Dulbecco's Modified Eagle Medium (DMEM), 5% fetal bovine serum (FBS) and incubated overnight. Upon confluency, cells were treated with extracted SPD which was already serially diluted in DMEM supplemented with 5% FBS. Cells with media only were used as negative control and empty well filled with media was used as blank. Cells were further incubated for 48 hours. After incubation, medium was decanted and replaced with 40 μ L MTT plus 100 μ L DMEM and cells were incubated for 3 h. Formazan crystals formed after

incubation were dissolved using 100% dimethyl sulphoxide (DMSO) and the optical absorbance was taken at 545 nm using Chromate (Awareness Technology Inc, USA). Cell viability was determined using the formula below. Non-linear regression was done to calculate 50% cytotoxic concentration (CC_{50}).

$$\text{Cell viability percentage} = \frac{OD_{\text{test well}} - OD_{\text{blank}}}{OD_{\text{negative control}} - OD_{\text{blank}}} \times 100$$

Antiviral test

Plaque reduction assay for antiviral activity was based on Souza *et al.* (2008) with modification in the virus concentration. Briefly, 2×10^5 cells/mL of Vero cells was seeded on 12-well plates supplemented with DMEM and 5% FBS and incubated overnight. After overnight incubation, cells were infected with 50 PFU (plaque forming unit) of HSV-1. After two hours adsorption period, infected cells were treated with 3 different concentrations for each formulation (with and without SPD). Pure SPD at 0.0025 mg (12.5 μ M) was used as experimental control while ACV at 0.001 mg (5 μ M) was used as positive control. Each well was overlaid with culture media (DMEM+ 5% FBS+ 1% methylcellulose) and further incubated for another 48 h. After 48 h incubation or until visible plaques appeared, all cells were stained with crystal violet and plaques were counted under microscope. Percentage of plaque reduction was calculated based on formula below:

$$\text{Plaque reduction percentage} = \frac{\text{Average plaque count}_{\text{non-treated infected cells}} - \text{average plaque count}_{\text{test well}}}{\text{Average plaque count}_{\text{non-treated infected cells}}} \times 100$$

Statistical analysis

One-way ANOVA or student t-test was conducted to analyse significant difference where difference was considered significant if p value < 0.05.

RESULTS

Cytotoxicity screening showed CC_{50} value for ointment and gel base were 0.84 mg/mL and 14.0 mg/mL respectively, while for SPD extracted from 20%-SPD ointment and 10%-SPD gel showed CC_{50} value of 0.09 mg/mL and 0.30 mg/mL respectively (Figure 1).

Since determination of what concentration to be used in antiviral activity is based on CC_{50} , therefore, different concentration used between SPD-ointment and SPD-gel was due to different CC_{50} value. For antiviral activity, both SPD-incorporated ointment and gel showed a significant antiviral activity against HSV-1. As depicted in Figure 2A and 2B, the formulation bases only showed less than 15% plaque reduction percentage at their highest non-toxic concentration. On the other hand, both SPD-incorporated ointment and gel showed high plaque reduction percentage at 86% and 98% respectively (Figure 3A and

3B). The actual SPD concentration in each formulation was also found to exert similar antiviral activity showed by purified form. For ointment, 20% SPD-ointment (SPD = 0.003 mg) showed 79% plaque reduction while 10% SPD-gel (SPD = 0.004 mg) showed 87% plaque reduction. Pure SPD at 0.0025 mg showed 81% plaque reduction (Figure 2C), which is in accordance with previous report where SPD at 0.0025 mg (12.5 μ M) was not toxic and showed high antiviral activity (Md Nor and Ibrahim, 2011). Further analysis showed in Table 1 suggested that the antiviral activity was still significant even after subtraction with the antiviral activity exerted by formulation base.

DISCUSSION

The original purpose of conducting antiviral screening for SPD-incorporated formulation is to evaluate whether SPD still retain the antiviral activity even after being mixed into a mixture of chemical components. The antiviral activities observed in both SPD-incorporated ointment and gel was in agreement with activity observed in pure SPD. In comparison to ACV, the activity of SPD was slightly lower than ACV. This is expected as ACV mode of action is specifically targeting viral DNA polymerase activity, which

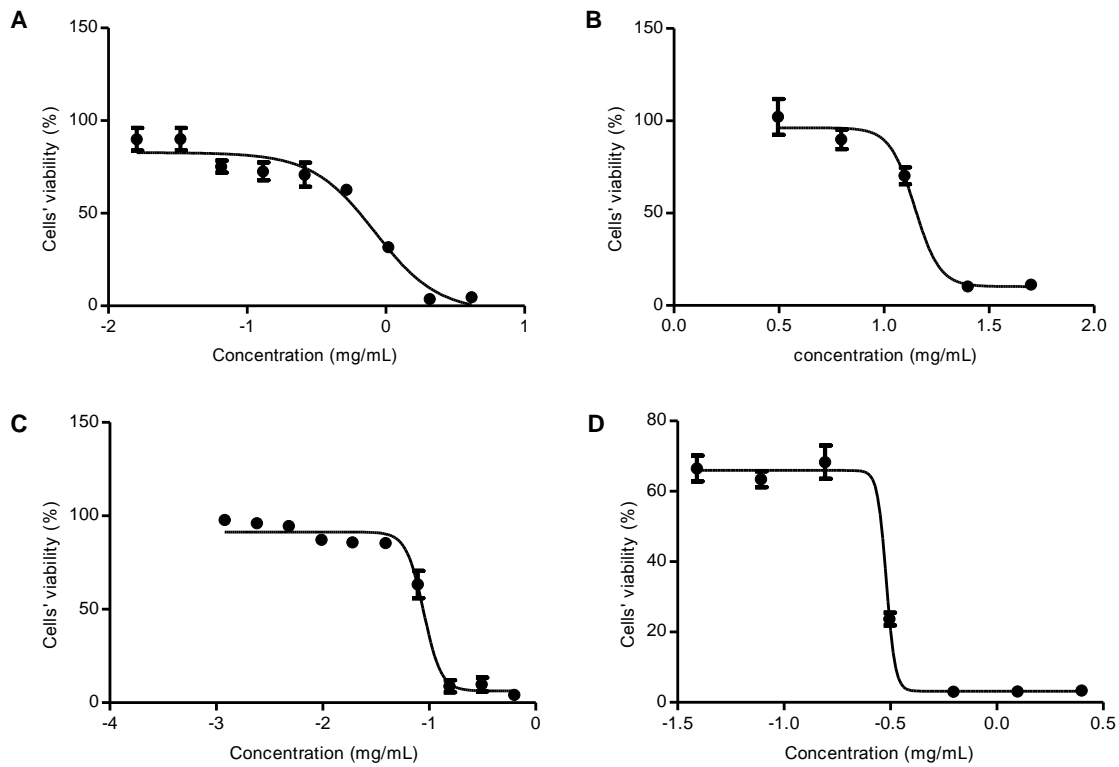


Figure 1: Cytotoxicity screening of formulation bases and SPD extracted from formulation. A, ointment base; B, gel base; C, 20% SPD-ointment and D, 10% SPD-gel. SD bar represented the data from at least three independent experiments.

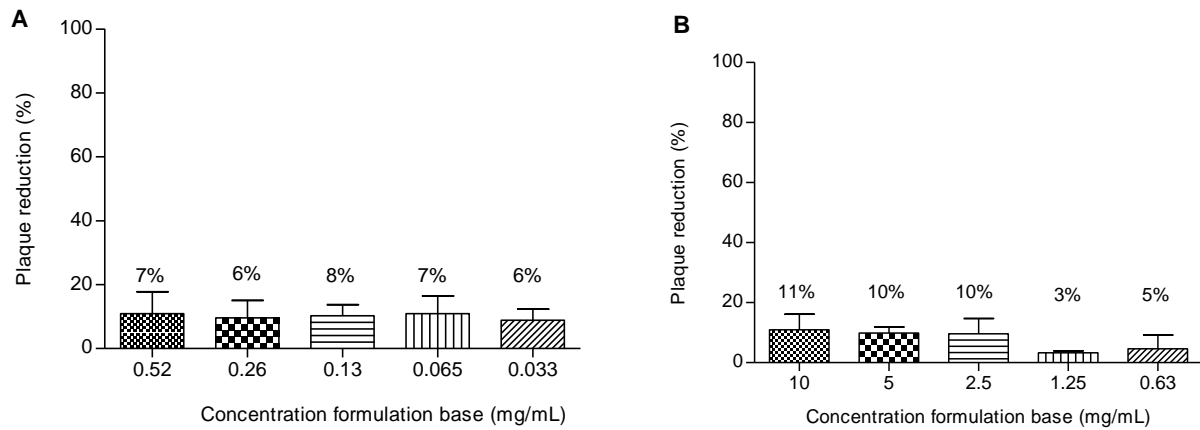


Figure 2: Plaque reduction percentage of formulation bases where A, ointment base and B, gel base.

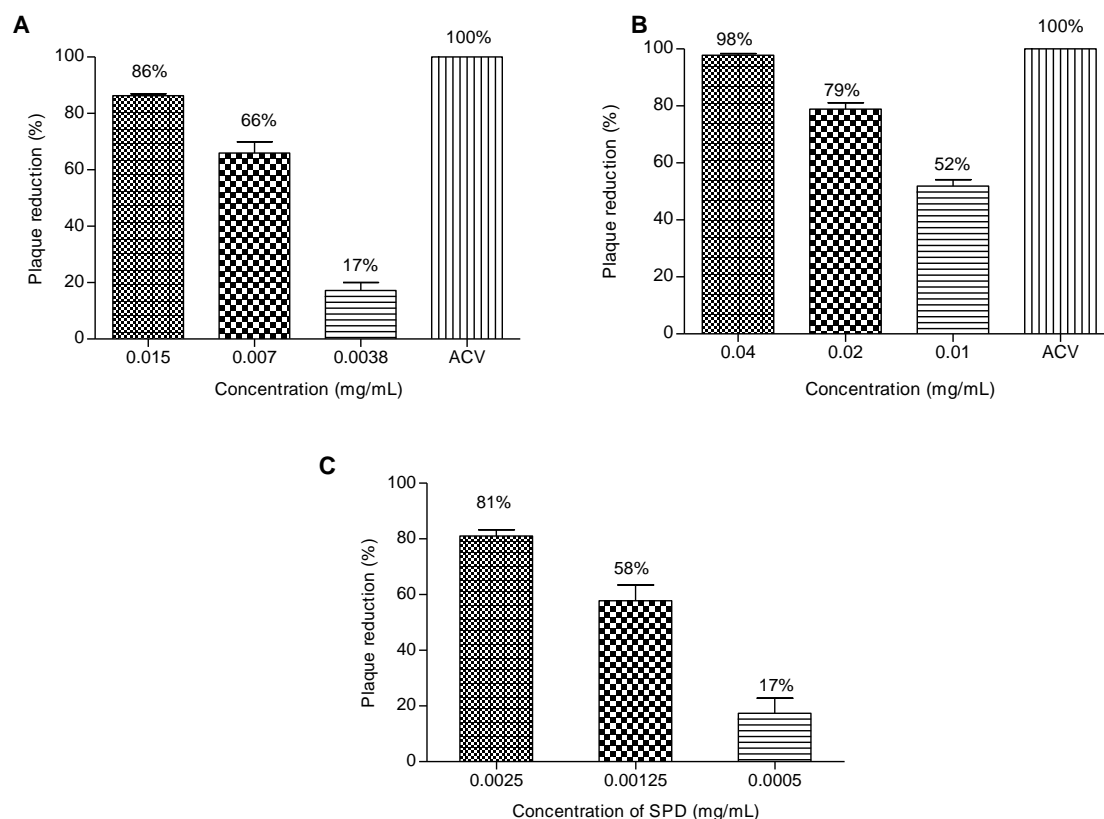


Figure 3: Plaque reduction percentage for SPD-incorporated formulations and pure SPD (crystal form) where A, 20% SPD-incorporated ointment; B, 10% SPD-incorporated gel and C, pure SPD. Concentration of positive control (ACV) was 0.001 mg/mL. SD bar represented the data from at least three independent experiments.

Table 1: SPD concentration and plaque reduction percentage after normalized with baseline activity. SD bar represented the data from at least three independent experiments.

SPD conc. in formulation (mg/mL)	% Plaque reduction formulation - % plaque reduction formulation base
20% ointment (0.015 mg/mL)	86 – 7 = 79%
10% gel (0.04 mg/mL)	98 – 11 = 87%

Note: SPD concentration was calculated based on original stock of 10 mg formulation. 20% ointment consist of 2 mg SPD, therefore SPD in 0.015 mg formulation is (0.015mg × 2 mg SPD/10 mg). 10% gel consist of 1 mg SPD, therefore SPD in 0.04 mg formulation is (0.04 mg × 1 mg SPD/10 mg). SD bar represented the data from at least three independent experiments.

is known to be one of the most efficient antiviral targets (Allaudeen *et al.*, 1982). However, virus can easily mutate which leads to resistance towards ACV (Christophers *et al.*, 1998) and also to other alternatives namely Foscarnet and Cidofovir (Burrel *et al.*, 2013). Antiviral drug with mechanism that does not directly targets the virus has less chances to cause virus mutation and become resistant to drug (Muller and Krausslich, 2009). Since SPD mechanism was not directly targeting the virus where it induces cell cycle arrest and apoptosis in virus-infected cells (Md Nor, 2015), SPD has a good potential as a new antiviral drug and is worth to be further studied.

Baseline antiviral activity observed in both formulation bases were expected since some of the components used in the formulation itself have antiviral potential, such as black seed oil against murine cytomegalovirus and Hepatitis C virus (Salem and Hossain, 2000; Barakat *et al.*, 2013) and lecithin against Hepatitis A virus (Ramadan and Asker, 2009). However, ability of SPD-incorporated ointment and SPD-incorporated gel to retain the antiviral activity after subtraction with antiviral in respective formulation bases proved the activity shown by both ointment and gel were not masked by the formulation components.

Since both 20%-SPD ointment and 10%-SPD gel retained the antiviral activity, both formulations will be further studied *in vivo*. Preparation of two different types of formulations was intended to test suitability of each formulation as topical antiviral. Emollient effect exerted by ointment and its immiscibility with water will allow it to remain on the skin without being easily washed out. This may help in prolonged exposure to the drug (Bajaj *et al.*, 2012). Meanwhile, gel has a non-greasy feature which is easily spread thus allowing it to thoroughly cover the affected area (Ajazuddin *et al.*, 2013). In conclusion, both SPD-incorporated ointment and gel were proven to retain the antiviral activity against HSV-1 and should be evaluated *in vivo*.

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