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Syzygium Polyanthum Inhibit Herpes Simplex Virus Type 1 (HSV-1) Replication Cycle *In Vitro*

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This study aims to investigate the antiviral activities of methanolic extract of *Syzygium polyanthum* towards Herpes simplex virus type 1 (HSV-1). Plaque reduction assays were carried out to evaluate the antiviral activity of *S. polyanthum* extract against HSV-1. Time dependent studies were conducted to evaluate the effect of delayed treatment and also the effect of different time exposure to *S. polyanthum* methanol extract againstHSV-1 antiviral activity. The effect on replication phases of HSV-1 was determined by time-of-addition and time-of-removal assays. Time-of-addition assay showed the extract inhibits 70% of the virus at 2 hours post infection (hpi). In time-of-removal assay, treatment with extract caused 20% reduction of plaque formation as early as 2 hpi and reached more than 80% after 16 hpi. Gene expression analysis by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) using selected primers was done to investigate the level of gene transcript at different replication phases of HSV-1 which included immediate early genes (UL54), early gene (UL30), and late gene (UL27). The result showed that transcript levels of these genes in infected cells treated with *S. polyanthum* extract were reduced compared to infected cells without treatment. This study showed that *S. polyanthum* extract has potential as anti-HSV-1 by the following modes: interruption of virus attachment and penetration into cells, direct damage to viral particle, reduction of viral progeny infectivity, and reduction of expression of HSV-1 genes at different phases of viral replication.

Keywords: *Syzygium polyanthum*; antiviral; herpes simplex virus type 1; plaque reduction assay

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Herpes simplex virus (HSV) is part of the alpha subfamily of human herpesviruses [1]. To multiply, these viruses rely on the host cell nucleus for DNA replication and transcription via RNA with subsequent synthesis of their gene products [2]. HSV's capability to recover from latency and initiate a vigorous replication cycle leads to its potential of causing persistent diseases that manifest as recurrent infection, viral shedding, and spreading to new hosts [3]. All herpesviruses have the ability to generate both lytic and latent infections. Replication of a virus in a host cell leads to lysis, releasing hundreds to thousands of genetic virions which is known as a lytic infection [4]. The virus remains dormant until a reactivation occurs, which results in recurrent infection and the spread of the virus to new host organism. In sensory neurons, the HSV latency-associated transcript helps in development of heterochromatin on the HSV lytic genes and the transcriptional silencing of these genes throughout latent infection with the virus [5].

The most common type of HSV is herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2). HSV-1 is typically spread by oral-to-oral contact and is responsible for the majority of occurrences of oral herpes, often manifested as cold sores around the mouth. HSV-2, which causes genital herpes, is commonly transmitted sexually via direct contact and usually causes itching, pain, and blisters around the genital [6]. Despite the fact that a comparatively small fraction of infected person's exhibits clinical symptoms, the significant proportion of the global population infected with these viruses results in a vast number of people suffering from HSV-related conditions [7]. In many advanced countries, HSV-1 has taken over as the main cause of genital herpes in young adults. According to a local study [8], the University Malaya Medical Centre in Kuala Lumpur, Malaysia, took samples from 274 people who might have had genital herpes between 1982 and 2008; out of the 118 genital herpes cases, 42.4% were caused by HSV-1 and 57.6% were caused by HSV-2. HSV-1 was more likely to be found in Malay and Indian patients than it was in Chinese patients; this could be because of differences in how individuals behave sexually between cultures.

Several antiviral medications can be prescribed by doctors for treating HSV which is usually first line antiviral agents such as acyclovir (ACV), valacyclovir, penciclovir, and famciclovir [9]. Unfortunately, cases of HSV resistance to ACV have been reported.

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Alarmingly, HSV resistance to ACV rose considerably from 3.8% in 2002–2006 to 15.7 % in 2007-2011 [10]. One of the cases is that the incidence of allogeneic HSCT (hematopoietic stem cell transplantation) with T-cell depletion has been rising, which is a risk factor for the establishment of drug-resistant HSV infections [11]. The rise of HSV strains resistant to existing medications, as seen in the first-line treatment ACV, had increased this issue [12]. Thus, plants extract has long been proposed to be an alternative source of antiviral chemicals useful to humans, and they continue to aid in the development of new therapeutic compounds [13].

Syzygium polyanthum is one of the plants that has been traditionally used as a remedy for diseases such as diabetes, obesity, gout, arthritis, and diarrhoea [14]. *S. polyanthum* is rich in bioactive compounds, including essential oils, terpenoids, tannins, and flavonoids [15]. These phytochemical compounds, which have strong antioxidant action, can aid in the inhibition of viral genome replication which disable the viral lipid envelope [16]. Several studies have found that *S. polyanthum* can significantly act as anti-microbial. It has been reported that alkaloids, saponin, terpenoids, and steroid compounds have a bacterio-static or bactericidal impact on wide variety of Gram-positive and Gram-negative bacteria [17]. Alkaloid compounds have also been reported to have antiviral activity against influenza virus by inhibiting viral proteins and releasing of certain interferons in the immune system to inhibit the virus replication [18]. Therefore, in this study, we investigated the potential effect of *S. polyanthum* methanol extract against HSV-1 viral replication *in vitro*.

The methanol extract of *S. polyanthum* is safe to cells, protects cells against viral infection, inhibits viral attachment and penetration, and also has virucidal effect [19]. *S. polyanthum* methanolic leaves extract demonstrated broad-spectrum activity against Gram-positive and Gram-negative bacteria. Qualitative tests for secondary metabolites in methanol extracts of *S. polyanthum* leaves revealed the presence of alkaloids, saponin, terpenoids, and steroid which may be responsible for the antibacterial and antiviral activity demonstrated [17]. Therefore, this study was done to further determine the antiviral effect of *S. polyanthum* methanol extract with regards to *in vitro* inhibition of different stages of HSV-1 replication.

EXPERIMENTAL

Plant Materials and Extraction

The methanol is widely used as a solvent to extract antimicrobial substances from plants [20]. Therefore, the obtained leaves samples of *S. polyanthum* was extracted with methanol (CH_3OH 100%; 1 L). Fresh-leaves of *S. polyanthum* were collected and the leaves were washed under running tap water and rinsed with distilled water. The leaves were then air dried for 48 hours, homogenized into a fine powder, and stored in air-tight plastic containers. The powder was then extracted using methanol solvent. 300 g of the plant powder was soaked in 1.5 L of methanol and kept in room temperature for 72 hours. The extract was filtered using Whatman No1 filter paper. The residue was further extracted twice with fresh methanol solvent. Then, all the filtrates were mixed. Methanol was removed from the extract using rotavapor and the extracts were kept for several days in an oven at 40°C for drying. After complete drying, the yield of extract was measured, and the extract was stored until used for study [21].

Cell Lines and Growth Conditions

Vero cell from American Type Culture Collection (ATCC) CCL-81 was used for both cytotoxicity, virus propagation, and antiviral test. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal bovine serum (FBS) was used for Vero cell maintenance throughout the experiment. Herpes Virus Type 1 (HSV-1) used in this study is a clinical isolate strain, a kind gift from the Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Briefly, the virus was propagated in Vero cells, harvested, and the virus titre was determined by plaque forming assay (PFA) [19].

Antiviral Activity

Time dependent studies were designed based on a single cycle of HSV-1 replication can take up to 24 hours to complete [4]. Antiviral assays are composed of time-of-addition assay and time-of-removal assay. The method was adapted from a previous study [22] with some modifications.

(a) Time-of-Addition Assay

Vero cells (2.0×10^5 cells/mL) were seeded in 24 well plate and incubated overnight in 5% CO_2, 37°C incubator. Then, Vero cells were infected with 50 plaque-forming unit (PFU) of HSV-1 for 2 hours at 37°C. At 2, 4, 6, 8, and 10 hours post infection (hpi), 0.015 mg/mL of *S. polyanthum* methanolic extract was added to the infected cells, followed by the addition of 1% methyl cellulose. The plate was incubated for 48 hours in 5% CO_2, 37°C incubator. Treatment with ACV was added on infected cells as a positive control, while infection without treatment was performed on infected cells as a negative control. Following incubation, cells were stained with crystal violet solution (0.4%, w/v) and incubated for 30 minutes at room temperature. Plaque reduction percentage (%) was calculated as below:

$$\frac{\text{No.plaque}_{\text{infected non-treated}} - \text{No.plaque}_{\text{infected with treatment}}}{\text{No.plaque}_{\text{infected non-treated}}} \times 100$$

(b) Time-of-Removal Assay

Vero cells (2.0 x 10^5 cells/mL) were seeded in 24 well plate and incubated overnight in 5% CO_2, 37˚C incubator. The media was discarded and washed with PBS solution. Then, Vero cells were infected with 50 PFU of HSV-1 for 2 hours at 37°C. Then, 0.015 mg/mL of *S. polyanthum* methanolic extract was administered to the infected cells. At 2, 4, 6, 8, 16, 18, 20, 22, and 24 hpi, cells treated with the *S. polyanthum* methanolic extract was removed and overlayed with 1% methyl cellulose. The plate was cultured for 48 hours in the 5% CO_2, 37˚C. Following incubation, cells were stained with crystal violet solution (0.4%, w/v) and incubated for 30 minutes at room temperature. Plaque reduction percentage (%) was calculated as below:

$$\frac{\text{No.plaque}_{\text{infected non-treated}} - \text{No.plaque}_{\text{infected with treatment}}}{\text{No.plaque}_{\text{infected non-treated}}} \times 100$$

Quantitative Real Time – Polymerase Chain Reaction (qRT-PCR)

HSV-1 RNA was harvested from the HSV-1 infected Vero cells. Viral RNA was extracted using GENEzol TM TriRNA RNA extraction kits. The real-time PCR assay was carried out by adding 3 mL of extracted HSV-1 RNA to the Qpcrbio SyGreen mixture (qPCRBio SyGreen One-Step Go Hi-ROX) which consisted of 2×Qpcrbio SyGreen 1-Step Mix (10 mL), RNAse Inhibitor (20 units), ddH_2O (4.4 mL), forward and reverse primers (0.8 mL). The specific primers for qRT-PCR are listed in Table 1, which represented all three phases of HSV-1 life cycle: immediate early phase (UL 54), early phase (UL 30), late phase (UL 27); and housekeeping genes (RPL- 32). RPL 32 was used as an internal control gene to normalize the amount of gene mRNA in the samples using qRT-PCR 23]. In HSV-1, RPL 32 controls viral DNA synthesis and late gene expression in the nucleus [24]. Theamplification was carried out using the Step One Plus Real-time PCR System (Applied Biosystems) with the following thermal cycling conditions: reverse transcription at 45°C for 10 minutes, polymerase enzyme activation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing at 65°C for 30 seconds followed by a final melt curve analysis using instrument default settings [25].

Statistical Analysis

The statistical software used was Prism 6 (GraphPad software, CA, USA). Values were expressed as means + standard errors of the mean.

RESULTS AND DISCUSSION

Numerous studies have identified the phytochemical composition of *S. polyanthum* which provides many beneficial effects in medical fields. Flavonoids and tannin, two effective antimicrobial metabolites found in *S. polyanthum*, have been shown to have antimicrobial effects in recent studies [26]. The leaves of *S. polyanthum* which consist of tannin components can prevent the MRSA growth from replicate by inhibit the cell wall development and cell membrane activity [27]. Alkaloids' potential to inhibit bacterial growth was associated with their ability to bind with DNA, hence blocking DNA synthesis and reverse transcriptase based on the result of agar diffusing assay [28]. Another research found that an ethanolic extract of *S. polyanthum* leaves was effective against *Shigella dysenteriae* with bactericidal concentrations ranging from 10% to 20% w/v [29]. *S. polyanthum* leaf essential oil significantly reduced the development of *Bacillus subtilis* [30]. Extracts of plants containing alkaloids have been used as several drugs that act as local anaesthetic such as morphine. Antiviral terpenoids such as monoterpenoids, isoborneol, and borneol, among others, have powerful activity on HSV-1 [32]. According to a study [33], saponin derived from plant extracts exerts potential anti-viral effects by cytopathic effect on HSV-1. Thus, the richness of phytochemical contents in *S. polyanthum* may contribute to anti-HSV-1 properties.

S. polyanthum methanolic extract inhibits all phases of viral replication in time-dependent assays. *S. polyanthum* methanolic extract concentration 0.015 mg/mL was administered to the HSV-1 infected cells within the varying length of time in time-of-addition assay (Figure 1). The virus plaque reduction at 2 hpi

Table 1. List of forward and reverse primers.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
UL54	GAC GGG TCT CCT GGG AAA C	ATA ATG GGG TCC TGG GGG C
UL30	CGC CCC GCT CTG TTT TAC	CCA GCC GAA GGT GAC GAA C
UL27	CGG TGG TTC GTC GTA TGG G	GGC GGC GTT GGG TTT TTC
RPL-32	AAC ATT CCA TCT CCT CCT CGG	TTG ACA TAC CGG TCT GAC TGG TGC

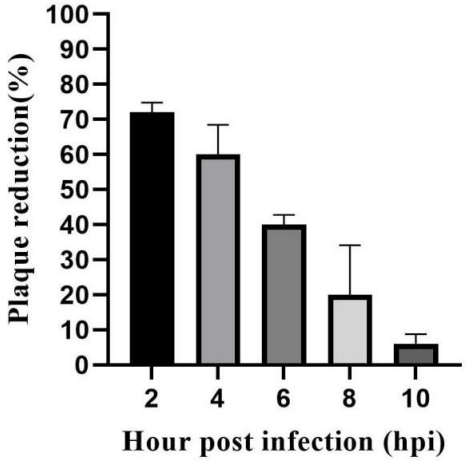

Figure 1. Time-of-addition assay. Graph shows antiviral activity of *S. polyanthum* methanolic extract when treatments were added at different time after infection. Data represent mean values ± SEM (n = 3).

when treated with *S. polyanthum* was high which is 72% that showed significant effect in inhibiting of HSV-1 replication. Prior to increasing of time until 10 hpi, the number of plaque formation increased when the *S. polyanthum* was treated which showed there was no antiviral activity in inhibiting the HSV-1 replication.

The time-of-removal assay was conducted to analyse the effect of *S. polyanthum* methanolic extract at a lower concentration which is 0.015 mg/mL when administered at various times starting at 2, 4, 6, 8, 16, 18, 20, 22, and 24 hpi (Figure 2). The virus plaque reduction at 2 hpi and 4 hpi when treated with *S. polyanthum* was low which is 20% and 36% that means the effectiveness of the plant by inhibiting the HSV-1 replication at early hour was significantly low. Upon the increasing of time, the inhibition of the *S. polyanthum* against HSV-1 replication was increased as the plant extract showed stronger effectiveness towards HSV-1 activity especially at 24 hpi at which time 100% of the extract inhibited the virus as there was none of the plaques were visible.

When a virus attaches to a host cell, it penetrates the cell and uncoating (disassembly) takes place, allowing the virus to replicate and produce new virions, which are released from the infected cell and infect other cells [34]. In this study, time-of-addition method investigated at which stage the viral life cycle is disrupted by antiviral compound [35]. *S. polyanthum* extract might interrupts with, for example, the anti-viral compounds of the extract which can inhibit viral replication when it is visible during the reverse transcription process during the viral replication cycle. After the reverse transcription process is done, the extract will no longer be allowed to prevent viral replication whether it is exposed based on time.

In time-of-addition assay, the concentration *S. polyanthum* extract used was 0.015 mg/mL which exhibited from highest antiviral activity in post treatment [19]. *S. polyanthum* extract was treated to HSV-1 infected cell based on the time interval in Figure 1 which the viral replication from the plaque was observed until 10 hpi. The result showed that the longer the viral absorption in the cell before treated, the less effective of the extract based on the increase of plaque formation at 10 hpi. This might cause at early hour, 2, and 4 hpi, the antiviral compound of the extract can slightly inhibit the viral particles when attached to the host cell, but as the time prolonged until 10hpi, the virions disassemble, the HSV-1 capsid remains intact, allowing the virions to continue to replicate [36]. Thus, the efficacy of this extract was not very effective to reduce the viral replication process in late phase. This can conclude that this assay was not parallel to the post treatment, as the extract only show the effectiveness in inhibiting virus attachment at early hour [19].

In time-of-removal assay, the concentration used was as same in time-of-addition assay. The *S. polyanthum* extract was administered after 2 hour of viral absorption and after that was removed based on the time interval in Figure 2. From the result, the percentage of plaque reduction increase from 2 hpi until 24 hpi. This suggests that *S. polyanthum* extract affects HSV-1 replication until late stage as the plaque reduction show 100% inhibition. At early phase, 2 until 8 hpi, the extract shows low effectiveness in antiviral activity. This might be due to the extract having weak antiviral activity for early phase of viral replication which the antiviral agents cannot inhibit the interaction of viral glycoprotein(s) gB or gC with HS proteoglycans (HSPGs) on the cell surface [37]. As the time increased, the extract showed significant effectiveness in late phase of viral replication. This could happen as the antiviral properties of the extract can inhibit the late phase of viral replication process which interfered with envelopment, progression or during viral genes were transcribing [25].

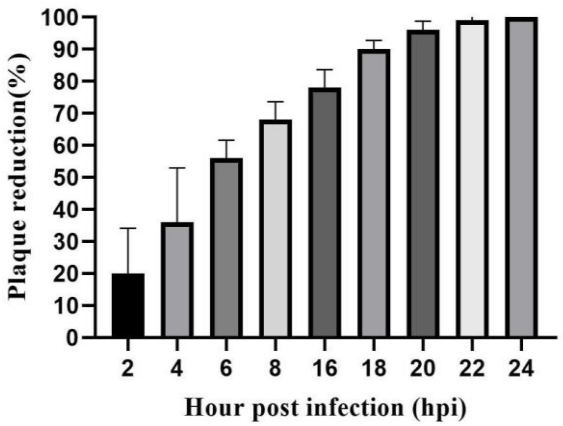

Figure 2. Time-of-removal assay
Treatment was removed at designated time point to evaluate the effect of different time of exposure of *S. polyanthum* methanolic extract. Data represent mean values ± SEM (n = 3).

Effect of *S. polyanthum* Methanolic Extract Treatment on HSV-1 Gene Expression

In order to further study the anti-HSV-1 mechanism of *S. polyanthum* methanolic extract, qRT-PCR analysis was conducted to monitor transcription levels of the viral genes including UL54, UL27, and UL30 within 24 hours of replication in *S. polyanthum* methanolic extract treated cells. During transcription and replication, HSV express immediate early genes, early genes, and late genes. The UL54 gene encodes the essential protein ICP27 of HSV. The HSV-1 protein ICP4 is required for host cell transcriptional reprogramming upon HSV-1 infection. UL27 gene encoding glycoprotein B (gB), which is a major target antigen in herpesviruses [38]. UL30 is DNA polymerase catalytic subunit, exhibits apurinic/apyrimidinic (AP) and 5′-deoxyribose phosphate (dRP) lyase activities [39].

Based on Figure 3, at 2 hpi, all genes were up-regulated when treated with the *S. polyanthum*. Prior at 8 hpi, only UL54 was up-regulated (less than 1-fold change) whereas the UL27 and UL30 were down-regulated. At 20 hpi, all gene were significantly up-regulated and even though all genes at 24 hpi were all up- regulated, UL27 and UL30 was less than 1-fold change.

qRT-PCR has been used as a technique to identify and quantify target RNAs by amplifying specific nucleic acid sequences in order to facilitate detection and comparison [40]. Gene expression can be studied using this technique in samples ranging in size from one to hundreds of thousands. In this study, we used two sample (treated and untreated) which were gene of interest and reference gene. The gene of interest used was UL54, UL30, and UL27; and RPL 32 as control gene. During transcription and replication, HSV express immediate early genes, early genes, and late genes [41]. HSV has five immediate-early proteins, including infected cell polypeptides 0, 4, and 27 (ICP0, ICP4 and ICP27) [42]. In the early stage of the disease, the UL54 encodes the vital HSV-1 protein ICP27, which can either elevate or repress gK expression. Upstream Binding Factor (UBF) can be relocated from the nucleus to viral replication compartments (VRCs) by ICP27, thereby facilitating viral replication of DNA [43]. ICP27 in HSV is involved in all phases of viral mRNA biogenesis from transcription to RNA processing finally to translation [44]. The viral DNA synthesis was reduced and viral replication proteins were less accumulated when ICP27 was absent [45]. Translation of viral transcripts, host gene expression suppression, cell cycle inhibition at G1 and apoptosis prevention are all possible effects of ICP27 [46].

UL30 is a DNA polymerase from the B sub-family that serves a variety of functions. In order to eliminate mismatched nucleotides, UL30 has polymerase and 3'- 5' DNA cleavage function. DNA polymerase is an important enzyme in the lytic stage of HSV infection. Transcription and replication use the viral DNA as a template [47]. Ensuring the growth of HSV-1 requires the presence of glycoprotein B (gB). After viral entry but before virus-specific proteins are expressed, gB is needed. The UL27 gene encoded it in the late stages of HSV-1 replication [48]. RPL32 was used as an internal control gene to normalize the amount of gene mRNA in the samples using qRT-PCR. In HSV-1, RPL32 controls viral DNA synthesis and late gene expression in the nucleus [49].

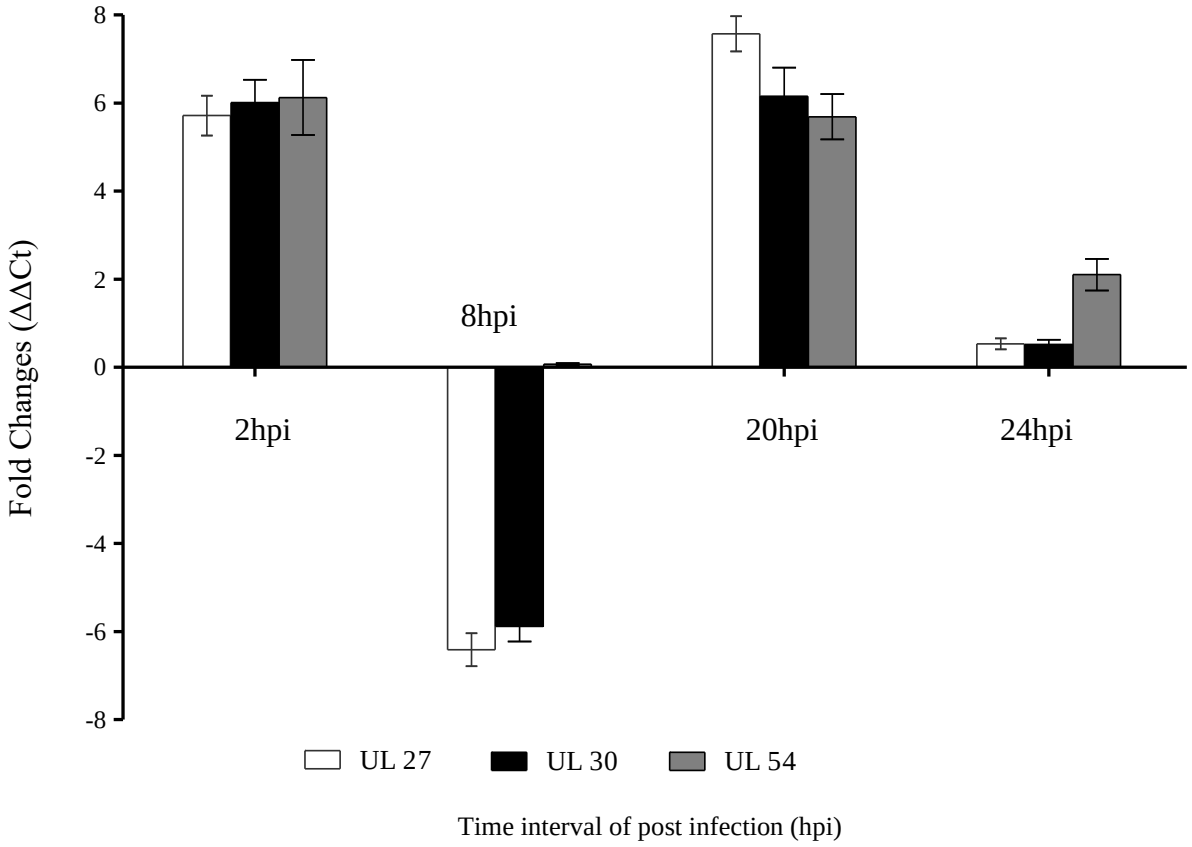

Figure 3. Gene expression activity of *S. polyanthum* extract against HSV-1 infection at 2, 8, 20, and 24 hpi which the HSV-1 treated with *S. polyanthum* methanolic extract to the expression of non-treated virus of UL27, UL30, and UL54 genes. Data represent mean values ± SEM (n = 3).

In this study, the effect of *S. polyanthum* extract was determined after the HSV-1 had already entered the cells and begun replicating. The expression of UL54, UL30, and UL27 that was infected by HSV-1 showed slight reduction in virulence. According to a study [50], typical replication cycle of the HSV-1 virus will have all of its relevant genes up-regulated at all times. The UL54 gene regulates viral gene expression at the post-transcriptional stage, which plays a role in the early-late phase [51]. At early phase, all genes at 2 hpi was significantly up-regulated which showed the treatment had no effect to the infection because the viral keep replicating until 7 hpi, however at 8 hpi, UL27 and UL30 were down-regulated as the extract can suppress DNA polymerase and gB which are needed for viral attachment. At 20 and 24 hpi, all three genes were up-regulated in treated infected cells at the late phase of replication compared to non-treated infected cells as it is considered to be the moment when the viral progeny is produced. None of the UL54 was down-regulated in all phases, thus, there was no effect of *S. polyanthum* extract on antiviral activity for this gene in viral replication as UL54 is responsible in increasing DNA replication and increasing the transcription of the late gene [19].

CONCLUSION

This study demonstrates the variable effects of *S. polyanthum* exact on HSV-1 replication in Vero cells. The extract had strong antiviral activities. In order to elucidate the mechanisms of inhibition, further studies are required to determine the underlying mechanisms that contribute to its antiviral activity.

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