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Cytotoxicity and anti-herpes simplex virus type-1 activity of *Kaempferia parviflora* extract

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Abstract

Kaempferia parviflora is a medicinal plant in the family Zingibeaceae. Phytochemicals of *K. parviflora* have anti-inflammatory, anti-cancer, anti-bacterial, anti-oxidant, and anti-viral activities. Here, the anti-herpes simplex virus type-1 (anti-HSV-1) activity of *K. parviflora* extracts was determined in an in vitro model. The plant extracts were prepared from dried rhizomes using different extraction solvents such as methanol, ethanol, acetone, ethyl acetate, dichloromethane and hexane. For phylogenetic identification of *K. parviflora* nucleotide sequence analysis of *K. parviflora* showed 99.82% and 99.45% homology with reference sequences of *K. parviflora* in the GenBank database for *trn*L intron and *trn*L-F intergenic spacer, respectively. For phytochemical analysis, the hexane extract of *K. parviflora* revealed the highest amount of total polyphenols (153.39 μg of gallic acid equivalent (GAE)/g extract). The methanolic extract contained the highest flavonoid contents at 176.81 μg of auercintin (QE)/g extract. The cytotoxicities of the *K. parviflora* extracts were determined using 3-[4,5-dimethylthiazol-2-yl]-2,5 duphynyl tetrazolium bromide (MTT) assays. The methanolic extract showed low toxicity in the mouse fibroblast L929 cell line, with a 50% cytotoxic concentration (CC_{50}) value of 25.27 ± 2.54 μg/mL. In addition, the hexane extract showed the lowest cytotoxic effect in Vero cells with a CC_{50} value of 41.77 ± 5.62 μg/mL. Regarding the anti-HSV-1 activity of *K. parviflora* extract, the ethanolic extract had the highest percentage of inhibition (52.57%) after 72 h of treatment at a concentration of 6.0 μg/mL; the 50% effective concentration (EC_{50}) value and TI value of the ethanolic extract were 4.49 ± 1.00 and 6.59, respectively.

Keywords: Anti-herpes simplex virus type-1, Cytotoxicity, Flavonoid contents, *Kaempferia parviflora*, Phylogenetic identification, Total polyphenol contents

1. Introduction

Herpes simplex viruses (HSVs) are important viral pathogens in humans that are relatively widespread in both developed and developing countries around the world [1]. HSVs are members of the family *Herpesviridae*, the subfamily *Alphaherpesvirinae* and the genus *Simplexvirus* [2]. HSVs can be divided into two types, i.e. herpes simplex virus type-1 (HSV-1) and herpes simplex virus type-2 (HSV-2) [3]. Herpes simplex virus type-1 (HSV-1) causes primarily mouth, eye, face, throat and central nervous system infections. Both HSV-1 and HSV-2 can also cause neonatal infections which are often severe [4,5]. Nowadays, several drugs are available against HSV, which include nucleoside analogues such as acyclovir (ACV) and its derivatives [6]. Nevertheless, the resistance of HSV to ACV has been observed, especially in immunocompromised patients [7]. Therefore, the development of new anti-herpetic drugs from natural products have gained much interest because they are an important source of anti-herpetic agents [8,9]. Recently, many medicinal plants have been found to possess anti-HSV activity such

as *Centella asiatica* L., *Phyllanthus urinaria* L., *Rhinacanthus nasutus* (Linn.) Kurz and *Solanum melongena* L [10-13]. Thus, *Kaemferia parviflora*, which has been reported to contain flavonoids as major compounds, is interesting to study for its anti-herpes simplex virus type-1 activity.

Kaempferia parviflora, also known as 'Krachaidum' in Thai, belongs to the Zingiberaceae family. This plant is a native to tropical Asia, including southern China, Thailand, Taiwan, Malaysia and India [14]. The genus *Kaempferia*, a native of India comprising more than 60 species, is distributed throughout the tropic and sub-tropic regions of Asia and Africa. A few species are grown as ground cover in the tropics or under cover in cold areas [15]. This plant is used for the treatment of allergy, hypertension, impotence, leucorrhoea, asthma, diarrhoea, stomach ache, flatulence and oral diseases [16]. Many recent studies have demonstrated the potential pharmacological activities of rhizome extracts such as anti-inflammatory [17], anti-microbial [18], anti-cancer [19], anti-H5N1 [20] and anti-oxidant [12] activity. The activity of *K. parviflora* extract may be due to its phytoconstituents, which have been extensively investigated. The major phytoconstituents isolated from the rhizome of *K. parviflora* include phenolic glycosides and many flavonoids such as 5-hydroxy-7-methoxyflavone, 5,7-dimethoxyflavone, flavanone, kaempferol and 3,5,7-trimethoxyflavone [21].

Phylogenetic analysis of plants is very important to identify the species of the plant materials. In Thailand, several *Kaempferia* species such as *K. grandifolia*, *K. galanga*, *K. marginata*, *K. elegans* and *K. roscoeana* can be found [22,23]. Therefore, it is very important to correctly identify the plant species before starting experiments. In previous studies, regions of chloroplast DNA (cpDNA) were used as genetic markers, such as *trn*L intron, *trn*L-F intergenic spacer (IGS), *psb*A-*trn*H and *pet*A-*psb*J for plant identification [24,25]. Non-coding regions can be used for phylogenetic identification, evolutionary studies and discrimination of different plant genera because non-coding regions of chloroplast DNA show considerable variation between species [26].

For the safety of *K. parviflora* extract use on normal cells or uninfected cells, the cytotoxicity of the plant extract was determined. Cytotoxicity assays by the 3-[4,5-dimethylthiazol-2-yl]-2,5 duphynyl tetrazolium bromide (MTT) method are rapid and sensitive. The procedure is based on a spectrophotometric assessment of cell viability [27]. In this research, the cytotoxicity and anti-HSV-1 activity from *K. parviflora* extract were investigated using the mouse fibroblast L929 cell line and African green monkeykidney cell line (Vero cells) as *in vitro* models. Thus, *K. parviflora* extract was evaluated in terms of phylogeneticidentification, phytochemical analysis and the cytotoxicity of *K. parviflora* extracts (using hexane, ethyl acetate, dichloromethane, acetone, ethanol and methanol as extraction solvents) on mouse fibroblast L929 cell lines and Vero cells.

2. Materials and methods

2.1 Plant materials

The dried rhizome plant material and powders of *K. parviflora* were purchased from Parinam Osot Ltd., Samut Sakhon province, Thailand.

2.2 Phylogenetic identification

For DNA extraction, total DNA of rhizomes from plant samples were ground to a dry powder using liquid nitrogen and a mortar. The total DNA was extracted using a NucleoSpin® Plant II kit and analysed by 0.8% (w/v) agarose gel electrophoresis. The quality and quantity of total DNA were determined using a Nanodrop spectrophotometer (BioDrop, UK). For polymerase chain reaction (PCR) amplification, the plant DNA was used as the DNA template in a PCR to amplify non-coding regions, i.e. *trn*L intron and *trn*L-F IGS. The PCR primers for these regions are shown in Table 1. PCR amplification mixtures were performed in a total reaction volume of 50 µL, which contained 10 µL of DNA template, 2.5 µL of 10 µM of C, D, E and F primers, 10 µL of sterile deionized water, and 25 µL of 2X Phusion flash PCR master mix. The amplification conditions were as follows; initial denaturation at 98°C for 30 sec, followed by 30 cycles of 98°C denaturation for 15 sec, 55 to 56°C annealing for 15 sec, and 72°C extension for 30 sec; this was followed by a final extension at 72°C for 1 min. The PCR reactions were applied in automate thermal cycling (Biometra, Germany). The PCR products were determined by 1.2% agarose gel electrophoresis.

The PCR product of *trn*L intron that contained a single band was purified by an E.Z.N.A.® Cycle Pure kit. Non-specific bands of *trn*L-F IGS were purified using an E.Z.N.A.® Gel Extraction kit. The purified PCR products were submitted to First BASE Laboratories Sdn Bhd (Malaysia) to perform direct nucleotide sequencing by BigDye® Terminator v3.1 cycle sequencing using *trn*L intron and *trn*L-F IGS primers, as shown in Table 1 and Figure 1. The nucleotide sequences of *trn*L intron and *trn*L-F IGS were analysed using BioEdit v7.2.5. program. Then, the nucleotide sequences were subjected to Nucleotide Blast analysis on the GenBank database and submitted to the GenBank nucleotide database.

Table 1 Nucleotide sequences of *trn*L intron and *trn*L-F intergenic spacer primers for PCR amplification.

Non-coding region	Primer	Sequence (5´ → 3´)	Direction	Reference
*trn*L intron	C	CGAAATCGGTAGACGCTACG	Forward	[28]
	D	GGGGATAGAGGGACTTGAAC	Reverse	[28]
*trn*L-F IGS	E	GGTTCAAGTCCCTCTATCCC	Forward	[28]
	F	ATTTGAACTGGTGACACGAG	Reverse	[28]

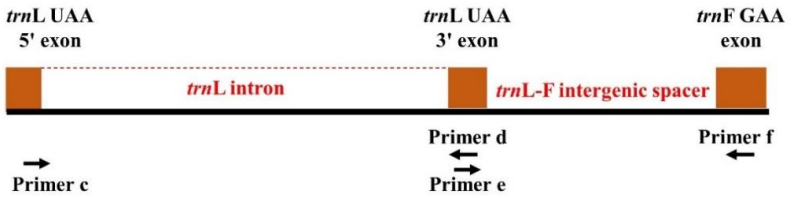

Figure 1 Positions and directions of two non-coding regions of chloroplast DNA, consist of *trn*L intron and *trn*L-F IGS.

2.3 Plant extraction

The powdered plant was soaked in six different solvents, i.e. methanol, ethanol, acetone, ethyl acetate, dichloromethane and hexane, at room temperature for seven days. The ratio of plant to solvent was 50:250 (g/mL). The suspension was then filtered and the solvents were evaporated using a rotary evaporator and dried to constant weight by vacuum evaporation. The dry material was dissolved in dimethyl sulfoxide (DMSO) before investigating cytotoxicity and anti-herpes simplex virus type-1 (HSV-1) activity.

2.4 Phytochemical analysis

Total polyphenols were determined according to [29] with minor changes. Briefly, 20 µL of extract (2 mg/mL of *K. parviflora* extract in DMSO) was mixed with 100 µL of Folin-Ciocalteau reagent (10% v/v) followed by incubated for 5 min. After that, 80 µL of a sodium carbonate solution (7.5% w/v) was added to the mixture. After that, the mixture was shaken and incubated at room temperature for 30 min. Then, the absorbance was measured with a microplate reader (SpectraMax® i3x, Molecular Devices, USA) at 765 nm. The content of total polyphenols is expressed as micrograms of gallic acid equivalents (GAE) per gram of extract.

To determine total flavonoid contents, the extract (50 µL of a solution 2 mg/mL), $NaNO_2$ (10 µL of a solution 5% w/v in deionized water) and aluminium trichloride (20 µL of a solution 10% w/v in methanol) were mixed. After that, the mixtures were slightly shaken by an orbital shaker and incubated for 5 min at room temperature. Next, NaOH (60 µL of a solution 1 M) was added to 70 µL of deionised water, then slightly shaken on an orbital shaker and incubated for 10 min. The absorbance was measured at 530 nm using a microplate reader and the total flavonoids are expressed as micrograms quercetin equivalents (QE) per gram of extract.

2.5 Cytotoxicity of K. parviflora extracts

The cytotoxicities of plant extracts were tested using the MTT assay on normal mouse fibroblast L929 cells (ATCC® CCL-1™) and Vero cells (ATCC® CCL-81™). Briefly, the cells were seeded into 96- well plates at a concentration of $1x10^4$ cells/mL and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. After that, the cells were incubated with various concentrations of six different solvents from *K. parviflora* at 0, 5, 10, 15, 20, 25, 50 and 100 µg/mL concentrations and acyclovir (0-250 µg/mL) as the positive control for 48 h. Then, the cells were washed with 100 µL of PBS (pH 7.4) and 100 µL of MTT solution (5 mg/mL in PBS) was added and incubated at 37°C for 4 h. Finally, the medium was replaced by 100 µL of DMSO to dissolve the MTT formazan crystals. Finally, the absorbance was measured using a microplate reader (SpectraMax® i3x, Molecular Devices, USA) at a wavelength of 540 nm and a reference wavelength of 620 nm. The percentage of cell viability was calculated using the formula:

$$\text{Cell viability (\%)} = \frac{(\text{average of absorbance value of untreated group} - \text{treated group})}{\text{average of absorbance value of untreated group}} \times 100$$

Then, the 50% cytotoxic concentration (CC_{50}) or the extract concentration that reduced the cell viability by 50% when compared to untreated controls was determined.

2.6 Anti-herpes simplex virus type-1 activity of K. parviflora extracts

The antiviral activity of *K. parviflora* extracts against HSV-1 was investigated by the plaque reduction assay, using a method described by Thongchuai *et al*., 2019 [13]. Three crude extracts using different extraction solvents of *K. parviflora* were evaluated for anti-HSV-1 activity (methanolic, ethanolic and hexane), which were considered due to the low cytotoxicity of the crude extracts in Vero cells. Vero cells were seeded onto 24-well culture plates at a density of $1x10^5$ cells/mL and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Then, 200 µL of culture medium was removed and the cell monolayer was infected with 100 µL of $4x10^7$ plaque forming units (PFU)/mL virus stock to yield 50 plaques per well. Next, the virus was allowed to be adsorbed for 1 h at 25°C andthen the 200 µL of solution was aspirated from the 24-well culture plates. After that, 200 µL of the non-cytotoxicconcentrations of methanolic and ethanolic extract at various concentrations (0.5-6.0 µg/mL), hexane extract (1.5- 15.0 µg/mL) and acyclovir (0.06-1.00 µg/mL) were added to each well. Then, the cells were overlaid with 200 µL of an overlay medium containing 0.375% methylcellulose. The 24-well culture plate was incubated for 72 h at 37°C in a humidified atmosphere with 5% CO_2. After that, the overlay medium was removed, and the infectedcell monolayer was fixed with 10% (w/v) formaldehyde. Finally, the virus plaques formed on Vero cells were stained with 0.1% (w/v) crystal violet. The number of plaques in extract-treated cells was compared with untreated controls. The inhibitory effect values of 50% effective concentration (EC_{50}) were determined. The percentage of inhibitory effect was calculated using the formula:

$$Inhibitory\ effect\ (\%) = 100 - \frac{(\text{Number plaques of treated group})}{(\text{Number plaques of untreated group})} \times 100$$

3. Results

3.1 Phylogenetic identification of K. parviflora

K. parviflora, also known as Krachai-dam in Thailand, belongs to the family Zingiberaceae. The plant materials used was dried rhizome of *K. parviflora* due to phytochemical compound enrichment in rhizomes. To identify the plant species, the quality and quantity of total DNA from *K. parviflora* analysed by a Nanodrop spectrophotometer. The results show the concentration of total DNA as 34.99 ng/µL and the purity of DNA as 1.80. After PCR amplification, *trn*L intron and *trn*L-F IGS primers successfully amplified *trn*L intron and *trn*L-F IGS in chloroplast DNA (cpDNA). PCR products of approximately 600 bp in *trn*L intron and 400 bp in *trn*L-F IGS were observed. However, PCR products in *trn*L-F IGS had non-specific bands. As seen in Figure 2, the purified PCR products in *trn*L intron and *trn*L-F IGS could be used for direct DNA sequencing due to the high PCR product yield and non-specific bands.

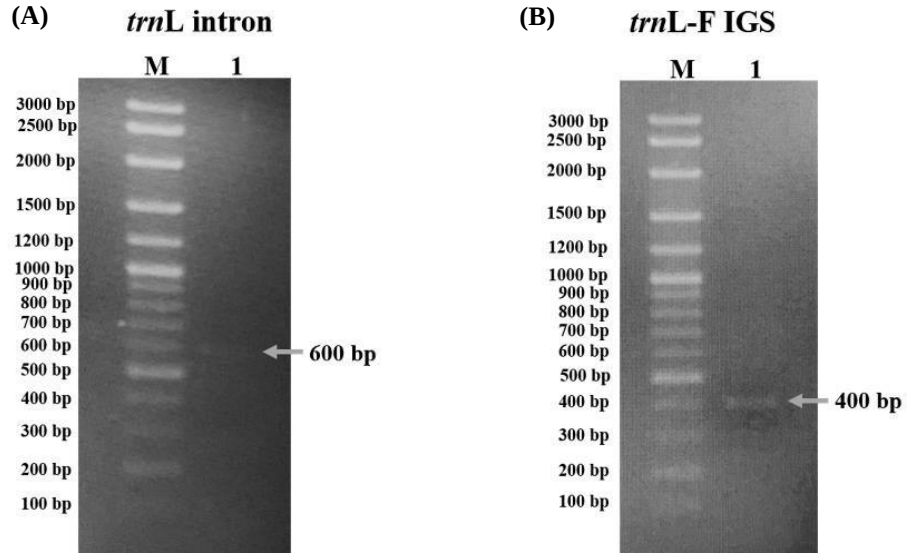

Figure 2 1.2% (w/v) agarose gel electrophoresis of purified PCR products in *trn*L intron (A) and *trn*L-F IGS (B) Lane M: VC 100 bp Plus DNA Ladder and Lane 1: purified PCR products of *K. parviflora.*

All nucleotide sequences of *trn*L intron and *trn*L-F IGS in this study were aligned with ClustalW using BioEdit sequence alignment editor v7.0.5, as shown in Figure 3A and 3B. The sizes of the PCR products were approximately 600 and 400 bp for *trn*L intron and *trn*L-F IGS, respectively. Then, the nucleotide sequences were subjected to nucleotide blast analysis on the GenBank database to identify the most closely related sequences. According to the nucleotide sequence analysis of *K.* parviflora in *trn*L intron, *K. parviflora* CMU01 in our study showed the highest homology with *K. parviflora* (99.82%) in the database, followed by *K. galanga*, *K. elegans*, *Curcuma longa* and *Curcuma phaeocaulis* (99.15%, 99.15%, 98.98% and 98.98%, respectively) (Table 2). Regarding the nucleotide sequence analysis of *K. parviflora trn*L-F IGS, *K. parviflora* CMU01 showed the highest homology with *K. parviflora* (99.45%) in the database, followed by *K. galanga*, *K. elegans*, *Curcuma longa* and *Curcuma phaeocaulis* (99.23%, 98.72%, 98.72% and 98.72%, respectively). The nucleotide sequences in obtained in this study were submitted to the GenBank nucleotide database. Accession numbers were obtained, as shown in Table 2.

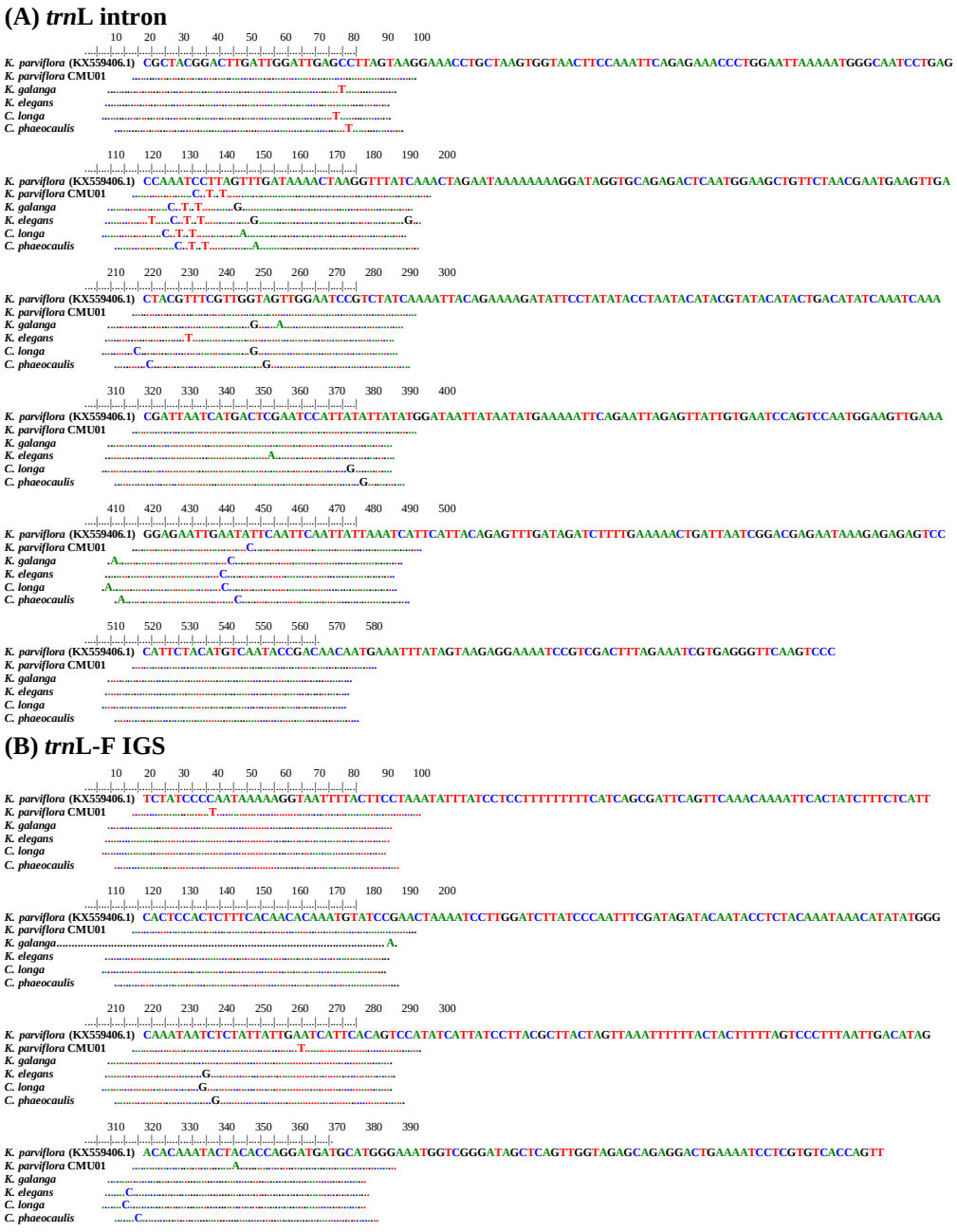

Figure 3 Comparison of nucleotide sequences in *trn*L intron (A) and *trn*L-F IGS (B) from *K. parviflora* (KX559406.1), *K. parviflora* CMU01, *K. galanga*, *K. elegans*, *C. longa* and *C. phaeocaulis.*

Table 2 BLAST analysis of plant material according to *trn*L intron and *trn*L-F IGS.

Best similar specie on GenBank database	Accession number	Similarity (%)	
		*trn*L intron *K. parviflora* CMU01 (MN747803.1)	*trn*L-F IGS *K. parviflora* CMU01 (MN747804.1)
Kaempferia parviflora	KX559406.1	99.82	99.45
Kaempferia galanga	NC_040851.1	99.15	99.23
Kaempferia elegans	NC_040852.1	99.15	98.72
Curcuma longa	MN711722.1	98.98	98.72
Curcuma phaeocaulis	MK621772.1	98.98	98.72

3.2 Phytochemical analysis

Colorimetric determinations highlighted the presence of total polyphenols and flavonoids of *K. parviflora* extracts. Under our experimental conditions, the phytochemical analysis revealed a particular phenolic composition of *K. parviflora* extracts. The hexane extract of *K. parviflora* showed the highest amount of total polyphenols (153.39 μg GAE/g extract), followed by the methanolic, acetone, ethyl acetate, ethanolic and dichloromethane extracts (109.23, 105.85, 105.32, 102.68 and 95.51 μg GAE/g extract, respectively) (Table 3). Regarding the flavonoid contents, the methanolic extract showed the highest flavonoid content of 176.81 μg QE/g extract, followed by the ethanolic, ethyl acetate, dichloromethane, acetone and hexane extracts (160.94, 153.49, 127.66, 123.82 and 38.10 μg GAE/g extract, respectively).

Table 3 Total polyphenols and total flavonoids in the six different solvent extractions from rhizomes of *K. parviflora*.

Extracts	Total polyphenol contents (μg GAE/g extract)	Total flavonoid contents (μg QE/g extract)
Methanolic extract	109.23 ± 9.29^{b}	176.81 ± 4.22^{a}
Ethanolic extract	102.68 ± 6.65^{b}	$160.94 \pm 11.86^{a, b}$
Acetone extract	105.85 ± 9.07^{b}	123.82 ± 7.76^{c}
Dichloromethane extract	95.51 ± 7.67^{b}	127.66 ± 2.76^{c}
Ethyl acetate extract	105.32 ± 8.05^{b}	153.49 ± 3.91^{b}
Hexane extract	153.39 ± 13.14^{a}	38.10 ± 1.87^{d}

GAE, gallic acid equivalent; QE, quercetin equivalent.
[a,b,c and d] Means in the same column with different letters are significantly different according to the Tukey HSD test ($p < 0.05$). Statistical evaluation by one-way ANOVA using Statistix.

3.3 Cytotoxicity of K. parviflora extracts

The cytotoxicity of *K. parviflora* extracts were measured using the MTT assay. In Table 4, the cytotoxic doses are expressed as the CC_{50} of the crude extracts using the L929 cell line. The results reveal that the acetone, dichloromethane, ethyl acetate and hexane extracts from *K. parviflora* showed greater toxicity than the ethanolic and methanolic extracts. The methanolic extract showed the least toxicity in the L929 cell line ($CC_{50} = 25.27 \pm 2.54$ μg/mL), while the hexane extract had the lowest cytotoxicity in Vero cells ($CC_{50} = 41.77 \pm 5.62$ μg/mL), followed by the methanolic, ethanolic, ethyl acetate, acetone and dichloromethane extracts, as shown in Table 4.

Table 4 The 50% cytotoxic concentration (CC_{50}) of rhizome extracts of *K. parviflora* in L929 cells and Vero cells.

Extraction solvents	CC_{50} (μg/mL)	
	L929 cell line	Vero cells
Methanolic extract	25.27 ± 2.54^{b}	$31.73 \pm 1.16^{b, c}$
Ethanolic extract	$22.46 \pm 1.86^{b, c}$	$29.58 \pm 3.07^{b, c}$
Acetone extract	$17.14 \pm 1.21^{c, d}$	24.80 ± 1.42^{c}
Dichloromethane extract	13.49 ± 1.50^{d}	20.56 ± 1.01^{c}
Ethyl acetate extract	13.15 ± 1.51^{d}	25.30 ± 0.79^{c}
Hexane extract	$19.55 \pm 2.86^{b, c, d}$	41.77 ± 5.62^{b}
Acyclovir (HSV drug)	42.87 ± 3.59^{a}	121.33 ± 11.31^{a}

[a,b,c and d] Means in the same column with different letters are significantly different according to the Tukey HSD test ($p < 0.05$).Statistical evaluation by one-way ANOVA using Statistix.

3.4 3.4 Anti-herpes simplex type-1 activity of K. parviflora extracts

The anti-viral effect of *K. parviflora* extracts against herpes simplex virus type-1 (HSV-1) was evaluated by the plaque reduction assay, which was the method for viral quantification. Vero cells were infected with HSV-1 and treated with various non-cytotoxic concentrations of *K. parviflora* extracts after viral attachment. In this study, the crude extracts of *K. parviflora* were investigated for anti-HSV-1 activity, including the methanolic, ethanolic and hexane extracts due to their low cytotoxicity in Vero cells. Acyclovir was used as the positive control, as acyclovir is a therapeutic drug for the treatment of HSV infection. The results reveal that the ethanolic extract had the highest percentage of inhibition at 52.57% after 72 h of treatment at a concentration of 6.0 µg/mL when compared with other extracts, as shown in Figure 4. Additionally, the plaque reduction assay demonstrated that the ethanolic extract was significantly the highest regarding the inhibition of HSV-1 with an EC_{50} of 4.49 ± 1.00 ($p < 0.05$) and a therapeutic index (TI) of 6.59, as shown in Table 5.

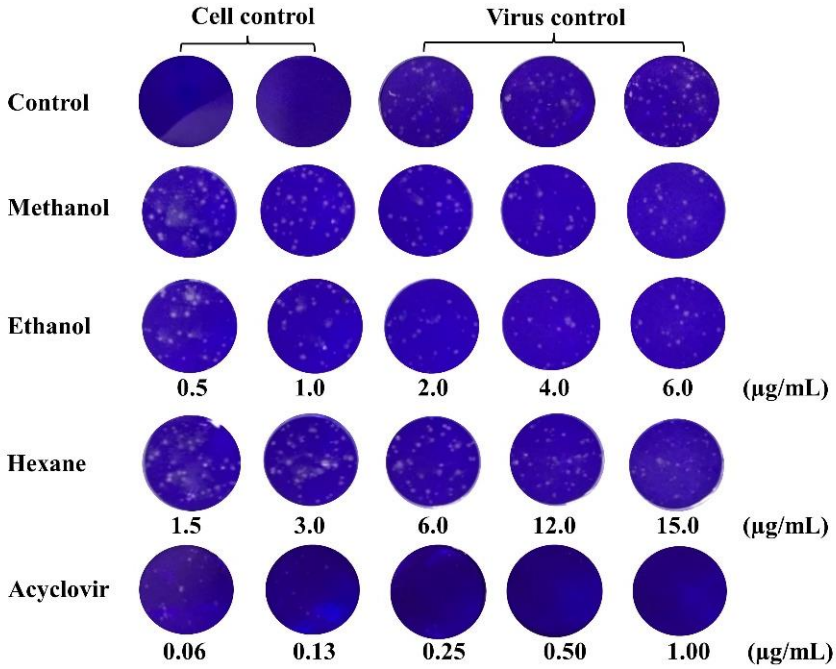

Figure 4 Plaque reduction assay and the effective concentration (EC_{50}) of the various extraction solvents of *K. parviflora* and acyclovir.

Table 5 50% cytotoxic concentration (CC_{50}), 50% effective concentration (EC_{50}) and therapeutic index (TI value) of the rhizome extracts of *K. parviflora* in Vero cells after viral attachment (n = 3).

Extraction solvents	CC_{50} (µg/mL)	EC_{50} (µg/mL)	TI value
Methanolic extract	31.73 ± 1.16[b]	> 6	-
Ethanolic extract	29.58 ± 3.07[b]	4.49 ± 1.00[b]	6.59
Hexane extract	41.77 ± 5.62[b]	12.42 ± 0.58[a]	3.36
Acyclovir (HSV drug)	121.33 ± 11.31[a]	0.11 ± 0.02[c]	1103.00

Note: TI value = therapeutic index (CC_{50}/EC_{50})

[a,b and c] Means in the same column with different letters are significantly different according to the Tukey HSD test ($p < 0.05$).Statistical evaluation by one-way ANOVA using Statistix.

4. Discussion

In this study, the phylogenetic identification of *K. parviflora trn*L intron and *trn*L-F IGS in the chloroplast genome was carried out and a DNA sequence database was established for plant species identification. *K. parviflora* belongs to the Zingiberaceae family, genus *Kaempferia*. In the total DNA extraction, the yield of total DNA was low since the DNA was degraded and contained high amounts of polysaccharide, polyphenols and secondary metabolites. The DNA purity (A_{260}/A_{280} ratio) of 1.8 indicates greater than 90% nucleic acid [30]. Furthermore, the band of the purified PCR product without a non-specific band indicates the specificity of the primer for *trn*L intron and *trn*L-F IGS. According to the nucleotide sequence analysis, the plant nucleotide sequences were compared to a database which percentage homology should be considered the highest percentage.

The results showed 99.82% and 99.45% homology with *K. parviflora* in the database for *trn*L intron and *trn*L-F IGS, respectively. It was found that transverse base substitution was observed in *trn*L intron. In contrast, both transverse and transitions base substitutions were found in *trn*L-F IGS. That may reveal the same genus but different species. In addition, it was found that *K. parviflora* CMU01 had similarities with other species, including *K. galanga* and *K. elegans.* Furthermore, *K. parviflora* CMU01 showed similarity with other genera in Zingiberaceae, including *C. longa* and *C. phaeocaulis*. *K. parviflora* species identification is difficult due to the morphological similarity between species and other genera in Zingiberaceae, such as *Curcuma* and *Boesenbergia* [25]. Techaprasan *et al*. (2010) studied the genetic variation of *Kaempferia* (Zingiberaceae) in Thailand based on chloroplast DNA (*psb*A-*trn*H and *pet*A-*psb*J) sequences [25]. In addition, Tsai *et* al. (2006) studied species identification using sequences of *trn*L intron and *trn*L-F IGS of the chloroplast genome among popular plants in Taiwan [24]. Non-coding regions in chloroplast DNA could be used to successfully amplify all the plants tested. Therefore, these previous reports suggest that chloroplast DNA can be used as a genetic marker, including *psb*A-*trn*H, *pet*A-*psb*J, *trn*L intron and *trn*L-F IGS, to identify plant species [24,25].

K. parviflora rhizome is known to contain flavonoids, phenolic glycosides, flavone, quercetin, kaempferol and flavonol glycosides [31]. The crude extracts of *K. parviflora* were determined as the total polyphenol and flavonoid contents since the main phytochemical constituents that have shown anti-HSV activity include polyphenol and flavonoids [32]. Therefore, the total polyphenol and flavonoid contents seem to be related to anti-HSV activity. According to the phytochemical analysis results, total polyphenols and flavonoid contents were determined as GAE and QE, respectively. The results showed that the hexane extract had the highest amount of total polyphenols (153.39 μg GAE/g extract). On the other hand, the methanolic extract had the highest total flavonoid content (176.81 μg QE/g extract). Nanasombat *et al*. (2014) reported that the total polyphenol and flavonoid contents of a methanolic extract of *K. parviflora* were 24.62 mg GAE/g extract and 17.20 mg QE/g extract, respectively [31]. The total polyphenol and flavonoid contents from this previous study were higher than those of *K. parviflora* extracts used in this study. This may be due to the concentrations of phenolic and flavonoid compounds in plants, influenced by many factors including the solvent, extraction process, time and temperature [21]. Tewtrakul *et al*. (2005) reported that methoxylated flavonoids were found in the hexane extract of *K. parviflora* rhizomes, such as 5-hydroxy-3,7,4'-trimethoxyflavone, 5-hydroxy-7,4'-dimethoxyflavone and 5-hydroxy-3,7,3',4' tetramethoxyflavone [33].

In addition, total polyphenol contents have been found in different solvent extracts, with major differences due to the extraction method [12]. The varieties of phytochemical bioactive compounds contained in plant materials have different solubility properties in different solvents. Therefore, the conditions for ideal solvent for extraction depend on the particular plant material and the target compounds that are to be isolated. A solvent of similar polarity to the solute will properly dissolve. The polarity, from least polar to most polar, of the solvents used in this study is hexane < dichloromethane < ethyl acetate < acetone < ethanol < methanol.

Among the phytoconstituents of *K. parviflora* extracts, flavonoids have been reported to possess antiviral activity against herpes simplex viruses. This antiviral activity appears to be associated with non-glycosidic compounds, and hydroxylation at the 3-position is apparently a prerequisite [34]. Previously, a natural plant flavonoid polymer with a molecular weight 2,100 Daltons was found to have antiviral activity against two strains of HSV-1 and HSV-2 [35]. Previous studies have reported that polyphenols have anti-HSV-1 activity [9,12]. In addition, the effect of polyphenol-rich extracts from *Pistacia vera* L. on anti-HSV-1 activity showed an EC_{50} and selectivity index (SI) of 0.4 mg/mL and 3, respectively. Polyphenol-rich extracts have been proposed to interfere with the viral attachment of HSV-1 [9].

According to the cytotoxicity of *K. parviflora* extracts, cytotoxicity tests are designed to determine the toxicity to normal cells of compounds either qualitatively or quantitatively. Mouse fibroblast L929 cell lines and Vero cells were incubated with different concentrations (ranging from 5 to 100 μg/mL) of *K. parviflora* extract, and cell viability was evaluated by the MTT assay. Previous studies suggested that the final concentration of 1% (v/v) DMSO has no toxic effects. Acyclovir was used as the positive control. It was found that the methanolic extract exhibited less toxicity than the ethanolic, acetone, dichloromethane, ethyl acetate and hexane extracts. However, all crude extracts from *K. parviflora* were toxic to L929 cells due to low CC_{50} values (Table 4). In addition, the cytotoxicities of *K. parviflora* on the Vero cells showed that the CC_{50} values of the crude extracts were less toxic than in L929 cell lines, as shown in Table 4. The results reveal that acyclovir is less toxic in Vero cells than in L929 cells. Regarding the anti-HSV-1 activity of *K. parviflora* extract, the ethanolic extract demonstrated a TI value approximately 1.96-fold higher than that of the other extraction solvents, which indicated that it was more effective and minimally toxic in Vero cells. Previous studies have found that the highly polar solvent extracts (such as methanolic and ethanolic extracts) revealed that anti-HSV activity since most anti-HSV-1 active compounds are soluble in polar solvents, including flavonoids and polyphenols [32,36]. In addition, previous studies have reported that terpenes could be isolated from the hexane extract of *K. parviflora* rhizomes, including germacrene D, borneol and α-copaene; these terpenes are soluble in non-polar solvents, such as hexane [37]. Interestingly, the terpenes possessed anti-HSV activity [36]. In previous reports, acetone, ethanol and methanol extracts of *P. urinaria* inhibited HSV-2 infection by disturbing the early stage of virus infection. The 50%

inhibitory concentration against HSV-2 infection (IC_{50}) values of the acetone, ethanol and methanol extracts were 4.3 ± 0.5, 5.0 ± 0.4 and 4.0 ± 0.9 μg/mL, respectively. All three extracts showed no cytotoxic effect against the African green monkey kidney cell line (Vero cell) at concentrations of 10.0 μg/mL or below [11]. Yoosook *et al*. (2000) reported that water extracts from Thai medicinal plants such as *C. asiatica* L, *M. cochinchinensis* Cornor and *M. indica* L. showed less toxicity than other plants in Vero cells, and a mixture of *C. asiatica* L and *M. indica* L. had an additive effect against HSV [10]. Thongchuai *et al*. (2019) reported that the methanolic extract from the stem of *R. nasutus* (Linn.) Kurz. showed low toxicity in Vero cells [13]. Moreover, several herbal medicines have been shown effective and safe for the treatment of patients with HSV-1 infection, such as *C. nutans* L. and *P. urinaria* L. extract. In this study, the most active crude extract could be applied for the treatment of patients with HSV-1 infection, including topical medication for skin conditions and oral medications. For further work, the most active crude extract could be studied regarding the effect of *K. parviflora* extract on HSV-1 protein expression using Western blot to identify specific proteins from HSV-1 infected cells. This information will provide a better understanding of the mechanism or pathway involved in host and HSV-1 protein expression.

5. Conclusions

The nucleotide sequences of *trn*L intron and *trn*L-F IGS of chloroplast DNA were successfully PCR amplified from total DNA extracted from *K. parviflora* samples. The PCR products were 600 and 400 bp for *trn*L intron and *trn*L-F IGS, respectively. In addition, the BLAST results of the nucleotide sequence analysis of *K. parviflora* samples showed 99.82% and 99.45% homology with *K. parviflora* in the database for *trn*L intron and *trn*L-F IGS, respectively. In the phytochemical analysis, the hexane extract of *K. parviflora* sample had the highest amount of total polyphenols (153.39 μg GAE/g extract), while the methanolic extract had the highest flavonoid content of 176.81 μg QE/g extract. According to the cytotoxicity results, methanolic extracts showed the least toxicity in the L929 cell line (CC_{50} = 25.27 ± 2.54 μg/mL) and the hexane extract had the lowest cytotoxicity in Vero cells (CC_{50} = 41.77 ± 5.62 μg/mL). Regarding the anti-HSV-1 activity of *K. parviflora* extract, the results showed that the ethanolic extract had the highest percentage inhibition of 52.57% after 72 h of treatment at the concentration of 6.0 μg/mL when compared with other solvent extracts. In addition, the results demonstrated that the ethanolic extract inhibited HSV-1 with an EC_{50} of 4.49 ± 1.00 and a TI value of 6.59.

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7. References

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