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journal homepage: www.elsevier.com/locate/apjtbFloral research <http://dx.doi.org/10.1016/j.apjtb.2015.12.014>Anti-herpes simplex virus activities of monogalactosyl diglyceride and digalactosyl diglyceride from *Clinacanthus nutans*, a traditional Thai herbal medicineSirada Pongmuangmul^{1,2}, Supaporn Phumiamorn³, Panchana Sanguansermisri¹, Nalin Wongkattiya⁴, Ian Hamilton Fraser⁵, Donruedee Sanguansermisri^{1,6*}¹Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand²Regional Medical Sciences Center Nakhonswan, Department of Medical Sciences, Ministry of Health, Nakhonswan 60130, Thailand³Institute of Biological Products, Department of Medical Sciences, Ministry of Health, Nonthaburi 11000, Thailand⁴Faculty of Science, Maejo University, Chiang Mai 50290, Thailand⁵School of Chemistry, Monash University, Victoria 3800, Australia⁶Centre of Excellence in Medical Biotechnology, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand

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ABSTRACT

Objective: To evaluate the monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) from *Clinacanthus nutans* (*C. nutans*) for their *in vitro* antiviral activities against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) by plaque reduction assay.**Methods:** MGDG and DGDG were extracted with chloroform from *C. nutans* leaves. MGDG and DGDG were separated from chloroform crude extract using column chromatography, characterized by thin layer chromatography and quantified by high performance liquid chromatography. The anti HSV-1 and 2 activity against pre-treatment and post-treatment of the compounds was evaluated using plaque reduction assay. The cytotoxicity of the extract and the compounds on Vero cells were performed by MTT assay.**Results:** MGDG and DGDG obtained by column chromatography showed identical profiles as standard MGDG and standard DGDG using thin layer chromatography and high performance liquid chromatography. MGDG and DGDG from *C. nutans* showed 100% inhibition of HSV-1 replication at the post step of infection at noncytotoxic concentration with IC₅₀ values of 36.00 and 40.00 µg/mL, and HSV-2 at 41.00 and 43.20 µg/mL, respectively. Moreover, MGDG and DGDG from *C. nutans* were demonstrated to have anti-herpes simplex activity at the same level as standard synthetic compounds. In contrast, pre-treatment of Vero cells with MGDG and DGDG before HSV-1 and HSV-2 infection did not show inhibitory effect against these viruses. MGDG and DGDG exhibited antiviral activity against HSV-1 with selectivity index of 26.00 and 23.00 and HSV-2 of 23.30 and 21.30.**Conclusions:** MGDG and DGDG from *C. nutans*, a traditional Thai herbal medicine illustrated inhibitory activity against HSV-1 and HSV-2, probably by inhibiting the late stage of multiplication, suggesting their promising use as anti-HSV agents.

1. Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) infections are distributed worldwide in humans with different degree of severity. It may lead to life-threatening conditions, especially in immunocompromised patients. HSV-1 is more frequently associated with orofacial mucocutaneous lesions. HSV-2 is more commonly associated with genital herpes. Herpes viruses can cause primary, and recurrent infections upon viral reactivation through stimuli such as sunlight, stress and weakened immunity [1,2]. The

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drug of choice of the prophylaxis and treatment of HSV infection is acyclovir, a nucleoside analog, which selectively inhibits HSV replication with low host cell toxicity [3,4]. However, the emergence of resistant viruses to commonly used antiviral drugs is a problem in the treatment, particularly in immunocompromised patients and infants [5–7]. Consequently, there is a need to search for new and more effective antiviral agents that can substitute or complement currently used antiviral drugs [8].

Medicinal plants are known to be a source of abundant of chemical compounds and traditionally used in healthcare in many countries. A large number of plants, either as extracts or as pure compounds, have been demonstrated to exhibit antiviral activity [9–11]. *Clinacanthus nutans* (Burm. f.) Lindau (*C. nutans*) is a medicinal plant in family Acanthaceae which is native to many tropical Asia countries including Thailand and often cultivated. *C. nutans* has been traditionally used as a medicine for topical treatment of skin rashes, insect and snake bite, HSV, and varicella-zoster virus lesions [12,13]. Clinical trials have reported the successful use of *C. nutans* topical treatment for relief of skin inflammation and insect bites, genital herpes and varicella-zoster lesions [14]. Crude extract of *C. nutans* leaves was shown to inhibit HSV-1 and HSV-2 activity [15]. In phytochemical investigation, flavonoids, steroids, triterpenoids, cerebrosides, two glycolglycerolipids, glycerides, sulfur-containing glycerides were isolated from this plant [16,17]. Trigalactosyl and digalactosyl diglycerides isolated from *C. nutans* have been shown to exhibit antiviral activity [18]. Synthetic glycolglycerolipids has also been shown to inhibit HSV [19].

In this study, the inhibition of HSV-1 and HSV-2 by two glycolglycerolipids: monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG), isolated from *C. nutans* and chloroform crude extract of *C. nutans* leaves were investigated using plaque reduction assay. Furthermore, the mode of the inhibitory activities was also determined by adding the compound in the different step of virus replication.

2. Materials and methods

2.1. Plant material and extraction

C. nutans plants were collected from Uthai Thani, Thailand by Warayu Leeprasert, Thai medicinal plant expert. Dried leaves were extracted with chloroform using a Soxhlet extraction apparatus at 60 °C for 8 h. Whole extracts were collected and the solvent evaporated to dryness with a rotary evaporator. The crude extracts were stored at –20 °C until used in tests.

2.2. Preparation of MGDG and DGDG

The crude extracts were partially purified on a column of silica gel 60 by exhaustive elution with 350 mL of each of the following elution steps: chloroform: acetone (9:1, 3:1 and 1:1), pure acetone and acetone: methanol (3:1) [20]. And 50 mL of each fraction was collected and evaporated for further identification of MGDG and DGDG.

Each fraction was verified for MGDG and DGDG by thin layer chromatography (TLC) and compared with standard MGDG and DGDG (Larodan AB, Sweden). The phase for MGDG identification consisted of chloroform: acetone: distilled water (30:60:2). Mobile phase for DGDG identification consisted of chloroform: acetone: methanol: glacial acetic acid: distilled water (100:40:20:30:10) [21].

Fractions which contained MGDG or DGDG were pooled and evaporated using rotary evaporator. Confirmation of MGDG was performed using TLC with 2 systems of mobile phase, chloroform: acetone: distilled water (30:60:2) and acetone: glacial acetic acid: distilled water (100:2:1) [22]. Confirmation of DGDG was performed using TLC with 2 systems of mobile phase, chloroform: methanol: glacial acetic acid: distilled water (85.0:15.0:10.0:3.5) [22] and chloroform: acetone: methanol: glacial acetic acid: distilled water (100:40:20:30:10) [21].

The quantity of MGDG and DGDG was observed and determined by high performance liquid chromatography (HPLC) using an Agilent 1100 equipped with photo diode array detector at 254 nm. MGDG and DGDG were separated at 30 °C using a silica column, size 200.0 mm × 4.6 mm, particle size 5 µm (hypersil silica). The flow rate was 1 mL/min and the mobile phase was hexane: iso-propanol (97.5:2.5) [23].

2.3. Viruses and cell lines

Vero cells (African green monkey kidney cells) were grown in monolayer cultured with Dulbecco's modified Eagle medium, supplemented with 5% fetal bovine serum. HSV-1 and HSV-2 were provided by Dr. Panadda Dhepakson, Medical Biotechnology Center, Department of Medical Science, Thailand. Virus stocks were prepared from infected cultures in Vero cells with maintenance medium (Dulbecco's modified Eagle medium, 2% fetal bovine serum and 1% antibiotic), propagated at 37 °C, 5% CO₂ and virus titer was determined by plaque assay in Vero cells.

2.4. Cytotoxicity assay

Cytotoxicity was evaluated by the MTT assay. A volume of 100 µL of Vero cells at concentration 2.5×10^5 cell/mL were seeded in 96-well plate and propagated at 37 °C with 5% CO₂ for 1 day. The cultured medium was replaced by fresh maintenance medium containing different concentrations of chloroform crude extract, extracted and purified MGDG or DGDG. The cells were incubated at 37 °C with 5% CO₂ for 2 days, and the MTT reagent (20 µL) was added to each well. After 4 h of further incubation, the formazan was solubilized by adding diluted HCl (0.04 mol/L) in isopropanol, and the absorbance was determined at 490 nm by Biotek ELISA plate reader with a reference wavelength of 620 nm. The 50% cytotoxic concentration (CC₅₀) causing visible morphological changes in 50% of Vero cells were determined [24].

2.5. Antiviral activity assay

The antiviral activity of glycolglycerolipid from Phaya Yaw was evaluated by plaque reduction assay as previously described [25]. The compounds were added at different stage during viral infection cycle in order to trace the mode of antiviral action.

2.6. Pre-treatment

The Vero cell monolayer was pretreated with chloroform crude extract, MGDG, DGDG, standard MGDG, standard DGDG and acyclovir at 37 °C for 24 h prior to virus infection. After washing, cells were infected with HSV-1 or HSV-2 at a multiplicity of infection of 0.1 at 37 °C for 1 h. The infected cells were washed, and overlaid with 3 mL of 1.2% nutrient methylcellulose and incubated at 37 °C for 48 and 96 h for HSV-

1 and HSV-2, respectively. The infected cells were fixed with 5% formaldehyde, stained with 0.5% crystal violet and then the number of plaques was counted. The percentage of inhibitory activity concentration was determined and the IC_{50} was determined from the curve relating the percentage of inhibitory activity concentration to the concentration of the samples.

2.7. Post-treatment

Confluent Vero cells were infected with 100 plaque forming unit of HSV-1 or HSV-2. After adsorption at 37 °C for 1 h, the infected cells were washed and overlaid with 3 mL of 1.2% nutrient methylcellulose containing chloroform crude extract, MGDG, DGDG, standard MGDG, standard DGDG and acyclovir in various concentration and incubated at 37 °C for 48 and 96 h for HSV-1 and HSV-2, respectively. The cells were then treated as described earlier for the viral plaque number reduction assay.

3. Results

3.1. Isolation and characterization of crude extract and MGDG and DGDG from *C. nutans*

Crude extract of *C. nutans* dried leaves was obtained after chloroform extraction with yield of 7%. The crude extract was then run through a silica gel column and sequentially eluted with 5 different eluants. Fractions of 50 mL were collected as per the method described by Diehl *et al.* [20]. After running the crude extract, MGDG was eluted from fractions 12–15 and DGDG was eluted from fractions 26–28. The pooled portions of each

fraction of MGDG and DGDG were preliminary identified by TLC (Figure 1) when using different mobile phase systems. The MGDG from *C. nutans* showed similar R_f as the standard MGDG (Figure 1a,b) as well as the DGDG from *C. nutans* and the standard DGDG (Figure 1c,d). The identification of MGDG and DGDG extracts were confirmed by HPLC. HPLC profiles obtained from MGDG and DGDG extracts were compared well to that of standard MGDG and DGDG (Figure 2).

3.2. Cytotoxicity

To examine the possible cytotoxic effects of chloroform extract, extracted and purified MGDG and DGDG from *C. nutans* on cell viability, Vero cells were incubated with different concentrations (100–15000 $\mu\text{g/mL}$) of the compounds for 48 h and cell viability was measured by MTT assay. As shown in Figure 3, extracted and purified MGDG and DGDG from *C. nutans* showed a lower toxic effect than chloroform crude extract. In Table 1, the CC_{50} for these compounds were reported. In this study, chloroform extract, extracted and purified MGDG and DGDG from *C. nutans* were investigated for the effectiveness against HSV-1 and HSV-2 infected Vero cells. In Vero cells, the CC_{50} of chloroform extract, extracted and purified MGDG and DGDG from *C. nutans* was between 520 and 960 $\mu\text{g/mL}$. Activity of the extracted and purified MGDG and DGDG from *C. nutans* was less toxic than the chloroform crude extract.

3.3. Anti HSV-1 and 2 activity

The effect of chloroform extract, MGDG and DGDG from *C. nutans* on HSV was evaluated for inhibition at different steps

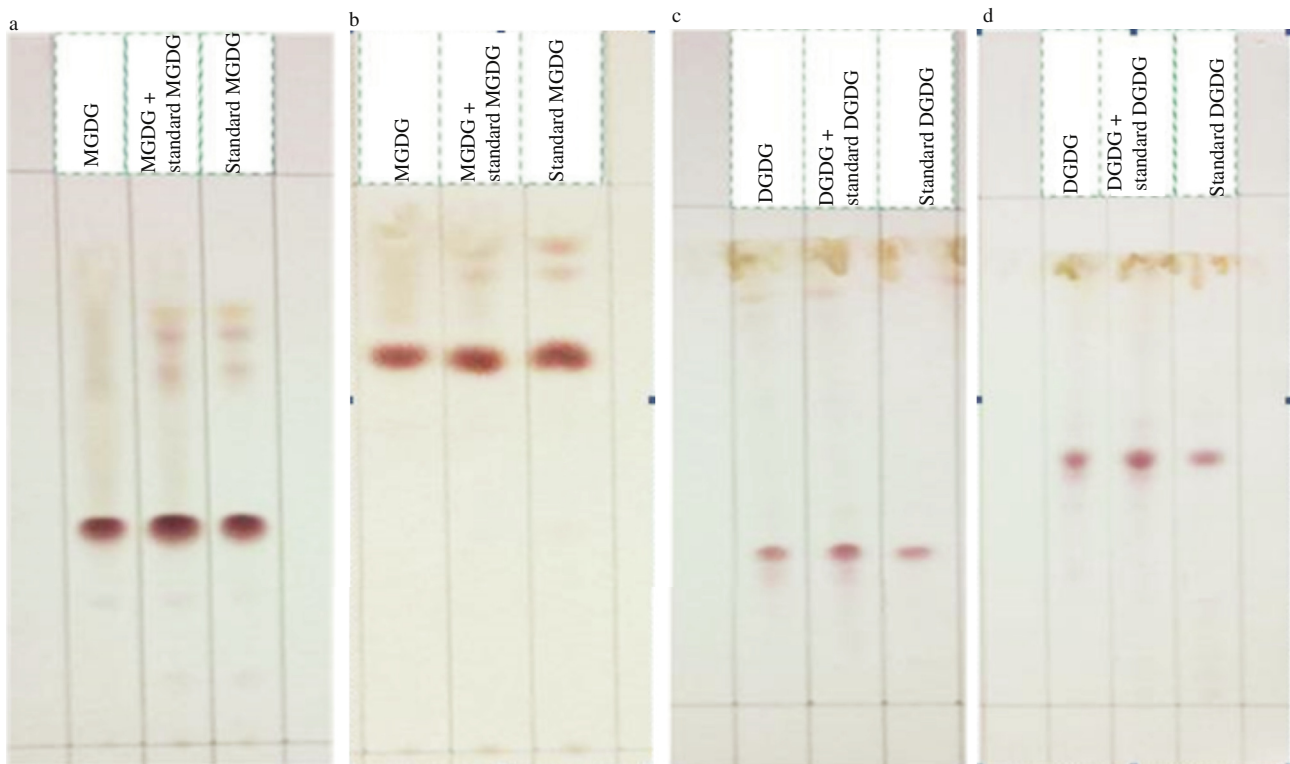


Figure 1. TLC chromatogram of extracted and purified MGDG from *C. nutans* and standard MGDG in chloroform: acetone: water (a) and in acetone: glacial acetic acid: water (b), extracted and purified DGDG from *C. nutans* and standard DGDG in chloroform: acetone: water (c) and in acetone: glacial acetic acid: water (d).

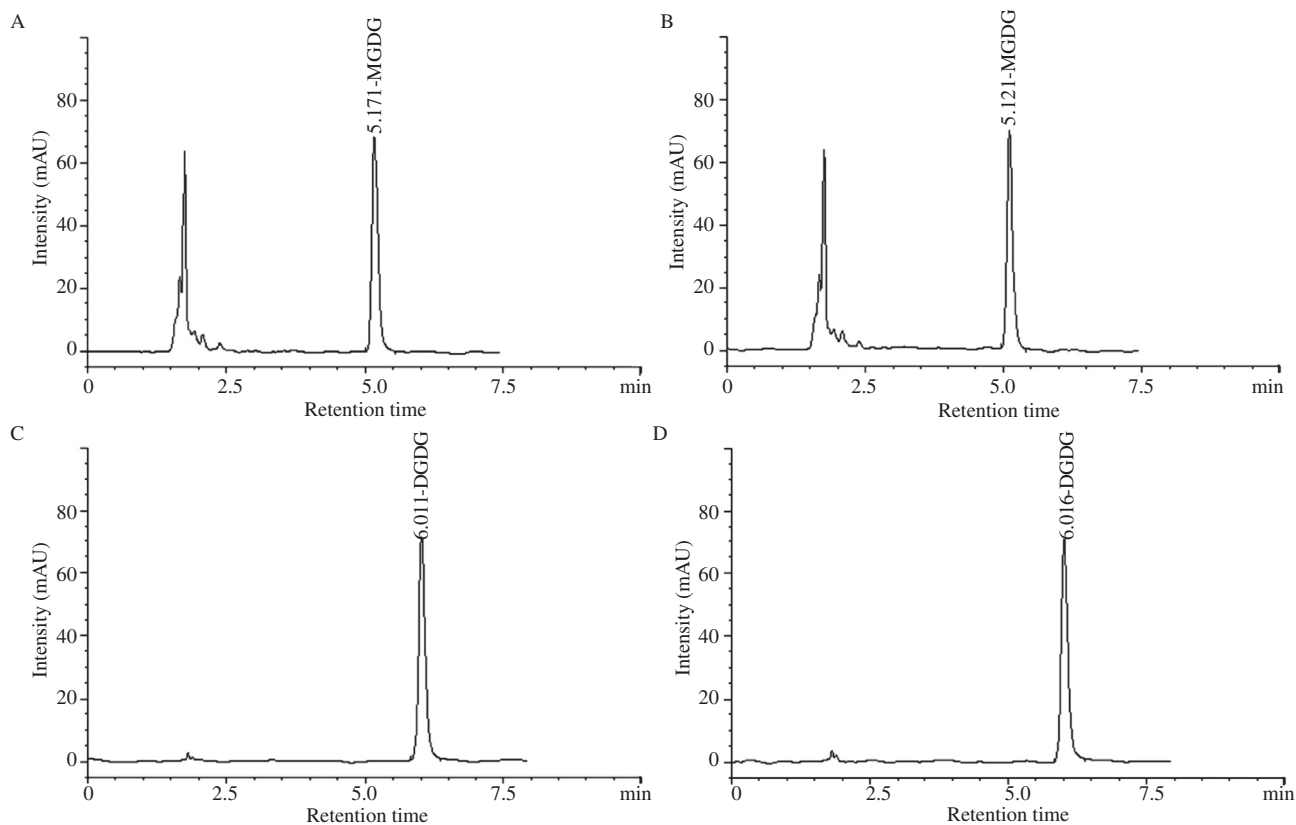


Figure 2. HPLC chromatogram. A: Extracted and purified MGDG from *C. nutans*; B: Standard MGDG; C: Extracted and purified DGDG from *C. nutans*; D: Standard DGDG.

of infection. Vero cells were pre-treated with chloroform crude extract, extracted and purified MGDG, extracted and purified DGDG, standard MGDG and standard DGDG in different concentrations at subtoxic levels. For post-treatment, the compounds were added to the cells after viral infection. Acyclovir was also used as a drug control in anti-HSV study. At the maximum non-cytotoxic concentrations of all compounds, the anti HSV-1 and HSV-2 activities with post-treatment showed 100% inhibition of plaque formation whereas pre-treatment showed less than 50% inhibition of plaque formation (Figure 4). The result indicated that chloroform extract, extracted

and purified MGDG and DGDG from *C. nutans*, standard MGDG and standard DGDG predominantly affected anti HSV-1 activities in post viral infection with IC₅₀ value of 115.00, 36.00, 40.00, 31.00 and 33.60 µg/mL, respectively and anti HSV-2 activities with IC₅₀ value of 140.00, 43.20, 33.40 and 36.80 µg/mL, respectively. In addition, acyclovir expressed the IC₅₀ value on post-treatment at 0.64 and 0.80 µg/mL for HSV-1 and HSV-2 (Table 1). In particular, extracted and purified MGDG and DGDG from *C. nutans* showed antiviral activity with selectivity indexes (CC₅₀/IC₅₀) on HSV-1 of 26.0 and 23.1 and on HSV-2 of 23.3 and 21.3, respectively (Table 2).

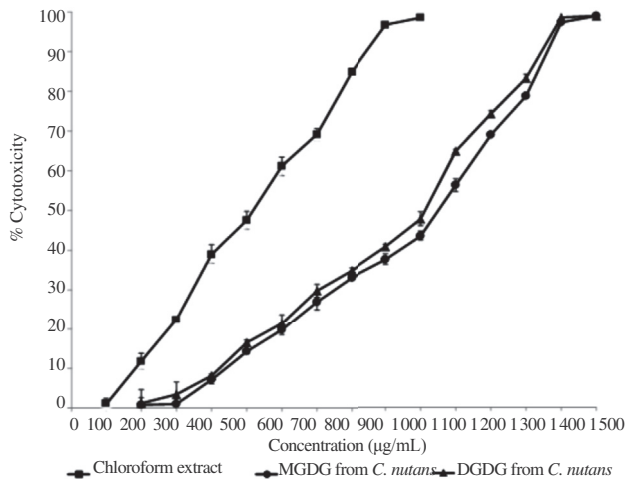


Figure 3. Determination of CC₅₀ of chloroform crude extract, extracted and purified MGDG and DGDG from *C. nutans* on Vero cells.

Table 1

Cytotoxicity, anti-HSV activity on post-treatment, and selectivity index of chloroform crude extract, extracted and purified MGDG and DGDG from *C. nutans*, standard MGDG, standard DGDG and acyclovir.

Compound	Cytotoxicity (CC ₅₀ , µg/mL)	Anti-HSV activity (IC ₅₀ , µg/mL)	
		HSV-1	HSV-2
Chloroform crude extract	523.00 ± 4.00	115.00 ± 4.00	140.00 ± 3.00
MGDG from <i>C. nutans</i>	955.00 ± 7.00	36.00 ± 5.00	41.00 ± 1.00
DGDG from <i>C. nutans</i>	922.00 ± 4.00	40.00 ± 3.00	43.20 ± 0.40
Standard MGDG	ND	31.00 ± 2.00	33.40 ± 0.40
Standard DGDG	ND	33.60 ± 0.20	36.80 ± 0.90
Acyclovir	ND	0.64 ± 0.07	0.80 ± 0.04

ND: No detection.

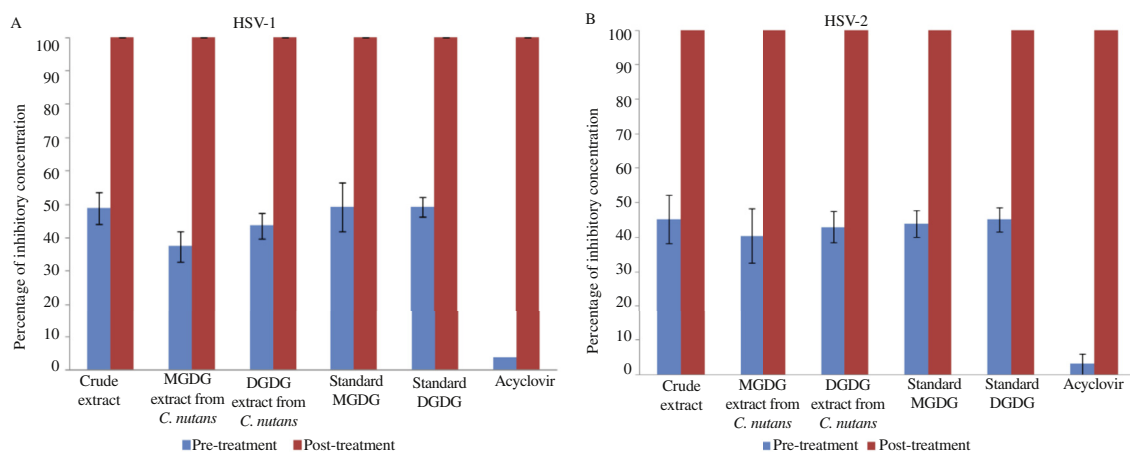


Figure 4. Mode of inhibitory activities of chloroform crude extract, extracted and purified MGDG and DGDG from *C. nutans* against HSV-1 (A) and HSV-2 (B) during different stages of the viral infection.

Viruses were treated with the maximal subtoxic concentration of the compounds. Acyclovir was used as a control in each experiment. Data represented the percentage of virus inhibition when compared to untreated controls as mean \pm SD.

Table 2

Selectivity index of crude extract, extracted and purified MGDG and DGDG from *C. nutans* on the replication of HSV-1 and HSV-2 on post-treatment.

Compound	Selectivity index (CC_{50}/IC_{50})	
	HSV-1	HSV-2
Chloroform crude extract	5.0 \pm 2.0	4.0 \pm 2.0
MGDG from <i>C. nutans</i>	26.0 \pm 1.0	23.3 \pm 0.9
DGDG from <i>C. nutans</i>	23.1 \pm 0.3	21.3 \pm 0.3

4. Discussion

For many generations, medicinal plants have been used by indigenous people for the treatment of many infectious diseases including HSV infection. Modern medicine currently uses acyclovir and other nucleoside derivatives have been used in the treatment of HSV infections. However, these are expensive and a large number of people, especially from developing countries, may not be able to afford those expensive drugs. Thus, some medicinal plants which can be found locally would be an alternative treatment.

In this study, the antiviral activity of chloroform crude extract, extracted and purified MGDG and DGDG from *C. nutans* were investigated for their antiviral activity against HSV-1 and HSV-2 on Vero cells. Cytotoxicity assay is an important part for evaluation of a potential antiviral agent because an extract or compounds should be selective for virus specific processes with little or no effects on host cellular metabolism.

Pre-treatment of Vero cells with chloroform extract, extracted and purified MGDG and DGDG for 24 h before viral infection demonstrated that these samples showed 50% protective effect against the HSV-1 and HSV-2 infection process. In contrast, the results showed that extracted and purified MGDG and DGDG from *C. nutans* demonstrated 100% inhibition of HSV-1 and HSV-2 replication at the post-infection step at subtoxic concentration levels. Moreover, extracted and purified MGDG and DGDG from *C. nutans* exhibited anti-HSV activity at the same level as the standard MGDG and DGDG

samples. The extracted and purified compounds displayed moderate anti HSV-1 and HSV-2 activity with IC_{50} values ranging from 36.00 to 43.20 μ g/mL. In addition, the chloroform crude extract of *C. nutans* has shown less effective against HSV-1 and HSV-2 than the extracted and purified MGDG and DGDG. It may postulate that MGDG and DGDG are one of the main contributors towards the inhibition of herpes virus replication. The selectivity index shows that extracted and purified MGDG and DGDG from *C. nutans* are selective for the inhibition of HSV-1 and HSV-2 infection. Similar to the acyclovir, extracted and purified MGDG and DGDG inhibit HSV-1 and HSV-2 replication. Acyclovir specifically inhibits the viral DNA polymerase during the replication cycle when new viral DNA is synthesized [26]. From this study, it could be said that the inhibitory activities against HSV-1 and HSV-2 of extracted and purified MGDG and DGDG from *C. nutans* are the most effective in the post step of infection. Even though the mechanism of anti-HSV activity of MGDG and DGDG is still not clear, a report showed that some monoglycerides showed antiviral activities against enveloped viruses by the destruction of viral envelopes [27].

Study of the synthetic monoglycosyl diglycerides also showed inhibitory activity against HSV-1 and HSV-2 in Vero cells [19]. This is in accordance with previous study that reported the inhibitory effect of *C. nutans* extract against HSV-1 and HSV-2 infection before viral entry to the host cells [15]. The active constituent from *C. nutans*, chlorophyll *a* and chlorophyll *b*, were also found to have anti-HSV activity before viral entry step [16]. Other investigators have also reported anti-HSV activities of hexane, dichloromethane and methanol extracts from *C. nutans* at the post step of infection [15]. It is interesting to note that this differs to a study where water and organic solvent extracts of *C. nutans* did not inhibit HSV replication in Vero cells [28,29].

In conclusion, this study has discovered the inhibitory activities of extracted and purified MGDG and DGDG from *C. nutans* against HSV-1 and HSV-2 probably in post viral entry step. *C. nutans* is a Thai herbal medicine used for herpes infection for many generations and the herb is generously available throughout Southeast Asia. MGDG and DGDG from *C. nutans* have been demonstrated to have anti-herpes simplex

activity at the same level as commercially available synthetic compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

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