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Anti HSV-1 activity of sulphoquinovosyl diacylglycerol isolated from *Spirulina platensis*

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ABSTRACT: Herpes simplex virus type 1 (HSV-1) causes a variety of infections in humans and can be found in many countries worldwide. Although the antiherpes drug, acyclovir, has been used very successfully, an acyclovir-resistant strain of HSV has recently been found in patients and drug toxicity has been reported with increasing use of it. As a potential alternative, it has been found that both a water soluble and a lipid extract of the microalga *Spirulina platensis* exhibit antiviral activity (HSV-1) in Vero cells. In this study, bioassay guided fractionation of a lipid extract from *S. platensis* led to the identification of sulphoquinovosyl diacylglycerol as the active antiviral agent. The chemical structure of this compound was confirmed by NMR and LC-mass spectroscopic analyses. Results from the study showed that the compound exhibited a remarkable activity against HSV-1 with an IC_{50} value of 6.8 µg/ml, which was comparable to that of acyclovir (1.5 µg/ml).

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) causes the vast majority of oral herpes (cold sores or fever blisters)[1]. Developing countries have a higher prevalence of infections than developed countries. Crowded living conditions increase the risk of HSV-1 infection, with prevalence rates approaching 100% in some populations[2–4]. Acyclovir, a synthetic drug of nucleoside analogue, has a remarkable effect against HSV-1 infection. It inhibits virus replication by acting on viral DNA synthesis[5,6]. Effective antiherpes synthetic drugs are continuously being developed. However, drug-resistant HSV infections have emerged due to the increase in the use of the drug[7]. Therefore, many researchers have attempted to search for new, more effective and inexpensive antiviral agents from natural sources.

Sulphoquinovosyl diacylglycerol (SQDG) was discovered in 1959[8]. The characteristic structural feature of SQDG is the unique head group constituent, sulphoquinovose, a derivative of glucose in which the 6-hydroxyl is replaced by a sulphonate group[9]. SQDG is a natural sulpholipid found in all photosynthetic plants, cyanobacteria, and algae[10]. It is located in thylakoid membranes where it appears to be involved in maintaining a constant ratio of anionic lipids. Besides its role in the thylakoid membrane, SQDG is an attractive compound for development of new drugs because of its extensive biological activities which include inhibitory effects on DNA polymerase and HIV-reverse transcriptase, P-selectin receptors, the AIDS virus, telomerase, and inflammation/proliferation[11–16].

Cyanobacterium, *Spirulina platensis*, is a rich source of valuable compounds such as the antioxidant phycocyanin and polyunsaturated fatty acids (linoleic acid and gamma-linolenic acid) which have an important role in human metabolic pathways, particularly as a precursor of one kind of prostaglandin[17,18]. Hayashi et al[19] were the first to report the anti HSV-1 activity of aqueous extracts from *S. platensis*. A water soluble fraction containing a sulphated polysaccharide called calcium spirulan isolated from *S. platensis* was found to inhibit a number of membrane viruses including HSV-1[19–21]. Our preliminary study revealed that both water soluble and non-polar extracts of *S. platensis* exhibited antiviral activity on HSV-1[22]. In the present work, we describe the isolation, purification, and characterization of the active ingredient in a non-polar extract of *S. platensis*.

MATERIALS AND METHODS

Extraction and isolation

Dried biomass of *S. platensis* (9 kg, Siam Algae Co., Ltd., Thailand) was extracted with 30 l of a 2:1 mixture of chloroform ($CHCl_3$) and methanol (MeOH) for 3 h at room temperature with continuous stirring. The collected filtrate was evaporated to give a crude lipid as a greenish brown gummy solid (630 g).

The crude lipid was dissolved with 30% MeOH in water solution and then partitioned with hexane. The hexane layer was evaporated to give 63 g of the hexane extract. The aqueous MeOH solution was then sequentially partitioned with ethyl acetate (EtOAc) and MeOH to give 535.5 g of EtOAc extract and 5.04 g of the MeOH extract. Each extract was subjected to bioassay for anti-herpes simplex virus activity. The EtOAc and MeOH extracts showing anti HSV-1 activity were chosen for further separation. The EtOAc extract was chromatographed on a Sephadex LH-20 (3.5 cm $\times$ 45 cm, eluted with MeOH). Ten fractions were obtained (50 ml per fraction), and we refer to these as F_E1–F_E10 (the first chromatography fractions). Because of their antiviral activity, F_E7–F_E10 were combined (6.43 g) and re-chromatographed on a Sephadex LH-20 (3 cm $\times$ 70 cm, eluted with MeOH) to give eighteen fractions, $F_{E'}1$–$F_{E'}18$ of 30 ml each (the 2nd chromatography fractions). The $F_{E'}5$–$F_{E'}8$ fractions were combined (1.95 g) based on their antiviral activity. The MeOH extract was also separated by a Sephadex LH-20 (3.5 cm $\times$ 45 cm, eluted with MeOH), yielding ten fractions (F_M1–F_M10). Each fraction was tested for anti HSV-1 activity.

General experimental procedures

^{1}H, ^{13}C, ^{1}H-^{1}H COSY, NOESY, HMQC, and HMBC experiments were carried out on a Bruker DRX operating at 400 MHz for protons and 100 MHz for carbon. The solvent for NMR spectra was deuterated chloroform ($CDCl_3$). LC-MS was performed by using a reversed-phase C18 (LICHROCART 250 $\times$ 4 mm) column. ESI-TOF mass spectra (ESI-TOF MS) were obtained from a Micromass LCT mass spectrometer.

Fatty acids analysis

Analyses were done using a modification of the method of Lepage and Roy[23]. The samples were subjected to direct transmethylation in 5% HCl in MeOH at 85 °C for 1 h, and heptadecanoic acid (C17:0, Sigma Co.) was added as an internal standard. Fatty acid methyl esters were analysed by gas chromatography (GC 17-A, Shimadzu) using a capillary column of fused silica glass (30 m $\times$ 0.25 mm, SP-2330, Supelco) with a film thickness of 0.20 µm. The column, injector, and detector temperatures were 205 °C, 250 °C, and 260 °C, respectively. The flow rate was 1.0 ml/min and the split ratio was 1:50.

Antiviral activity

Anti HSV-1 activity of *Spirulina* extracts was tested against HSV-1 (HF strain), using a colorimetric microtitre plate assay that determines host cell growth by measuring cellular protein content[24]. HSV-1 was maintained in the Vero cell line (kidney fibroblast of an African green monkey), which was cultured in Eagle's minimum essential medium with the addition of 10% heat-inactivated foetal bovine serum and antibiotics. The test samples were put into wells of a microtitre plate at final concentrations ranging from 20–50 µg/ml. The viral HSV-1 (30 PFU) was added, followed by plating of Vero cells (1×10^5 cells/ml). The final volume was 200 µl. After incubation at 37 °C for 72 h in a 5% CO_2 atmosphere, cells were fixed by 50% trichloroacetic acid and stained with 0.05% sulphorhodamine B in 1% acetic acid. Optical density was measured at 510 nm by a microplate reader. The growth of host cells infected with virus and treated with extract was compared with control cells infected with virus only. Acyclovir was used as the reference compound for antiviral activity.

Determination of cytotoxicity assay

A 190 µl volume of Vero cell suspension containing 1×10^5 cells/ml and 10 µl of tested compound was added to each well in triplicate. Elliptine and 10% DMSO were used as positive and negative controls, respectively. The cells were incubated at 37 °C for 72 h in a 5% CO_2 atmosphere. After incubation, the cytotoxicity was determined by a colorimetric method[24]. The compound was subjected to serial dilution to determine the IC_{50} value, which is the concentration of the compound which inhibits cell growth by 50% compared with untreated cells. A compound is non-toxic on the growth of Vero cells if $IC_{50} > 50$ µg/ml.

RESULTS AND DISCUSSION

Crude lipid extract of *S. platensis* exhibited antiviral activity with an IC_{50} value of 25.1 µg/ml, whereas the EtOAc and MeOH layers (of crude lipid) showed antiviral activity with respective IC_{50} values of 20.9 and 45.7 µg/ml. However, the hexane extract did not possess any antiviral activity. It was concluded that the active compound contains relatively polar moiety, and is not soluble in hexane. Hernández-Corona et al[25] also reported that the extracts with high polarity solvents such as MeOH and MeOH-H_2O 3:1 from *S. maxima* showed antiviral activity against HSV-2[25].

After the EtOAc and MeOH extracts were chromatographed on the Sephadex LH-20 column, no antiviral activity was observed in any of the fractions (F_M1–F_M10) of MeOH extract or fractions F_E1–F_E2 of EtOAc extract (data not shown). This suggests that the active compound might be degraded during purification. The fractions F_E3–F_E5 of EtOAc extract

Table 1 Antiviral activity of Sephadex LH-20 column chromatography fractions and re-chromatographed fractions of EtOAc extract of *S. platensis*.

1st chromatog. fractions		2nd chromatog. fractions	
Fraction	Anti HSV-1 IC_{50} (µg/ml)	Fraction	Anti HSV-1 IC_{50} (µg/ml)
F_E3	36.80 ± 3.21	$F_{E'}3$	36.61 ± 3.86
F_E4	30.83 ± 3.42	$F_{E'}4$	15.82 ± 1.87
F_E5	39.35 ± 2.81	$F_{E'}5$	7.71 ± 1.44
F_E6	7.59 ± 1.21	$F_{E'}6$	2.42 ± 1.01
F_E7	3.01 ± 0.84	$F_{E'}7$	0.97 ± 0.36
F_E8	0.84 ± 0.31	$F_{E'}8$	4.35 ± 0.21
F_E9	1.55 ± 0.41	$F_{E'}9$	24.31 ± 2.07
F_E10	18.31 ± 2.07	$F_{E'}10$	42.80 ± 3.92
		$F_{E'}11$	37.30 ± 2.54

Values are expressed as mean $\pm$ SD ($n = 3$).

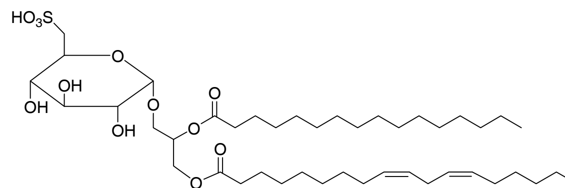

Fig. 1 Structure of sulphoquinovosyl diacylglycerol.

showed antiviral activity with an IC_{50} value above 30 µg/ml, whereas fractions F_E6–F_E10 of EtOAc extract showed strong antiviral activity (Table 1). The fractions F_E7–F_E10 of EtOAc were combined and re-chromatographed on Sephadex LH-20, and each fraction was subjected to test for anti HSV-1 activity. No antiviral activity was observed in the early fractions ($F_{E'}1$–$F_{E'}2$) and the fractions $F_{E'}12$–$F_{E'}18$ whereas the $F_{E'}5$–$F_{E'}8$ fractions exhibited effective antiviral activity (Table 1). $F_{E'}5$–$F_{E'}8$ fractions were then combined for use in chemical investigation and rechecked for anti HSV-1 activity. The combined $F_{E'}5$–$F_{E'}8$ fraction showed a remarkable activity against HSV-1 with an IC_{50} value of 6.8 µg/ml, comparable to the reference drug, acyclovir, which has a IC_{50} value of 1.5 µg/ml. In this study, crude lipid, the three extracts (hexane, EtOAc and MeOH), and all fractions of EtOAc and MeOH extracts were non-toxic against the growth of Vero cells at a maximum concentration of 50 µg/ml of samples.

Chemical investigations of combined $F_{E'}5$–$F_{E'}8$ fraction EtOAc extract led to the isolation of an unknown compound. The structure of the compound was elucidated by extensive analyses of NMR and ESITOF-MS spectral data, as well as by data comparison with previous reports [14]. The ^{1}H-NMR and ^{13}C-NMR spectra of the compound suggest that the compound has characteristic signals of glycolipid and sugar. The HMQC and COSY data revealed that the sugar is quinovose which contains a sulphonic acid on C6. The compound is a known sulphonic acid containing glycolipid. To clarify the structure of long-chain fatty acids, the compound was subjected to methanolic HCl treatment and fatty acids methyl ester were compared with the authentic fatty acid standards, i.e. palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (18:0), linoleic acid (C18:2), and linolenic acid (C18:3) by gas chromatography. Results showed that the compound contained palmitic acid and linoleic acid groups. The ESITOF-MS results for the compound established a molecular formula of $C_{43}H_{78}O_{12}S$ and a molecular weight of 818 Da (Fig. 1). The structure of the compound was identified as a SQDG that has two hydrophobic tails consisting of glycerol-bearing fatty acids connected to a hydrophilic moiety, and hence this molecule has an amphiphilic character.

SQDG is reported to have an important role in photosynthetic processes of cyanobacteria [26,27]. The aqueous and methanolic extracts of cultured cyanobacteria of several genera such as *Microcystis*, *Nodularia*, *Oscillatoria*, *Scytonema*, *Lyngbya*, and *Calothrix* showed antiviral activity against influenza virus A [28]. Biological activity of SQDG of some cyanobacteria (*Phormidium* and *Lyngbya*) was reported to possess both antiviral (HIV-1) and anti-tumour activity [12,29]. Loya et al [12] postulated that the lipophilic groups of SQDG interact with the hydrophobic core of the enzyme whereas the negatively charged sulphonate moiety in quinovose was important and may interact with the positively charged side chains on the enzyme [12]. Matsumoto et al [11] investigated the relationship between the structure and function of the β-SQDG-C18:0 vesicles to identify the forms that are effective for DNA polymerase inhibition. They found that the sulphonate moiety in quinovose was important in inhibiting the polymerase activity [11]. However, the cyanobacteria *Microcystis*, *Phormidium*, and *Lyngbya* also produce a wide variety of toxins implicated in animal and human poisoning incidents [30,31]. To date, attention has focused on the cyanobacteria *Spirulina* which has been widely used as a protein source and food supplement and cultivated for mass production in several countries including Thailand. Although many health benefits of *Spirulina* have been claimed by algal manufacturers, scientific evidence is scarce. Although water soluble

polysaccharides of *Spirulina* were previously found to inhibit the growth of HSV-1[19–21], there has been no report on anti HSV-1 activity of lipid extract from *Spirulina*. This work suggests that SQDG of *Spirulina* may help prevent viral infection in Vero cells.

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