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Epidermal and Blood Vessel Barrier Functions of Glucosylceramides and Digalactosyldiacylglycerols Isolated from Yellow Strawberry Guava

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Abstract: Strawberry guava is the fruit of *Psidium littorale*, which grows in tropical regions. Few studies have examined the hydrophobic compounds and biological activities of this fruit. Therefore, we purified lipophilic compounds of strawberry guava and examined their effects on epidermal and blood vessel barrier functions as well as their anti-melanogenic activity. Lipophilic compounds were isolated by silica gel column chromatography followed by reversed-phase HPLC with MeOH from an EtOH extract of the fruit. Isolated compounds were identified by comparing NMR and MS spectra with those of reference values. The effects of these compounds on epidermal barrier function were evaluated by measuring transepidermal water loss (TEWL) using reconstructed human epidermal keratinocytes (RHEKs). Blood vessel barrier function was examined using dye permeability through human umbilical vein endothelial cell (HUVEC) layers. Anti-melanogenic activity was assessed by theophylline-induced melanogenesis in B16 melanoma cells. We isolated six glucosylceramides (GlcCers) and three digalactosyldiacylglycerols (DGDGs). Only GlcCer[t18:1(8*Z*)/23:0] significantly lowered TEWL in RHEKs, while GlcCer[t18:1(8*Z*)/24:0] induced a slight reduction. Regarding the permeability of the HUVEC layer, GlcCer[d18:2(4*E*,8*Z*)/16:0] and DGDG (1,2-dilinolenoyl-3-digalactosylglycerol) significantly suppressed dye permeability and this effect was accompanied by the expression of VE-cadherin, which facilitates cell-to-cell adhesion. GlcCers and DGDGs did not exhibit anti-melanogenic activity. Therefore, strawberry guava containing specific GlcCers and DGDGs may promote epidermal and blood vessel barrier functions.

Keywords: yellow strawberry guava; *Psidium littorale*; glucosylceramide; digalactosyldiacylglycerol; transepidermal water loss; blood vessel permeability

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1. Introduction

Yellow strawberry guava (Myrtaceae) is a tropical plant that grows in Florida, Hawaii, Mexico, the West Indies, Jamaica, the Bahamas, Burazil, and Uruguay as well as China and Japan. In Japan, it is cultivated in the Southern Izu peninsula, which has a warm climate. The fruit is edible and processed into jam, vinegar, and juice. Phloroglucinols and phenolic glycosides have been isolated from the fruit [1], while formyl-phloroglucinols [2], megastigmane glycosides [3], lignans [3], flavonoids [4], benzophenones [5], and diphenylmethane glycosides [5] have been identified in the leaves. The anti-glycation effect [6] of its fruit extract and the inhibitory activity of its leaf extract against glycolytic enzymes [7] have been reported as the biological activities of strawberry guava. Based on limited chemical and biological findings on strawberry guava, we purified and identified the lipophilic constituents of strawberry guava fruit and successfully isolated glucosylceramides (GlcCers) and digalactosyldiacylglycerols (DGDGs).

Skin ceramides (Cers) are sphingolipids that are mainly present in the stratum corneum (SC) and play pivotal roles in epidermal hydration and barrier functions [8]. Twelve major Cers have been identified as intracellular SC lipids [9]. Among Cers, ω-hydroxy Cer, which has a very long fatty acid chain such as Cer [EOS], exhibits a stronger barrier function [10]. In terms of major plant-derived Cers, GlcCer is present in a wide variety of botanical resources [11]. The findings of clinical studies indicate the potential of rice-derived GlcCers to reduce transepidermal water loss (TEWL), which increases skin moisture levels and enhances barrier function [12]. Increases in epidermal ceramide production may be one of the mechanisms by which GlcCer supplementation facilitates epidermal barrier function [13]. In our previous study on GlcCers in rice, we isolated 13 GlcCers, which consisted of multiple molecules comprising different types of sphingoid bases and free fatty acid chains of multiple lengths from the gummy byproducts of rice bran oil [14]. Moreover, we clarified the structure–activity relationships of rice GlcCers for epidermal barrier function [15] and anti-melanogenic activity [14].

Other than skin barrier function, blood vessel barrier function is important for the transportation of water, proteins, and other nutrients between the blood and extravascular tissues [16]. Impairments in endothelial barrier function have been shown to induce lung and heart failure [17]. Hypervascular permeability is caused by type I allergy and induces edema and flair [18]. Sphingosine-1-phosphate [19] and Cers [20] have been shown to play pivotal roles in the regulation of vascular permeability.

Based on these findings, it was predicted that strawberry guava-derived GlcCers and DGDGs might exhibit epidermal and blood vessel barrier functions. We herein examined the effects of GlcCers and DGDGs [21,22] derived from strawberry guava on skin and blood vessel barrier functions as well as their anti-melanogenic activity.

2. Results

*2.1. Effects of GlcCers (**1**–**6**) and DGDGs (**7**–**9**) on TEWL in Reconstructed Human Epidermal Keratinocytes (RHEKs)*

In evaluations using RHEKs, the application of 10 μM GlcCer[t18:1(8Z)/23:0] (**3**) for 7 days induced a reduction in TEWL (Figure 1). GlcCer[t18:1(8Z)/24:0] (**4**) also decreased TEWL to a similar extent as **3**; however, a significant difference from the control was not observed. GlcCer[d18:2(4*E*,8*Z*)/16:0] (**1**) and GlcCer[t18:1(8Z)](**2**, **5**, **6**) did not reduce TEWL. Furthermore, DGDG (**9**) did not decrease TEWL, while DGDG (**7**) and DGDG (**8**) slightly increased TEWL at an earlier stage in the culture period. The strawberry guava extract (10 μg/mL) significantly reduced TEWL on day 7.

*2.2. Effects of GlcCers (**1**–**6**) and DGDGs (**7**–**9**) on the Permeability of the Human Umbilical Vein Endothelial Cell (HUVEC) Layer and VE-Cadherin Expression*

Among the GlcCers isolated from strawberry guava, 10 μM GlcCer [d18:2(4*E*,8*Z*)/16:0] (**1**) suppressed dye transmission through the HUVEC layer without cytotoxicity (Figure 2). Among DGDGs, only 1,2-dilinolenoyl-3-digalactosylglycerol (**7**) reduced the permeability of the HUVEC layer. No cytotoxicities were observed for GlcCers (**1**, **2**, **4**–**6**) or DGDGs (**7**–**9**), whereas GlcCer[t18:1(8Z)/23:0] (**3**) was cytotoxic.

We examined the effects of GlcCer [d18:2(4*E*,8*Z*)/16:0] (**1**) and 1,2-dilinolenoyl-3-digalactosylglycerol (**7**), which enhanced blood vessel barrier function, on VE-cadherin expression. The results obtained showed that 10 μM GlcCer (**1**) and DGDG (**7**) significantly increased the protein expression of VE-cadherin (Figure 3).

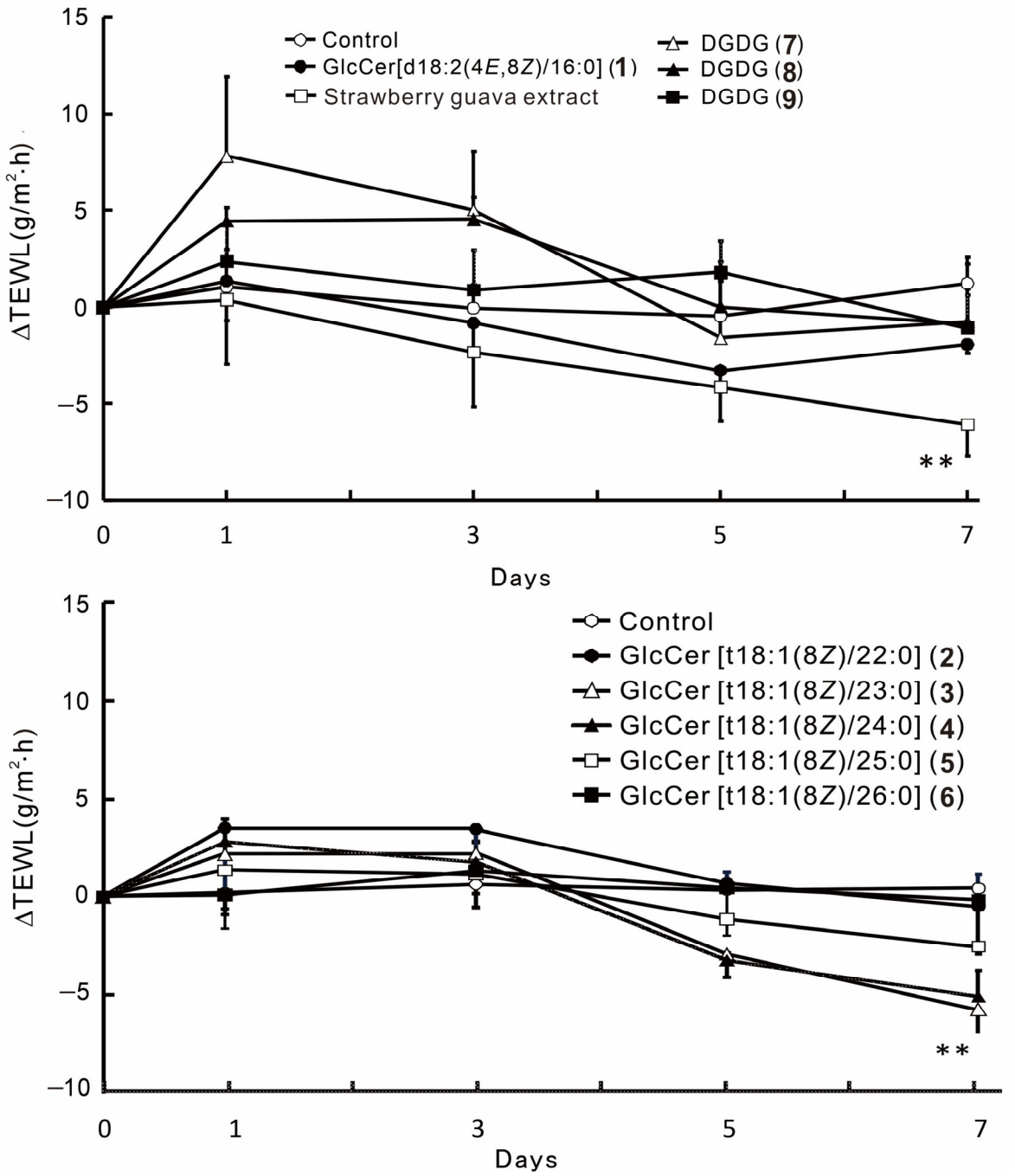


Figure 1. Effects of GlcCers (**1**–**6**), DGDGs (**7**–**9**), and strawberry guava extract on TEWL in RHEKs. The RHEK model was cultured for 7 days to assess TEWL. TEWL was measured before and 1, 3, 5, and 7 days after the application of treatments. Each value represents the mean ± S.E. (N = 3–4). Asterisks denote significant differences from the control group, **: $p < 0.01$.

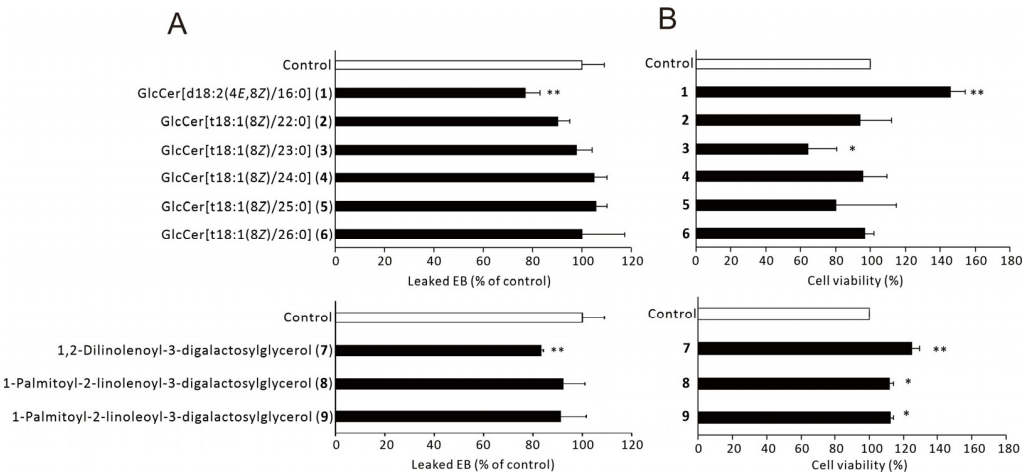


Figure 2. Effects of GlcCers (**1**–**6**) and DGDGs (**7**–**9**) on dye permeability in the HUVEC layer. (**A**) The amount of leaked EB from HUVECs. HUVECs were cultured on collagen-coated Millicell® culture inserts for 7 days to form a cell layer. After the pre-culture, the medium was changed to that containing 10 μM GlcCer or DGDG. After a culture for 24 h, the permeability assay was performed. (**B**) Cytotoxicity test. HUVECs were cultured with 10 μM GlcCer or DGDG for 24 h and MTT assay was performed. Each value represents the mean ± S.E. (N = 3–4). Asterisks denote significant differences from the control group, *: $p < 0.05$, **: $p < 0.01$.

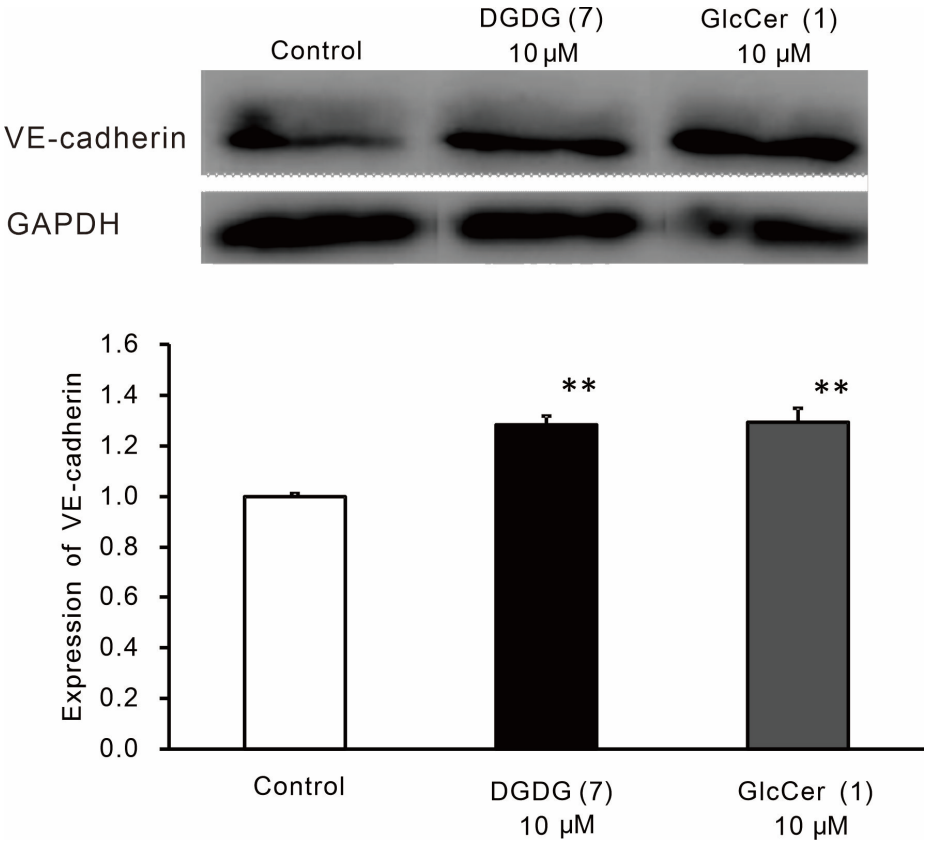


Figure 3. Effects of GlcCer [d18:2(4*E*,8*Z*)/16:0] (**1**) and DGDG (**7**) on VE-cadherin expression in HUVECs. Each column represents the mean with the SE (N = 3). Asterisks denote a significant difference from the control **: $p < 0.01$.

*2.3. Effects of GlcCers (**1**–**6**) and DGDGs (**7**–**9**) on Theophylline-Induced Melanogenesis in B16 Melanoma Cells*

Among the GlcCers and DGDGs, 10 μM DGDG (**9**) slightly (approximately 25%) suppressed melanin production without cytotoxicity (Table 1). The other DGDGs (**7**, **8**) did not inhibit the production of melanin. GlcCers (**3**–**6**) weakly suppressed melanogenesis but were cytotoxic at the same concentration.

Table 1. Anti-melanogenic effects of GlcCers against theophylline-induced melanogenesis and cell viability of B16 melanoma cells.

	Inhibition (%) and Cytotoxicity			
Sample Conc. (μM)	0	1	3	10
GlcCer[d18:2(4*E*,8*Z*)/16:0] (**1**)	0.0 ± 6.6 (100.0 ± 0.5)	−6.5 ± 5.7 (99.6 ± 1.2)	0.5 ± 4.9 (100.0 ± 1.1)	−0.7 ± 9.1 (100.2 ± 0.9)
GlcCer[t18:1(8*Z*)/22:0] (**2**)	17.1 ± 4.0 (94.0 ± 5.8)	−5.9 ± 4.2 (115.1 ± 8.3)	1.6 ± 5.1 (86.4 ± 5.1)	11.2 ± 1.5 (81.9 ± 6.3)
GlcCer[t18:1(8*Z*)/23:0] (**3**)	0.0 ± 2.3 (100.0 ± 0.6)	17.4 ± 1.9 ** (96.4 ± 1.6)	10.8 ± 4.3 (90.8 ± 0.6 **)	21.4 ± 2.6 ** (85.5 ± 0.1 **)
GlcCer[t18:1(8*Z*)/24:0] (**4**)	0.0 ± 3.1 (100.0 ± 1.0)	19.9 ± 1.8 ** (68.4 ± 0.6 **)	16.4 ± 2.8 ** (64.0 ± 0.8 **)	16.6 ± 1.0 ** (70.8 ± 0.4 **)
GlcCer[t18:1(8*Z*)/25:0] (**5**)	0.0 ± 4.0 (100.0 ± 1.4)	5.7 ± 1.7 (83.5 ± 0.3 **)	14.4 ± 1.4 ** (87.9 ± 0.4 **)	14.7 ± 0.4 ** (85.8 ± 0.6 **)
GlcCer(t18:1(8*Z*)26:0) (**6**)	0.0 ± 1.9 (100.0 ± 4.3)	15.3 ± 0.6 ** (79.8 ± 3.1 **)	22.8 ± 1.4 ** (67.0 ± 2.9 **)	17.8 ± 0.8 ** (85.1 ± 10.7)
1,2-Dilinolenoyl-3-digalactosylglycerol (**7**)	13.0 ± 4.1 (98.5 ± 0.8)	8.5 ± 1.1 (98.8 ± 1.6)	1.9 ± 1.1 (98.7 ± 0.7)	4.8 ± 5.1 (96.7 ± 1.1)
1-Palmitoyl-2-linolenoyl-3-digalactosylglycerol (**8**)	1.8 ± 5.0 (99.0 ± 0.6)	0.3 ± 2.6 (100.9 ± 0.5)	4.4 ± 2.0 (100.9 ± 0.6)	10.4 ± 2.1 (100.7 ± 1.1)
1-Palmitoyl-2-linoleoyl-3-digalactosylglycerol (**9**)	11.5 ± 4.8 * (99.0 ± 1.1)	15.2 ± 2.5 ** (99.6 ± 0.2)	12.5 ± 1.0 * (101.4 ± 0.5)	25.9 ± 1.5 ** (97.6 ± 2.0)
	0	30	100	300
Kojic acid	0.0 ± 4.4 (100.0 ± 1.6)	9.6 ± 5.9 (96.0 ± 1.3)	25.9 ± 3.2 ** (96.5 ± 0.4)	50.4 ± 0.7 ** (99.5 ± 1.8)

B16 melanoma cells were cultured with GlcCer, DGDG, or kojic acid and theophylline (2 mM) for 72 h. Each value represents the mean ± S.E. (N = 4). Asterisks denote significant differences from the control *: $p < 0.05$, **: $p < 0.01$. Values in parentheses indicate the viability of B16 melanoma cells.

3. Discussion

We herein confirmed that the fruit of strawberry guava contained GlcCers and DGDGs as lipophilic compounds. GlcCers are also present in other fruits, such as pineapples [23] and peaches [24]. These fruit-derived GlcCers are used in dietary supplements that claim to have skin barrier and moisturizing effects as functional foods in Japan. The molecular composition of pineapple GlcCers is similar to that of rice GlcCers, which contain GlcCer[d18:2(4*E*,8*Z*)] as a major GlcCer [15]. The composition of GlcCers in peaches currently remains unknown. Strawberry guava GlcCers differed from rice GlcCers including GlcCer[d18:2(4*E*,8*Z*)/16:0] (**1**). In contrast, the major GlcCer of strawberry guava is GlcCer[t18:1(8*Z*)]. This is also the main type of GlcCer present in the fruit of *Citrus unshiu* (Satsuma mandarin) [25] and has been detected in large amounts in grains, such as wheat [26] and rice [15]. Therefore, we herein compared the barrier function of GlcCer[t18:1] with free acid chains of different lengths ranging from C22 to C26 with that of GlcCer [d18:2(4*E*,8*Z*)]. In addition to GlcCers, we isolated three types of DGDGs. Besides their effects on skin barrier function, DGDGs have been reported to exert anti-denaturation [27], anti-hyaluronidase [27], anti-inflammatory [28], and anti-angiogenic [29] effects. However,

DGDGs are a mixture of multiple molecules, and the activities of a single molecule of DGDG currently remain unclear. Based on previous findings, we examined the biological functions of strawberry guava GlcCers and DGDGs in in vitro epidermal water evaporation and blood vessel permeability models of the epidermal barrier and blood vessel barrier, respectively.

To assess the effects on the epidermal barrier, we used RHEKs and measured TEWL. The results obtained showed that GlcCer[t18:1(8*Z*)/23:0] (**3**) promoted epidermal barrier function. GlcCers with fatty acid chains of different lengths, such as GlcCer[t18:1(8*Z*)/22:0] (**2**), GlcCer[t18:1(8*Z*)/24:0] (**4**), GlcCer[t18:1(8*Z*)/25:0] (**5**), and GlcCer[t18:1(8*Z*)/26:0] (**6**), did not decrease TEWL. We previously showed that GlcCer[d18:2(4*E*,8*Z*)] with fatty acid chain lengths between C18 and C26 exerted fatty acid length-dependent effects on barrier function in the RHEK model [30], whereas GlcCer[t18:1(8*Z*)] did not. The present study is the first to demonstrate that some t18:1 types of GlcCers reduced TEWL in an in vitro epidermal model.

On the other hand, DGDGs did not suppress TEWL in RHEKs. DGDGs are glycolipids that play a vital role in photosynthesis in plants [31]. They are also crucial for the tolerance of wheat to environmental heat stress [32]. Moreover, in the market of dietary supplements, wheat DGDG and GlcCer complexes have been used to prevent skin dehydration [22]. However, the results obtained in the in vitro experiment in the present study do not support this clinical effect because the three types of DGDGs examined did not exert positive effects. Therefore, further studies are needed to assess and identify other types of DGDGs with the potential to suppress TEWL.

Vascular permeability is a distinct mechanism in blood vessels that maintains the exchange between blood vessels and peripheral tissues [33]. Excessive increases in vascular permeability, particularly in the endothelium, have been reported in many diseases, such as type I skin allergy [34], rosacea [35] swelling [36], and various types of inflammation [37]. Endothelial permeability is also recognized as an endothelial barrier function [38] that maintains vascular homeostasis. HUVEC layers are frequently used to examine changes in endothelial barrier function [39,40]. In the present study, we investigated the effects of GlcCers and DGDGs on HUVEC permeability. The results obtained showed that GlcCer[d18:2(4*E*,8*Z*)/16:0] (**1**) suppressed vascular permeability. According to previous findings on Cers and GlcCers, C6 ceramide, a simple type of Cer with no glucose, enhanced the endothelial permeability of HUVECs [41]. Other studies showed that Cers appeared to induce vascular leakage, whereas sphingosine-1-phosphate enhanced endothelial integrity [42]. In addition, C8 ceramide was found to induce pyroptosis, a type of cell death, in HUVECs [43]. Based on these findings, simple Cers are considered to enhance vascular permeability or be harmful to vascular cells. Therefore, the present results showing that plant-derived GlcCers suppressed vascular permeability provide novel insights into the field of anti-inflammatory compounds.

In terms of DGDGs in HUVECs, only DGDG (**7**) suppressed vascular permeability. Various inhibitory effects on macrophage activation have been reported as the anti-inflammatory effects of DGDGs [44–47]. Marine microalga-derived DGDGs [44] strongly suppressed nitric oxide (NO) production from lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. DGDGs consisting of very long fatty acid chains suppressed NO production. Similarly, de Los Reyes et al. reported that other marine microalga-derived DGDGs inhibited TNF-α production in LPS-stimulated human THP-1 macrophages [45]. These DGDGs commonly have 18:5/18:4 or 18:5/16:1 fatty acids in their structures. Regarding plant-derived DGDGs, a Perilla-derived DGDG (1-*O*-9*Z*,12*Z*-octadecadienoyl-2-*O*-9*Z*,12*Z*,15*Z*-octadecatrienoyl-3-*O*-(β-D-galactopyranosyl-(1′→6″)-α-D-galactopyranosyl)-sn-glycerol) suppressed NO production in LPS-stimulated RAW264.7 macrophages [46]. In addition, the oral administration of an oat-derived DGDG was found to attenuate ethanol-induced brain inflammation in mice [47]. As described above, some types of DGDGs exerted anti-inflammatory effects in vitro and in vivo; however, the effects of DGDGs on blood vessel permeability remain unknown. Therefore, the present results confirmed the effects

of DGDGs on blood vessel barrier function, which contributed to their anti-inflammatory effects. Moreover, GlcCer[d18:2(4*E*,8*Z*)/16:0] (**1**) and DGDG (**7**) both increased the expression of VE-cadherin, which promotes cell-to-cell adhesion [48]. Therefore, these lipids in strawberry guava appear to suppress blood vessel permeability by strengthening cell-to-cell adhesion. In the case of colitis, intestinal GlcCer promoted epithelial cell adhesion through colon-tight junctions [49] and increased VE-cadherin expression. Based on these findings, GlcCer enhances blood vessel epithelial cell adhesion.

We also examined the anti-melanogenic effects of GlcCers and DGDGs in strawberry guava. The results obtained revealed that many GlcCers and DGDGs did not exert anti-melanogenic effects and only DGDG (**9**) weakly suppressed B16 melanin production. Furthermore, the inhibitory effects of **9** were weaker than those of GlcCer [d18:2(4*E*,8*Z*)/18:0], GlcCer [d18:2(4*E*,8*Z*)/20:0], and Cer [t18:0/20:0] [14]. Therefore, strawberry guava extract does not exhibit anti-melanogenic activity, and strawberry guava GlcCers were not anti-melanogenic.

In conclusion, we purified and identified six GlcCers and three DGDGs as lipophilic compounds from yellow strawberry guava fruit. Among them, GlcCer[t18:1(8*Z*)/23:0] (**3**) exhibited epidermal barrier function, while GlcCer[d18:2(4*E*,8*Z*)/16:0] (**1**) and DGDG (**7**) and enhanced blood vessel barrier function. These compounds appear to contribute to the beneficial effects of strawberry guava fruit on health. This report revealed the purification processes and biological activities of GlcCers and DGDGs from strawberry guava for the first time, providing new insight into lipophilic compounds in berries and fruits.

4. Materials and Methods

4.1. Experimental Instruments

The following instruments were used to obtain spectroscopic data: ^{1}H NMR spectra, JNM-ECS400 (400 MHz) spectrometers; ^{13}C NMR spectra, JNM-ECA400 (100 MHz) spectrometers (JEOL Ltd., Tokyo, Japan); and ESIMS and HRESIMS, Exactive Plus mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

A single-channel automated Smart Flash EPCLC-AI-580S system (Yamazen Corporation, Osaka, Japan) with an evaporative light scattering detector (ELSD-100X, Yamazen Co.) was used for silica gel flash chromatography.

The following instruments were used for reverse-phase HPLC: a refractive index (RI) detector, RID-20A; an LC pump, LC-9A; and a column oven, CTO-10AC (Shimadzu Co., Kyoto, Japan).

4.2. Reagents

Amberlite^{TM} XAD-4 was purchased from Organo Corporation (Tokyo, Japan). Dulbecco's modified Eagle's medium (D-MEM), a penicillin (100 units/mL) and streptomycin (100 µg/mL) mixture, phosphate-buffered saline (PBS) (−), dimethyl sulfoxide (DMSO), Evans blue (EB), kojic acid, and theophylline were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Fetal bovine serum (FBS) was supplied by Biosera (Boussense, France). Endothelial cell growth medium (EGM)-2 containing 2% FBS, 0.04% hydrocortisone, 4% human fibroblast growth factor (hFGF)-β, 0.1% vascular endothelial growth factor (VEGF), 0.1% R3-insulin-like growth factor (IGF), 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, and 0.1% heparin was from Lonza (Basel, Switzerland). The radioimmunoprecipitation assay (RIPA) buffer, BCA protein assay kit, protease phosphatase inhibitor cocktail, and ECL Plus Western blotting substrate were obtained from Thermo Fisher Scientific Inc. (MA, USA). Super Signal Can get signal solutions 1 and 2 were purchased from Toyobo Co., Ltd. (Osaka, Japan). Anti-VE-cadherin (AF6265) was supplied by Affinity Bioscience (Cincinnati, OH, USA). Anti-GAPDH (MA5-16738) was purchased from Invitrogen (Carlsbad, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was obtained from Abcam, Inc. (Cambridge, UK) and HRP-conjugated goat anti-rabbit IgG from Merck Millipore (Darmstadt, Germany).

4.3. Cells

The RHEK model (LabCyte EPI-MODEL) and assay medium were obtained from Japan Tissue Engineering Co., Ltd. (Gamagori, Japan). HUVECs were purchased from Lonza Japan. The cells were cultured with EGM™-2 BulletKit™ (EBM™-2 containing 0.1% hEGF, 2% FBS, 0.1% VEGF, 0.4% hFGF-B, 0.04% hydrocortisone, 0.1% R3-IGF-1, 0.1% ascorbic acid, and 0.1% GA-1000) in a T75 flask at 37 °C under a 5% CO_2 atmosphere. The cells were used to assess the blood vessel permeability of the HUVEC layer and cytotoxicity.

The mouse skin melanoma cell line B16 (JCRB0202) was purchased from the Health Science Research Resources Bank (Osaka, Japan). The cells were sub-cultured in D-MEM supplemented with 10% FBS and a penicillin (100 units/mL) and streptomycin (100 μg/mL) mixture at 37 °C under a 5% CO_2 atmosphere. The cells were used to assess melanogenesis.

4.4. Preparation of GlcCer and DGDG

Yellow strawberry guava, the fruits of *Psidium littorale*, were collected in South Izu town, Shizuoka, Japan, in October 2022. The plant material was identified by one of the authors (T.M.). A voucher specimen (November 2022. Oryza-01) was deposited in our laboratory. Yellow strawberry guava fruit (4.61 kg) cultivated in the South Izu area of Shizuoka, Japan, was crushed and extracted with EtOH (17.8 L) at 70 °C. The extracted solution was filtered and the solvent was evaporated and concentrated to obtain the EtOH extract (462 g). The extract (462 g) was applied to an Amberlite™ XAD-4 column (20–60 mesh, 2.23 kg) and eluted with H_2O (10 L), MeOH (10 L), and EtOH (10 L). The EtOH eluent was evaporated to obtain the sugar-eliminated EtOH extract of strawberry guava (SESG, 2.36 g). SESG (2.36 g) was subjected to silica gel flash chromatography (Universal silica premium 2 L, 55 g, mesh size: 30 μm, Yamazen Co., Osaka, Japan) and fractionated with a mixture of hexane and ethyl acetate (9:1→7:3→1:1), ethyl acetate, and a mixture of chloroform and MeOH (9:1→7:3→1:1). The flow rate of the flash chromatograph apparatus equipped with an ELSD detector was 10 mL/min.

The chloroform and MeOH (7:3) fraction (145 mg) was purified by InertSustain C18 (ϕ20 × 250 mm, GL Science, Tokyo, Japan) with a mixture of MeOH and tetrahydrofuran (9:1). The flow rate was 9 mL/min, and an RI detector was used for detection. GlcCer[d18:2(4*E*,8*Z*)/16:0] (**1**, 5.1 mg), GlcCer[*t*18:1(8*Z*)/22:0] (**2**, 5.7 mg), GlcCer[*t*18:1(8*Z*)/23:0] (**3**, 8.0 mg), GlcCer[*t*18:1(8*Z*)/24:0] (**4**, 8.1 mg), GlcCer[*t*18:1(8*Z*)/25:0] (**5**, 21.2 mg), and GlcCer[*t*18:1(8*Z*)/26:0] (**6**, 5.6 mg) were obtained (Figure 4A). The same fraction (150.1 mg) was purified under the same conditions with MeOH as the solvent to obtain 1,2-dilinolenoyl-3-digalactosylglycerol (**7**, 19.6 mg), 1-palmitoyl-2-linolenoyl-3-digalactosylglycerol (**8**, 21.4 mg), and 1-palmitoyl-2-linoleoyl-3-digalactosylglycerol (**9**, 23.2 mg) (Figure 4B). Chemical structures (Figure 5) were identified by ^{1}H-, ^{13}C-NMR, ESIMS, and HRESIMS spectra with the reference values listed in a previous study [50–53].

GlcCer[d18:2(4E,8Z)/16:0] (**1**): Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S1; negative-ion ESIMS *m/z* 713 $[M - H]^-$; negative-ion HRESIMS *m/z* 712.5348 $[M - H]^-$ (calcd for $C_{40}H_{74}NO_9^-$, 712.5358).
GlcCer[t18:1(8Z)/22:0] (**2**): Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S2; negative-ion ESIMS *m/z* 815 $[M - H]^-$; negative-ion HRESIMS *m/z* 814.6381 $[M - H]^-$ (calcd for $C_{46}H_{88}NO_{10}^-$, 814.6403).
GlcCer[t18:1(8Z)/23:0] (**3**): Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S3; negative-ion ESIMS *m/z* 829 $[M - H]^-$; negative-ion HRESIMS *m/z* 828.6536 $[M - H]^-$ (calcd for $C_{47}H_{90}NO_{10}^-$, 828.6559).
GlcCer[t18:1(8Z)/24:0] (**4**): Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S4; negative-ion ESIMS *m/z* 843 $[M - H]^-$; negative-ion HRESIMS *m/z* 842.6694 $[M - H]^-$ (calcd for $C_{48}H_{92}NO_{10}^-$, 842.6716).
GlcCer[t18:1(8Z)/25:0] (**5**): Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S5; negative-ion ESIMS *m/z* 857 $[M - H]^-$; negative-ion HRESIMS *m/z* 856.6838 $[M - H]^-$ (calcd for $C_{49}H_{94}NO_{10}^-$, 856.6862).

GlcCer[t18:1(8Z)/24:0] (**6**): Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S6; negative-ion ESIMS *m/z* 871 $[M - H]^-$; negative-ion HRESIMS *m/z* 870.7007 $[M - H]^-$ (calcd for $C_{50}H_{96}NO_{10}^-$, 870.7029).
DGDG (1,2-dilinolenoyl-3-digalactosylglycerol) (**7**): Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S7; positive-ion ESIMS *m/z* 960 $[M + H]^+$; positive-ion HRESIMS *m/z* 959.5713 $[M + H]^+$ (calcd for $C_{51}H_{84}O_{15}Na^+$, 959.5702).
DGDG (1-palmitoyl-2-linolenoyl-3-digalactosylglycerol) (**8**) Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S8; positive-ion ESIMS *m/z* 938 $[M + H]^+$; positive-ion HRESIMS *m/z* 937.5869 $[M + H]^+$ (calcd for $C_{49}H_{86}O_{15}Na^+$, 937.5859).
DGDG (1-palmitoyl-2-linoleoyl-3-digalactosylglycerol) (**9**): Amorphous white powder; for ^{1}H and ^{13}C NMR spectroscopic data, see Table S9; positive-ion ESIMS *m/z* 940 $[M + H]^+$; positive-ion HRESIMS *m/z* 939.6021 $[M + H]^+$ (calcd for $C_{49}H_{88}O_{15}Na^+$, 939.6015).

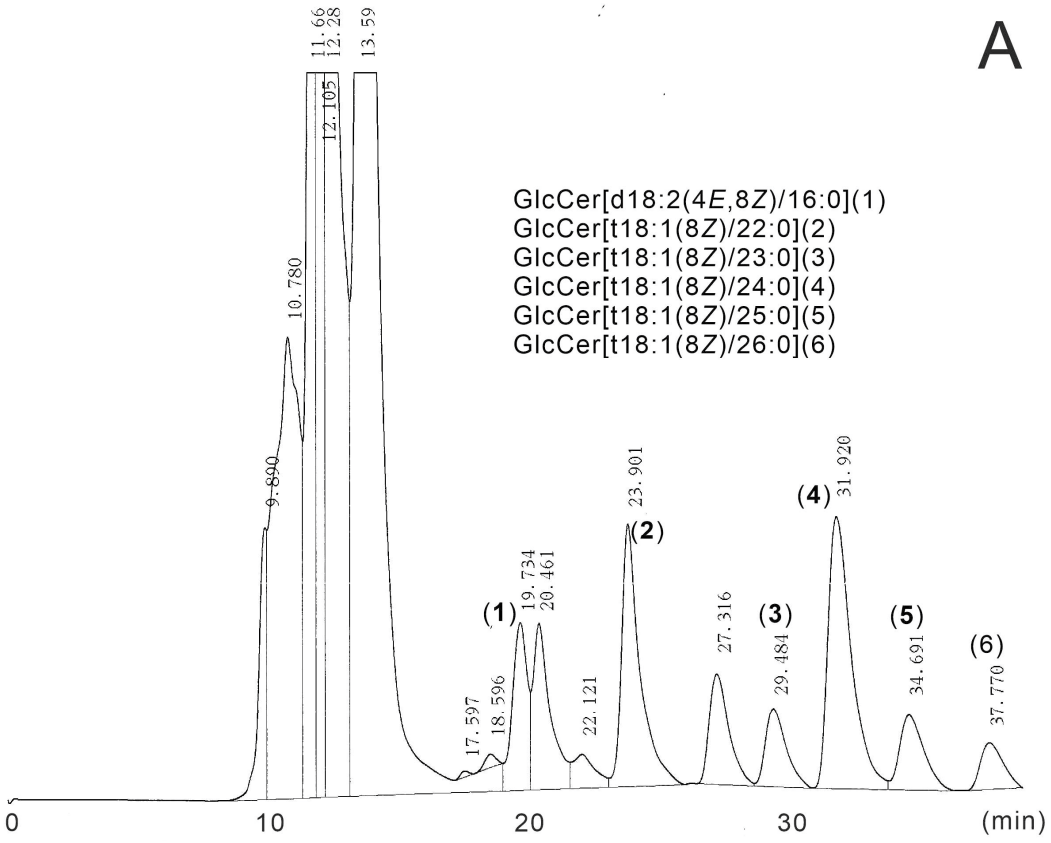


Figure 4. *Cont.*

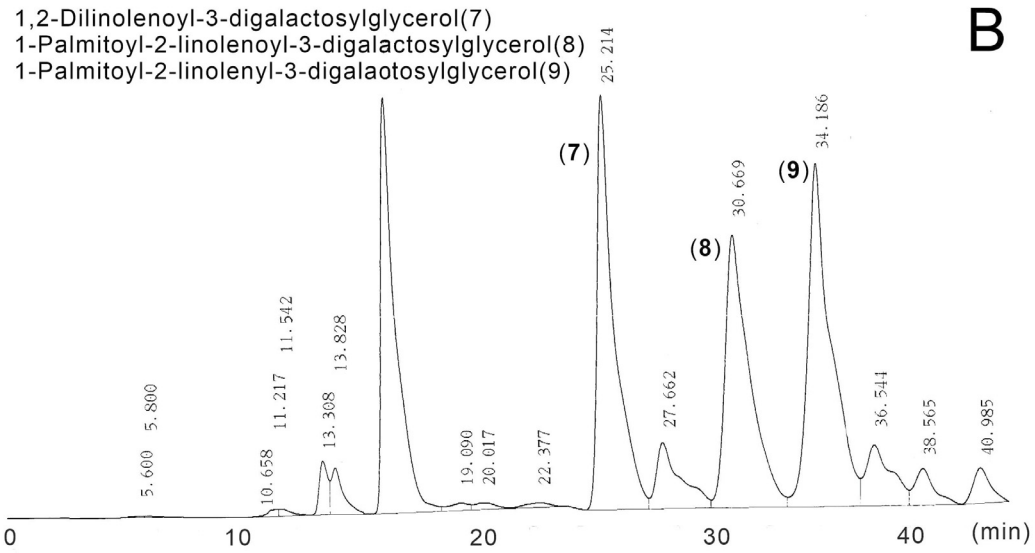


Figure 4. Preparative HPLC chromatograms of GlcCers (**A**) and DGDGs (**B**) from strawberry guava fruit.

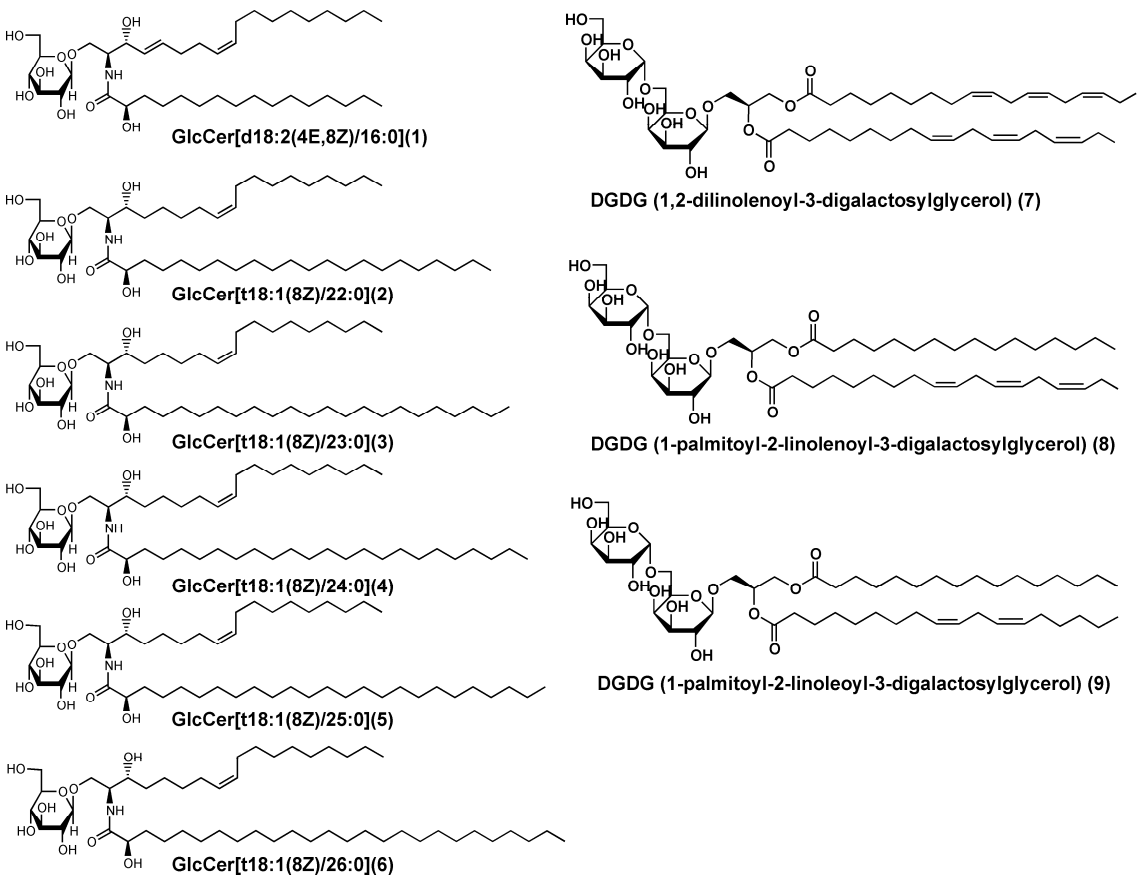


Figure 5. Chemical structures of GlcCers (**1**–**6**) and DGDGs (**7**–**9**) isolated from yellow strawberry guava fruit.

4.5. RHEK Culture and TEWL Measurement

RHEK models [15] were placed onto a 24-well culture plate, and assay medium was added under the cup. After incubating the plate at 37 °C under a 5% CO_2 atmosphere for 1 day, the RHEK model was treated with a solution of 10 μM GlcCer or DGDG or 10 μg/mL of strawberry guava extract (final DMSO concentration: 0.1%). The RHEK model

was cultured for 7 days for TEWL measurements. TEWL was assessed by Tewitro TW24 (Courage + Khazaka) before and 1, 3, 5, and 7 days after the treatment of samples. The RHEK model was placed on a thermal insulation mat (HIENAI Mat; Cosmo Bio Co., Ltd., Tokyo, Japan), which consistently kept the entire bottom surface at 32 °C, without a lid for 5 min before measurements. TEWL was evaluated for 30 min by inserting the sensor of Tewitro TW24 into each well of the RHEK model while maintaining the bottom surface at 32 °C, and the mean value for the last 10 min was used in the analysis. The measurements were performed under sterile conditions.

4.6. Evaluation of Effects on the Blood Vessel Permeability of the HUVEC Layer and Cytotoxicity

According to a previously described method [39], HUVECs (3.2×10^5 cells/mL) suspended in 200 µL/well of EGM™-2 BulletKit™ were seeded onto hanging collagen-coated Millicell® cell culture inserts (24-well, pore size 0.4 µm PET), 500 µL/well of EGM™-2 BulletKit™ was added, and the cells were cultured for 7 days to form a cell layer. After the preculture, the medium on the cells was changed to a new one containing 10 µM GlcCer or DGDG (final concentration of DMSO: 0.1%). After a 24 h culture, a permeability assay was performed.

PBS (200 µL/well) containing 0.1% EB and 0.1% bovine serum albumin was added to each upper insert. PBS (500 µL/well) was also added to the lower chamber, and the plate was incubated for 30 min. The PBS (200 µL) in the lower chamber was transferred to a 96-well plate, and the absorbance was measured at 620 nm. The ratio of permeability was calculated by referring to the control group.

In the cytotoxicity study, 100 µL of cells (2.5×10^5 cells/mL) was seeded onto a 96-well plate and cultured with 10 µM GlcCer or DGDG for 1 day. MTT solution (5 mg/mL, 10 µL) was added to each well and incubated for 6 h. After removing the medium, DMSO (100 µL) was added to dissolve formazan products. The absorbance of wells at 570 nm was measured (reference wavelength: 660 nm).

4.7. Western Blot Analysis (WB) of VE-Cadherin

According to the method described by Guo et al. [39], HUVECs were harvested and homogenized in RIPA buffer containing a protease phosphatase inhibitor cocktail and EDTA (0.5 M). The solution was centrifuged (14,000× *g*, 4 °C, 15 min) to obtain the supernatant. After adding sample loading buffer (62.5 mM Tris-HCl, 5% 2-mercaptoethanol, 2% SDS, 25% glycerol, and 0.01% bromophenol blue) to the supernatant, which was adjusted to 1.0 mg/mL protein content, the mixture was heated at 95 °C for 5 min. Electrophoresis was performed on 10% SDS gels. Polyvinylidene difluoride membranes were used for the transcription of separated proteins and 5% skim milk for blocking. The following primary antibodies were used: VE-cadherin (1:500) and GAPDH (1:5,000). The secondary antibodies were as follows: HRP-conjugated goat anti-rabbit IgG (1:10,000) and HRP-conjugated goat anti-mouse IgG (1:10,000). ImageQuant LAS500 (Global Life Sciences Technologies Japan K.K., Tokyo, Japan) and ECL Plus Western blotting substrate were used for detection.

4.8. Melanin Production in B16 Melanoma Cells

The evaluations of GlcCers and DGDGs were performed according to the method described by Miyasaka et al. [14]. Briefly, B16 melanoma cells (5×10^4 cells/mL) were seeded on a 48-well culture plate (200 µL). After incubation for 24 h, GlcCer or DGDG and theophylline (2 mM) were added and incubated for 72 h. The medium was then removed from the 48-well plate and distilled water (100 µL/well) was added. The cells were sonicated and 6 M NaOH (20 µL/well) was added. The absorbance of the sonicated solution was measured with a microplate reader (measurement wavelength: 405 nm, reference wavelength: 660 nm).

To evaluate cytotoxicity, MTT solution (5 mg/mL) dissolved in PBS (−) was added (10 µL/well) to the 96-well plate and incubated for 4 h. The medium was removed, and DMSO (100 µL/well) was added to dissolve formazan crystals. The absorbance

was measured with a microplate reader (measurement wavelength: 570 nm, reference wavelength: 660 nm).

4.9. Statistical Analysis

The results are expressed as the mean and S.E. The significance of differences was examined using a one-way ANOVA, followed by Dunnett's test, and $p < 0.05$ was considered to be significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12071421/s1, Table S1: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of GlcCer **1** (pyridine-d_5). Table S2: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of GlcCer **2** (pyridine-d_5). Table S3. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of GlcCer **3** (pyridine-d_5). Table S4. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of GlcCer **4** (pyridine-d_5). Table S5. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of GlcCer **5** (pyridine-d_5). Table S6. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of GlcCer **6** (pyridine-d_5). Table S7. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of DGDG **7** (pyridine-d_5). Table S8. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of DGDG **8** (pyridine-d_5). Table S9. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of DGDG **9** (pyridine-d_5).

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